PLANTS' RESPONSES TO NOVEL ENVIRONMENTAL PRESSURES

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PUBLISHED IN: Frontiers in Plant Science

frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-88945-402-0
DOI 10.3389/978-2-88945-402-0

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PLANTS' RESPONSES TO NOVEL ENVIRONMENTAL PRESSURES

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Levander, a native Mediterranean plant to the dry and heat Mediterranean regions, expanding northwards due to global change. Here, an urban planting in Copenhagen.

Image: Alessio Fini.

Plants have been exposed to multiple environmental stressors on long-term (seasonal) and short-term (daily) basis since their appearance on land. However, the frequency and the intensity of stress events have increased much during the last three decades because of climate change.

Plants have developed, however, a multiplicity of modular and highly integrated strategies to cope with challenges imposed by novel, usually harsher environments. These strategies include migration, acclimation and adaptation. Twelve articles in this research topic exactly focus on the relative significance of these response mechanisms for the successful acclimation of plants to a wide range of novel environmental pressures. Four articles , additionally, explore how plants respond to severe stress conditions resulting from the concurrent action of multiple stressors. Ten articles mostly examine how morpho-anatomical, physiological and biochemical-related traits integrate when plants suffer from 'novel' threats, such as solid, gaseous, and electromagnetic pollutants. Suitable physiological indicators for developing conservation strategies are described in the last two works. This research topic highlights that bottom-up, as well as, top-down approaches will be necessary to develop in near future in the study of plants' responses to environmental pressures.

Citation: Fini, A., Tattini, M., Esteban, R., eds. (2018). Plants' Responses to Novel Environmental Pressures. Lausanne: Frontiers Media. doi: 10.3389/978-2-88945-402-0

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frontiers in Plant Science

EDITORIAL
published: 23 November 2017
doi: 10.3389/fpls.2017.02000

Editorial: Plants' Responses to Novel Environmental Pressures

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Keywords: adaptation, acclimation, drought, migration, multiple stress, pollution, warming

Editorial on the Research Topic

Plants' Responses to Novel Environmental Pressures

Multiple environmental stressors, on both short-term and long-term basis, challenged early land plants since they colonized the land, around 450 million years ago. Emerged lands were far more sensitive to changes in climate than oceans, and plants have displayed during evolution an extraordinary ability to survive (i.e., to adapt) dramatic variations in environmental conditions. This has occurred through acclimation, adaptation, or migration to new environments (Bussotti et al., 2014). Migration is a very long process, as plants do not possess the "flight" strategy displayed by other living organisms, and it is further hampered by environmental, biological, and spatial barriers (Bussotti et al., 2014). For example, high UV-load may hasten the upward migration of tropical alpine plants. Barnes et al., in this special issue, tested the hypothesis that more effective photoprotective mechanisms may explain the greater upward migration potential of exotic compared to native species. After surveying the leaf epidermal UV-A-transmittance (inversely related to the accumulation of flavonoids and related UV-absorbing compounds) in 54 species, the authors found that plasticity in this parameter poorly explained the different migration potential of native and exotic species, despite significant intra-specific differences. Rull and Vegas-Vilarrubia also present a remarkable case study of the impact of barriers on plant migration. Authors draw the attention on "The Lost World," the neotropical Guayana highlands. This pristine habitat, which never suffered from direct human pressure, has been recently exposed to the indirect pressure of human activities, such as global warming and altered rainfall regime. Migration toward higher altitudes is prevented in this habitat because of the flat tops of the highlands. Species with higher phenotypic and physiological plasticity are better suited to survive the challenges associated to climate change. The relative significance of environmental/ and "spatial" (barrier) factors on vegetation dynamics has been explored, at large level of scale, by Chen et al. Authors give compelling evidence that environmental factors are primary drivers of shrub species turnover, whereas barriers mostly hamper the turnover of trees. Wang Z. et al. have also explored traits, such as the germination potential, responsible for effective species dispersal. It is shown that this trait is under tight control of environmental pressures, on short-term scale level, whereas phylogenic constrains become prevalent on long-term basis.

It is not surprising to observe that plants, due to their sessile nature and their slow migration speed, have developed a complex suite of modular and highly integrated mechanisms to match the key constrains encountered within the species distribution range, broadly the acclimation/adaptation syndrome (Lichtenthaler, 1998; Demmig-Adams et al., 2014; Esteban et al., 2015). The ability to adjust morpho-physiological and biochemical traits at different time scales (i.e., over seconds at the chloroplast and cellular level, or over days, growing seasons, or even generations at the whole-canopy level) constitutes the "flight strategy of sessile organisms" (Potters et al., 2007; Karban, 2008).

OPEN ACCESS

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *10 October 2017*
Accepted: *09 November 2017*
Published: *23 November 2017*

Citation:
Fini A, Tattini M and Esteban R (2017) Editorial: Plants' Responses to Novel Environmental Pressures. Front. Plant Sci. 8:2000. doi: 10.3389/fpls.2017.02000

Investigating three ecotypes of *Arabidopsis thaliana*, Adams et al. evidenced the relevant effects of native habitat on anatomical and functional traits of leaves. Interestingly, while the ecotype from the central portion of the distribution range showed the highest plasticity to environmental changes, accessions from the Northern and Southern portions showed highly distinct adaptive traits to cope with cool temperatures and drought, respectively. Similarly, to survive to the harsh Mediterranean climate, plants have developed a wide range of adaptive traits, including a fine tuning of phenological phases to maximize carbon gain, while avoiding frost damage in winter (Cleland et al., 2007), and specific morpho-physiological characteristics to cope with summer drought (Bussotti et al., 2014). Consistently, Fernandez-Marín et al. pointed out that photoprotective mechanisms of Mediterranean flora are highly adapted to the environmental pressures associated to Mediterranean climate, namely drought, excess light, and high temperature, with α-tocopherol and violaxanthin-cycle pigments being more responsive to such stresses in Mediterranean than in other flora. It is worth noting, that the priming driven by stressful conditions enable plants, not only animals, to acclimate successfully to recurrent stress episodes, i.e., living organisms have the capacity to remember stress events (Conrath et al., 2006), as pointed out, in this special issue, by Matsubara et al. The extraordinary capacity to acclimate and adapt to their growing environment is observed indeed at the individual scale, as well as at larger scales (e.g., cenosis and ecosystem): de la Riva et al. provided rationale of such biodiversity, and highlighted that different biogeochemical niche partitioning allows species to coexist through divergent leaf nutrient composition and resource uptake.

It is not surprising indeed that several habitats threatened by the most severe environmental pressures are biodiversity hotspots. For example, Mediterranean ecosystems, where plants are simultaneously challenged by high light, heat, and drought stress of increasing severity, represent just 2% of the earth's land area, but account for 16% of the world's plant species (Myers et al., 2000). Similarly, mountain ecosystems and nutrient-poor soils are biodiversity hotspots highly endangered by climate change (Lambers et al., 2013). Although land plants have long history of successful response to the challenges imposed by a changing environment, climate change in the Anthropocene has peculiarities, which can turn old stresses into something "novel" to plants.

First, the unprecedented rate at which climate changes may exceed the rate of genetic adaptation and migration in most species (Klein et al., 2013), with some functional groups being more affected than others. Exploring the effect of changes in night temperatures on different plant functional types, growth forms, and economic purposes (C3 vs. C4 vs. CAM; herbaceous vs. woody; crop vs. no-crop), Jing et al. highlighted that the large variation in the acclimation capacity of the different plant species may yield different fitness under future climate scenarios. Wang D. et al., evaluating long-term response to heat stress in C3 and C4 species, offered evidence that repeated, even transient heat stress episodes can affect plant community structure, vegetation dynamics, biodiversity, and ecosystem functioning on long-term basis. Interestingly, plasticity during stress acclimation does not only vary among functional groups, species, accessions and ecotypes, but also between male and female plants in dioecious species. The topic is explored by Jiang et al., who found important trade-offs between growth in male and female individuals of *Salix paraplexia* growing under poor nutrient availability, with females investing more in growth than males. The authors concluded that a moderate increase in female willow species in restoration programs could increase the resistance and resilience of willow populations to early sporadic desertification. Similarly, on the dioecious palm *Chamaerops humilis*, Morales et al. found that female individuals were more sensitive than males to winter photoinhibition. These results may be, therefore, helpful for restoration decisions focused on sex selection.

Secondly, unprecedented biotic and abiotic stress events, such as heat waves, drought spells, (introduced) pest outbreak, and human disturbance, often occur simultaneously and interact with each other. While literature is full of experiments evaluating plant response to single stressors, research investigating the interaction among co-occurring stressors is scarce (Dieleman et al., 2012). Trying to fill this gap, the research by Top et al. focused on the effects of high temperatures and water availability on secondary metabolites in leaves of *Quercus rubra*. In comparison to plants growing under favorable climate, the plants challenged by stress factors produced more tannins with lower degree of complexity, i.e., with reduced chain length. This may have relevant consequences on both carbon and nutrient recycling and, as consequence not only on the short-time "adaptation" of species to adverse environmental pressures associated to climate change, but also in the regulation of herbivore dynamics. How physiological and metabolic adjustments triggered by the acclimation to abiotic stresses affect plant response to pests is indeed intriguing. Ederli et al. investigated the influence of drought stress on *Nezara viridula* infestation and nymph growth performance in *Vicia faba.* Authors highlighted a role for salicylic acid signaling in both drought stress and *N. viridula* infestation, whereas ABA-signaling is either activated by drought or down-regulated by *N. viridula* infestation, thus offering new insights about how plants respond to concurrent abiotic and biotic stressors. The interaction effects of UV-B, light quality and quantity, heat and drought stresses, on plant defenses against herbivores also constitute the main issue explored in the review article by Escobar-Bravo et al. UV-B can interact positively with high photosynthetic active radiation, blue light, high temperature, and water deficit to increase plant performance and constitutive chemical defenses. Nonetheless, fresh assimilated carbon available to the synthesis of secondary metabolites may be severely constrained at severe heat and drought stress, and alternative carbon sources are required for their biosynthesis. It is suggested that UV-B radiation, possibly in combination with high blue light, may enhance plant defense against herbivores, especially in high density planting, as herbivores arthropods take advantage in far red-to-far red-enriched environments.

Besides host–pathogen interaction, the relationship between plants and beneficial microorganisms is highly sensitive to the novel pressures imposed by global change (Compant et al., 2010). For example, there is evidence that this beneficial interaction may be favored by rising CO_2 and mild drought,

but hastened by severe disturbances, such as urbanization (Fini et al., 2011). Understanding the plant-microbe relation under the climatic pressure associated to climate change, is crucial for deeper insights into stress-mitigation mechanisms. Meena et al. reviewed the abiotic stress responses and the microbe-mediated mitigation through multi-omics strategies. Authors offered an exhaustive view of the key roles of multi-omics approaches, such as transcriptomics, proteomics, metabolomics, and phenomics in providing a better understanding of the complex interaction of plant-microorganisms.

Thirdly, the release of gaseous, solid, and electromagnetic pollution from human activities has increased greatly since the industrial age, and constitutes a novel "oxidative" threat for plants. Primary pollutants, such as nitrogen oxides and carbon monoxide, are highly reactive and can either direct injury plants or combine in the troposphere into secondary pollution, a hard-to-solve issue for plant community health. The input of reactive nitrogen in the atmosphere has fostered nitrogen deposition and acid precipitation, with detrimental effects on biodiversity on global scale (Sala et al., 2000). Mao et al. investigated the effects on nitrogen addition and altered snowfall pattern at different scales, from the intra-specific variability of leaf traits to the functional diversity of the whole community. Authors found positive effect of nitrogen addition on the overall growth of plant communities, but highlighted that annual plants shifted faster than perennials to more nitrophilic functional traits (e.g., low leaf mass per area), which may determine a change of dominant species in plant communities. Ozone is a secondary gaseous pollutant markedly influencing plant communities. Cotrozzi et al. explored the interaction between ozone and drought stresses on the evergreen broad-leafed *Quercus ilex*. It is shown, that plants challenged by water deficit display decreased capacity to activate phytohormone-related signaling pathways, and hence proper warning signals, when successively exposed to acute O_3 stress. Ozone is formed in the troposphere when nitrogen oxide reacts with volatile organic compounds (VOC). A fraction of total VOCs in the atmosphere has biogenic origin (BVOC, mainly isoprene and monoterpenes) and primarily emitted by plants. After reviewing why biogenic isoprene emission may become relevant in a warmer and drier climate, Fini et al. focused on its metabolic roles, with particular reference to hygrophilic species. Most isoprene emitters are hygrophylic and display a constitutive suite of morpho-anatomical and physiological features unable to efficiently counter the challenges imposed by harsh climates (Valladares et al., 2000; Tattini et al., 2006; Matesanz and Valladares, 2014). Authors noted that isoprene emission in response to drought stress largely depends on the strategies adopted by plants to cope with the scarcity of water, and may not be a good proxy of its leaf internal concentration. For example, Martinez-Sancho et al. showed that contrasting xylem anatomy and hydraulics in the evergreen isohydric *Pinus sylvestris* and in the deciduous anisohydric broadleaf *Quercus robur*, yielded different stomatal sensitivity to dehydration. Oak adjusted vessel size to maintain high stomatal conductance and carbon uptake, while avoiding embolism under increasing aridity during drought. Pine, instead, relied more on stomatal closure to prevent excessive water loss. Indeed, these contrasting stomatal behaviors determine the ratio between the biosynthesis and the emission of isoprenoids and open new questions about their protective roles under drought stress of increasing severity. The effect of moderate drought on volatiles emission by two ecotypes of *Arundo donax*, a fast-growing, isoprene emitter has been evaluated by Haworth et al. They investigated the methyl-erythritol and the methionine pathways, linked to carotenoid/isoprene and glutathione biosynthesis, respectively. Authors suggested that both cycles are independent chloroplastic pathways, both aimed at preserving photosynthetic capacity and enhancing tolerance to moderate drought, and no trade-off occurs between the two pathways.

Solid pollutants from human activities include heavy metals, black carbon, some polycyclic aromatic hydrocarbons, and the much less explored nanoparticles. Lead is one of the most abundant pollutants, readily absorbed through the root system of edible plants and entering the food-chain, with adverse consequences on public health worldwide. Wang Y. et al. examined the mechanism underlying root proteome in response to Pb in radish. Key enzymes in response to Pb relate with glycolysis, citric acid cycle, and the scavenging of reactive oxygen species. The response of floating and submerged leaves of *Potamogeton nodosus* to Ag toxicity was examined by Shabnam et al.. They showed that the antioxidant machinery of floating leaves is responsible for their superior Ag tolerance as compared to submerged leaves, and provide new insights to assist effective bioremediation of water bodies. Besides, the rising release of nanoparticles to the environment from household, agricultural, industrial, and healthcare products pose urgent need to understand their effect on plant growth, development, and physiology. Marslin et al. reviewed this topic and found that exposition to nanoparticles induces a ROS signaling that activate secondary metabolic pathways. Due to their sensitivity to environmental disturbances, including air pollution, and their capillary distribution in cities, urban trees can be effective low-cost indicators of air quality in urban sites. Ferreira et al., using a combination of bioindication and geostatistical methods, showed that the analysis of tree bark is a precise, still inexpensive tool for assessing the spatial distribution of airborne solid pollutants within a city as well as citizens' health risking areas lacking traditional pollution monitoring networks. Electromagnetic pollution is a novel pressure imposed by human activities impose to plant communities, still research has only focused on its effects on animals. Sztafrowski et al. analyzed the effect of magnetic fields on gene expression in *Solanum tuberosum* and *A. thaliana*, to evaluate if electromagnetic fields trigger similar effects on gene transcription as those observed in humans.

Understanding plant response to the unprecedented challenges associated to rapid climate change has been the aim of this Research Topic. Such knowledge is urgently needed to develop effective conservation strategies, and to set-up long-term monitoring networks aimed at observing the impact of multiple stress pressures on forest ecosystems. Suitable physiological indicators for this purpose, such as carbon isotope composition and photosystem II photochemistry efficiency, are well described in Bussotti and Pollastrini. Biochemical markers can also assist

monitoring and guide restoration programs. This may be the case of methionine, suggested by Taïbi et al. as a suitable marker for screening drought tolerant ecotypes of *Pinus halepensis*. We have provided information regarding plants responses to abiotic and biotic stresses from multiple organization levels (molecular, cell, organism, and ecosystem) giving new evidence that may help to better understand how plants will cope with stress factors and novel pressures imposed by the global warming and anthropogenic pressure. However, this topic is wide, which and has been highlighted in other research topics in Frontiers in Plant Science, namely "water stress responses in plants," "mechanisms of abiotic stress responses and tolerance in plants," or "photosynthesis within changing climate conditions." These three are only examples of the 90 research topics published regarding "climate change" or of the 450 concerning "plant responses to stress." Nonetheless, further experimentation is urgently needed to understand and predict the interactions that underpin the special capacity of plants to fine-tune their physiology and (secondary) metabolism to successfully respond to the severe challenges imposed by a rapidly changing climate, particularly in most fragile ecosystems worldwide. This requires approaches at different levels of scale, using bottom-up as well as top-down approaches, not only to breed "resistant" crops and/or selected varieties, but also to additionally develop strategies for promoting both biodiversity conservation and ecosystem functionality.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

RE received a Juan de la Cierva-incorporación grant IJCI-2014-21452 funded by Spanish Ministry of Economy and Competitiveness (MINECO) and a research stay grant "Jose Castillejo" CAS 17/00146 funded by the Ministry of Science and Education.

ACKNOWLEDGMENTS

We thank all the authors and reviewers that have contributed to this Research Topic.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 22 August 2017
doi: 10.3389/fpls.2017.01451

UV Screening in Native and Non-native Plant Species in the Tropical Alpine: Implications for Climate Change-Driven Migration of Species to Higher Elevations

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Ongoing changes in Earth's climate are shifting the elevation ranges of many plant species with non-native species often experiencing greater expansion into higher elevations than native species. These climate change-induced shifts in distributions inevitably expose plants to novel biotic and abiotic environments, including altered solar ultraviolet (UV)-B (280–315 nm) radiation regimes. Do the greater migration potentials of non-native species into higher elevations imply that they have more effective UV-protective mechanisms than native species? In this study, we surveyed leaf epidermal UV-A transmittance (T_{UVA}) in a diversity of plant species representing different growth forms to test whether native and non-native species growing above 2800 m elevation on Mauna Kea, Hawaii differed in their UV screening capabilities. We further compared the degree to which T_{UVA} varied along an elevation gradient in the native shrub *Vaccinium reticulatum* and the introduced forb *Verbascum thapsus* to evaluate whether these species differed in their abilities to adjust their levels of UV screening in response to elevation changes in UV-B. For plants growing in the Mauna Kea alpine/upper subalpine, we found that adaxial T_{UVA}, measured with a UVA-PAM fluorometer, varied significantly among species but did not differ between native (mean = 6.0%; $n = 8$) and non-native (mean = 5.8%; $n = 11$) species. When data were pooled across native and non-native taxa, we also found no significant effect of growth form on T_{UVA}, though woody plants (shrubs and trees) were represented solely by native species whereas herbaceous growth forms (grasses and forbs) were dominated by non-native species. Along an elevation gradient spanning 2600–3800 m, T_{UVA} was variable (mean range = 6.0–11.2%) and strongly correlated with elevation and relative biologically effective UV-B in the exotic *V. thapsus*; however, T_{UVA} was consistently low (3%) and did not vary with elevation in the native *V. reticulatum*. Results indicate that high levels of UV protection occur in both native and non-native species in this high UV-B tropical alpine environment, and that flexibility in UV screening is a mechanism employed by some, but not all species to cope with varying solar UV-B exposures along elevation gradients.

Keywords: alpine, elevation gradient, epidermal UV-A transmittance, flavonoids, Hawaii, native species, non-native species, ultraviolet radiation

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *11 April 2017*
Accepted: *04 August 2017*
Published: *22 August 2017*

Citation:
Barnes PW, Ryel RJ and Flint SD (2017) UV Screening in Native and Non-native Plant Species in the Tropical Alpine: Implications for Climate Change-Driven Migration of Species to Higher Elevations. Front. Plant Sci. 8:1451. doi: 10.3389/fpls.2017.01451

INTRODUCTION

Many plant species are migrating in response to ongoing changes in climate and additional shifts in geographic ranges are expected in the future, though the rates of movement will likely vary substantially with growth form (e.g., herbaceous vs. woody plants; IPCC, 2014). For species in montane environments, recent climate change-induced shifts in species distributions toward higher elevations have been documented in temperate and tropical locations (Benavides et al., 2016; Dolezal et al., 2016). Over the past 100–200 years, many non-native (i.e., introduced or alien) species have colonized high altitude environments (Pyšek et al., 2011) and in several temperate mountain ranges in North America and Europe, non-native species appear to be migrating to higher elevations to a greater degree than native species (Wolf et al., 2016; Dainese et al., 2017). These findings suggest that, at least along elevation gradients, non-native species have higher migration potentials than native species, though this may depend upon levels of disturbance and local habitat heterogeneity (Suding et al., 2015; Averett et al., 2016). This upward migration of species inevitably exposes plants to novel combinations of biotic and abiotic environmental conditions, including ultraviolet (UV) radiation (280–400 nm), with the potential for significant negative impacts on native alpine biodiversity (Chapin and Körner, 1994; Savage and Vellend, 2015; Cuyckens et al., 2016).

Because of differences in atmospheric conditions (primarily optical depth of the atmosphere and the thickness of the stratospheric ozone layer) and prevailing solar angles, the levels of solar UV-B radiation (280–315 nm) generally increase with decreasing latitude and increasing altitude (Caldwell et al., 1980; Blumthaler et al., 1997; McKenzie et al., 2001). Consequentially, tropical alpine environments experience some of the highest UV-B irradiances on the Earth's surface. UV-B radiation is known to induce a number of potentially deleterious effects in plants, including disruption of the integrity and function of important macromolecules (DNA, proteins, and lipids), oxidative damage, partial inhibition of photosynthesis and growth reduction (Albert et al., 2011; Jansen and Bornman, 2012; Hideg et al., 2013). However, plants have evolved photosensory mechanisms to detect UV (Tilbrook et al., 2013; Jenkins, 2014) and then protect and repair sensitive targets from direct and indirect UV-induced injury (Jansen et al., 1998; Britt, 1999) such that the negative effects of ambient UV-B on plant growth and productivity are typically small or difficult to detect under field conditions (Ballaré et al., 2011). Nonetheless, UV-B is generally considered to be an important selective force in the evolution and adaptation of the tropical alpine flora (Lee and Lowry, 1980; Robberecht et al., 1980; Caldwell et al., 1982). To what extent UV-B limits the ability of plant species to migrate into alpine environments or expand their ranges within the alpine, however, is not known.

One of the most important and widespread protective responses of plants to UV radiation involves the induction and synthesis of flavonoids, hydroxycinnamic acids (HCAs) and related phenylpropanoids that function as "UV sunscreens" and antioxidants (Searles et al., 2001; Agati et al., 2012; Schreiner et al., 2012). Flavonoid biosynthesis is influenced by UV-B, UV-A (315–400 nm), and visible radiation (400–700 nm) (Flint et al., 2004; Siipola et al., 2015) and appears to be mediated, at least in part, by the UV-B photoreceptor UV RESISTANCE LOCUS 8 (UVR8) (Morales et al., 2013). The accumulation of flavonoids and related UV-absorbing compounds in epidermal tissue decreases epidermal UV transmittance (Mazza et al., 2000; Bidel et al., 2007) and is a primary mechanism by which plants acclimate to changing UV environments, including alterations resulting from stratospheric ozone depletion and climate change (Caldwell et al., 1983; Bornman et al., 2015).

This UV screening response entails measurable energetic and growth costs (Snell et al., 2009; Hofmann and Jahufer, 2011) and varies within and among plant species (e.g., Qi et al., 2010; Randriamanana et al., 2015). Some of the interspecific variation in UV screening can be attributable to growth form differences in leaf structure and cellular distributions of UV-absorbing compounds (i.e., vacuole vs. cell wall; Day et al., 1992, 1993). For example, in a study using micro-probes to measure UV penetration into the foliage of a diverse group of plants in the North American Rocky Mountains, Day et al. (1992) found that the leaf epidermis of herbaceous dicots (forbs) was less effective in attenuating UV-B than that of grasses and woody dicots. The accumulation of UV-absorbing compounds and resultant changes in leaf optical properties are also highly plastic traits in many species (Wargent et al., 2015) and have been shown to vary in relation to natural elevation/latitudinal UV-B gradients (Robberecht et al., 1980; Rozema et al., 1997; Ruhland et al., 2013). In many cases, these differences in UV protection can account, at least in part, for the differential UV-B sensitivities of high- vs. low-elevation taxa (Barnes et al., 1987; Sullivan et al., 1992; Ziska et al., 1992; but see Nybakken et al., 2004).

At present, very little is known whether native and non-native plant species differ in their tolerances to UV-B and levels of UV protection. The apparent greater propensity for non-native species to migrate to higher elevations than native species may indicate that non-native species are capable of adjusting their UV protection more effectively, either through greater phenotypic plasticity or more rapid genetic adaptation, than native species. Indeed, the success of non-native species in general is often attributed, in part, to their high degree of phenotypic plasticity to environmental change (Richards et al., 2006; Davidson et al., 2011). If non-native species exhibit greater phenotypic plasticity to UV-B change than native species, one would expect greater variation in UV protective mechanisms along elevational/UV-B gradients relative to native species, and non-native species would display similar or perhaps even higher levels of UV protection than native species in the high UV-B alpine environment. Alternatively, many non-native species possess functional traits (physiology, allocation, growth rate, etc.) that allow for high rates of resource acquisition and performance (Van Kleunen et al., 2010; Funk, 2013) and these traits can come at a cost in reduced tolerance to abiotic stress in harsh conditions, such as occurs in the alpine (Brock and Galen, 2005). Tolerance to UV-B is often cross-linked with tolerance to other abiotic stresses such as drought (Bandurska et al., 2013) and defense against pests and pathogens (Mewis et al., 2012; Zavala et al., 2015). It is thus conceivable that non-native species may invest less in UV protection than native species and may be more rather than

less sensitive to UV-B than native species. If this is the case, the invasion of high elevation habitats by non-native species may be governed less by UV-B tolerance than by other plant attributes, such as life history traits and competitive abilities. In support of this hypothesis, Wang H. et al. (2016) reported that non-native populations of *Triadica sebifera* exhibited greater sensitivity to elevated UV-B than native populations under controlled conditions. Whether these differences in UV tolerance were the result of differences in UV-absorbing compounds and UV screening was not investigated. Taken together it is therefore difficult to predict, *a priori*, whether non-native species that occur in high elevation environments would exhibit similar, higher or lower levels of UV protection than their native counterparts.

In the study described here, we characterize the leaf optical properties of a suite of native and non-native plant species of different growth forms growing in the tropical alpine and upper subalpine of Mauna Kea, Hawaii to test whether differences in epidermal UV transmittance (measured as the screening of UV-A radiation) exist between native vs. non-native species. Additionally, we examine UV screening of a native and a non-native species along an elevation gradient spanning 2600–3800 m to determine if these species differ in their abilities to adjust their levels of UV protection in response to natural variation in UV-B exposure. This study examines the ability of native and non-native plant species to cope with extreme natural levels of UV-B, and thus provides insights into the role that UV-B may play in influencing climate change-induced upward range expansions in mountains.

MATERIALS AND METHODS

Survey of Native and Non-native Alpine Species

Studies were conducted on 19 common native (8) and non-native (11) wild species growing on native volcanic soil in un-shaded habitats on the south slope of Mauna Kea, Hawaii, United States (19°45′N, 155°27′W) ca. 2800–3900 masl from early to mid-June (**Table 1**). This elevation range includes the upper subalpine and alpine vegetation zones of Mauna Kea (Gerrish, 2013). For all species, we sampled plants that were growing at, or near, their approximate peak elevations as determined from floristic surveys (**Table 1**; Wagner et al., 1999). We were limited in the amount of the Mauna Kea alpine/upper subalpine vegetation we could sample in due to logistical constraints (i.e., there are very few roads on the mountain) and out of deference to the indigenous Hawaiian culture that considers the mountain to be sacred. The majority of our sampling was therefore conducted within the south-facing slope of the 212 km^2 Mauna Kea Forest Reserve, including sites along the Mauna Kea access road leading from the Mauna Kea Information Visitor's Center (2800 masl) to the summit (ca. 4200 masl). Within this area, we selected species for study that were easily accessible (they occurred within ca. 1 km of the Mauna Kea Access Road), relatively abundant (at least 10 individuals present per sampling location), and which were suitable for measurement (large enough leaves to fit the sampling chamber and green in color; see below).

The primary goal of this study was to compare UV screening in native and non-native species growing in this tropical alpine environment. We recognized, however, that there was a diversity of plant growth forms in the Mauna Kea alpine, and results from previous studies (e.g., Day et al., 1992) have shown that UV screening can vary significantly among plant growth forms. Thus, we also wished to compare UV screening among plant growth forms to determine if any potential differences in UV screening between native and non-native species could be attributable to growth form differences. We attempted to survey species representing all of the major growth forms present on Mauna Kea [i.e., woody dicots (trees and shrubs), herbaceous dicots (forbs), and grasses]. We did not examine any conifers (none occurred at our study site) and we also did not sample any cushion plants, rosettes or succulent growth forms that are often found in alpine life zones (Körner, 2003) but which are rare or absent in the Mauna Kea alpine (Gerrish, 2013). As a consequence of the sampling limitations described above, the native species tested were mostly woody species (i.e., five of the eight species were shrubs or trees) whereas all of the non-native species were herbs (forbs or grasses; **Table 1**). There are no non-native woody species in the Mauna Kea alpine (Gerrish, 2013). Thus, plant growth form in this study is, to a certain degree, inherently confounded with native vs. non-native status. Also, the native species sampled were all perennials, whereas the non-natives included both annuals and perennials. One species of native fern was sampled. Nomenclature follows Wagner et al. (1999) for the angiosperms and Palmer (2003) for the fern as per recent updates by Wagner et al. (2012).

Measurements of UV screening [epidermal UV-A transmittance (T_{UVA}); see below] were taken on 10 plants/species selected haphazardly at each sampling location with two to three leaves measured per plant. There was no systematic pattern of leaf sampling within each plant (i.e., we made no attempt to isolate the effect of leaf position or age on T_{UVA}). Rather, we haphazardly selected several leaves among the healthy, mature leaves on an individual plant shoot. Preliminary analyses (ANOVA) showed no significant effect of leaf sample number on T_{UVA}. Thus, data were averaged within a plant and subjected to an arcsine transformation (Zar, 1999) to normalize data prior to statistical analysis. We used individual one-way ANOVAs (SAS JMP, Cary, NC, United States) to test for species, growth form, and native vs. non-native effects. In the ANOVA testing for the effect of species on T_{UVA}, the individual plant was the unit of replication. For the other ANOVAs (growth form and native vs. non-native comparisons) we averaged values within a species such that the individual species was the unit of replication. Significant differences were determined at $P < 0.05$.

Elevation Gradient Study

One native and non-native species were selected for additional study to explore whether UV screening varied with elevation and prevailing levels of solar radiation. For this study, we sampled the native shrub, *Vaccinium reticulatum*, and the non-native forb, *Verbascum thapsus*, across the entire elevational range of both species (762–3352 masl for *V. reticulatum*; 100–3962 masl for

TABLE 1 | Characteristics of the native and non-native (introduced) species sampled for epidermal UV-A transmittance in the Mauna Kea, Hawaii alpine/subalpine.

Species	Family	Growth form	Elevation sampled (m)	Elevation range (m)
Native species				
Leptecophylla tameiameiae	Epacridaceae	Shrub	3429	60–3230
Chenopodium oahuense	Amaranthaceae	Shrub	2774	0–2520
Geranium cuneatum	Geraniaceae	Shrub	3444	1480–3250
Sophora chrysophylla	Fabaceae	Shrub/tree	2914	450–3240
Vaccinium reticulatum	Ericaceae	Shrub	3353	640–3700
Stenogyne microphylla	Lamiaceae	Vine/forb	2774	1200–2700
Cystopteris douglasii	Woodsiaceae	Fern	3962	1500–3000+
Trisetum glomeratum	Poaceae	Grass	3962	750–4090
Non-native species				
Malva parviflora	Malvaceae	Forb	2774	0–2270
Verbascum thapsus	Scrophulariaceae	Forb	3962	1550–2350
Taraxacum officinale	Asteraceae	Forb	3962	NA
Oenothera stricta	Onagraceae	Forb	2774	1200–2740
Heterotheca grandiflora	Asteraceae	Forb	2774	10–2270
Verbascum virgatum	Scrophulariaceae	Forb	2774	NA
Rumex acetosella	Polygonaceae	Forb	3962	1115–2840
Hypochaeris radicata	Asteraceae	Forb	3429	1100–2800
Senecio madagascariensis	Asteraceae	Forb	2914	NA
Poa pratensis	Poaceae	Grass	3962	1220–4025
Anthoxanthum odoratum	Poaceae	Grass	2914	840–2140

Nomenclature, native vs. non-native classifications, growth form and elevation ranges are from Wagner et al. (1999) and Palmer (2003), according to recent updates. NA, not available.

V. thapsus) during June. We chose these species because they could often be found growing in close proximity throughout much of this elevation gradient, which was essential for sampling purposes (measurements were conducted in the dark as indicated below). Sampling locations were located on the south slope of Mauna Kea along a transect that generally corresponded to that used by Nullet and Juvik (1997) in a study characterizing elevation changes in UV-B, photosynthetically active radiation (PAR; 400–700 nm), and total shortwave (SW) radiation (300–3000 nm). In their study, Nullet and Juvik (1997) measured UV-B using a broadband sensor (Robertson-Berger Model 501A Biometer) that provided a measure of biologically effective UV-B (UV-B_{ERY}) weighted according to the human erythemal action spectrum. PAR was measured with a LiCor Model LI190SB quantum sensor and SW radiation was measured with an Eppley PSP pyranometer. Nullet and Juvik (1997) collected radiation data at 10 elevations ranging from sea level (0 m) to 4230 masl on Mauna Kea near midday under clear skies in June and then they adjusted their data to correspond to a solar zenith angle of 10°. At each of their sampling elevations, these investigators reported values of UV-B_{ERY}, PAR, and SW relative to those at the Mauna Kea summit. We used polynomial regression models [second-order for UV-B and fourth-order for PAR and SW (R^2 = 0.98–0.99); SAS JMP] to establish relationships between elevation and these three measures of relative solar irradiance. We then used these regression models to calculate relative clear sky UV-B_{ERY}, PAR, and SW for the sampling elevations used in our study. Least square regression and correlation (multivariate) analyses in JMP were used to examine relationships between T_{UVA}, elevation, and solar radiation. For these regression models we tested linear and polynomial models (second, third, and fourth order) and selected the model that explained the largest amount of variation in the data (i.e., the highest value of R^2).

Measurements of Leaf Optical Properties

Non-invasive measurements of epidermal T_{UVA} were made on adaxial (upper) surfaces of healthy, fully expanded leaves with a field-portable pulse amplitude modulation (PAM) chlorophyll fluorometer (UVA-PAM; Gademann Instruments, Würzburg, Germany). This instrument provides estimates of epidermal T_{UVA} by measuring the fluorescence yield of chlorophyll (F_o; $\lambda > 650$ nm) induced by UV-A (375 nm) and blue (BL; 470 nm) radiation, as outlined by Kolb et al. (2005) and following the precautions and procedures of Barnes et al. (2008). This technique is based on the premise that both UV-A and BL can induce chlorophyll fluorescence and that reductions in the penetration of UV to the mesophyll (e.g., from UV-absorbing compounds in the epidermis) will reduce UV-A-induced chlorophyll fluorescence (F_{UVA}). Fluorescence induced by BL (F_{BL}), which is not absorbed by UV pigments, serves as a reference to account for variation in chlorophyll content and distribution in the mesophyll. Ideally, calculations of epidermal UV transmittance using this technique are based on the F_{UVA}/F_{BL} of epidermis-free leaf samples. As it is usually not possible to readily remove the epidermis for most species, F_{UVA}/F_{BL} values are normally expressed relative to a blue plastic standard (Heinz Walz GmbH, Effeltrich, Germany), which has emission

properties similar to an epidermis-free green leaf. Such was the case in this study. The epidermal T_{UVA} reported here should therefore be considered as approximations of the true transmittances for these species.

Measurements of T_{UVA} by the UVA-PAM generally exhibit strong, positive correlations with direct measurements of epidermal UV transmittance (in both the UV-B and UV-A) made from epidermal peels (Markstadter et al., 2001), and this technique has been widely used to investigate UV sunscreen protection in a diversity of plant species and conditions (see reviews of Barnes et al., 2015; Julkunen-Tiitto et al., 2015; and references therein). However, while measurements of T_{UVA} made by the UVA-PAM are generally correlated with epidermal UV-B transmittances (T_{UVB}), the specific relationships between T_{UVB} and T_{UVA} can vary with species, depending on the type of UV-absorbing compounds employed (e.g., flavonoids vs. HCAs; Bilger et al., 2001). Thus, while epidermal T_{UVA} measurements made with the UVA-PAM can serve as reasonable estimates of the overall UV screening of leaves (including T_{UVB}, which is technically much more difficult to measure in the field than T_{UVA}), we are unable to precisely relate levels of UV-A screening to that for UV-B in the species surveyed in this study. Also, the presence of anthocyanins in the epidermis can introduce errors in determining T_{UVA} with the UVA-PAM by affecting the penetration of the reference (F_{BL}) beam (Barnes et al., 2000; Pfündel et al., 2007). To avoid these errors, we restricted our sampling to include only plants with green leaves (i.e., leaves that had no visible reddish coloration which would be indicative of anthocyanin accumulation).

Previously, we reported that several of our study species (*V. thapsus* and *Oenothera stricta*) exhibited diurnal changes in epidermal T_{UVA} at this study location, with absolute values of T_{UVA} decreasing 1–3% from predawn to midday and then increasing to predawn levels at sunset (Barnes et al., 2008). Although diurnal changes in T_{UVA} are now known to occur in many species (Barnes et al., 2016a), it is unknown if all the species studied here undergo these diurnal changes. To allow for comparisons among species in the alpine survey we therefore measured T_{UVA} of all species under clear skies during midday (10:00 to 14:00 h local time). These values thus represent maximum levels of UV screening (minimum T_{UVA}) for all species regardless of whether or not they adjust T_{UVA} throughout the day. For the elevation study, we did not want diurnal changes in T_{UVA}, which could potentially vary in magnitude with temperature and sky conditions (Barnes et al., 2016a,b), to confound elevation/UV-B effects on UV screening. For this study, we therefore measured T_{UVA} ca. 1 h prior to sunrise (predawn). These values thus represent the "baseline" level of UV screening within each species.

RESULTS

Survey of Native and Non-native Alpine Species

Significant variation in daily minimum epidermal T_{UVA} existed among the plant species measured in the alpine/upper subalpine on Mauna Kea (**Figure 1**; ANOVA, $F_{18,79} = 14.8$; $P < 0.0001$). Mean values of T_{UVA} at midday ranged from a low of 2.6% in *Senecio madagascariensis* to a high of 11.5% in *Poa pratensis* (**Figure 1**). However, when averaged at the species level we detected no significant difference (ANOVA; $F_{1,17} = 0.05$; $P = 0.83$) between native and non-native taxa (**Figure 2A**). Similarly, we found no significant effect (ANOVA; $F_{2,15} = 0.82$; $P = 0.46$) of growth form on T_{UVA}, when averaged at the level of species (**Figure 2B**). However, data were variable and forbs represented a disproportionate fraction of the species tested ($n = 10$) as compared to grasses ($n = 3$) and woody plants (trees and shrubs; $n = 5$). The single fern species was excluded from the growth form analysis. We found no consistent patterns in T_{UVA} in growth forms between native and non-native species but replication was insufficient to test for statistical differences (**Figure 2C**). Even though measurements were taken over a range of elevations (2774–3962 masl), we found no significant relationship between T_{UVA} and elevation for the species sampled ($r = 0.20$; $P = 0.42$; not shown). The average sampling elevation of native species (3326 masl) was also not significantly different (ANOVA; $F_{1,17} = 0.02$; $P = 0.89$) than that for the non-native species (3291 masl).

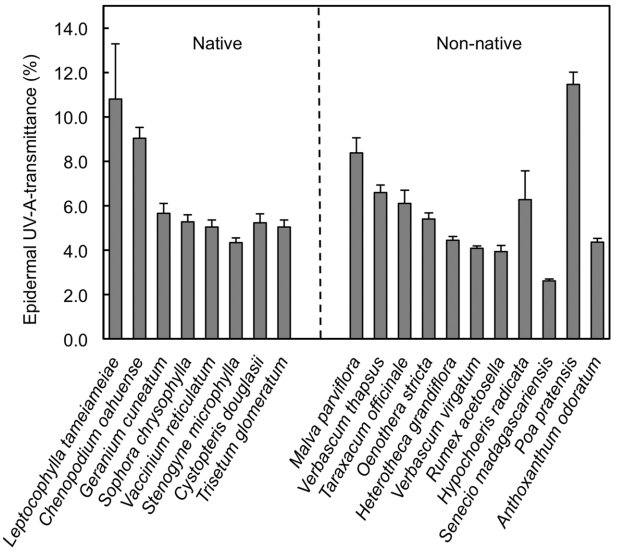

FIGURE 1 | Midday adaxial epidermal UV-A transmittance in native and non-native plant species in the alpine and upper subalpine zones of Mauna Kea, Hawaii. Data are means + SE ($n = 10$). The order of species reflects that in **Table 1** and is based, generally, on similarity in growth form.

Elevation Gradient Study

Along an elevation gradient spanning 2600–3800 m we found a strong negative relationship ($R^2 = 0.96$; $P < 0.001$ for linear regression model) between elevation and predawn adaxial T_{UVA} in the non-native *V. thapsus* but predawn T_{UVA} did not vary ($R^2 = 0.02$; $P = 0.87$ for linear regression model) with elevation in the native shrub *V. reticulatum* (**Figure 3A**). However, *V. reticulatum* maintained 2-4 times higher predawn levels of

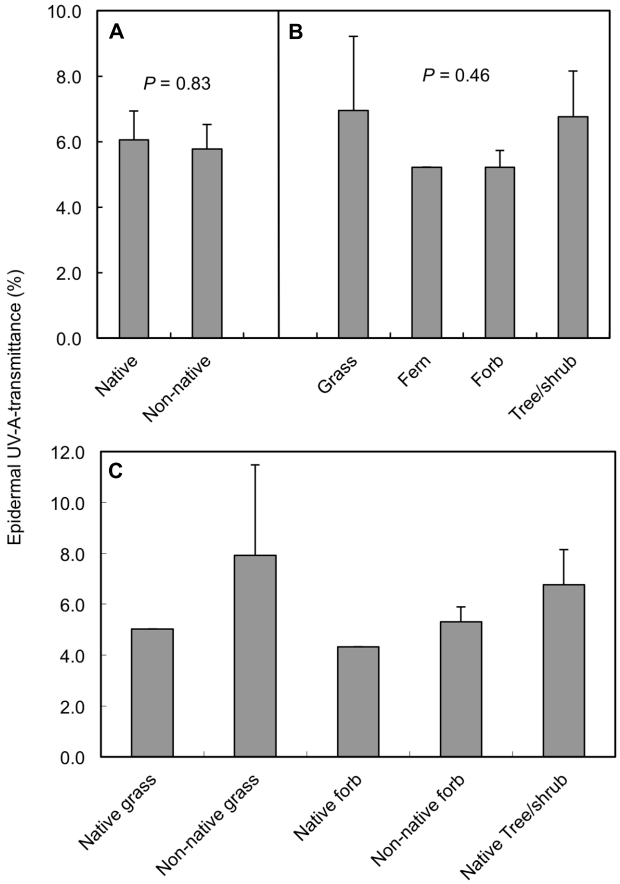

FIGURE 2 | Mean midday adaxial epidermal UV-A transmittance of plant species in the alpine and upper subalpine zones of Mauna Kea, Hawaii grouped according to native vs. non-native status **(A)**, growth form **(B)**, and growth forms within native and non-native categories **(C)**. Data are means + SE with $n = 8$ and 11 for native and non-native species in panel **(A)**; $n = 3$, 1, 10, and 5 for grass, fern, forb, and tree/shrub growth forms, respectively in panel **(B)**; and $n = 1$–5 in panel **(C)** (see **Table 1** for additional information). P values for the effect of native vs. non-native status **(A)** and growth form **(B)** are from individual one-way ANOVAs; the one fern species was not included in the growth form ANOVA.

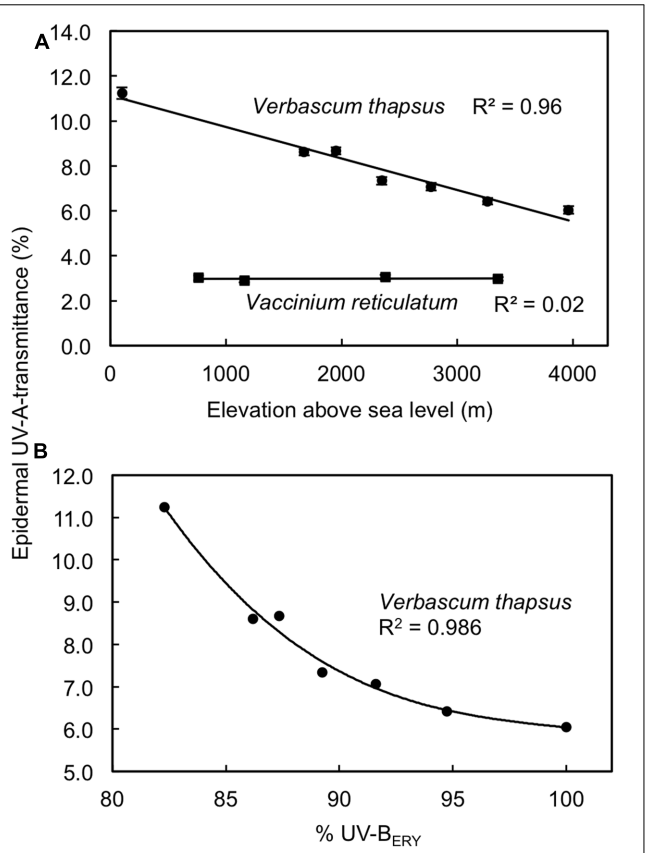

FIGURE 3 | Relationships between elevation and predawn adaxial epidermal UV-A transmittances in *Vaccinium reticulatum* (native species) and *Verbascum thapsus* (non-native species) **(A)**, and the relationship between midday summer clear sky erythemal UV-B irradiance (UV-B_{ERY}) and predawn adaxial epidermal UV-A transmittance in *V. thapsus* **(B)**. Data are means ± SE ($n = 10$). Linear regression models in panel **(A)** were significant for *V. thapsus* ($P < 0.001$; $y = 11.13$–$0.0014x$) but not for *V. reticulatum* ($P = 0.87$). The best-fit line relating epidermal UV-A transmittance to %UV-B_{ERY} for *V. thapsus* in panel **(B)** is a third-order polynomial model [$y = 32.2 - 0.28x + 0.022(x - 90.2)^2 - 0.0007(x - 90.2)^3$], which is significant at $P = 0.003$. UV-B_{ERY} data are expressed relative to the value at the highest sampling elevation on Mauna Kea as per data from Nullet and Juvik (1997).

UV screening (mean T_{UVA} = 2.8-3.1%) than *V. thapsus* (mean T_{UVA} = 6.0-11.2%) at similar elevations, based on estimates from regression models. For *V. thapsus*, predawn T_{UVA} exhibited a non-linear, negative relationship ($R^2 = 0.986$; $P = 0.003$ for third-order polynomial regression model) with relative peak daily clear sky UV-B_{ERY} along this elevation gradient (**Figure 3B**). Similar relationships were found with daily maximum clear sky total SW irradiance ($R^2 = 0.982$; $P < 0.001$ for a linear model; not shown) and PAR ($R^2 = 0.948$; $P = 0.003$ for a second-order polynomial model; not shown) though the relative irradiance changes in these two wavebands over this sampling gradient were less (5% for PAR and 12% for SW) than that for UV-B_{ERY} (ca. 20%). There were no significant relationships between predawn T_{UVA} and these relative measures of solar radiation (UV-B_{ERY}, PAR and SW) for *V. reticulatum* ($R^2 < 0.02$; $P > 0.8$; not shown).

DISCUSSION

Results from our survey of 19 species representing 13 different plant families indicate that significant interspecific variation exists in maximum (midday) levels of UV screening for plants growing in the Mauna Kea alpine/upper subalpine. Overall, however, epidermal T_{UVA} was low for all taxa (means ranged from 2.6 to 11.5%). Further, we found no significant differences in UV screening between native and non-native species. Most of the native species in our study were woody dicots (five of the eight species sampled) whereas the majority of the non-native species were herbaceous dicots (9 of 11 species sampled). Therefore, our comparison of native vs. non-native species was, to some degree, confounded with growth form effects. Day et al. (1992) reported significantly greater leaf epidermal UV transmittance in herbaceous dicots ($n = 7$ species) than woody

dicots ($n = 3$ species) for plants growing in a temperate subalpine meadow in Wyoming, United States (3310 m elevation). By comparison, we detected no significant effect of growth form on leaf optical properties in this tropical alpine environment. Our study was similar in size and scope to the study of Day et al. (1992) in that the majority (74%) of the species we tested were also herbaceous and most of these (71%) were forbs. Thus, even though differences in growth form composition existed between native and non-native species in our study, these differences appeared to have little effect on overall levels of UV screening between these two categories of species. It seems that growth form effects on T_{UVA} are less pronounced in the extreme UV-B conditions in the tropical alpine than in lower UV-B environments, which occur at lower elevations and higher latitudes.

Eldredge and Evenhuis (2003) reported that of the 2311 species of vascular plants known to occur in the Hawaiian Islands ca. 50% (1160) of these species are non-native (non-indigenous) in origin. Only a small fraction (73 species or ca. 3%) of these species occurs in the alpine/upper-subalpine of Mauna Kea though the relative floristic composition of non-native species in this life zone (54%) is comparable to that of the Hawaiian Islands in general (Gerrish, 2013). In our study, we examined about one-third of the native and non-native species of the Mauna Kea alpine/upper subalpine (8 native and 11 non-native species). Our results are therefore derived from sizable and comparable fractions of the native and non-native flora present in this habitat and there is no reason to believe that our findings would have differed had we sampled a greater number of species in the Mauna Kea alpine.

While it is seldom possible to determine the exact origin of non-native species, the non-native species included in our study clearly originated from lower elevation, higher latitude sites where UV-B exposure would be considerably less than our sampling locations. The majority of species (8 of 11) are listed in floras as originating from Europe or Eurasia. More detailed distributions of several species indicate they typically originate north of 30° latitude (Weber, 2017). The remaining species also originate outside the low-latitude tropics: *Heterotheca grandiflora* from coastal California (Munz, 1968), *O. stricta* from southern South America (Robberecht and Caldwell, 1983), and *S. madagascariensis* from South Africa (as determined by phylogenetic analysis; Le Roux et al., 2006). Whereas, it is possible that these species were pre-adapted to the extreme UV-B conditions in the tropical alpine, it seems more likely that they either (1) evolved higher UV screening over the course of their invasion and colonization of montane environments in Hawaii, or (2) that they possess high degrees of phenotypic plasticity in UV tolerance that then enabled them to acclimate to a wide range of UV-B conditions. Some of the non-native species in our study have arrived in Hawaii relatively recently and are highly invasive pests (e.g., *S. madagascariensis*) whereas others have been on the island sufficiently long enough to be considered "naturalized" (e.g., *P. pratensis* and *Rumex acetosella*) (Wagner et al., 1999; USDA, 2017). Thus, the non-native species in our study have experienced various periods of time since their introductions and these historical differences may have influenced the degree to which their adjustments in UV protection reflect genetic changes in populations vs. phenotypic plasticity.

In general, the success of non-native, invasive species is often attributed to their high levels of phenotypic plasticity, which then enables them to cope with a wide array of environmental conditions (Davidson et al., 2011). Many of the non-native species in our study, regardless of the timing of their introductions, may have achieved levels of UV protection that are similar to those of the native alpine species via phenotypic adjustment (i.e., acclimation) to the high UV-B irradiances in this alpine environment. In the case of *V. thapsus*, this is an herbaceous weed at many elevations in the temperate zone and this species has been found to exhibit a high degree of phenotypic plasticity rather than rapid evolution over the course of its invasions (Parker et al., 2003). Findings from the current study revealed that predawn T_{UVA} varied in a linear fashion (1.3% change in relative T_{UVA} per 100 m) with elevation in *V. thapsus* whereas this was not the case for the native *V. reticulatum* (**Figure 3A**). Using a similar approach, but conducting measurements at midday over a narrower elevation range (ca. 800 m), Ruhland et al. (2013) reported linear decreases in T_{UVA} with increasing elevation (9.7% per 100 m) for the native shrub *Artemisia tridentata* in Wyoming, United States. Because of the short distances between their sampling sites, these authors attributed the elevation variation in UV screening in *A. tridentata* to phenotypic plasticity rather than ecotypic differentiation. In a growth chamber study, Beckmann et al. (2012) found similar levels of phenotypic plasticity in native (German) and non-native (New Zealand) populations of *Hieracium pilosella* in morphological and growth responses to UV-B, though some genetic differentiation also occurred between these two populations. Thus, at present it is not clear whether non-native species (or populations) exhibit greater phenotypic plasticity in UV protection than native plants and further study on a greater number and diversity of species is needed to adequately test this hypothesis. It is conceivable, however, that phenotypic plasticity in UV protection in *V. thapsus* is one factor that has aided the invasion of this particular species in Hawaii.

In this study, we focused on the attenuation of incoming UV by the epidermis (i.e., UV screening) as this mechanism provides the first line of defense against the potentially deleterious effects of UV-B. However, UV protection in plants involves not only UV screening but other factors as well, such as levels of antioxidant compounds, DNA repair and leaf thickness, that all serve to protect and repair sensitive targets from direct and indirect UV-induced injury (Britt, 1999; Jacques et al., 2009; Majer et al., 2014; Robson et al., 2015). In some cases, these other mechanisms of UV protection have been shown to vary with elevation. For example, Wang Q. W. et al. (2016) found that differential sensitivity to UV radiation between high vs. low elevation populations and species of *Arabidopsis* growing in the Hakkado Mountains, Japan, was attributable, in part, to population differences in DNA damage and repair. Wildi and Lütz (1996) reported that total antioxidant levels increased with elevation in several species in the Austrian Alps, but whether these differences were due to elevation changes in UV, temperature, or other factors was not assessed. Moreover,

while the attenuation of UV within the leaf is predominantly influenced by UV-absorbing compounds, the surface features of leaves (e.g., trichomes and waxes) can also influence leaf optical properties (Karabourniotis et al., 1992; Holmes and Keiller, 2002) and in some cases these traits have been shown to vary with elevation (e.g., Pilon et al., 1999). One of the species in our study (*V. thapsus*) possesses pubescent leaves and it is conceivable that the trichomes of this species are also involved in UV screening. We did not test whether these hairs absorb or reflect UV nor did we evaluate whether there were elevation changes in the levels of pubescence in this species. Increases in pubescence would add to the UV-filtering effect of the epidermis and thus further decrease T_{UVA} but only if the leaf hairs possessed UV-absorbing compounds. Pubescence would likely have no effect on T_{UVA}, as measured with the UVA-PAM, if the hairs primarily reflect UV, as they would also reflect visible (including blue light) radiation (Holmes and Keiller, 2002). In this situation, the ratio of F_{UVA}/F_{BL} and thus T_{UVA} would be unaffected by variation in trichome density.

Because of their known function in UV protection and potential value in plant systematics, a large number of studies have examined elevation changes in the levels of flavonoids (and related phenolic compounds) and/or UV screening in a variety of plant species (e.g., Caldwell et al., 1982; McDougal and Parks, 1984; Barnes et al., 1987; Rau and Hofmann, 1996; Alonso-Amelot et al., 2007; González et al., 2007; Rieger et al., 2008; Bernal et al., 2013; Ruhland et al., 2013; Cirak et al., 2017; and others). Some of these studies have further tested the linkage between elevation variation in flavonoids/UV screening and UV tolerance. In one of the most extensive studies to date, Sullivan et al. (1992) examined 33 species collected along a 3000 m elevation gradient in Maui, Hawaii, and found a significant, inverse relationship between elevation and negative effects of UV-B on growth for plants growing in a greenhouse. A companion study by Ziska et al. (1992) showed that greater sensitivity to UV-induced partial inhibition of photosynthesis in a subset of greenhouse-grown low elevation species ($n = 4$) was associated with lower constitutive levels of UV-absorbing compounds relative to high elevation taxa. Thus, even though our study only examined UV screening, our findings imply that non-native species would not be more or less prone to UV-induced injury than native species under the extreme UV-B conditions in this tropical alpine environment. Furthermore, our findings that non-native species possess levels of UV screening that are comparable to those of native species do not support the general expectation that non-native species invest more heavily into resource acquisition and growth at the expense of stress tolerance mechanisms than native species (Van Kleunen et al., 2010). Rather, it appears that the low resource, highly stressful environment of the alpine serves as a strong filter of plant species (Alexander et al., 2011; Gerrish, 2013) and functional traits (Funk et al., 2016) such that native and non-native species in this environment exhibit little difference in UV defense.

Elevation gradients, such as the one in our study, are complex gradients where a number of environmental factors (e.g., solar radiation, temperature, precipitation) change in concert (Körner, 2007). Studies such as ours linking elevation changes in UV screening to changes in UV-B are therefore, correlative at best, and other environmental factors may contribute to this variation. Indeed, it is well known that low temperatures can increase UV-absorbing compounds and UV screening in leaves (e.g., Bilger et al., 2007) and some have found elevation changes in flavonoids to be more strongly influenced by changes in temperature than UV (Albert et al., 2009). Nonetheless, studies along elevation gradients can provide insights into how plants might respond to the changes in UV-B that occurs with migration to higher elevations as a consequence of climate change. In our study, we found a strong negative relationship between clear sky erythemal UV-B and T_{UVA} in *V. thapsus*, but we also detected strong relationships with PAR and total SW radiation. In general, radiant fluxes of biologically effective UV-B increase proportionally more with elevation than those of UV-A, PAR or SW in temperate and tropical mountains (Caldwell et al., 1980; Piazena, 1996; Blumthaler et al., 1997; McKenzie et al., 2001). Such appears to be the case for this elevation gradient in Hawaii (Nullet and Juvik, 1997). Thus, while migration to higher elevations exposes plants to increases in solar radiation in all wavebands, the relative changes are greatest for biologically effective UV-B. These elevation gradients in UV-B can be further accentuated by the presence of clouds. For example, because of a persistent, dense cloud layer at ca. 2000 masl that results from trade-wind inversions, the differences in UV-B between the alpine and sea level differ considerably from the eastern, wind-ward side of Mauna Kea to the western, lee-ward side of the mountain. From continuous UV measurements, Nullet and Juvik (1997) reported that monthly erythemal UV-B, when averaged over all sky conditions, was actually 55–103% greater at 3400 masl than at a windward sea level location, depending on time of year (summer vs. winter), in comparison to the ca. 20% difference in clear sky UV-B between these elevations. Plant species that occur below the cloud layer on the moist, eastern side of Mauna Kea, and which migrate to elevations above this cloud layer would therefore likely require greater acclimation to UV-B than would those migrating comparable elevations on the drier, eastern side of this mountain.

CONCLUSION

Our findings indicate that high levels of UV screening are not restricted to plant species native to the high UV-B conditions of the tropical alpine and that plasticity in epidermal UV transmittance is a mechanism employed by some, but not all, species to cope with varying solar UV exposures. Whether this plasticity in UV screening is a general feature of non-native species is unknown, but our findings do suggest that many terrestrial plants will be able to tolerate the increased levels of UV-B radiation as they migrate to higher elevations as a consequence of climate change.

AUTHOR CONTRIBUTIONS

PB and RR collected and analyzed the data. PB wrote the manuscript with the participation of SF. PB, RR, and SF

designed the studies and all were involved in securing funding for the research. RR died before the final draft of the manuscript was completed but he assisted with the preparation of early drafts.

FUNDING

This research was supported by the US Department of Agriculture UV Monitoring Program (USDA-CSREES No. 2004-34263-14270 to Utah State University via subcontract with Colorado State University), the Utah Agricultural Experiment Station, the Loyola University J.H. Mullahy Endowment in Environmental Biology and a Loyola University Faculty Research Grant.

ACKNOWLEDGMENTS

We appreciate the assistance of M. Abbene, V. Del Bianco, A. Dickhute, S. Kelly, and L. Ducas. Logistical support in Hawaii was provided by J. Juvik, S. Juvik, R. Koehler, D. Byrne, and Mauna Kea Support Services.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

MINI REVIEW
published: 25 January 2017
doi: 10.3389/fpls.2017.00081

Potential Responses of Vascular Plants from the Pristine "Lost World" of the Neotropical Guayana Highlands to Global Warming: Review and New Perspectives

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The neotropical Guayana Highlands (GH) are one of the few remaining pristine environments on Earth, and they host amazing biodiversity with a high degree endemism, especially among vascular plants. Despite the lack of direct human disturbance, GH plants and their communities are threatened with extinction from habitat loss due to global warming (GW). Geographic information systems simulations involving the entire known vascular GH flora (>2430 species) predict potential GW-driven extinctions on the order of 80% by the end of this century, including nearly half of the endemic species. These estimates and the assessment of an environmental impact value for each species led to the hierarchization of plants by their risk of habitat loss and the definition of priority conservation categories. However, the predictions assume that all species will respond to GW by migrating upward and at equal rates, which is unlikely, so current estimates should be considered preliminary and incomplete (although they represent the best that can be done with the existing information). Other potential environmental forcings (i.e., precipitation shifts, an increase in the atmospheric CO_2 concentration) and idiosyncratic plant responses (i.e., resistance, phenotypic acclimation, rapid evolution) should also be considered, so detailed eco-physiological studies of the more threatened species are urgently needed. The main obstacles to developing such studies are the remoteness and inaccessibility of the GH and, especially, the difficulty in obtaining official permits for fieldwork.

Keywords: global warming, plant responses, acclimation, adaption, migration, habitat loss, extinction

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Tobias Gebauer, University of Freiburg, Germany
Zhenzhu Xu, Chinese Academy of Sciences (CAS), China

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *08 November 2016*
Accepted: *13 January 2017*
Published: *25 January 2017*

Citation:
Rull V and Vegas-Vilarrúbia T (2017) Potential Responses of Vascular Plants from the Pristine "Lost World" of the Neotropical Guayana Highlands to Global Warming: Review and New Perspectives. Front. Plant Sci. 8:81. doi: 10.3389/fpls.2017.00081

INTRODUCTION

While pristine biomes and ecosystems are not subject to direct human pressure, they can be indirectly affected by anthropogenic forcing as expressed through ongoing global change, especially global warming (GW). The potential influence of GW on ecosystem composition, structure and function in pristine environments has been considered to be a novel and somewhat "unexpected" element that should be accounted for in conservation programs (Rull and Vegas-Vilarrúbia, 2006). Ecological and evolutionary studies of pristine areas are not only useful for discovering and protecting potentially endangered endemic and often unique species but also for unraveling the origin of biodiversity and its patterns on Earth. Additionally, such research

increases the understanding of the long-term ecological functioning of ecosystems that are only subject to natural forcing. Untouched biomes are very rare, and the eventual consequences of GW could make them disappear. Therefore, it is urgently necessary to concentrate efforts on their study and conservation.

The neotropical Guayana Highlands (GH), the Lost World of Arthur Conan Doyle (1912), are so remote and inaccessible that they have remained virtually untouched until now. The GH have been considered a natural laboratory for addressing important and long-standing issues in neotropical ecology, evolution, and biogeography (Rull, 2010). The potential consequences of GW on GH vascular plants have been estimated given the 21st-century warming projected by the IPCC (Nogué et al., 2009; Safont et al., 2012; Vegas-Vilarrúbia et al., 2012). Emphasis has been placed on upward migration and eventual extinction by habitat loss, but other aspects such as the adaptation and acclimation potential of plants have not been fully explored. This paper reviews the results of the research that has been performed to date and provides perspectives on future research into the less developed aspects, acclimation and adaptation, and the potential genetic and ecological mechanisms that are involved. Studies of the potential consequences of GW for untouched biomes and ecosystems are uncommon, and the GH could serve as a pilot survey.

THE GUAYANA HIGHLANDS

The GH are composed of the flat summits of the ∼60 sandstone table mountains (locally called tepuis) in the neotropical region of Guayana (**Figure 1**), whose unique biotic features define the Pantepui biogeographic province (Huber, 1994; Berry et al., 1995). The total extent of these summits is ca. 6000 km^2, and they range from 1500 to 3014 m in elevation (Huber, 1995a). This study is centered on Venezuela, the country to which most tepuis belong. The GH emerge from the surrounding lowlands as an archipelago of islands suspended in the air and are characterized by outstanding plant diversity and levels of endemism similar to those of oceanic islands (Berry and Riina, 2005; Rull, 2009b). A database built with the currently available floristic information revealed the occurrence of 2433 species (>4000 species per 10,000 km^2), of which 618 (ca. 25%) are endemic to Pantepui (Safont et al., 2012). Considering that many tepui summits remain largely unexplored (Huber, 1995b), these numbers are extraordinary in a global context (Barthlott et al., 1999). These mountain islands are remote and mostly inaccessible; only a few summits can be reached by foot. The indigenous people inhabiting the region call these table mountains "tepuis" and consider their summits to be the home of gods, from which humans are forbidden. There are no economically profitable resources on the tepui summits, and activities, such as agriculture, livestock husbandry, forestry or mining are prevented by the particular geologic, edaphic, and biotic conditions. The only activities that have been developed in the GH are tourism and scientific exploration, and access is mainly by helicopter (Huber, 1995b). There is no tourist infrastructure on the GH (visitors should camp on the summits), so their pristinity is maintained.

The GH are protected by several designations including national parks, a Biosphere Reserve and a World Heritage Site, and the tepuis themselves were declared a natural monument in 1990 (Huber, 1995c). However, the existing framework is not sufficient to guarantee their conservation. For example, the latest environmental evaluation of the Canaima National Park, the most emblematic protected area in the region, concluded that while the conservation status of the tepuian summits is good, the level of threat is high, especially to biodiversity (Novo and Díaz, 2007). The report also identified several obstacles to a suitable conservation policy, namely, the insufficient level of funding; the scarcity, high turnover and incomplete training of the personnel responsible daily field surveillance; the enormous size of the park and the remoteness of many protected areas; the diversity of actors and stakeholders, including governmental, private and indigenous interests, and the absence of a mechanism of exchange among them; and the lack of a definite management plan for the park, although some proposals exist (Bevilacqua et al., 2009). The Guiana Shield Facility (GSF), an international conservation organization that works in the Guayana/Guiana Shield countries, considers the tepuis to be priority areas for protection (Huber and Foster, 2003; Bernard et al., 2011), but specific actions have not yet been attempted. Furthermore, many tourist activities are illegal or out of control, and the first signs of human disturbance are beginning to appear. For example, in the Roraima (**Figure 1**), the most visited tepui, vegetation trampling and other direct impacts are not uncommon, and recent studies have documented the introduction of invasive plants and the contamination of water by fecal bacteria (Safont et al., 2014; Fernández-Delgado et al., 2016). Urgent actions are needed to control this particular problem (Rull et al., 2016), which, fortunately, is an exceptional situation, and most of the tepuian summits remain virtually untouched.

RANGE SHIFTS AND HABITAT LOSS

Despite their general pristinity and the paucity of direct human impact, the GH vascular flora is considered to be severely threatened by climate change (Rull and Vegas-Vilarrúbia, 2006). GW is considered a major threat to mountain flora worldwide as plants may respond by shifting their altitudinal distributions, leading to changes in the diversity and composition of their communities as well as the reduction, fragmentation or loss of their habitat. An increasing number of studies show that this is already occurring and warn of the potential threat of GW to summit species and communities (e.g., Thuiller et al., 2005; Pauli et al., 2007, 2012; Colwell et al., 2008; Kelly and Goulden, 2008; Lenoir et al., 2008; Grabherr et al., 2010; Kreyling et al., 2010; Walther, 2010; Dirnböck et al., 2011; Engler et al., 2011; Feeley et al., 2011; Sheldon et al., 2011; Gottfried et al., 2012; Jump et al., 2012). Tropical mountains are of particular concern because of their high biodiversity and levels of endemism (Davis et al., 1997; Myers et al., 2000; Malcom et al., 2006; Laurence et al., 2011; Nogué et al., 2013). Indeed, the extinction of an endemic species is a global extinction.

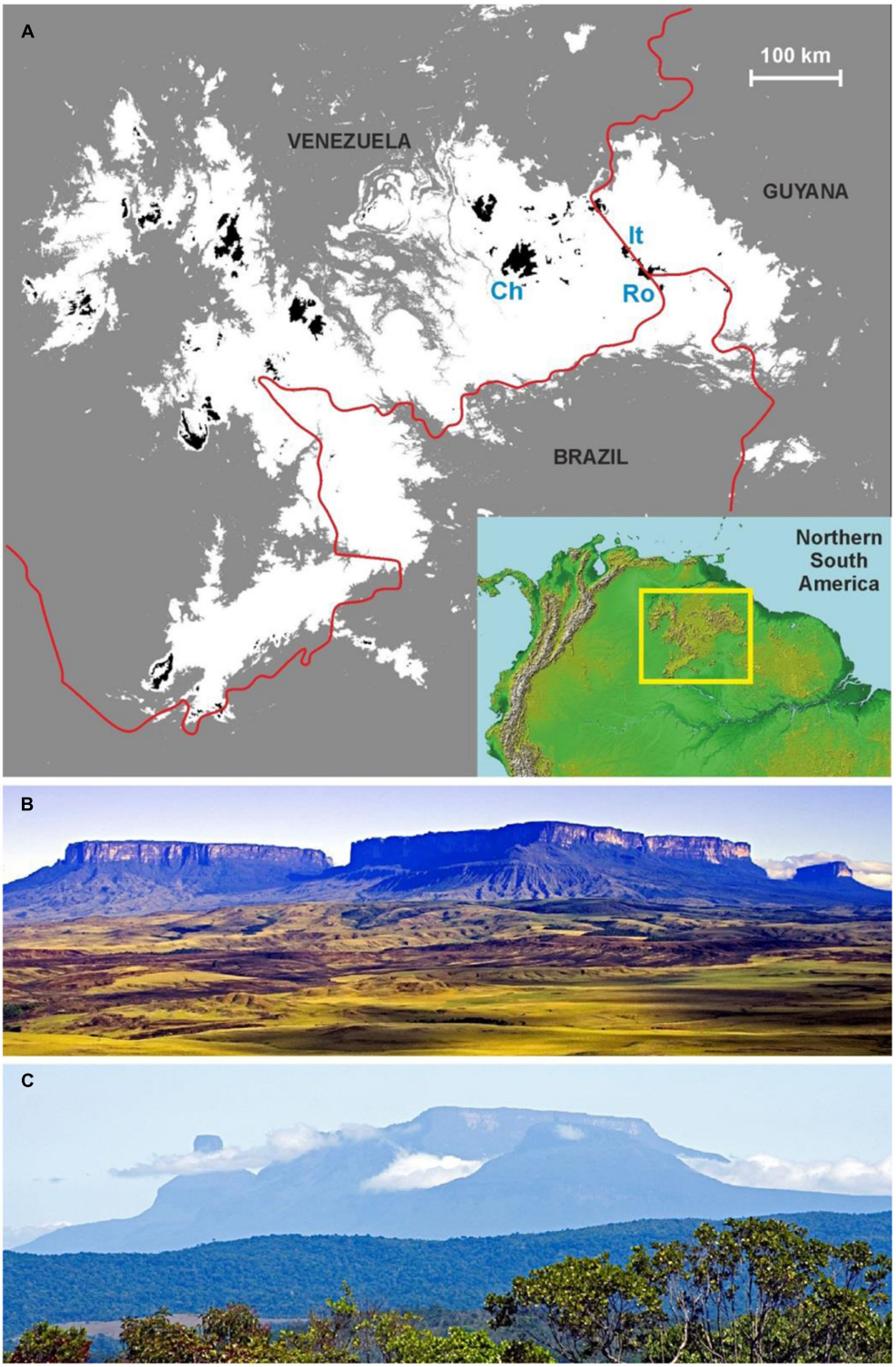

FIGURE 1 | Map of the study location and example of tepuian massifs. (A) Map with the studied area in the geographical context of northern South America. The Amazon and Orinoco lowlands are in gray; the Guayana Highlands (GH) region in white with the GH proper (i.e., situated above 1500 m in elevation) in black. Ch – Chimantá massif, It – Ilú-Tramén massif, Ro – Roraima tepui. Red lines are the international borders. **(B)** The Roraima (right) and the Kukenán (left) tepuis. The summit of the Roraima has a surface area of 34.38 km^2 and a maximum elevation of 2723 m, whereas the Kukenán summit has a surface area of 20.63 km^2 and a maximum elevation of 2650 m (Huber, 1995a). **(C)** The Ilú-Tramén massif, whose summit has a surface area of 5.63 km^2 and a maximum elevation of 2700 m (Huber, 1995a) (Photos: V. Rull).

In the GH, the risk of extinction by habitat loss due to the projected GW in this century has been addressed using the altitudinal range shift (ARS) method (Rull and Vegas-Vilarrúbia, 2006) and climate envelope distribution models (CEDM) (Rödder et al., 2010). Both methods use geographic information systems (GIS) techniques and have been applied to estimate the expected habitat reduction for Pantepui species assuming that these species will respond to GW by upward migration at rates similar to that of the warming. Using the ARS method, the present potential distribution area of the Pantepui habitat was first plotted in a digital elevation model (DEM), and its reduction was simulated for a given increase in temperature and an average adiabatic lapse rate of −0.6°C per 100 m of elevation, which is characteristic of the Guayana region (Huber, 1995a). The reduction in the number of species was then calculated using the previously developed species-area relationship for the GH based on empirical data (Nogué et al., 2009). Simulations were run for the more optimistic (B1) and the more pessimistic (A2) scenarios in the third IPCC assessment report (Houghton et al., 2001), which predicted warmings of 2.0 and 4.0°C, respectively, for northern South America by the end of this century. Under these conditions, the ARS method yielded estimates of 1700 (B1)–1824 (A2) species (75–79% of the total) at risk of habitat loss, of which 209 (B1)–406 (A2) (28–54%) were endemics. An individual species-by-species analysis based on their specific estimated reductions in habitat confirmed that 166–343 endemic species (22–46%) were threatened by habitat loss (Nogué et al., 2009). All of these species are listed in Nogué et al. (2009). In addition, virtually all of the analyzed species showed a fragmented potential distribution by the end of the century regardless of the IPCC scenario (Nogué et al., 2009). More sophisticated CEDM produced similar results that supported previous ARS estimates (Rödder et al., 2010), and the CEDM-generated maps of the potential habitat by the end of this century were nearly identical to those obtained by ARS. However, CEDM maps were not used to estimate the number of species at risk of habitat loss.

The ARS was re-run under the new predictions of the fourth IPCC assessment report (Solomon et al., 2007), but the estimated number of endangered endemic species did not change significantly (Safont et al., 2012). However, this report considered a three-step warming: (I) 1.0°C (for both B1 and A2) by 2011–2030, (II) 2.0°C (B1) to 2.5°C (A2) by 20146–2065, and (III) 2.5 (B1) to 4.0°C (A2) by 2080–2099, which facilitated a preliminary hierarchization of the endangered species. The ARS analysis was individually run for the three warming phases, and the 35 endemic species (6%) losing their potential habitat at stage I were considered the most threatened, followed by the 140 (B1)–184 (A2) (23–30%) predicted to lose their habitat at stage II and, finally, the 184 (B1)–307 (A2) (30–50%) that will be at risk of habitat loss by the end of the century (stage III). An exhaustive list of these species is provided by Safont et al. (2012).

CONSERVATION STRATEGIES

The above species-level risk analysis was complemented by defining conservation priority categories using an index called the environmental impact value (EIV) for each species, which considered the taxonomic level of endemism (i.e., family, genus, species), the degree of endemism (i.e., local tepui endemism, general Pantepui endemism, tepuian district endemism), the characteristic of keystone species (when a given species is fundamental to the existence of its community), and the geographical and altitudinal ranges of the species (Safont et al., 2012). Prioritizing species according to extinction threat may be a more cost-effective way to invest conservation resources than comprehensive plans intended to preserve all species at once or only the most charismatic (Pärtel et al., 2005; Jiménez-Alfaro et al., 2010). The EIV defined 10 (B1)–13 (A1) priority conservation categories; species deserving immediate conservation actions were those estimated to lose their habitat during stage I with the highest EIV scores, which corresponded to priority categories 1–3 for both the B1 and A2 scenarios. A complete list of all priority categories is provided in Safont et al. (2012).

In general, the best strategy for long-term biodiversity conservation is considered to be *in situ* conservation, notably the enhancement of the degree of protection or designation of new protected areas (Frankel et al., 1995; Primack, 2002). However, the danger of habitat loss due to GW in the absence of direct human impacts, as is the case in the GH, cannot be addressed with *in situ* practices alone, so *ex situ* measures seem to be required to preserve the biodiversity and ecosystems of the Pantepui. It has been estimated that, by the end of this century, the Pantepui area will be reduced by >80% and that the suitable habitat for Pantepui species will disappear from more than 35 (58%) tepuis. The largest remaining patch will be located in the Chimantá massif (**Figure 2**), which will represent nearly the half of the total remaining Pantepui area (Vegas-Vilarrúbia et al., 2012). This patch can be considered a potential refuge for future GH flora, which would contain resistant species from lower altitudes and other species that will eventually persist in microrefugia (Rull, 2009a). Maps of the potential Pantepui area remaining by 2100, in which eventual *in situ* actions should be concentrated, are available in Vegas-Vilarrúbia et al. (2012).

Among the available *ex situ* techniques, germplasm banks, living plant collections and managed relocation have been discussed (Rull and Vegas-Vilarrúbia, 2006; Nogué et al., 2009; Safont et al., 2012). Germplasm preservation seems to be especially feasible as it will allow for the restoration of species when needed, provided the suitable environmental conditions are present. Classical seed collection and banking as well as the creation of botanical gardens have already been suggested as suitable methods for preserving endangered species for restoration purposes. However, in these procedures, special care should be taken to preserve genetic diversity as much as possible (Vitt et al., 2010; Hardwick et al., 2011). Managed relocation is a more controversial approach due to the likelihood of unexpected ecological consequences in recipient ecosystems (Hunter, 2007; McLachlan et al., 2007; Davidson and Simkanin, 2008; Hoegh-Guldberg et al., 2008; Ricciardi and Simberloff, 2009; Seddon et al., 2009), so to minimize potential ecological impacts, relocated species may be restricted to selected areas along an elevational transect according to their individual requirements

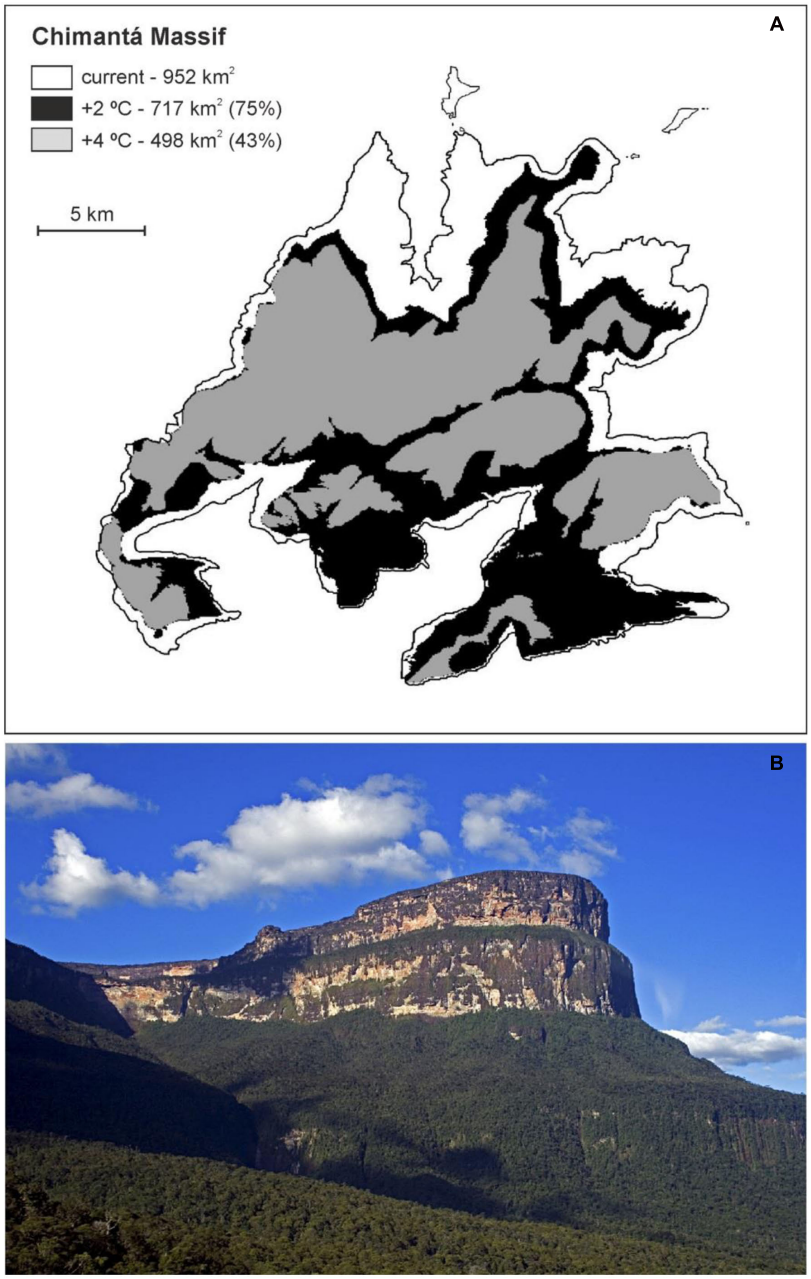

FIGURE 2 | Pantepui habitat reduction (in %) on the Chimantá massif. (A) Geographic information systems (GIS)-generated map of the summit contour of the Chimantá massif (>1500 m in elevation), indicating the Pantepui habitat area for warmings of 2°C (black area) and 4°C (gray area). **(B)** NE cliffs of the Chimantá massif (Photo: V. Rull).

and isolated from autochthonous communities, i.e., in artificial microrefugia. The northern Andes have been proposed as a possible recipient area as species would be allowed to migrate upwards due to the occurrence of suitable terrain above 3000 m in elevation (Rull et al., 2009). Potential relocations must be informed by carefully planned ecological studies to ensure that the creation of novel ecosystems for stressed populations do not damage the recipient ecosystem (Safont et al., 2012).

SOME PROBLEMS AND NEW PERSPECTIVES

There are several issues with the habitat loss estimations that have been performed in the GH to date. First, environmental factors other than temperature could act as drivers of species range shifts. Second, the migration capacity of species could be limited by the lack of suitable substrates or topographical

barriers. Third, the estimation methods that have been used assume that all species will respond to warming by migrating upwards at the same rates. Therefore, the available potential habitat loss estimates should be considered preliminary and as a first step toward more realistic projections that consider all relevant environmental factors as well as the ecological features of the species of concern that could affect their individual responses to climate change.

In terms of climatic factors other than temperature, precipitation intensity and distribution is predicted to undergo significant shifts worldwide during this century, but such changes seem to be of relatively little relevance to the studied region. On the one hand, the 0–5% reduction in mean annual rainfall forecasted by the IPCC by the end of this century (Solomon et al., 2007; Stocker et al., 2013) is almost negligible considering the perhumid climate of the tepui summits, where precipitation ranges from 2500 to 4000 mm per year with little seasonal variation (Huber, 1995a). On the other hand, temperature has been recognized as the main factor controlling the altitudinal distribution of vascular plant species in Pantepui (Huber, 1995a) as in many other areas (e.g., Guisan and Theurillat, 2000; Thuiller et al., 2005). The projected increase in the concentration of atmospheric CO_2 is another factor that should be considered, and evaluating its effects requires detailed eco-physiological studies of selected species, such as those in the priority categories mentioned above. Unfortunately, such studies are not available and are difficult to perform due to logistic and bureaucratic constraints (see below). The influence of local topographical and edaphic factors (e.g., slope orientation, substrate availability, microclimatic conditions, and vegetation structure) might be relevant to the control of altitudinal plant migration and could be understood with intensive fieldwork and the use of GIS tools. Again, acquiring detailed knowledge of these aspects has been hindered by the difficulty of developing ecological studies atop the tepuis, but the need for these types of studies is indisputable and methods that are applicable globally, such as those developed by the GLORIA (Global Observatory Research Initiative in Alpine environments) project (Pauli et al., 2005) have been encouraged (Rull et al., 2009).

Increasing the autoecological knowledge of the potential responses of threatened plant species is imperative. Current predictive models for the GH assume homogeneous responses, but this should be considered approximative as different species may respond to warming in different ways. Species with higher phenotypic plasticity might be able to tolerate warming by accommodating their morphological, physiological or life history traits to changing climates. Examples include changing photosynthesis rates and growth in response to increasing atmospheric CO_2 concentrations or modifying phenological features, such as growth and flowering times and duration, in response to changing meteorological seasonality (Ainsworth and Rogers, 2007; Gunderson et al., 2010; Medeiros and Ward, 2013; Liancourt et al., 2015). The combined effects of warming, water stress and elevated CO_2 should also be taken into account (e.g., Xu and Zhou, 2006; Xu et al., 2014, 2016). At the ecosystem level, such changes could influence the resilience of species to climate change and, consequently, their competitive ability, symbiotic interactions and fitness, which may affect community composition and ecological functioning (Kimball et al., 2012). According to Becklin et al. (2016), phenotypic plasticity may alleviate the effects of climate change but may not guarantee long-term persistence, especially if environmental change exceeds the variability that species have experienced historically. The species that are unable to accommodate to GW in this way, should adapt, in an evolutionary sense, to the new environmental conditions or migrate; otherwise, they will become extinct *in situ*. The potential occurrence of rapid evolutionary adaptation, i.e., genetic change, to GW is a controversial issue (Merilä, 2012) that remains difficult to demonstrate (Franks et al., 2007). A necessary condition for species to undergo rapid evolutionary change in response to climate change is sufficient genetic variation in the traits under selection pressure (Becklin et al., 2016), but such knowledge of the GH does not exist for the same reasons mentioned above and should be urgently addressed in light of the priority categories established for the more threatened species (Safont et al., 2012).

In addition to the remoteness and low accessibility of the GH, a recurrent obstacle to a sound assessment of the plant species and communities threatened with extinction by habitat loss is the difficulty obtaining fieldwork and sampling permits. This problem was first noted several years ago (Rull and Vegas-Vilarrúbia, 2008; Rull et al., 2008), but the situation has not changed. The lack of sufficient environmental management tools (Novo and Díaz, 2007) and the difficulty of establishing effective control mechanisms in a region as vast as the GH are serious challenges to adequate protection policies (Rull et al., 2016). Currently, visits to most of the tepuis are prohibited, and scientific surveys, especially those related to genetic studies, are subjected to serious restrictions to prevent biopiracy (Rull and Vegas-Vilarrúbia, 2008). These restrictions should be relaxed to allow a proper appraisal of the potential effect of GW on GH species and their ecosystems. Current estimates based on the available databases and methods, such as ARS and CDEM, are all that can currently be accomplished. Without new and more detailed ecological and physiological studies of selected GH plants and ecosystems, the background information required for optimizing conservation practices will remain unknown. In these conditions, the use of remote sensing techniques would be useful, especially those involving radiation measurements related to ecophysiological features such as energy balance or photosynthesis (Jones et al., 2003). Such measurements might be done using conventional satellite imagery or newly developed techniques as for example Unmanned Aerial Vehicles (UAV), also known as drones (Salamí et al., 2014).

AUTHOR CONTRIBUTIONS

VR and TV contributed equally to the ideas expressed here. VR wrote the manuscript and TV provided additional insights.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 October 2016
doi: 10.3389/fpls.2016.01533

Effects of Environment and Space on Species Turnover of Woody Plants across Multiple Forest Dynamic Plots in East Asia

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Species turnover is fundamental for understanding the mechanisms that influence large-scale species richness patterns. However, few studies have described and interpreted large-scale spatial variation in plant species turnover, and the causes of this variation remain elusive. In addition, the determinants of species turnover depend on the dispersal ability of growth forms. In this study, we explored the large-scale patterns of woody species turnover across the latitude gradient based on eight large stem-mapping plots (covering 184 ha forest) in East Asia. The patterns of woody species turnover increased significantly with increasing latitude differences in East Asia. For overall woody species, environment explained 36.30, 37.20, and 48.48% of the total variance in Jaccard's (β_j), Sorenson's, (β_s), and Simpson's dissimilarity (β_{sim}). Spatial factors explained 47.92, 48.39, and 41.38% of the total variance in β_j, β_s, and β_{sim}, respectively. The effects of pure spatial and spatially structured environments were stronger than pure environmental effects for overall woody species. Our results support the hypothesis that the effect of neutral processes on woody species turnover is more important than the effect of the environment. Neutral processes explained more variation for turnover of tree species, and environmental factors explained more variation for the turnover of shrub species on a large scale. Therefore, trees and shrubs should be subjected to different protection strategies in future biodiversity conservation efforts.

Keywords: climate, environmental, latitudinal gradient, neutral processes, growth forms

OPEN ACCESS

Edited by:
Alessio Fini,
University of Florence, Italy

Reviewed by:
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *05 July 2016*
Accepted: *29 September 2016*
Published: *13 October 2016*

Citation:
Chen Y, Yuan Z, Li P, Cao R, Jia H and Ye Y (2016) Effects of Environment and Space on Species Turnover of Woody Plants across Multiple Forest Dynamic Plots in East Asia. Front. Plant Sci. 7:1533. doi: 10.3389/fpls.2016.01533

INTRODUCTION

Species turnover pattern (or beta diversity) is a basic pattern in biogeography and macroecology (Gaston, 2000), and it provides fundamental insights into mechanisms of community assembly, especially on a large scale (Anderson et al., 2011). However, species turnover patterns have received less attention than alpha diversity (Koleff et al., 2003), and most studies on species turnover have been conducted locally (Kraft et al., 2011; Wang et al., 2012a). On a large scale, recent studies have demonstrated that species spatial turnover is a key factor for both plants and animals (Kreft and Jetz, 2007; Qian and Ricklefs, 2008). Stuart et al. (2012) have found that the environmental dissimilarity coefficient of lizards and frogs increased with changing geographical distance and habitat; Qian et al. (2009) have shown that the similarity coefficients of North American mammals and African amphibians decrease with increasing spatial distance. However, the studies on the

patterns of species turnover across latitudes are relatively few. Moreover, in the few studies about the patterns of species turnover across latitudes, a great majority is based on the space-filling species of range maps. The drawbacks of this method include coarse spatial resolutions; spatial heterogeneity among species composition has been smoothed out, and spatial autocorrelation has increased (Wang et al., 2012b).

The underlying mechanisms governing large-scale species turnover are ambiguous and poorly studied (De Cáceres et al., 2012) despite several and recent efforts to describe large-scale species turnover patterns of plant (De Cáceres et al., 2012; Tang et al., 2012a,b; Wang et al., 2012b; Xu et al., 2015). Several research studies have indicated that species coexistence is attributed to different environmental factors; species have different resources, time, and space to achieve coexistence (Jia et al., 2015; Escudero and Valladares, 2016). Alternatively, neutral processes state that species coexistence results from biogeographic barriers and low dispersal abilities (Hubbell, 2001; Jia et al., 2015). Moreover, results vary because of differences in the methods, including the spatial extents and the measures of species turnover. Therefore, the relative role of the environment and neutral processes in governing species turnover may vary (Liu et al., 2015; Valladares et al., 2015).

The dispersal ability of plant species is frequently predicted to influence species turnover, but only limited tests have been performed to confirm this; the results of these tests have been inconsistent (Qian, 2009; Elgar et al., 2014). For example, Bin et al. (2010) studied the Dinghushan plot and indicated that shrubs had weaker dispersal limitations than arbors and subarbors. Guèze et al. (2013) studied seven villages in the southwestern Amazon and indicated that the floristic patterns of large trees were explained more by environmental variables; those of small trees were explained more by geographical distances. Moreover, the large-scale dispersal ability among growth forms has been studied to a lesser degree, especially in terms of mechanism.

China, which possesses various tropical and temperate areas, provides ideal environments for investigating large-scale biodiversity patterns (Fang et al., 2012). Over the past decade, the Chinese Forest Biodiversity Monitoring Network[1] established large stem-mapping plots located along a latitudinal gradient from temperate to subtropical and tropical forests (De Cáceres et al., 2012; Erickson et al., 2014; Song et al., 2015). Furthermore, two large stem-mapping plots were established by the Forestry research institution in Taiwan (Song et al., 2015). Here, we exploit this opportunity and analyze the turnover of woody species across temperate, subtropical, and tropical forests based on eight large stem-mapping plots.

The objectives of the current study are as follows: (1) to obtain an integrated analysis of the similarity of species composition among eight large stem-mapping plots; (2) to identify the patterns of species turnover along the latitude difference using three measures of species turnover; and (3) to assess the relative influence of environmental and neutral processes on species turnover of overall woody species and growth forms.

[1]http://www.cfbiodiv.org/

MATERIALS AND METHODS

Study Sites

In this study, eight large stem-mapping plots were selected, as follows: the Lianhuachi, Fushan, Xishuangbanna, Dinghushan, Badagongshan, Gutianshan, Tiantongshan, and Changbaishan plots. Distributions of these eight plots are shown in **Figure 1**. The latitude of the plots cross ranges from 21.61 to 42.38°, and the average elevation ranges from 350.0 to 1405.5 m. The community type includes tropical rain forests, subtropical evergreen broad-leaved forests, and temperate deciduous broad-leaved forests. Census methodologies were the same for all plots: all trees with a diameter at breast height $\geq$1 cm were tagged and identified.

Species and Environment Variables

In the study, we used data on the species catalog, the longitude and latitude of the plots, topographical factors [mean slope (MS, °), mean elevation (ME, m), highest elevation (HA, m), lowest elevation (LE, m)], and climate factors [mean annual precipitation (MAP, mm), relative humidity (RH, %), mean annual temperature (MAT, °C), and the mean temperature of the warmest (MTWM, °C) and coldest months (MTCM, °C)]. The species catalog was obtained from literature. Longitude and latitude, topographical factors, and climate factors were obtained from the Chinese Forest Biodiversity Monitoring Network or literature (Su et al., 2007; Hao et al., 2008; Lan et al., 2008; Lin et al., 2011; Yuan et al., 2011; Song et al., 2015). The topographical factors of the Tiantongshan plot were obtained according to the topographic map in the Biodiversity Monitoring Network; and calculation methods were based on the methods by Harms et al. (2001) and Valencia et al. (2004). Information on geography and climate for the eight study sites are shown in **Table 1**.

The eight plots differed in established time, region, and researchers. Thus, plant identification varied among plots. Thus, the species names in the plots were checked based on the Flora Reipublicae Popularis Sinicae (1959–2004) and Catalogue of Life China (2013). Concurrently, all species were divided into three types, namely, trees, small trees, and shrubs, based on the records of the Flora Reipublicae Popularis Sinicae (1959–2004).

Studies that used 20 m × 20 m subplots as basic units have shown that the species–area curves of all the forest community types in China are smooth for about 6 ha, which includes most of the species in the region (Hao et al., 2008; Lan et al., 2008; Zhu et al., 2008; Yang et al., 2011). In this study, every plot has an area greater than 20 ha. Thus, every plot can reflect the profile of the species composition in the region. Furthermore, the smallest plot area is 20 ha, and the largest is 25 ha; thus, the effect of area difference on large-scale species composition among plots is negligible.

Measurement of Species Turnover Rate

Species turnover rate is the rate of dissimilarity among species composition across all possible plot pairs along the environmental gradient. The slope of the relationship between the species turnover and environmental divergence measures

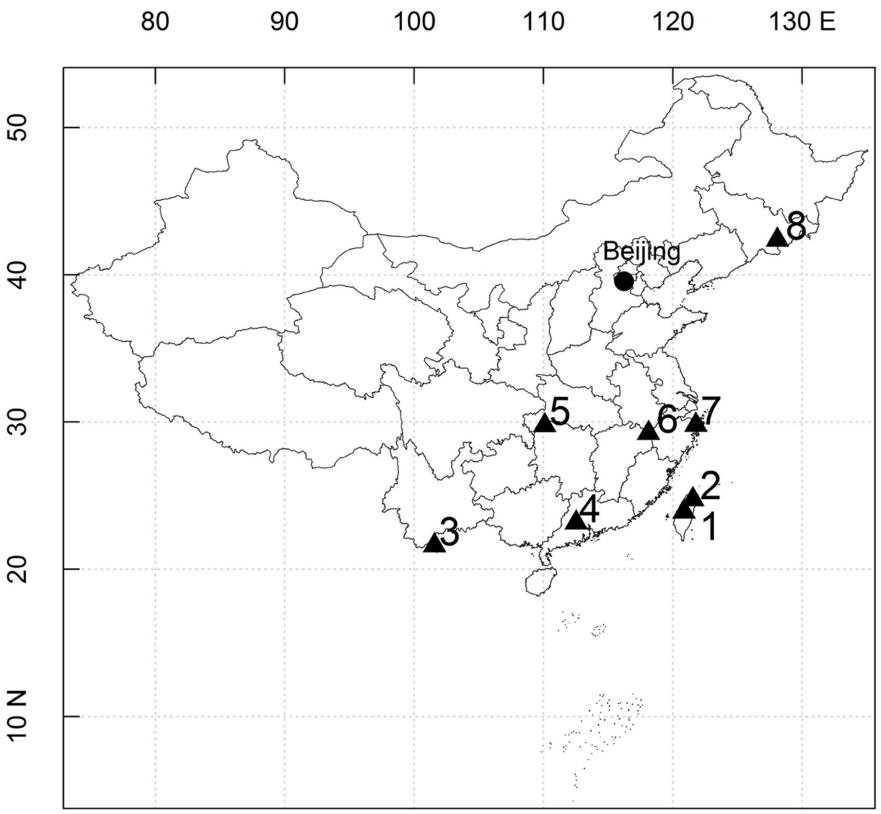

FIGURE 1 | Location of eight dynamics plots in East Asia. (1) Lianhuachi plot; (2) Fushan plot; (3) Xishuangbanna plot; (4) Dinghushan plot; (5) Badagongshan plot; (6) Gutianshan plot; (7) Tiantongshan plot; (8) Changbaishan plot.

species turnover rate. Jaccard's index (β_j; Jaccard, 1912), Sorenson's index (β_s; Sorensen, 1948), and Simpson's index (β_{sim}; Lennon et al., 2001) measure turnover rate of species composition. β_j and β_s are two widely employed indices and are independent of α-diversity (Jost, 2007). β_{sim} controls for local gradients in species richness (Lennon et al., 2001; Wang et al., 2012b).

$$\beta_j = 1 - c/(a+b+c) = (a+b)/(a+b+c)$$
$$\beta_s = 1 - 2c/(a+b+2c) = (a+b)/(a+b+2c)$$
$$\beta_{sim} = 1 - c/[\min(a,b)+c] = \min(a,b)/[\min(a,b)+c]$$

where a and b are the numbers of species only occurring in the focal and neighboring plots, respectively, and c is the number occurring in both.

Data Analysis

We used detrended correspondence analysis (DCA) for the ordination of plots. DCA is an effective method in vegetation analysis. In our study, we conducted DCA using a plot-species matrix using relative abundance data to analyze the similarity of the species composition among plots.

TABLE 1 | Information on geography and climate for eight study sites.

Plot name	Plot size (ha)	Latitude (°)	Longitude (°)	Mean elevation (m)	Mean slop (°)	Mean annual temperature (°C)	Mean annual Precipitation (mm)	Relative humidity (%)
Lianhuachi plot	25	23.90	120.87	756.00	35.00	25.0	2211.0	95.0
Fushan plot	25	24.75	121.55	675.00	10.00	18.3	4237.0	95.0
Xishuangbanna plot	20	21.61	101.57	789.21	11.33	21.8	1493.0	85.0
Dinghushan plot	20	23.17	112.52	350.00	40.00	20.9	1985.0	80.3
Badagongshan plot	25	29.77	110.09	1405.50	36.00	11.5	2105.4	90.0
Gutianshan plot	24	29.25	118.12	580.60	37.00	15.3	1963.7	92.4
Tiantongshan plot	20	29.80	121.78	447.25	31.50	16.2	1374.7	82.0
Changbaishan plot	25	42.38	128.08	801.50	2.23	3.6	700.0	78.0

Environmental variables: We used the climate and topographical factors to determine the environmental divergence between pairs of sites: MAP, RH, MAT, MTWM, MTCM, MS, ME, MA, and LE. All environmental variables were normalized as: $x' = (x - \text{mean}(x))$ / standard deviation (x), where x is a variable. Several studies have shown that most environmental variables are highly correlated with each other in China (Wang et al., 2012a). To avoid strong multicollinearity in regression models, we did not use original environmental variables as response variables. Instead, we conducted a principle component analysis (PCA) for all environmental variables and extracted the first six PC axes, which contained 95.19% of the total variance in the original environmental variables. In addition, longitude and latitude data of all the plots as shown in **Table 1**. Latitude difference is the difference in latitude values between the plot-pair.

Spatial variables: In our study, principal coordinates of neighbor matrices (PCNM) were used to obtain the spatial variable as a response variable based on the latitude and longitude of each plot. PCNM variables represent the spatial relationship among sites more accurately than Euclidean distance matrix and geographic coordinates (Legendre et al., 2009).

To account for effects of variation in γ-diversity, we explored the relationship between β- and γ-diversities using simple regression, with significance at $p < 0.05$. γ-Diversity is the number of species occurring in each plot-pair. Moreover, a null modeling approach was used to calculate the β-deviation according to Kraft et al. (2011). β-Deviation is currently the most widely used method (Myers et al., 2013).

To explore the influence of environment and space on β-diversity, we adopted multiple generalized linear models (GLM) using the calculated β_j, β_s, and β_{sim} as dependent, and aforementioned environment (MAP, RH, MAT, MTWM, MTCM, MS, ME, MA, and LE) and PCNM variables as response variables. The adjusted R^2 of a model is used to represent the explanatory power of environmental and PCNM variables on species turnover. To avoid overestimation resulting from a large number of response variables, we compared the values of the Akaike information criterion (AIC) to select the best environmental model and spatial model separately. For models with the same number of variables, the model with the smallest AIC was selected. Model selection stopped when any added variable increased the AIC (Tang et al., 2012b).

Partial regression analyses were used to further compare the effects of environmental and spatial processes. In the partial regression analyses, β_j, β_s, and β_{sim} were dependent, and environment variables (MAP, RH, MAT, MTWM, MTCM, MS, ME, MA, and LE) and PCNM variables were the response variables. Partial regression divided the variance in species turnover index into four parts: pure spatial effects, pure environmental effects, spatially structured environmental effects, and residual variance.

All analyses were conducted in R 2.15 (R Core Development Team[2]). DCA and PCA were performed using the "vegan" package (Oksanen et al., 2007). PCNM was performed using the "pcnm" package (Blanchet et al., 2008).

[2]http://www.Rproject.org

RESULTS

Structural Characteristics of Plant Community

A total of 1025 woody species were included in the final dataset, encompassing 355 genera in 100 families. We classified all plots into three vegetation types based on their thermal characteristics and water availability, as follows: tropical rain forest, subtropical evergreen broad-leaved forest, and temperate deciduous broad-leaved forest (**Figure 2A**). The species diversity differed significantly among forest types. Among the eight plots, the Xishuangbanna plot belonged to the tropical rain forest; therefore, its species diversity is the highest among all plots (Overall: 365, tree: 239, small tree: 89, shrub: 37). The Changbaishan plot belonged to the temperate deciduous broad-leaved forest. Therefore, its species diversity is the lowest among all plots (Overall: 52, tree: 26, small tree: 9, shrub: 17; **Figure 2B**).

The result of plot ordination is shown in **Figure 3**. The similarity between the Lianhuachi and Fushan plots is the highest in species composition. The similarities among the Dinghushan, Badagongshan, Gutianshan, and Tiantongshan plots are highest in species composition. The similarities between the Changbaishan and the other seven plots are the smallest because of far distance and large environmental differences.

Patterns of Species Turnover

β_j, β_s, and β_{sim} increased significantly with increasing latitude difference of overall species, trees, small trees, and shrubs. The species turnover rates that crossed the latitudinal difference were 0.030, 0.056, and 0.076 for β_j, β_s, and β_{sim} of overall woody species; 0.030, 0.055, and 0.070 for β_j, β_s, and β_{sim} of trees; 0.038, 0.071, and 0.102 for β_j, β_s, and β_{sim} of small trees; and 0.020, 0.038, and 0.050 for β_j, β_s, and β_{sim} of shrubs, respectively (**Figure 4**). The species turnover rate of shrubs was less than that of trees and small trees along the latitudinal difference. The number of species declined significantly with increasing latitude of overall woody species, trees, small trees, and shrubs.

Relationship between β- and γ-Diversity

γ-diversity did not significantly affect β_j, β_s, and β_{sim} for overall species, trees, small trees, and shrubs (**Figures 5A–D**). Moreover, β-deviation increased along latitudinal difference for overall species ($R^2 = 0.66$), trees ($R^2 = 0.58$), small trees ($R^2 = 0.67$), and shrubs ($R^2 = 0.54$; **Figure 5E**). Therefore, after correcting the differences in species pool size (γ-diversity), β-diversity had significant difference along latitudinal difference.

Determinants of Species Turnover

The first six PC axes and four PCNM variables were used to assess the effects of the environment and space. The best environmental models and spatial models are shown in **Table 2**. For overall woody species, environment explained 36.30, 37.20, and 48.48%

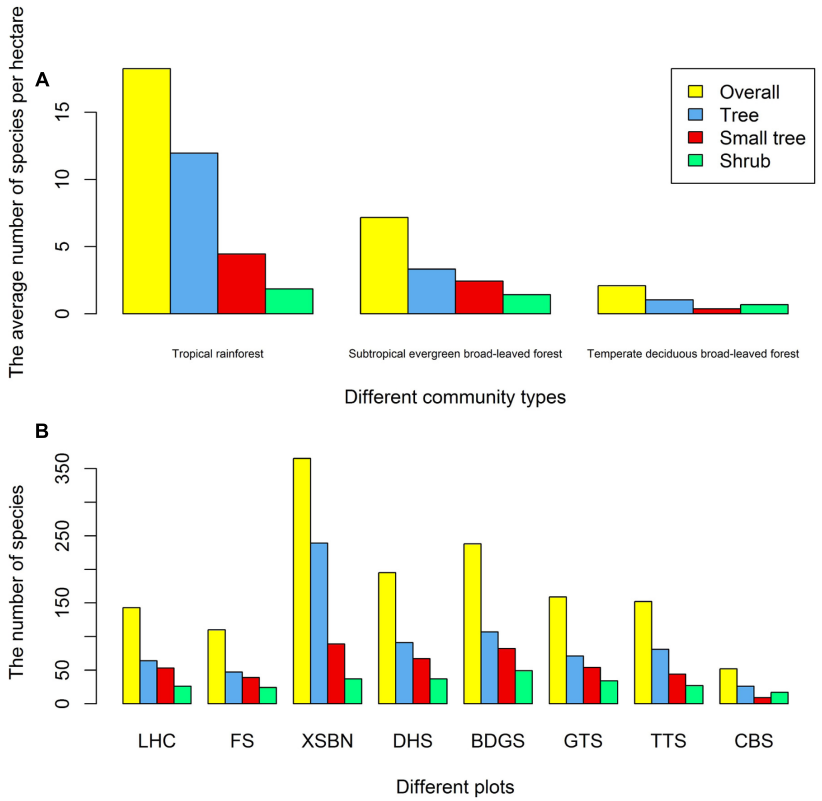

FIGURE 2 | Number of species in different community types (A) or different plots (B). Abbreviations: LHC, Lianhuachi plot; FS, Fushan plot; XSBN, Xishuangbanna plot; DHS, Dinghushan plot; BDGS, Badagongshan plot; GTS, Gutianshan plot; TTS, Tiantongshan plot; CBS, Changbaishan plot.

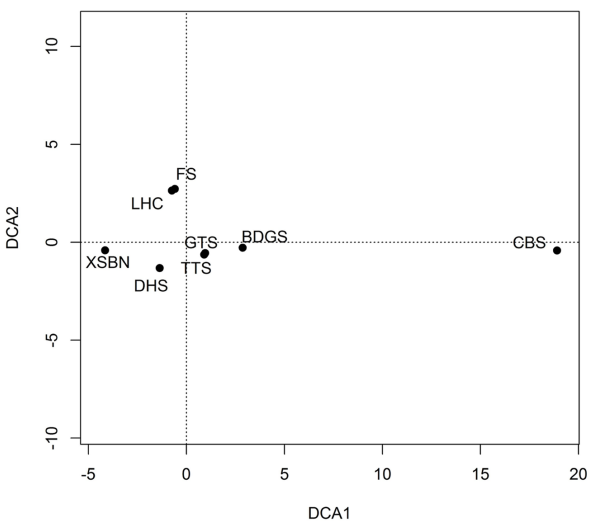

FIGURE 3 | DCA analysis of plant species composition among eight dynamics plots. The plot codes are the same as in **Figure 2**.

of the total variance in β_j, β_s, and β_{sim}, respectively; and spatial factors explained 47.92, 48.39, and 41.38% of the total variance in β_j, β_s, and β_{sim}, respectively.

Partial regressions indicated that the effects of pure spatial and spatially structured environments on β_j, β_s, and β_{sim} were stronger than those of pure environmental factors for overall woody species (**Figure 6**). For trees and small trees, the effects of pure spatial and spatially structured environmental factors on β_j, β_s, and β_{sim} were stronger than those of pure environmental factors, whereas the effects of pure environmental factors on β_j, β_s, and β_{sim} were stronger than those of pure spatial and spatially structured environmental factors for shrubs. Moreover, the effects of pure environmental factors on β_j, β_s, and β_{sim} for trees were the lowest, and the effects of pure environmental for shrubs were the highest.

DISCUSSION

Large stem-mapping plots have been established in different regions worldwide (Erickson et al., 2014). Many scholars have studied large stem-mapping plots, such as functional traits (Liu et al., 2016) and species coexistence mechanisms (Aiello-Lammens et al., 2016). However, most of these studies were conducted in local areas, and integrated large-scale analyses have been poorly studied. Various ecosystems worldwide are interconnected. Thus, integrating comparative large scale analyses can reveal the rules of forest community distributions

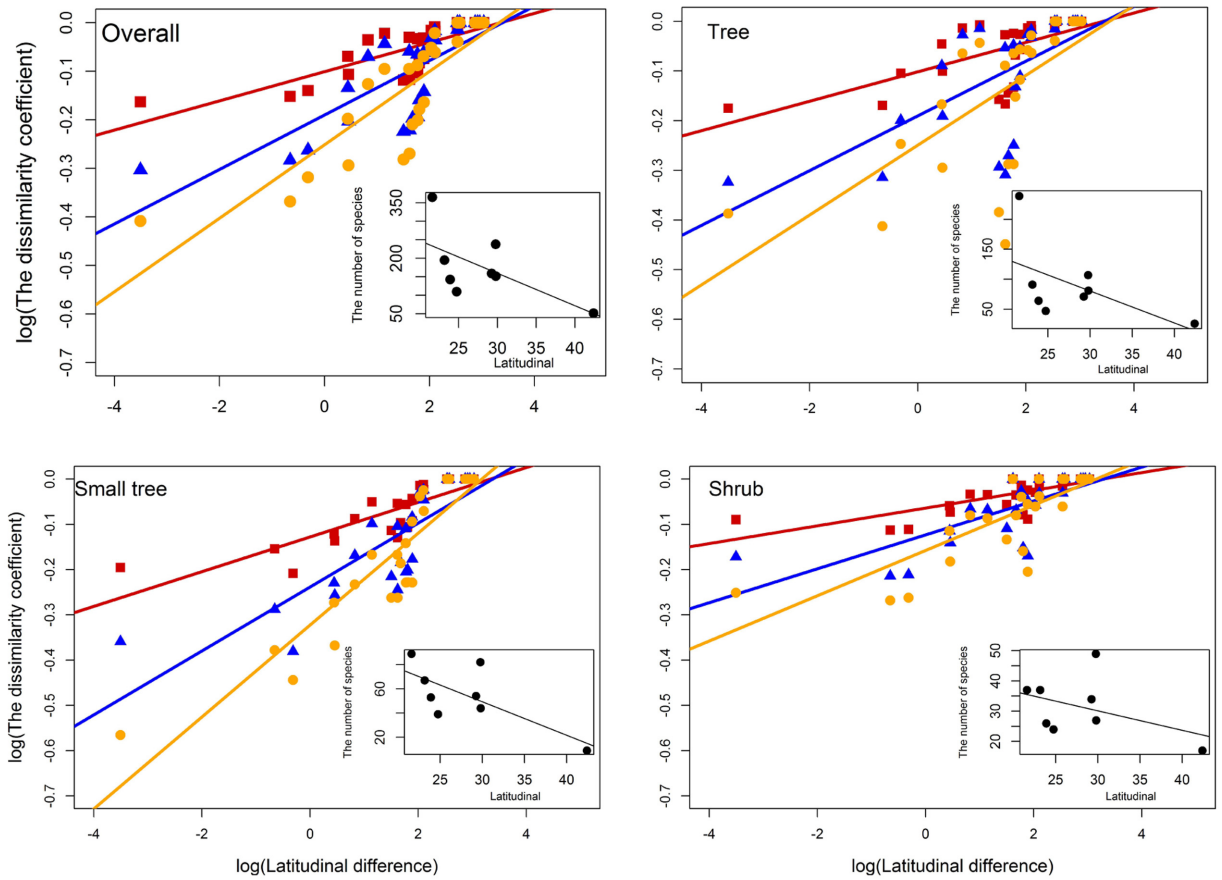

FIGURE 4 | Patterns of overall species turnover (β_j: $R = 0.5895$*; β_s: $R = 0.5824$***; β_{sim}: $R = 0.6929$***), trees (β_j: $R = 0.4009$***; β_s: $R = 0.4011$***; β_{sim}: $R = 0.4144$***), small trees (β_j: $R = 0.6859$***; β_s: $R = 0.6764$***; β_{sim}: $R = 0.8175$***), and shrubs (β_j: $R = 0.5250$***; β_s: $R = 0.5247$***; β_{sim}: $R = 0.6021$***) in different forest dynamics plots along the latitude difference.** Red square and line represent β_j, blue triangle and line represent β_s, and yellow dot and line represent β_{sim}. The subset shows the relationship between the number of species and latitudinal across multiple forest dynamics plots. Latitude difference is the difference in latitude values between the plot-pair.

and species coexistence mechanisms. In this study, we used comparative analysis to analyze the species composition similarities of eight large forest plots. As expected, smaller distances between the plots tended to increase similarity in species composition. Moreover, species diversity decreased with increasing latitude.

Several studies have shown that species diversity generally increases with decreasing latitude (Rodríguez and Arita, 2004; Qian and Ricklefs, 2007; Kraft et al., 2011; Wang et al., 2012b). However, different methods, including the sampling scale and measures of species turnover, have been used in different studies. Sampling scale in previous studies usually were small plots (e.g., 10 m × 10 m or 20 m × 20 m), and the sampling scales in this study were 20 or 25 ha plots. Moreover, measures of species turnover in previous studies usually used one method. To avoid the error caused by using different methods of measurement, three different methods were employed to measure the species turnover rate. Based on the forest dynamics plots in this study, the patterns of species turnover increased significantly with increasing latitude differences for β_j, β_s and β_{sim} of overall woody species, trees, small trees, and shrubs, respectively. These results are consistent with those obtained in previous studies (Rodríguez and Arita, 2004; Qian and Ricklefs, 2007; Kraft et al., 2011; Wang et al., 2012b). The reasons for the increase in species turnover rate with increasing latitude differences are complicated; among the reasons, latitudinal gradients in climatic tolerance and sampling effect of the species pool are the most reported (Wang et al., 2012b; Morin and Lechowicz, 2013). The hypothesis of latitudinal gradients in climatic tolerance claims that species are more climatically tolerant in high than in low latitudes. Lower climatic tolerance may further lead to narrower niche breadths in tropical than in temperate mountains, thereby decreasing the likelihood of co-occurrence of different species and increasing the species turnover rate (Wang et al., 2012b; Morin and Lechowicz, 2013).

The sampling effect hypothesis claims that variation in β-diversity across broad biogeographic gradients is more likely to be driven by γ-diversity than by differences in the mechanisms of community assembly (Kraft et al., 2011; Myers et al., 2013; Xu et al., 2015). In the present study, species pool did not significantly affect the pattern of species turnover for overall

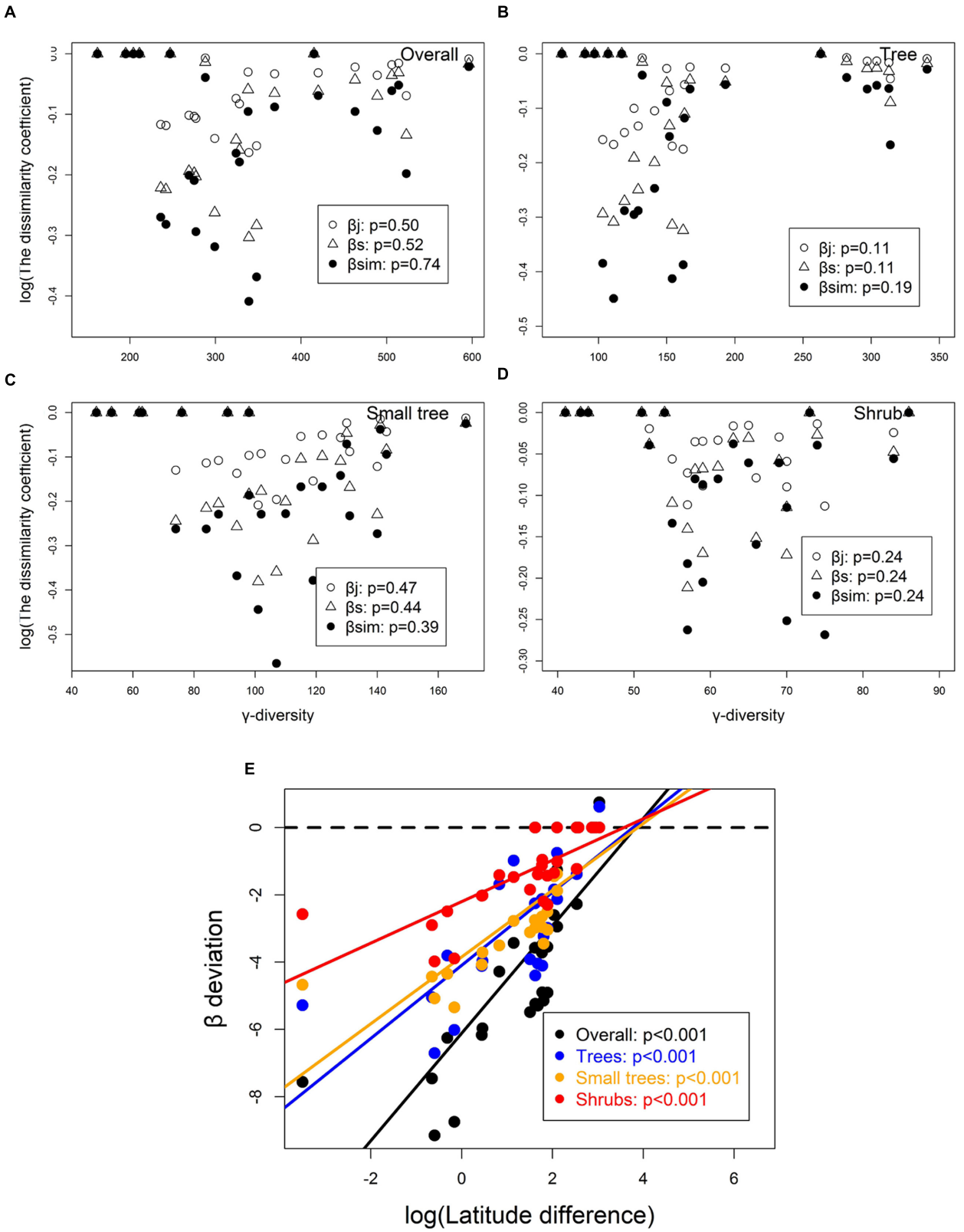

FIGURE 5 | Relationship between β- and γ-diversities for (A) overall, (B) tree, (C) small tree, and (D) shrub. Open dots represent β_j, gray triangles represent β_s, and solid dots represent β_{sim}. A standard effect size of β-diversity deviations from a null model that corrects for γ dependence across with latitude difference **(E)**.

TABLE 2 | Relationships between β-diversity and PC variables or PCNM variables.

Life form	β_j			β_s			β_{sim}		
	Factors	Cum.R^2 (%)	AIC	Factors	Cum.R^2 (%)	AIC	Factors	Cum.R^2 (%)	AIC
Overall	PC1	11.10	−80.616	PC1	10.90	−50.891	PC1	12.32	−42.065
	PC2	28.10	−85.255	PC2	29.60	−56.134	PC2	37.80	−50.095
	PC5	34.00	−86.624	PC5	35.00	−57.362	PC5	45.74	−52.804
	PC6	36.30	−86.753	PC6	37.20	−57.461	PC6	48.48	−53.359
	PCNM1	39.13	−90.451	PCNM1	39.96	−61.168	PCNM1	33.30	−48.176
	PCNM2	41.13	−90.479	PCNM2	42.35	−61.327	PCNM3	34.45	−49.732
	PCNM3	47.92	−92.794	PCNM3	48.39	−63.361	PCNM4	41.38	−50.795
Tree	PC1	6.32	−71.364	PC1	6.60	−42.659	PC1	5.99	−33.010
	PC2	13.61	−71.578	PC2	15.10	−44.235	PC2	20.34	−36.432
	PC5	19.77	−73.657	PC5	20.70	−45.193	PC5	29.79	−38.863
	PC6	27.83	−75.618	PC6	28.10	−46.919	PC6	36.70	−40.764
	PCNM1	39.68	−82.812	PCNM1	40.95	−54.586	PCNM1	34.28	−42.316
	PCNM2	43.77	−83.741	PCNM2	45.34	−55.698	PCNM2	38.02	−42.949
	PCNM3	50.14	−86.026	PCNM3	51.31	−57.863	PCNM3	45.93	−45.654
							PCNM4	50.70	−47.267
Small tree	PC1	11.23	−72.495	PC1	11.12	−43.853	PC1	13.80	−33.389
	PC2	42.73	−82.998	PC2	44.75	−55.320	PC2	49.05	−46.165
	PC4	44.49	−82.963	PC4	46.24	−55.187	PC5	53.47	−47.682
	PC5	49.07	−84.412	PC5	50.53	−56.557			
	PCNM1	29.86	−77.619	PCNM1	30.95	−50.415	PCNM1	27.67	−37.949
	PCNM2	30.38	−78.921	PCNM2	32.00	−49.923			
	PCNM3	36.18	−79.337	PCNM3	36.83	−50.994			
Shrub	PC1	11.09	−98.995	PC1	10.95	−67.546	PC1	13.51	−57.945
	PC2	26.46	−103.040	PC2	27.30	−71.928	PC2	28.71	−62.075
	PC3	33.55	−104.830	PC3	34.69	−73.872	PC3	32.50	−62.649
	PC5	36.84	−105.360	PC5	37.90	−74.391	PC5	35.72	−63.132
	PCNM1	17.76	−101.020	PCNM1	17.93	−69.67	PCNM1	18.49	−59.484

species, trees, small trees, and shrubs. These results indicated that the sampling effect hypothesis may not be applicable to the latitudinal pattern of species turnover, at least in this system. The results are inconsistent with those obtained in previous studies (Kraft et al., 2011; Myers et al., 2013; Xu et al., 2015). This inconsistency may be due to the use of latitude difference (not latitude) in our study. Latitude difference is similar to the distance between plots. The relationship between β- and γ-diversity is complicated. Some new methods have been proposed to measure and interpret β-diversity, such as multivariate pairwise distances (Bennett and Gilbert, 2016). Therefore, further analysis is needed to distinguish the effects of γ-diversity in controlling latitudinal gradients of β-diversity.

Most ecological patterns and processes in nature are scale-dependent (Bin et al., 2010; Báez et al., 2015). On a local scale, observed patterns and processes on the Changbaishan plot (Yuan et al., 2011), Lianhuachi plot (Lin et al., 2011), and Gutianshan plot (Legendre et al., 2009) showed that the diversity of forests are generally equally governed by environmental and neutral processes. On a large scale, Wang et al. (2012b) showed that spatial rather than environmental processes were the primary determinants for the latitudinal gradient in β-diversity of woody species in China. Qian et al. (2005) studied eastern Asia and eastern North America and found that environmental and neutral processes contributed equally to latitudinal β-diversity. Our results supported the hypothesis that the effect of neutral processes on species turnover of woody species in East Asia is more important than that of environmental processes. Moreover, our results are consistent with those of Wang et al. (2012b). This is likely because the study areas in our study and Wang et al. (2012b) are the same. Our study area and the obtained topographical environment characteristics have greater difference with the study of Qian et al. (2005). Therefore, our results are not consistent with those of Qian et al. (2005), but our results are not exact opposites.

The mechanisms causing patterns of species turnover may differ among trees, small trees, and shrubs (Qian, 2009; Guèze et al., 2013). This study shows that the effect of neutral processes is more important than environmental processes for trees, and that the effects of the environment for trees are minimal. However, the effects of the environment are more important than neutral processes for shrubs, and the effects of environmental factors on shrubs are greatest among trees, small trees, and shrubs. These findings may be because the dispersal abilities, such as shape, weight, number, and germination period of seeds, vary

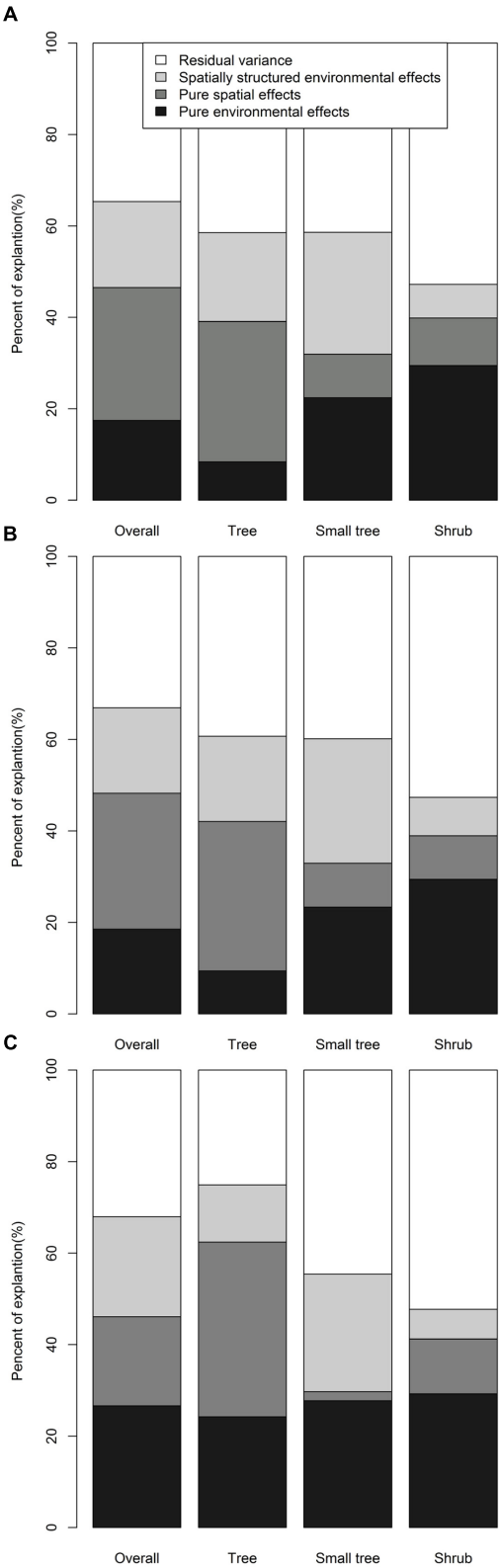

FIGURE 6 | Partial regression analyses for the effects of environmental and spatial processes on the (A) β_j, (B) β_s, and (C) β_{sim} of species composition.

among trees, small trees, and shrubs (Qian, 2009; Farrell et al., 2012). This phenomenon is also related to shrubs being more dependent on the local environmental heterogeneity (such as aspect and humidity) and canopy species distribution than other plants (Kristiansen et al., 2012). Thus, trees are more affected by neutral processes, and shrubs are more affected by environmental processes. These results contrast with the findings obtained by Guèze et al. (2013), who determined that environmental processes explained more variation for large trees than for small trees. This discrepancy may result partly from the use of spatial extents and environmental variables in the two studies. Guèze et al. (2013) focused only on seven villages in the southwestern Amazon and only used soil factors as environmental variables. By comparison, our study covered 184 ha plots and three temperature belts in East Asia. In particular, our environmental variables included climatic and topographical factors. Thus, at a large scale, neutral processes explained more variation for turnover of tree species, and environmental processes explained more variation for turnover of shrub species.

Chang et al. (2013) studied a subtropical broad-leaved plot in Taiwan and showed that better environmental data could reverse the conclusions about community assembly processes. Furthermore, this conclusion is also supported by some studies (Jia et al., 2015). In this study, environmental variables included climate and topographical factors. Soil, as one of the important environmental factors, was not considered. However, previous studies found that soil variables influence species distribution at small spatial extents (Sarr et al., 2005). Moreover, light environmental processes and soil microorganisms are two important factors that are often ignored in community ecology studies. Therefore, future research should consider more and better environmental factors and distinguish the effects of neutral and environmental processes on a community assembly.

CONCLUSION

The patterns of woody species turnover increased significantly with increasing latitude differences in East Asia. Our results support the hypothesis that the effect of neutral processes is more important than the effect of environmental processes on species turnover of woody species. However, the mechanisms underlying such patterns of species turnover may differ among trees, small trees, and shrubs. Neutral processes explained more variation for turnover of tree species. Environmental processes explained more variation for turnover of shrub species at a large scale. Therefore, trees and shrubs should receive different protection strategies in future biodiversity conservation efforts.

AUTHOR CONTRIBUTIONS

YY originally formulated the idea, YC and ZY developed methodology, PL, RC and HJ conducted fieldwork, YC and ZY performed statistical analyses and wrote the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 17 October 2016
doi: 10.3389/fpls.2016.01532

Phylogeny, Seed Trait, and Ecological Correlates of Seed Germination at the Community Level in a Degraded Sandy Grassland

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Seed germination strongly affects plant population growth and persistence, and it can be dramatically influenced by phylogeny, seed traits, and ecological factors. In this study, we examined the relationships among seed mass, seed shape, and germination percentage (GP), and assessed the extent to which phylogeny, seed traits (seed mass, shape, and color) and ecological factors (ecotype, life form, adult longevity, dispersal type, and onset of flowering) influence GP at the community level. All analyses were conducted on the log-transformed values of seed mass and arcsine square root-transformed values of GP. We found that seed mass and GP were significantly negatively correlated, whereas seed shape and GP were significantly positively correlated. The three major factors contributing to differences in GP were phylogeny, dispersal type, and seed shape (explained 5.8, 4.9, and 3.1% of the interspecific variations independently, respectively), but GP also influenced by seed mass and onset of flowering. Thus, GP was constrained not only by phylogeny but also by seed traits and ecological factors. These results indicated that GP is shaped by short-term selective pressures, and long-term phylogenetic constrains. We suggest that correlates of phylogeny, seed traits, and ecology should be taken into account in comparative studies on seed germination strategies.

Keywords: seed dispersal, seed mass, seed shape, life form, phylogeny

OPEN ACCESS

Edited by:
Massimiliano Tattini,
National Research Council, Italy

Reviewed by:
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *29 May 2016*
Accepted: *29 September 2016*
Published: *17 October 2016*

Citation:
Wang Z, Wang L, Liu Z, Li Y, Liu Q and Liu B (2016) Phylogeny, Seed Trait, and Ecological Correlates of Seed Germination at the Community Level in a Degraded Sandy Grassland. Front. Plant Sci. 7:1532. doi: 10.3389/fpls.2016.01532

INTRODUCTION

Seed germination, a central seed trait, is a key bottleneck in plant life history, potentially affecting the distribution and abundance of species in communities (Guo et al., 2000; Souza and Fagundes, 2014). Most life history traits are correlated (Moles and Leishman, 2008; Norden et al., 2009), and some traits may affect seed germination percentage. Understanding how life-history traits are related to germination percentage is crucial for understanding the evolution and ecology of seed germination strategies. Although relationships between life-history traits and germination have received considerable attention, most studies have focused on individual species without scaling up to a community level (but see Liu et al., 2007; Xu et al., 2014). Furthermore, variation in germination strategies among species in a community may support higher diversity by allowing temporal partitioning of environmental resources and providing a buffer against extinction (Chesson, 2000; Gremer and Venable, 2014). Therefore, understanding germination strategies of seeds within

a single community has implications for trait evolution as well as population and community dynamics.

Seed mass has long been regarded as an important aspect of plant reproductive biology (Baker, 1972; Moles et al., 2005, 2007). Seed mass influences many different life-history stages in plants. For instance, small-seeded species have low rates of seedling survivorship (Moles and Westoby, 2004), and they tend to have high seed output but short lifespans (Moles and Westoby, 2004). Relationships between seed mass and germination are less well understood than relationships between seed mass and other life-history stages. Differences in seed mass among species are due in part to different levels of starch and endosperm nutrients, and may influence germination percentage (Chen et al., 2002; Soriano et al., 2011). Studies have shown that seed mass may affect germination percentage (Reich, 1994; Paz et al., 1999; Bu et al., 2007; Xu et al., 2014). Regardless, it is widely accepted that large seeds generally have a higher germination percentage than small seeds. However, there is little information on the relationship between seed mass and germination in arid or semi-arid ecosystems, especially, the degraded grasslands in arid environments.

Seed shape, which varies in three dimensions, is another important trait that affects germination (Weiher et al., 1999; Tekrony et al., 2005). Theoretical studies have predicted that species with elongate and flat seeds should germinate more frequently than those with round seeds (Grime et al., 1981). Elongate and flat seeds are more likely to experience post-dispersal predation than round seeds, and should therefore show prompt germination to avoid risks of mortality. However, strong empirical data supporting this prediction are lacking (Liu et al., 2007).

It is not yet fully known how seed germination percentage is related to various factors. Germinability has been predicted to be associated with phylogeny, seed traits, and ecological factors (Bu et al., 2007; Norden et al., 2009). These hypotheses can be tested by answering two questions. Which factors of phylogeny, seed traits, and ecology affect seed germination? Which factors are most significant in explaining seed germination percentage? Although there has been research on the relationship between seed traits and germinability, there is limited information on the proportion of germination variation among species that could be attributed to the species' phylogenetic background, seed traits, and ecological factors. The relative importance of phylogeny, seed traits such as mass, shape and color, and ecological factors such as ecotype, life form, adult longevity, dispersal type, and onset of flowering in determining seed germination percentage need to be determined for degraded sandy grassland ecosystems.

The Horqin Sandy Land located in the arid zone of Inner Mongolia, China, is a well-known grassland in northeastern China (Zhu and Chen, 1994; Wang et al., 2003). In the last few decades, it has been affected by desertification, likely the consequence of over-cultivation, intensive fire wood collection, and overgrazing. Mobile dunes, semi-stabilized dunes, and stabilized dunes are distributed over some areas of the steppe. In degraded sandy grasslands, seedlings are vulnerable to desiccation, long periods of drought, high temperatures, and overgrazing (Liu et al., 2007; Zhao et al., 2011). Seed germination strategies are, in theory, some of the most effective strategies for plants inhabiting arid environments, which are typically characterized by highly variable and unpredictable environment (Philippi, 1993). Plants inhabiting arid degraded sandy grasslands have developed different strategies for seed germination to respond to the novel environmental pressures via long-term natural selection (Li et al., 2006). However, in recent years, many studies have focused on the relationship between seed morphology and vegetation processes (Liu et al., 2007; Yan et al., 2009; Ma et al., 2010; Zhao et al., 2011), but the link between the germination and seed traits, ecological factors at community level in this highly disturbed and stressed environment has not been elucidated. Studies of the relationships between seed germination and phylogeny, seed traits, and ecological factors would provide insights into seed germination strategies in unpredictable environments, especially in degraded sandy grasslands.

In the present study, we assessed relationships between germination percentage and phylogeny, seed traits, and ecological factors for 109 species in a degraded sandy grassland at the community level. The following questions were addressed: (1) whether species with small seeds would have a higher germination percentage than species with larger seeds, (2) whether species with elongate and flat seeds would have a higher germination percentage than those with round seeds, and (3) what proportion of germination variation among species could be attributed to the species' phylogeny, seed traits (mass, shape, and color), and ecological factors (ecotype, life form, adult longevity, dispersal type, and onset of flowering).

MATERIALS AND METHODS

Study Site

The study grassland is located in the Horqin Sandy Land in northeastern China (118° 35′–123° 30′ E, 42° 41′–45° 45′ N), which has a continental semiarid monsoonal climate with a very cold winter and a warm summer. Mean annual temperature is 6.3°C; the lowest and the highest monthly mean temperatures are −14.0°C in January and 23.0°C in July, respectively. Mean annual precipitation is 288 mm, of which 70–80% is received in June, July, and August. The prevailing winds are from the northwest from March to May and from the southeast from June to September. Annual mean wind velocity ranges from 3.0 to 4.4 m s^{-1}. The soils are sandy, loose, and very susceptible to wind erosion. Ninety percent of the total area has been desertified. The landscape is characterized by mobile, semi-stabilized, and stabilized dunes.

Seed Collection

Mature seeds of 109 species growing in different habitats were collected from August to November (at the time of seed dispersal). Seeds were collected from 15 to 25 plants per species. Seeds from one species were thoroughly mixed to minimize the contribution from single plants. Air-dried seeds were stored in paper bags in the laboratory.

Germination Experiment

For each species, five replicates of 50 seeds were placed on filter paper in Petri dishes (9 cm diameter). The filter paper was moistened with distilled water and the dishes were placed in temperature and light-controlled incubators (14:10 h, light: dark cycle; photosynthetic photon flux density (PPFD) of 25–30 μmol m^{-2} s^{-1} at seed level). The daytime temperature was kept at 28°C and the night temperature was 16°C, to approximate the mean daily maximum and minimum temperature in 3–5-cm-deep soil during rainy days from May to August. Each dish was inspected daily, and germinated seeds were counted and removed. The seeds whose radicles had emerged were considered to have germinated. Counting continued until no germination occurred for five successive days. Seeds that had not germinated were checked for viability. Germination percentage (GP) was calculated as the percentage of viable seeds that germinated.

Seed Mass

Seed mass, a measure of size, was represented by the average weight of single seed (Liu et al., 2007). For each plant species, seed mass was the average air-dried weight of 100 seeds, calculated from five replicates.

Seed Shape

The seed shape was calculated as the variance of three main perpendicular dimensions, i.e., length, width, and height (Thompson et al., 1993; Moles et al., 2000). Seed dimension variance is a standard index to describe seed shape (Thompson et al., 1993). The variance was calculated as follows, using the average of 10 seeds for each species:

$$\text{variance} = \sqrt{\frac{\left(1-\frac{\text{width}}{\text{length}}\right)^2+\left(1-\frac{\text{height}}{\text{length}}\right)^2+\left(1-\frac{\text{height}}{\text{width}}\right)^2}{3}}$$

The above equation gives a value for seed shape, such that spherical seeds have a variance of 0 and elongated or flattened seeds have a variance up to 0.33 (Thompson et al., 1993). In other words, larger variance values are associated with flatter seeds and smaller values with rounder seeds.

Statistical Analysis

To determine to what extent phylogenetic, seed traits (mass, shape, and color), and ecological factors (ecotype, life form, adult longevity, dispersal type, and onset of flowering) influence seed germination, a one-way, two-way, and factorial analysis of variance (ANOVA) were conducted (Mazer, 1989, 1990; Figueroa and Armesto, 2001). The one-way ANOVAs estimated the effect of each factor on the GP variance independently. Two-way ANOVAs were performed on the same data to assess the interactions between each pair of factors. Factorial ANOVAs provided two types of information (Figueroa and Armesto, 2001): individual factor effects and association effects. We calculated the proportion of the variance contributed by each factor to the complete ANOVA model to assess the combined effects of all factors by performing a series of factorial ANOVAs that estimated the effects all factors but one (incomplete model). We compared each incomplete ANOVA with the complete factorial ANOVA. The difference between the proportion of the total sum of squares (ss) of the complete model (its R^2) and the R^2 of the incomplete model represented the proportion of the total ss explained by the removed factor. If, in the complete ANOVA, a given class variable has a lower R^2 value than in the incomplete ANOVAs from which a different variable has been deleted, the increase in the R^2 value of the first variable would be due to an association (or correlation) or strong interaction with the second variable (see Mazer, 1989, 1990). In other words, when two variables were strongly associated, the ss of each variable would account for a higher proportion of the total ss of the model when the other was not included in the model.

GP was arcsine square root transformed and seed masses were log transformed to improve normality and stabilize variances. All analyses were performed with SPSS 18.0 software.

We grouped species into the following categories prior to analysis:

Phylogenetic group. To examine effects of phylogeny on germination, the 109 angiosperm species were grouped by order according to the APG III system of plant classification (Angiosperm Phylogeny Group III 2009): Liliales, Asparagales, Poales, Ranunculales, Zygophyllales, Malpighiales, Fabales, Rosales, Geraniales, Malvales, Caryophyllales, Ericales, Gentianales, Solanales, Lamiales, Asterales, and Apiales.

Life form. Species were assigned to the following three major growth categories: woody plants, graminoid plants, and forbs.

Adult longevity. Species were grouped into two classes: annual (including a few biennials) or perennial.

Seed mass. The mean seed mass of each species was assigned to a class: < 0.1 mg, 0.1–0.3 mg, 0.31–1.0 mg, 1.1–3.0 mg, 3.1–9.0 mg, 9.1–30 mg, or > 30 mg (Moles et al., 2000).

Seed shape. Each species was classified into one of six categories based on our observations and relevant literature (Liu, 1992; Gordon, 1998; Liu et al., 2005, 2014): (1) spheroid or nearly spheroid; (2) cylindrical, tubular, or conical; (3) ellipsoid, broad-ellipsoid, narrow-ellipsoid, ovoid, elongate-ovoid, or obovoid; (4) trigonous or prismatic; (5) oblate-discoid, lenticular, or plane; or (6) fusiform, acicular, or linear.

Dispersal model. Species were classified into five groups according to the morphological features of their seeds: (1) zoochorous species, defined as having awns, spines, or hooks to adhere to animals (epizoochory), or having fleshly or arillate fruits for animals to eat (endochory); (2) anemochorous species, defined as having membranous wings, hairs, bracts, a persistent or inflated perianth, or a pappus; (3) ombrohydrochorous species, defined as those wherein the seeds produce mucilage upon being wetted; (4) autochorous species, which are ballistically dispersed from explosively dehiscing capsules that throw the seeds some distance from the parent plant; or (5) barochorous species, defined as those lacking any obvious dispersal mechanism or disperser reward (Navarro et al., 2009; Liu et al., 2014).

Onset of flowering. Species were assigned to one of three groups: (1) early, where flowering begins in May; (2) middle,

where flowering begins in June; or (3) late, where flowering begins in July and August.

Seed color. Each species was classified into one of seven color categories based on our observations and relevant literature (Liu, 1992; Gordon, 1998; Liu et al., 2005, 2014): (1) light brown, brown, dark brown, or nut brown; (2) light reddish brown, reddish brown, or dark reddish brown; (3) light yellowish brown, yellowish brown, or dark yellowish brown; (4) pale yellow, yellow, orange, or reddish yellow; (5) light green, green, or dark green; (6) gray, grayish white, or grayish black; or (7) black.

Ecotype. Each species was assigned to an ecotype category based on the seed collection site: weed, steppe plant, meadow plant, or psammophyte.

RESULTS

Patterns of Seed Mass and Shape

Seed mass ranged from 0.011 mg in *Solanum nigrum* L. to 130.758 mg in *Tribulus terrestris* L., with a mean of 4.988 mg. The frequency of seed mass classes had an approximately normal distribution (**Figure 1A**). Seven species had a weight of < 0.1 mg, 14 species of 0.1–0.3 mg, 32 species of 0.3–1.0 mg, 26 species of 1.1–3.0 mg, 18 species of 3.1–9.0 mg, 9 species of 9.1–30.0 mg, and 3 species of > 30 mg.

According to the calculated mean seed shape variance and direct observations, the seed shapes of 109 species could be divided into six groups, of which 68.8% (75 species) were close to spherical to ovoid (**Figure 1B**).

Relationships between Seed Mass, Seed Shape, and GP

There was a significant negative correlation between seed mass and GP ($P = 0.007$, $R^2 = 0.074$,**Figure 2A**), i.e., smaller seeds had higher germination percentages than larger ones. Seed shape was positively and significantly associated with germination percentage ($P = 0.003$, $R^2 = 0.089$,**Figure 2B**), i.e., the elongate- and flat- seeded species had higher GPs than round-seeded species (**Figure 3A**).

Effects of Phylogeny and Life History Attributes on GP

A series of ANOVAs was used to determine whether the variance in GP among 95 species (excluding species with no germination and with only one representative per order) was correlated with the assessed factors. One-way ANOVAs showed that five factors (phylogenetic group, seed mass, seed shape, dispersal type, and onset of flowering) had statistically significant effects on GP (**Table 1**, **Figures 3A–C**). Dispersal type explained the highest percentage of the variation in GP when considered singly (61.2%), and onset of flowering, seed shape, phylogenetic group, and seed mass explained 60.6%, 59.9%, 35.5%, and 17.6% of the variation in GP, respectively (see **Table 1**). Further, the remaining four factors (life form, adult longevity, ecotype, and seed color) did not have significant effects and together explained 11.5% of the variation in GP (**Table 1**).

Two-way ANOVAs detected no significant interaction effects of any pair of factors that had separate effects on GP in the one-way ANOVAs (**Table 2**). In addition, the two-way ANOVAs revealed nonrandom associations between factors because the proportion of the total variance in GP explained by each of the factors (the R^2 value) in the two-way ANOVAs was less than in the one-way ANOVAs (**Table 2**).

Factorial ANOVAs revealed an independent effect of each factor on GP (**Table 3**). The difference between the proportion of the ss (R^2 value) of the complete and incomplete models can represent the proportion of the ss explained by the removed factor (**Table 3**), so we could systematically estimate the source of variance in GP. Variance was explained by phylogeny (5.9%),

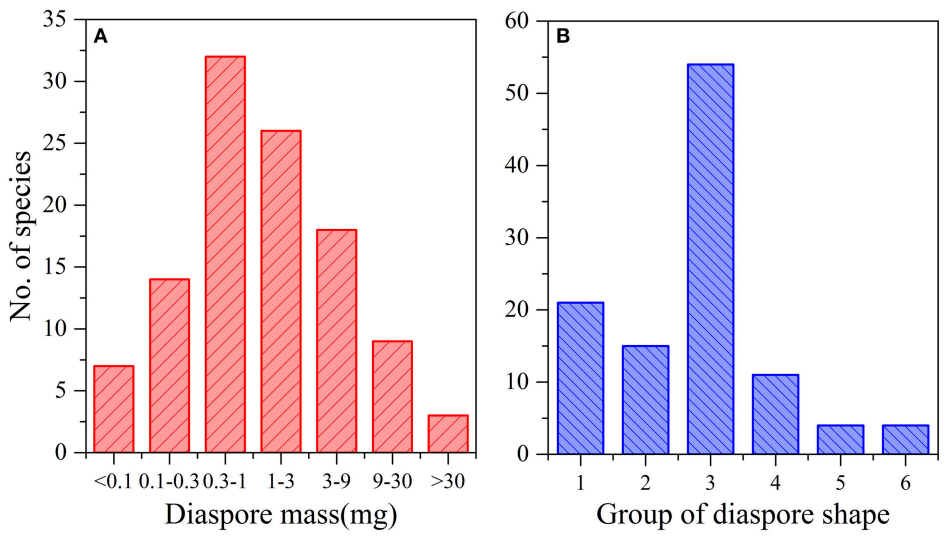

FIGURE 1 | Frequency distribution of seed mass (A) and seed shapes (B).

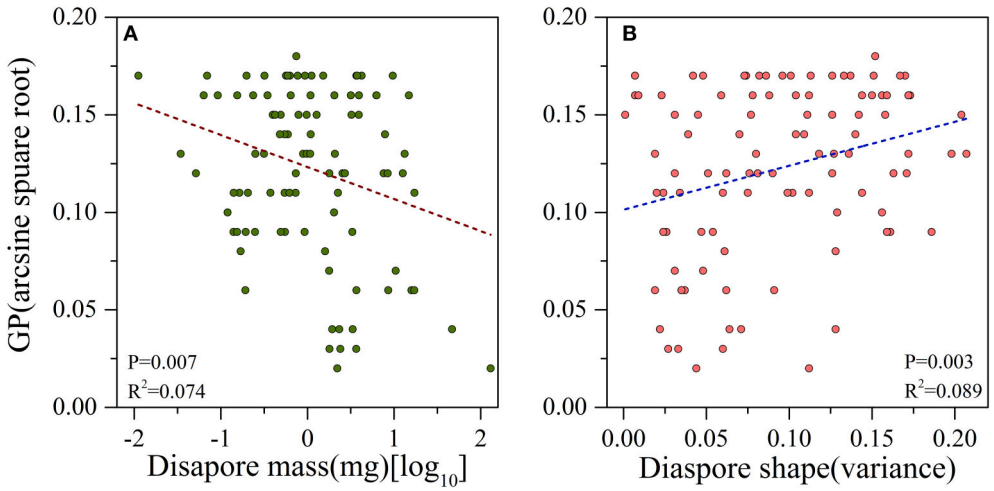

FIGURE 2 | Linear relationships between (A) log-transformed diaspore mass and arcsine square root-transformed germination percentage (GP); (B) variance of diaspore shape and arcsine square root transformed GP.

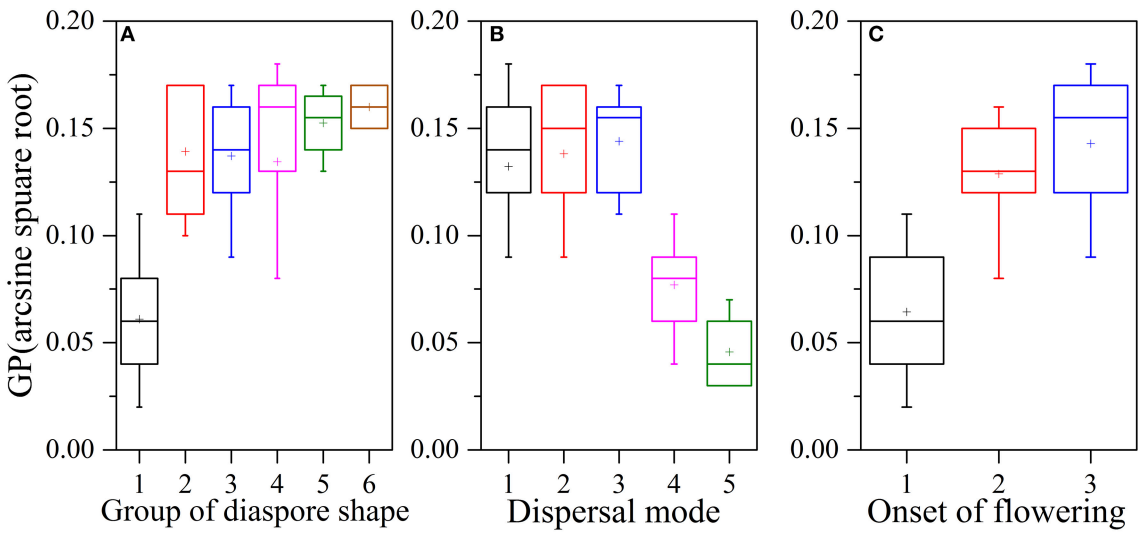

FIGURE 3 | Relationships between seed shape, dispersal type, onset of flowering and arcsine square root-transformed germination percentage. Plots of **(A)** seed shape [(1) spheroid or nearly-spheroid; (2) cylindrical, tubular, or conical; (3) ellipsoid, broad-ellipsoid, narrow-ellipsoid, ovoid, elongate-ovoid, or obovoid; (4) trigonous or prismatic; (5) oblate-discoid, lenticular, or plane; (6) fusiform, acicular, or linear] and GP, **(B)** dispersal type [(1) zoochorous, (2) anemochorous, (3) ombrohydrochorous, (4) barochorous, (5) autochorous] and GP, and **(C)** onset of flowering [(1) early, where flowering begins in May; (2) middle, where flowering begins in June; (3) late, where flowering begins in July and August] and GP. Boxplots showing mean (+), median (−), quartiles, and outliers (–).

dispersal type (4.9%), seed shape (3.1%), onset of flowering (2.4%), and seed mass (2.2%) (**Table 3**).

DISCUSSION

GP in the degraded sandy grassland was affected by phylogeny, seed traits and ecological factors that interacted strongly in a factorial model. **Figure 4** shows a model of the significant correlations found between mean GP and five independent variables analyzed in this study. Phylogenetic relatedness, dispersal type, and seed shape, in that order, were the three factors with the greatest contribution to the factorial model (14% of the variance in GP). Seed mass and onset of flowering combined explained only 4.6% of the variation in GP.

Several studies have advocated using phylogenetic correlates of plant species within a community to explain variance in ecological or other traits among taxa (Miles and Dunham, 1993; Figueroa and Armesto, 2001; Zhang et al., 2004; Norden et al., 2009; Xu et al., 2014). Our results clearly showed that phylogenetic groups significantly influenced GP for the species in the degraded sandy grassland. Previous research showed a phylogenetic pattern of seed germination in plant species of

temperate rain forests (Figueroa, 2003), alpine meadows (Bu et al., 2007; Xu et al., 2014), and arid and semiarid zones (Wang et al., 2009). We think that variation in GP may be closely dependent on phylogeny, i.e., inherent traits of species may play a remarkable role in evolution. In Sheffield, England, germination rates of species in the same families were more similar than those of species in different families (Grime et al., 1981). Germination behavior might be more similar in closely related species than in distantly related species regardless of ecological factors. Our results suggest that phylogeny should be taken into account when investigating the role of natural selection in the regulation of germination or other life history attributes.

Our results indicated that GP was also correlated with seed traits such as shape and mass. The most significant and interesting finding of our study was the significantly negative relationship between GP and seed mass (**Figure 1A**, **Table 1**). This result was inconsistent with other studies that showed that seed mass was significantly positively correlated with GP (Vanmolken et al., 2005; Wu and Du, 2007; Galíndez et al., 2009). Traditionally, large and heavy seeds have better GP (Wu and Du, 2007), emergence, seedling survival, and growth than small seeds because of their larger reserves (Bonfil, 1998), which is consistent with the idea that heavy seed mass confers a competitive advantage, as assumed by the competition-colonization model (Rees et al., 2001). However, our results showed that small-seeded species have a higher GP than large-seeded ones in the degraded sandy grassland. There are several possible interpretations for the contradiction. Firstly, our study contained species with a range of growth forms from a wide range of families, and the species in Asteraceae, Poaceae, and Fabaceae were dominant in this study site. Asteraceae species had a lighter average seed mass (mean mass 1.59 mg; 73% were less than 1.00 mg in our study site) than the average of all species (mean mass 4.90 mg), and they had a higher GP (>80%). Legume seeds, which tend to have a hard seed coat, typically had a greater average seed mass (mean mass 11.30 mg) than species without a hard seed (4.30 mg). Hard seeds often require scarification to breach the test and do not immediately germinate (Smıkal et al., 2014). Secondly, the Horqin sandy land is characterized by Aeolian sandy soils (coarsely textured with a loose structure), and seeds seem to be more easily buried in sand ecosystems than in others. Small seeds may become more deeply buried in open, loose, sandy soil, reducing seed germination. Thirdly, certain small-seeded species in this study, such as *Chloris virgata* Sw., *Euphorbia humifusa* Willd., *Datura stramonium* L., and *Solanum nigrum*, germinate immediately after dispersal ((Liu et al., 2004a), b). Finally, it is well known that environmental factors are crucial from seed germination (Tielbörger and Petrü, 2010; Cendán et al., 2013; Li et al., 2015). Germination strategy is can be considered a coevolutionary strategy to the extreme environmental conditions in the degraded sandy grasslands. Plants in the degraded sandy grassland of Horqin Sandy Land are exposed to a highly disturbed and stressed environment due to overgrazing, wind, and drought. The small-seeded species should have greater seed density, higher germination percentage, and lower seedling survival at

TABLE 1 | Results of one-way ANOVAs showing the effects of phylogenetic group, seed mass, seed shape, dispersal type, adult longevity, life form, onset of flowering, and ecotype on germination percentage among species.

Source of variation	df	*F*	Sig.	R^2
Phylogenetic group	12	3.761	0.000^{***}	0.355
Seed mass	6	3.126	0.008^{**}	0.176
Seed shape	5	26.565	0.000^{***}	0.599
Dispersal type	4	35.526	0.000^{***}	0.612
Onset of flowering	2	70.779	0.000^{***}	0.606
Adult longevity	1	0.564	0.454^{ns}	0.006
Life form	2	0.054	0.947^{ns}	0.001
Ecotype	3	1.733	0.166^{ns}	0.054
Seed color	6	0.839	0.543^{ns}	0.054

df, degrees of freedom; R^2, proportion of GP variance explained by each factor. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{ns}P > 0.05$.

TABLE 2 | Results of two-way ANOVAs showing the independent effects of one of two main factors that have significant effects in one-way ANOVAs, and interaction effects of the pairs of factors on germination percentage.

Source of variation	Effect of A				Effect of B				Effect of A × B			
(A/B)	df	*P*	*F*	R^2	df	*P*	*F*	R^2	df	*P*	*F*	R^2
PG/SM	12	0.008^{*}	2.613	0.274	6	0.163^{ns}	1.609	0.084	23	0.975^{ns}	0.470	0.095
PG/SS	12	0.095^{ns}	1.676	0.085	5	0.000^{***}	17.057	0.360	17	0.752^{ns}	0.738	0.053
PG/DT	12	0.451^{ns}	1.009	0.056	4	0.000^{***}	16.499	0.306	15	0.991^{ns}	0.319	0.022
PG/OF	12	0.351^{ns}	1.131	0.058	2	0.000^{***}	34.036	0.290	15	0.832^{ns}	0.639	0.041
SM/SS	6	0.067^{ns}	2.084	0.049	5	0.000^{***}	18.512	0.362	16	0.103^{ns}	1.567	0.098
SM/DT	6	0.196^{ns}	1.484	0.035	4	0.000^{***}	20.988	0.329	14	0.129^{ns}	1.514	0.083
SM/OF	6	0.062^{ns}	2.104	0.049	2	0.000^{***}	47.329	0.365	9	0.064^{ns}	1.902	0.066
SS/DT	5	0.109^{ns}	1.878	0.037	4	0.033^{*}	2.765	0.044	5	0.299^{ns}	1.239	0.025
SS/OF	5	0.040^{**}	2.454	0.045	2	0.000^{***}	8.864	0.065	5	0.331^{ns}	1.170	0.022
DT/OF	4	0.001^{***}	5.425	0.081	2	0.000^{***}	8.480	0.063	5	0.981^{ns}	0.145	0.003

df, degrees of freedom. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{ns}P > 0.05$. PG, phylogenetic group; SM, seed mass; SS, seed shape; DT, dispersal type; OF, onset of flowering.

TABLE 3 | Factorial ANOVAs for the independent effects of each main factor and their associations.

Source of variation	df	*F*	*P*	R^2
COMPLETE MODEL				
Phylogenetic group	12	1.573	0.122^{ns}	0.058
Seed mass	6	1.212	0.312^{ns}	0.022
Seed shape	5	2.006	0.089^{ns}	0.031
Dispersal type	4	3.946	0.006^{**}	0.049
Onset of flowering	2	3.858	0.026^{*}	0.024
Model	29	8.923	0.000^{***}	0.799
PHYLOGENETIC GROUP REMOVED				
Seed mass	6	1.290	0.272^{ns}	0.026
Seed shape	5	1.761	0.131^{ns}	0.030
Dispersal type	4	2.877	0.028^{*}	0.039
Onset of flowering	2	7.212	0.001^{***}	0.049
Model	17	12.955	0.000^{***}	0.741
SEED MASS REMOVED				
Phylogenetic group	12	1.640	0.100^{ns}	0.062
Seed shape	5	1.744	0.136^{ns}	0.027
Dispersal type	4	3.513	0.011^{*}	0.044
Onset of flowering	2	5.210	0.008^{**}	0.033
Model	23	10.742	0.000^{***}	0.777
SEED SHAPE REMOVED				
Phylogenetic group	12	440.898	0.172^{ns}	0.057
Seed mass	6	1.433	0.465^{ns}	0.019
Dispersal type	4	0.951	0.001^{***}	0.065
Onset of flowering	2	4.922	0.001^{***}	0.050
Model	24	9.669	0.000^{***}	0.768
DISPERSAL TYPE REMOVED				
Phylogenetic group	12	1.112	0.365^{ns}	0.048
Seed mass	6	0.824	0.555^{ns}	0.018
Seed shape	5	2.622	0.031^{*}	0.047
Onset of flowering	2	4.487	0.015^{*}	0.032
Model	25	8.301	0.000^{***}	0.750
ONSET OF FLOWERING REMOVED				
Phylogenetic group	12	2.063	0.032^{*}	0.083
Seed mass	6	1.560	0.173^{ns}	0.031
Seed shape	5	3.436	0.008^{**}	0.058
Dispersal type	4	4.279	0.004^{**}	0.057
Model	27	8.567	0.000^{***}	0.775

For each main factor, R^2 is the proportion of the sum of squares attributed to the main effect. To calculate the proportion of the variance explained by only one of the main factors, the R^2 of the incomplete model with the factor removed was subtracted from the R^2 of the complete model. df, degrees of freedom. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{ns}P > 0.05$.

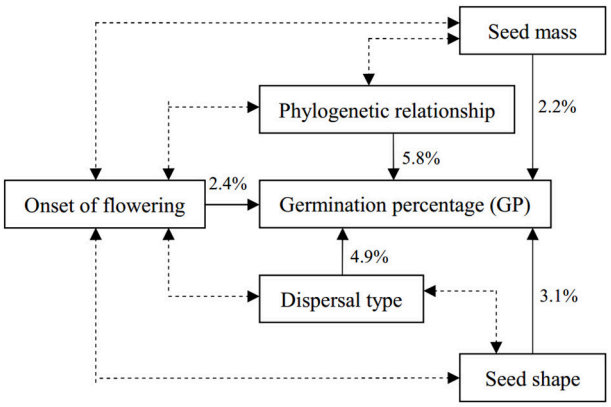

FIGURE 4 | Factorial model of factors explaining variation in GP among species in degraded sandy grassland, considering the smallest number of possible independent effects. Percentages indicate the proportion of the variance explained independently by each factor. Double arrows indicate stronger associations between factors, see text.

potential recruitment sites (Liu et al., 2004a, 2007). The large-seeded species have greater seedling establishment but a lower seed density and germination percentage (Liu et al., 2004a,b; Moles and Westoby, 2004; Li et al., 2006; Liu et al., 2007), even under favorable conditions. Thus, small-seeded species tend to produce large number of light seeds for long-distance dispersal to escape from unfavorable spatial conditions, and large-seeded species tend to be dormant to spread the risk of encountering unfavorable temporal conditions. Similarly, Rees (1996) found that seed germination was negatively related to seed size in a British grassland community with high overgrazing and stated that this was consistent with heavy seeds having better establishment success and suffering higher levels of unfavorable conditions (Rees, 1996).

We found that species with elongate and flat seeds had higher GPs than species with round seeds (**Figure 1B**). Our results indicated that the pattern between seed shape and GP found by Grime et al. (1981) applies to our study region. Interestingly, our study indicated that GP is more strongly correlated with seed shape than with seed mass in the Horqin sandy land (**Figure 1**, **Table 1**). We hypothesize that phylogeny may be responsible for this pattern because seeds of Poaceae and Asteraceae, which are often elongated or flattened, tend to germinate easily (Grime et al., 1981; Liu et al., 2003).

Ecological factors, such as seed dispersal type and onset of flowering, significantly influenced GP and could independently explain 5.9% and 2.4% of the total variance in GP in our study area, respectively (**Tables 1, 3**). Seeds of well-dispersed species germinated more frequently (**Figure 2A**), with anemochorous, zoochorous, and ombrohydrochorous species having higher GPs than either barochorous or autochorous species (**Figure 2A**). These results are consistent with those reported by Bu et al. (2007). One possible interpretation for this relationship between GP and dispersal type is that seed dispersal may be adapted to avoid or minimize sibling competition and place seeds at "safe sites," where they can successfully germinate and establish (Gutterman, 1994; Lu et al., 2013; Bolmgern and Eriksson, 2015). In most germinated seeds, restricted (barochorous) dispersal can lead to competition among the siblings produced by a fecund maternal plant (Cheplick, 1996; Fragoso et al., 2003). The dispersal potential of seeds with different dispersal types varies greatly, with both wind- and vertebrate-dispersed seeds potentially having a greater dispersal distance from the parent than seeds of barochorous and autochorous species. In some vertebrate-dispersed seeds, germination may be promoted by

passage through the animal's digestive system (Navarro et al., 2009). Thus, seeds with long dispersal distances (anemochorous, zoochorous, and ombrohydrochorous species) may germinate more frequently than seeds with short-range unassisted dispersal (which are more likely to experience sibling competition). Early onset of flowering may be correlated with early dispersal and germination, and thus a longer period of juvenile growth. A long juvenile period that includes the unfavorable season increases the risk of mortality before reproduction. These selection pressures involve tradeoffs that may hinder simple, direct effects of onset of flowering on germination. Our results revealed that most of the late-flowering species belong to Poaceae and Asteraceae. These species had a higher GP in the degraded sandy grassland community, which explains why onset of flowering species had a significant effect.

In this study, we found that ecological factors such as ecotype, life form, and adult longevity had insignificant effects on GP. In other words, the difference in GP between woody, graminoid, and forb species, and between annuals and perennials, was not significant. Previous studies have presented conflicting evidence of the relationships between life form, adult longevity, ecotype, and seed germination strategies. For example, Xu et al. (2014) indicated that there were no significant effects of life form (between annual and perennial) on GP in eastern Tibetan plateau species (Xu et al., 2014), while Rees suggested that perennials tended to have higher germination percentage than annuals based on a large grass data set (Rees, 1994). Bu et al. (2008) found life form (woody, graminoid, and forb) and adult longevity (annual and perennial) had a significant effect on seed germination time in eastern Qinghai-Tibet plateau species (Bu et al., 2008). The effects of life form and adult longevity on seed germination might vary by habitat or flora.

In this study, we provided insights into correlations between seed germination and phylogeny, seed traits, and ecological factors in a degraded sandy grassland of Horqin Sandy Land. Our most important finding is that seed germination strategies in this habitat were affected by the interactions of phylogeny, seed traits, and ecological factors. Phylogeny could explain the biggest proportion in variance of GP, which suggests that there are strong phylogenetic constraints on germination. Smaller seeds, elongate and flat seeds, seeds with long dispersal distances (anemochorous, zoochorous, and ombrohydrochorous species), and seeds from late-flowering species had higher GPs. Moreover, we found that phylogeny had a close association and interaction with seed size, shape, and dispersal strategy (**Tables 1–3**). Thus, we suggest that phylogeny may constrain seed germination, and because seed mass, shape, dispersal, and flowering time may be related to phylogeny, they also constrain germination. Therefore, phylogeny and life-history attributes could not be considered separately. Seed germination, like any other trait, is shaped by short-term selective pressures and long-term phylogenetic constraints.

CONCLUSION

In this study of the Horqin Sandy Land, a degraded sandy grassland in northeastern Inner Mongolia, China, we found that (1) GP was negatively and significantly correlated with seed mass, (2) species with elongate and flat seeds had higher GPs than species with round seeds, and (3) phylogenetic relatedness, dispersal type, and seed shape, in that order, were the three factors with the greatest contribution to the complete model (14% of the variance in GP). Furthermore, (1) GP, like any other trait, is shaped both by short-term selective pressures and long-term phylogenetic constraints, and (2) comprehensive studies at the community level that use multivariate approaches are needed to enhance our understanding of the evolutionary and ecological forces shaping germination strategies.

AUTHOR CONTRIBUTIONS

BL designed the research and collected and analyzed the data. ZW analyzed the data and drafted the manuscript. LW and ZL revised English language and contributed to paper writing. QL and YL assisted with data collection. All authors took part in writing the manuscript.

FUNDING

Funding was provided by the Key Project of Chinese National Programs for Fundamental Research and Development (No. 2013CB429903) and National Natural Science Foundation of China (No. 31570448) and the New Century Excellent Talents Program of Fujian Province (K8015053A).

ACKNOWLEDGMENTS

We thank Hongmei Wang and Yongming Luo for field and laboratory assistance. We would like to thank Editage [http://online.editage.cn/] for English language editing.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 25 July 2016
doi: 10.3389/fpls.2016.01026

Habitat Temperature and Precipitation of *Arabidopsis thaliana* Ecotypes Determine the Response of Foliar Vasculature, Photosynthesis, and Transpiration to Growth Temperature

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Acclimatory adjustments of foliar vascular architecture, photosynthetic capacity, and transpiration rate in *Arabidopsis thaliana* ecotypes (Italian, Polish [Col-0], Swedish) were characterized in the context of habitat of origin. Temperatures of the habitat of origin decreased linearly with increasing habitat latitude, but habitat precipitation was greatest in Italy, lowest in Poland, and intermediate in Sweden. Plants of the three ecotypes raised under three different growth temperature regimes (low, moderate, and high) exhibited highest photosynthetic capacities, greatest leaf thickness, highest chlorophyll *a/b* ratio and levels of β-carotene, and greatest levels of wall ingrowths in phloem transfer cells, and, in the Col-0 and Swedish ecotypes, of phloem per minor vein in plants grown at the low temperature. In contrast, vein density and minor vein tracheary to sieve element ratio increased with increasing growth temperature – most strongly in Col-0 and least strongly in the Italian ecotype – and transpirational water loss correlated with vein density and number of tracheary elements per minor vein. Plotting of these vascular features as functions of climatic conditions in the habitat of origin suggested that temperatures during the evolutionary history of the ecotypes determined acclimatory responses of the foliar phloem and photosynthesis to temperature in this winter annual that upregulates photosynthesis in response to lower temperature, whereas the precipitation experienced during the evolutionary history of the ecotypes determined adjustment of foliar vein density, xylem, and transpiration to temperature. In particular, whereas photosynthetic capacity, leaf thickness, and foliar minor vein phloem features increased linearly with increasing latitude and decreasing temperature of the habitats of origin in response to experimental growth at low temperature, transpiration rate, foliar vein density, and minor vein tracheary element numbers and cross-sectional areas increased linearly with decreasing precipitation level in the habitats of origin in response to experimental growth at high temperature. This represents a situation where temperature acclimation of the

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Matthew Paul, Rothamsted Research, UK
Robert Turgeon, Cornell University, USA

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *22 May 2016*
Accepted: *30 June 2016*
Published: *25 July 2016*

Citation:
Adams WW III, Stewart JJ, Cohu CM, Muller O and Demmig-Adams B (2016) Habitat Temperature and Precipitation of Arabidopsis thaliana Ecotypes Determine the Response of Foliar Vasculature, Photosynthesis, and Transpiration to Growth Temperature. Front. Plant Sci. 7:1026. doi: 10.3389/fpls.2016.01026

apparent capacity for water flux through the xylem and transpiration rate in a winter annual responded differently from that of photosynthetic capacity, in contrast to previous reports of strong relationships between hydraulic conductance and photosynthesis in other studies.

Keywords: *Arabidopsis thaliana*, ecotypic variation, foliar vasculature, phloem, photosynthesis, temperature acclimation, transpiration, xylem

INTRODUCTION

Linking natural variation in the genome and in gene expression to phenotypic variability in response to different environmental factors is a goal among researchers working on the model species *Arabidopsis thaliana* (Alonso-Blanco and Koornneef, 2000; Koornneef et al., 2004; Tonsor et al., 2005; Alonso-Blanco et al., 2009; Koornneef and Meinke, 2010; El-Soda et al., 2014; Provart et al., 2016). Clinal variation in the genome, growth parameters, multiple life history traits, and responses to, and tolerance of, low (freezing and chilling) and high temperature, drought, and irradiance (ultraviolet, visible, and far-red) in this species has been documented in numerous studies and typically presented in the context of a geographic gradient in one or more environmental factors (e.g., temperature, photoperiod, precipitation). Altitude has been the primary geographic variable in some studies (Ward and Strain, 1997; Montesinos-Navarro et al., 2011, 2012; Picó, 2012; Wolfe and Tonsor, 2014; Botto, 2015; Luo et al., 2015; Singh et al., 2015; Vidigal et al., 2016), whereas a combination of altitude and distance from the ocean was examined in a few others (Montesinos et al., 2009; Lewandowska-Sabat et al., 2012; Kang et al., 2013). Variation (especially genetic) along longitudinal gradients has also been considered in a number of studies (Schmuths et al., 2004; Beck et al., 2008; Reininga et al., 2009; Panthee et al., 2011; Samis et al., 2012; Zuther et al., 2012; Brachi et al., 2015), and edaphic, biotic, and/or environmental factors were shown to correlate with genomic variation and life history traits in several studies (Lasky et al., 2012; Brachi et al., 2013; Manzano-Piedras et al., 2014). The vast majority of studies focused on variation in traits that vary with latitude of origin (Li et al., 1998; Stenøien et al., 2002; Caicedo et al., 2004; Stinchcombe et al., 2004, 2005; Alonso-Blanco et al., 2005; Hannah et al., 2006; Li et al., 2006; Hopkins et al., 2008; Kipp, 2008; Tonsor et al., 2008; Zhen and Ungerer, 2008; Jansen et al., 2010; Chiang et al., 2011; Nägele et al., 2011, 2012; Zuther et al., 2012; Barah et al., 2013; Debieu et al., 2013; Distelbarth et al., 2013; Kurbidaeva et al., 2015), most likely because both photoperiod and temperature vary extensively along north-south transects.

Two European populations of *A. thaliana*, separated by 2298 km of latitudinal distance, have been the focus of collaboration among multiple laboratories. First described by Ågren and Schemske (2012), the southern ecotype stems from Castelnuovo di Porto (42°07′ north latitude) in central Italy and the northern ecotype from Rödåsen (62°48′ north latitude) in central Sweden (**Figure 1**). In an initial 5-year characterization in the field (Ågren and Schemske, 2012), reciprocal transplants established strong local adaptation through differences in fitness (survival, fecundity) between the two ecotypes in each site. Subsequent studies using recombinant inbred lines of the two parental lines examined some of the underlying genetics responsible for those fitness tradeoffs and other life history traits (Ågren et al., 2013; Dittmar et al., 2014; Oakley et al., 2014; Postma and Ågren, 2015). Quantitative trait locus mapping was used to identify genes underlying flowering in the two populations under both vernalizing and non-vernalizing conditions (Grillo et al., 2013) and additional insights into adaptive and maladaptive dominant and recessive genes were gained through crosses and backcrosses using the two parental lines (Oakley et al., 2015). Furthermore, the impact of leaf damage (Akiyama and Ågren, 2012) and germination timing (Akiyama and Ågren, 2014) on fitness was evaluated in the Swedish ecotype in the field. Moreover, the Swedish ecotype is relatively tolerant of freezing stress, whereas the Italian ecotype, with a non-functional C-repeat binding factor 2 (part of the CBF family of transcription factors that play a prominent role in acclimation to low temperature), is much less tolerant of freezing (Gehan et al., 2015). This latter finding led to a wider exploration of the role of CBF modification in the acclimation of multiple *A. thaliana* ecotypes to warmer climates (Monroe et al., 2016).

From a functional standpoint, both ecotypes were shown to exhibit acclimation to experimental growth temperature and light environment, with more pronounced acclimatory adjustments in chloroplast pigments and tocopherols, photosynthesis, plant and leaf structure, and foliar vasculature (particularly the phloem) in the Swedish compared to the Italian ecotype (Cohu et al., 2013a,b, 2014a; Adams et al., 2014a; Stewart et al., 2015, 2016). The current study was undertaken to place acclimatory adjustments in leaf architecture (and especially foliar vascular features), photosynthetic capacity, and transpiration in response to growth under controlled experimental conditions in growth chambers at three different temperatures (low, moderate, and high) in the context of ecotypic latitude of origin and the environmental conditions prevailing at each respective site of origin. Furthermore, these ecotypes were used to illuminate relationships among various vascular metrics. In addition to the northern and southern European ecotypes from Sweden and Italy, the widely used Columbia (Col-0) line was included as an ecotype from a latitude intermediate to those of the Swedish and Italian ecotypes. Derived from seed collected in the Landsberg an der Warthe region (outside the current city of Gorzów Wielkopolski in Poland; Rédei, 1992; Koornneef and Meinke, 2010; latitude of 52°42′ N from El-Lithy et al., 2004), the Col-0 ecotype stems from a site almost equidistant in latitude between the sites from which the Swedish and Italian ecotypes originate

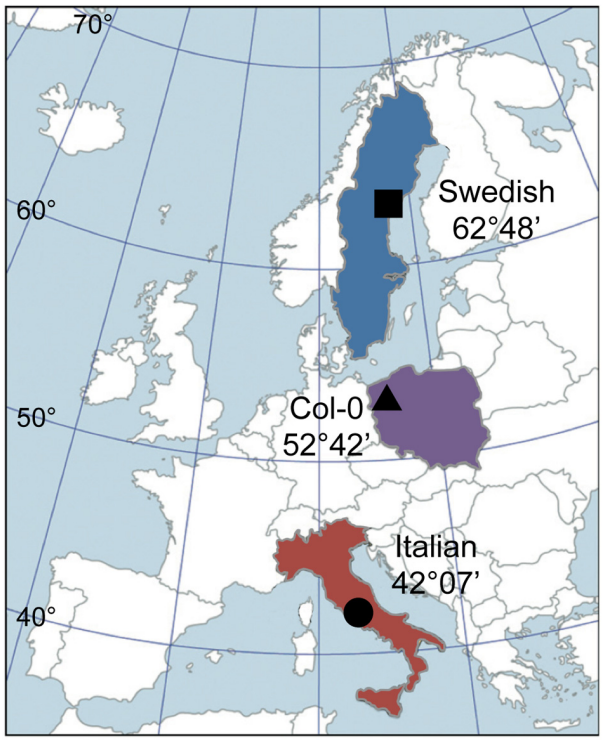

FIGURE 1 | Map showing the locations and latitudes from which the three ecotypes of *Arabidopsis thaliana* employed in this study originated. Squares (Swedish ecotype), triangles (Col-0), and circles (Italian ecotype) are used to designate each ecotype in all of the following figures, and the colors are used to illustrate each habitat/ecotype in **Figures 2**, **3**, **5**, and **10**. Redrawn after Ågren and Schemske (2012).

(**Figure 1**); there are 1176 km of latitude between the Italian and Polish sites and 1122 km of latitude between the Polish and Swedish sites. All three habitats of origin are low altitude sites near major bodies of water: the Swedish site is just over 80 m above sea level and less than a kilometer from the Gulf of Bothnia (Baltic Sea), the Polish site is just short of 60 m above sea level and approximately 160 km from the Baltic Sea, and the Italian site is approximately 225 m above sea level and 35 km from the Tyrrhenian Sea (Mediterranean Sea). The symbols pinpointing the location of the sites of origin (a circle for the Italian site, a triangle for the Polish site, and a square for the Swedish site; **Figure 1**) are used to designate the ecotypes originating from these three sites in all subsequent figures. In addition, the colors used to identify the countries of origin (red for Italy, purple for Poland, and blue for Sweden) are also used to identify the three sites/populations in **Figures 2**, **3**, **5**, and **10**.

These three ecotypes of *A. thaliana* thus allow for characterization of phenotypic plasticity that has presumably evolved in response to conditions experienced by these distinct populations from the three sites – with otherwise remarkably similar features – along an extensive latitudinal gradient. In order to evaluate the acclimation potential within each ecotype, plants were grown in growth chambers at different temperatures spanning more than 20°C. Given the many generations over which the Col-0 line has been maintained under controlled laboratory conditions since its original collection in 1955 (Rédei, 1992), there was a possibility that this ecotype would no longer exhibit traits aligned with its habitat of origin. However, based on the results of the present study, this does not seem to be the case for the evaluated features.

MATERIALS AND METHODS

Plant Material, Climatological Information, and Growth Conditions

Three ecotypes of *Arabidopsis thaliana* (L.) Heynhold were investigated. Seed of Col-0 was obtained from *The Arabidopsis Information Resource*[1] for comparison with the Swedish and Italian ecotypes (see Stewart et al., 2015 for a detailed description of the latter two ecotypes). Climatological information on the habitats from which the three ecotypes originated was obtained from http://www.worldclim.org/ (Hijmans et al., 2005) and the photoperiods prevailing in each site were estimated from the US Naval Observatory's website[2], all of which are summarized in **Figures 2** and **3**. Plants were grown in 2.8 l pots (one plant per pot) containing Canadian Growing Mix 2 (Conrad Fafard Inc., Agawam, MA, USA) under controlled conditions in growth chambers (Conviron E-15, Winnipeg, Canada), receiving water daily and nutrients every other day. Multiple plants of each ecotype were grown under three independent day/night air temperature regimes of 8°C/12.5°C (low or cool), 25°C/20°C (moderate), and 35°C/25°C (high or hot) under a common photon flux density of 400 μmol m^{-2} s^{-1} (provided by a mixture of cool white fluorescent bulbs and incandescent bulbs) during a 9 h photoperiod as described in Cohu et al. (2013b) and Stewart et al. (2016). Through evaluation of the response of some of these metrics to other combinations of temperature and light, it was determined that the greatest impact of temperature on the metrics examined could be seen at this intermediate light intensity (a higher growth light intensity resulted in saturation of many of the responses so that the differential impact of temperature was less distinguishable; see Cohu et al., 2013b). The experimental design thus consisted of three ecotypes by three growth temperature regimes, although the majority of data presented focus on the low and high temperature regimes (three ecotypes by two growth temperature regimes). In addition to evaluating the various metrics in relationship to each other, some metrics were also evaluated in relationship to the latitude of ecotypic origin as well as the temperatures and precipitation experienced by each ecotype in its natural habitat. The radiative heating experienced by the plants when lights were on in the growth chambers resulted in leaf temperatures (determined with a fine thermocouple; Wescor TH-65 meter, Logan, UT, USA) of 14°C, 28°C, and 36°C during the photoperiods. These daytime leaf temperatures are used to identify the three temperature regimes throughout the manuscript and are also used as the independent variable in **Figure 7**. Fully expanded leaves from

[1]https://www.arabidopsis.org

[2]http://aa.usno.navy.mil/data/docs/Dur_OneYear.php

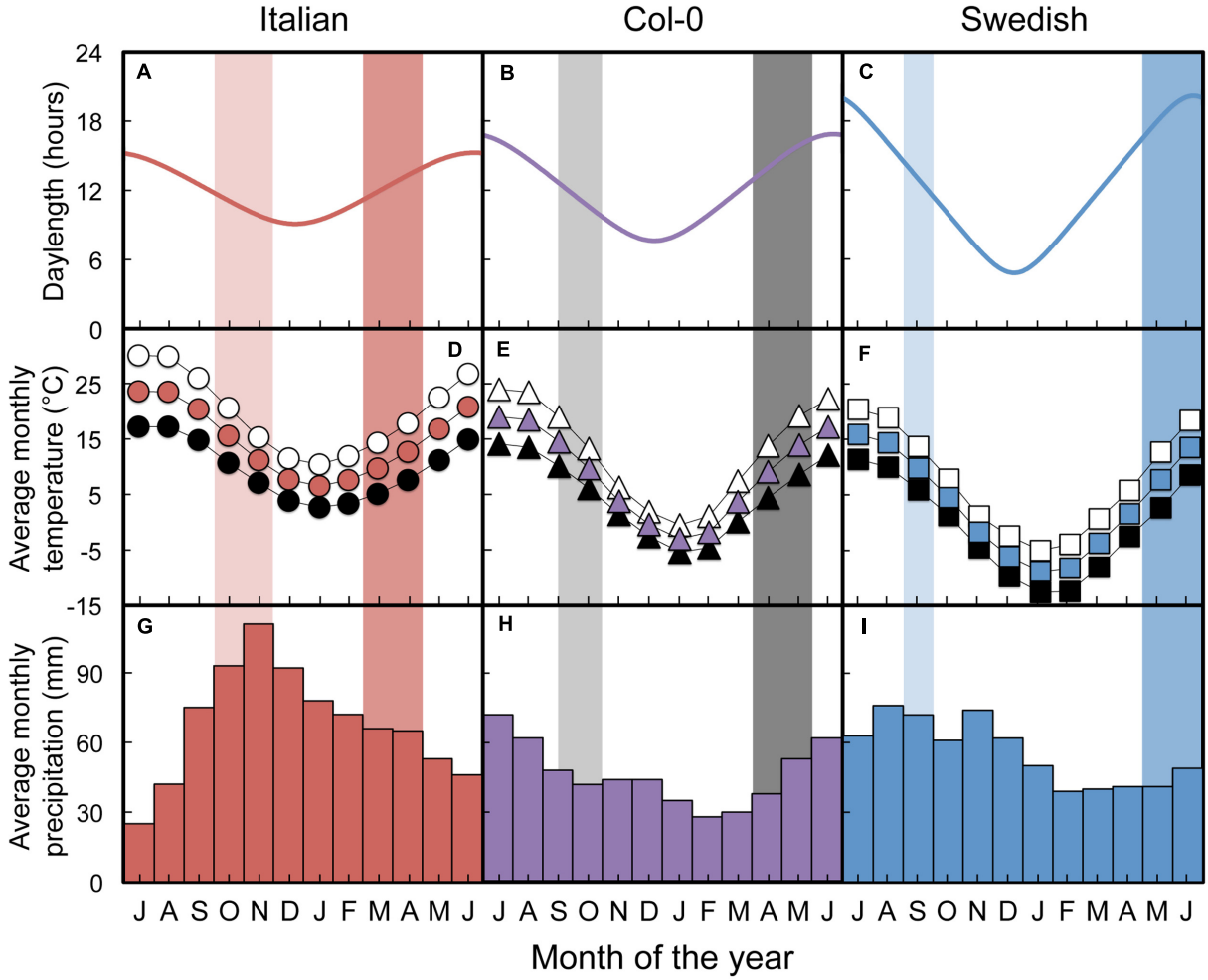

FIGURE 2 | (A–C) Photoperiod, (D–F) average monthly high temperature (open symbols), average monthly temperature (colored symbols), and average monthly low temperature (black symbols), and (G–I) average monthly precipitation for the sites from which the Italian (A,D,G), Col-0 (B,E,H), and Swedish (C,F,I) *A. thaliana* ecotypes were obtained. The periods of germination (light shade) and bolting through flowering (moderate shade) are also shown behind the data across all three panels for each habitat based upon observations reported by Grillo et al. (2013) for the Italian and Swedish ecotypes. Each year starts with the month of July and ends with the month of June.

three to five non-flowering plants were evaluated for each ecotype and growth temperature.

Leaf Metrics

Vein density (mm vein length per mm^2 leaf area), vein architectural features (phloem parenchyma [transfer] cell wall ingrowths and numbers and cross-sectional areas of the different xylem and phloem cells), leaf thickness, pigment levels (high pressure liquid chromatography; Shimadzu Corporation, Kyoto, Japan), photosynthetic capacity (light- and CO_2-saturated rate of photosynthetic oxygen evolution at 25°C; Hansatech oxygen electrode systems, King's Lynn, Norfolk, UK), and transpiration rate (LCi Portable Photosynthesis System, ADC Bioscientific Ltd., Hoddesdon, Herts, England, UK) during exposure to the growth photon flux density of 400 μmol m^{-2} s^{-1} at a leaf temperature of 28.7 ± 1.6°C (mean ± SD; $n = 29$) and a vapor pressure deficit of 2.74 ± 0.07 kPa (mean ± SD; $n = 29$) were determined as described in Amiard et al. (2005), Dumlao et al. (2012), Cohu et al. (2013a,b), Adams et al. (2014a), and Stewart et al. (2015, 2016). Vein metric analysis was limited to the minor veins (3rd and 4th order veins) as described in detail in Cohu et al. (2013a,b). Photosynthetic oxygen evolution capacity in a water-saturated atmosphere was utilized instead of CO_2 exchange because the former is unaffected by multiple resistances to CO_2 diffusion (e.g., stomatal, cuticular, mesophyll, chloroplastic) into the sites of carboxylation (Delieu and Walker, 1981), thus providing a measure of maximal electron transport capacity to match the various vascular metrics that serve as proxies for the capacity to load and export sugars from the leaves. The moderate conditions chosen for determination of transpiration rate proved to be sufficient to elucidate the variation present among the ecotypes that developed under the different growth temperature regimes without inducing stomatal closure that might result from employment of a higher light intensity, higher temperature, or

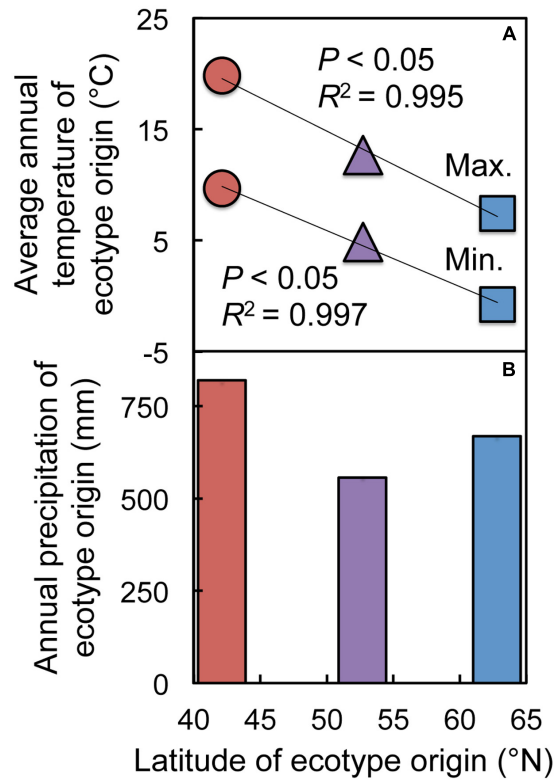

FIGURE 3 | Average annual (A) maximum and minimum temperatures and (B) precipitation for the sites from which the Italian (red circles and column), Col-0 (purple triangles and column), and Swedish (blue squares and column) *A. thaliana* ecotypes were obtained.

greater vapor pressure difference between the leaves and the atmosphere. Some of the data presented are derived from the experiments described in Cohu et al. (2013a,b), Adams et al. (2014a), and Stewart et al. (2016), although many of the metrics have not been evaluated previously (e.g., leaf thickness, pigment data) or comprehensively across all three ecotypes in the context of latitude and habitat environmental conditions, and data from Col-0 grown under high temperature were not included in any of the previous studies. Vein densities (n = 3 to 4 plants), photosynthetic capacities (n = 3 to 4 plants), transpiration rates (n = 4 to 5 plants), and pigment data (n = 3 to 4 plants) are presented as mean values ± SD (with the exception that **Figure 8H** represents a scatter plot of all data points), whereas leaf thickness (three cross-sections from each of 3 to 4 plants) and all of the vein architectural features (n = 7 to 10 minor veins per plant from 3 to 4 plants) are presented as mean values ± SE. With the exception of **Figures 5** and **10** that depict the calculated difference between metrics from plants grown under the low compared to the high temperature regimes and utilize the country of origin color coding (see **Figure 1**), the symbols in **Figures 4–12** are color coded according to the foliar chlorophyll *a*/*b* ratio and resulting subtle differences in visual appearance of the plants grown under the different temperature regimes (**Figure 7H**): blue-green (highest chlorophyll *a*/*b* ratio) for the low growth temperature regime, green (intermediate chlorophyll *a*/*b* ratio) for the intermediate growth temperature regime, and olive-green (lowest chlorophyll *a*/*b* ratio) for the high growth temperature regime.

Statistical Analyses

Data were subjected to linear regression analysis and two-way analysis of variance for comparison of multiple means to discriminate between the effects of growth temperature, ecotype, and response of the ecotypes to temperature using Pro 11.0.1 JMP software (SAS Institute Inc., Cary, NC, USA).

RESULTS

In their respective original habitats, the Italian ecotype experiences the smallest annual variation in photoperiod (from just over 9 h of daylight on the winter solstice to about 14 h at the end of seed set in April) during an annual life cycle, whereas the Swedish ecotype experiences photoperiods ranging from just under 5 h in December to 20 h at the end of June (**Figures 2A–C**). We estimate that the Col-0 ecotype germinates from mid-September through October, overwinters as a rosette, and flowers and sets seed in April and May, and thus experiences photoperiods intermediate to those of the Italian and Swedish ecotypes ranging from about 7.6 h at winter solstice to close to 17 h at the end of its life cycle. The range of temperatures experienced by each ecotype during the growing season in its native habitat increases with increasing latitude, from a differential between mean monthly minima and maxima of 18°C (2.7°C to 20.6°C) at the Italian site, to 24.7°C (−5.0°C to 19.7°C over the estimated growing period) at the Polish site, and 31°C (−12.6°C to 18.4°C) at the Swedish site (**Figures 2D–F**). Average annual temperature maxima and minima in the three sites vary linearly with latitude (**Figure 3A**). Precipitation patterns, on the other hand, do not follow a latitudinal gradient (**Figures 2G–I** and **3B**), but nonetheless present a range of variation among the habitats of origin: during the respective growing seasons, the habitat of the Italian ecotype receives the most precipitation (577 mm over seven months), followed by the Swedish site (529 mm over 10 months), with the Polish site receiving the least precipitation (362 mm over the estimated growing period of nine months).

Photosynthetic capacity (**Figures 4A,B**) and leaf thickness (**Figures 4C,D**) of the three ecotypes grown in climate-controlled growth chambers under two different experimental temperature regimes increased linearly with either increasing latitude of origin (**Figures 4A,C**) or decreasing average annual temperature in each respective habitat of origin (**Figures 4B,D**), particularly for cool-grown leaves. These linear increases in photosynthetic capacity and leaf thickness for the cool-grown leaves were paralleled by linear increases in several features of the foliar phloem, including the cross-sectional area per minor vein of the sugar-loading and -exporting sieve elements (**Figures 4E,F**), the number of phloem cells per minor vein that facilitate sucrose loading into sieve elements (**Figures 4G,H**), and increases in cell membrane length (and presumably membrane area) resulting from invagination of

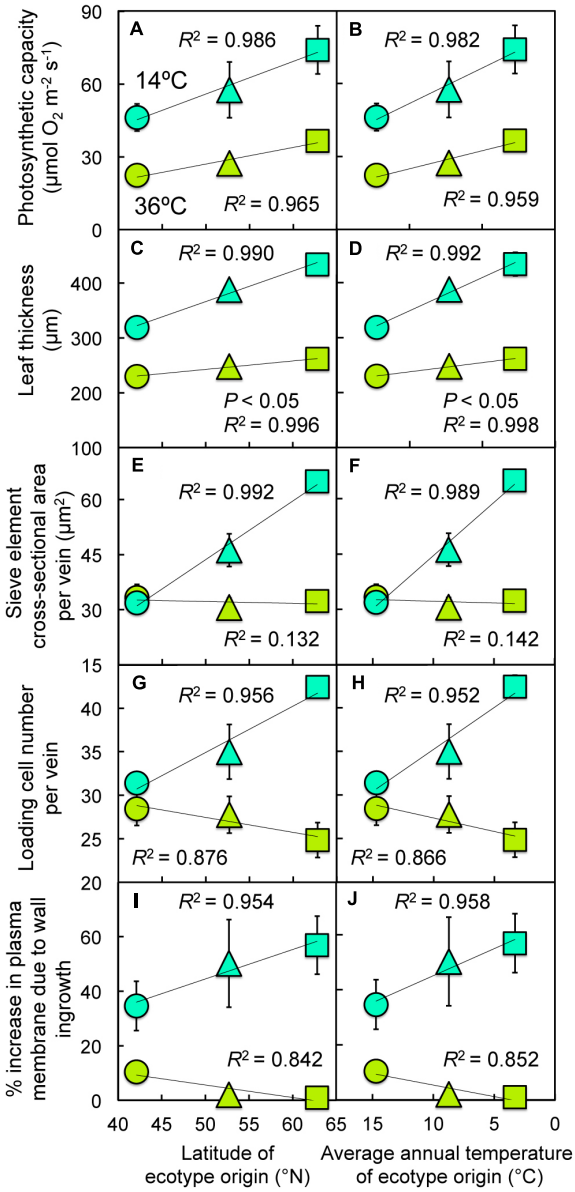

FIGURE 4 | Relationship between (A,B) photosynthetic capacity (light- and CO_2-saturated rate of photosynthetic oxygen evolution), (C,D) leaf thickness, (E,F) cross-sectional sieve element area per minor vein, (G,H) number of cells (phloem parenchyma, companion, and sieve element) contributing to the loading of sucrose per minor vein, or (I,J) the estimated percent increase in cell membrane length due to wall ingrowth in minor vein phloem transfer cells and (A,C,E,G,I) latitude of ecotype origin or (B,D,F,H,J) average annual temperature at the site from which each ecotype was obtained for leaves of Italian (circles), Col-0 (triangles), and Swedish (squares) *A. thaliana* ecotypes grown at cool (14°C = blue-green symbols) or hot (36°C = olive-green symbols) leaf temperature. Colors inspired by the relative foliar level of chlorophyll *a* to chlorophyll *b* under each growth temperature regime (see **Figure 7H**).

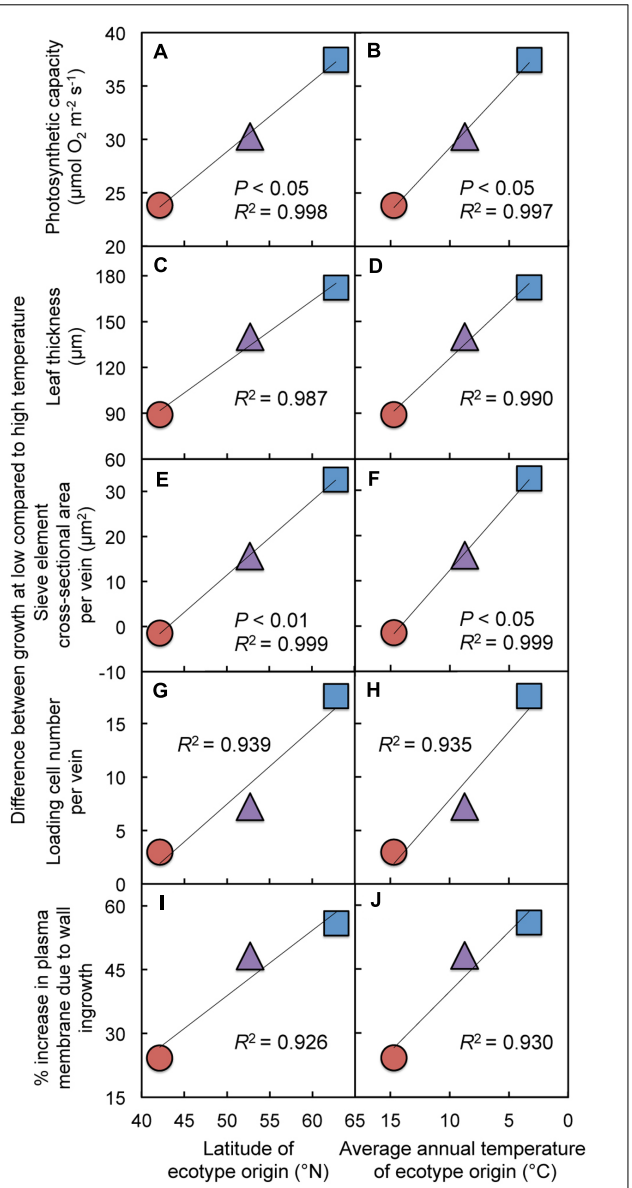

FIGURE 5 | Differences between growth at high (36°C) compared to low (14°C) temperature in (A,B) photosynthetic capacity, (C,D) leaf thickness, (E,F) sieve element cross-sectional area per minor vein, (G,H) number of cells (phloem parenchyma, companion, and sieve element) contributing to the loading of sucrose per minor vein, and (I,J) the estimated percent increase in cell membrane length due to wall ingrowths in minor vein phloem transfer cells for leaves of Italian (red circles), Col-0 (purple triangles), and Swedish (blue squares) *A. thaliana* ecotypes as a function of (A,C,E,G,I) the latitude of ecotype origin or (B,D,F,H,J) average annual temperature at the site from which each ecotype was obtained.

phloem parenchyma cell walls (**Figures 4I,J**). In contrast to the responses of cool-grown leaves, hot-grown leaves did not display such increases in phloem features (**Figures 4E–J**). There were also significant ecotypic differences in photosynthetic capacity, leaf thickness, and sieve element cross-sectional area per vein, significant ecotype × temperature interactions for the latter two parameters as well as for the number of loading cells per minor vein, and all of these features varied significantly with growth temperature in controlled growth chambers (**Table 1**).

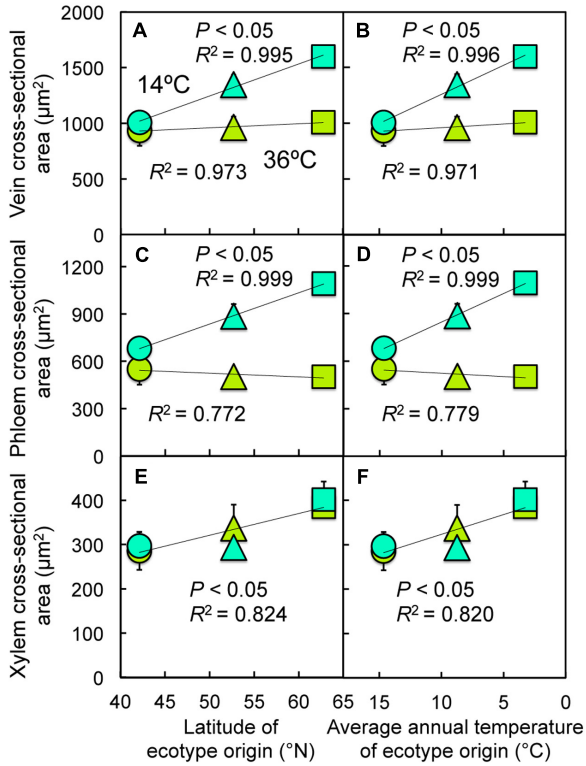

FIGURE 6 | Relationship between (A,B) cross-sectional area per minor vein, (C,D) cross-sectional area of phloem per minor vein, or (E,F) cross-sectional area of xylem per minor vein and (A,C,E) latitude of ecotype origin or (B,D,F) average annual temperature at the site from which each ecotype was obtained for leaves of Italian (circles), Col-0 (triangles), and Swedish (squares) *A. thaliana* ecotypes grown at cool (14°C = blue-green symbols) or hot (36°C = olive-green symbols) leaf temperature.

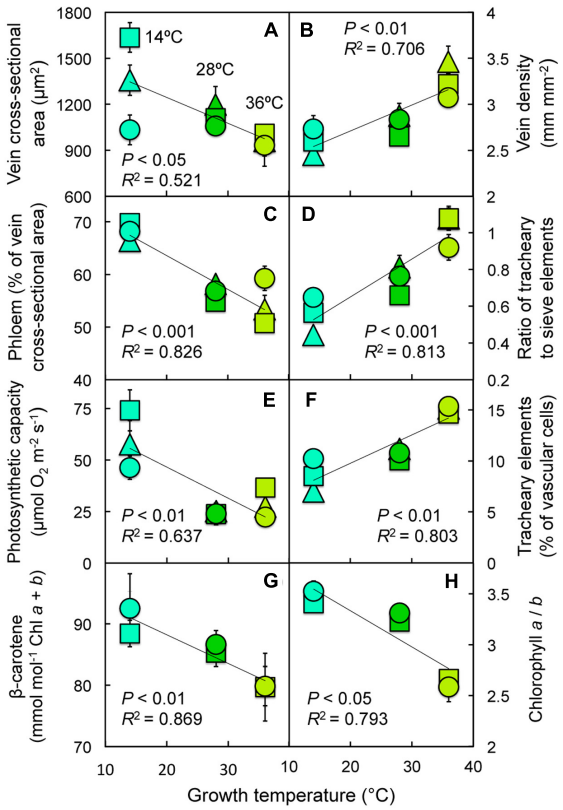

FIGURE 7 | Relationship between (A) cross-sectional area per minor vein, (B) minor vein density, (C) the fraction of minor vein area comprised of phloem, (D) the minor vein ratio of tracheary to sieve elements, (E) photosynthetic capacity, (F) the fraction of minor veins comprised of tracheary elements, (G) the level of β-carotene relative to chlorophyll, or (H) the ratio of chlorophyll *a* to *b* and growth temperature during leaf development for leaves of Italian (circles), Col-0 (triangles), and Swedish (squares) *A. thaliana* ecotypes grown at cool (14°C = blue-green symbols), moderate (28°C = green symbols), or hot (36°C = olive-green symbols) leaf temperature.

When expressed as the difference between hot-grown and cool-grown leaves (as a measure of the degree of responsiveness to experimental growth temperature under controlled conditions), photosynthetic capacity, leaf thickness, and minor vein phloem metrics exhibited linear increases with increasing latitude and also with decreasing temperature in the habitat of origin (**Figure 5**). While linear regressions for photosynthetic capacity (**Figures 5A,B**) and cross-sectional area of sieve elements comprising the minor veins (**Figures 5E,F**) with latitude or average annual temperature at ecotype origin were significant, the phloem features of phloem cell numbers and cell wall ingrowth levels, together serving as proxies for total phloem cell membrane area, were not. In particular, leaves of Col-0 exhibited levels of phloem transfer cell wall ingrowths that fell slightly below the line (**Figures 5I,J**) and numbers of phloem loading cells (**Figures 5G,H**) that fell slightly above the line; it is thus attractive to speculate that these two features together do effect a level of total phloem cell membrane area adjustment in the Col-0 ecotype in line with that of the other two ecotypes.

Foliar minor vein cross-sectional area in (experimentally) cool-grown leaves increased linearly with increasing latitude (**Figure 6A**) and decreasing annual temperature in the respective habitats of origin (**Figure 6B**), and this was due to increases in the cross-sectional areas of both the phloem (**Figures 6C,D**) and xylem (**Figures 6E,F**). Both of the latter metrics varied significantly as a result of growth temperature, while minor vein cross-sectional area also varied significantly among ecotypes and the phloem cross-sectional area showed significant ecotype × temperature interactions (**Table 1**). However, for (experimentally) hot-grown leaves, only the cross-sectional area of the xylem increased with increasing latitude (**Figure 6E**) and decreasing annual temperature (**Figure 6F**) in the respective habitats of origin; these latter trends did not differ between (experimentally) cool- and hot-grown leaves, but there was a significant difference in xylem cross-sectional area among the ecotypes (**Table 1**).

Furthermore, a number of foliar features also exhibited significant, linear relationships with temperature during (experimental) plant growth across ecotypes (**Figure 7**; **Table 1**),

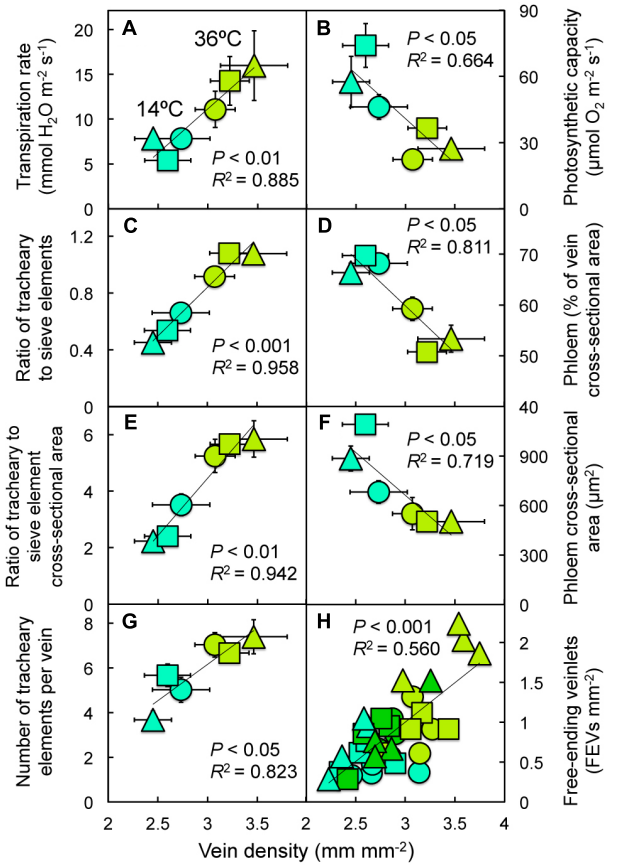

FIGURE 8 | (A) Transpiration rate, (B) photosynthetic capacity, (C) the minor vein ratio of tracheary to sieve elements, (D) the fraction of minor vein area comprised of phloem, (E) the minor vein ratio of tracheary to sieve element cross-sectional area, (F) the cross-sectional area of minor veins comprised of phloem, (G) the number of tracheary elements per minor vein, and (H) the number of free-ending veinlets per unit leaf area for leaves of Italian (circles), Col-0 (triangles), and Swedish (squares) *A. thaliana* ecotypes grown at cool (14°C = blue-green symbols) or hot (36°C = olive-green symbols) and, for (H), moderate (28°C = green) leaf temperature as a function of minor vein density.

with some features exhibiting positive and others negative correlations. While cross-sectional area of the minor veins decreased with increasing growth temperature under controlled experimental conditions (**Figure 7A**), due in large part to decreases in phloem tissue (**Figure 7C**), vein density (minor vein length per leaf area) increased with increasing growth temperature (**Figure 7B**), as did the ratio of tracheary to sieve elements in minor veins (**Figure 7D**) and the fraction of vascular cells per minor vein represented by tracheary elements (**Figure 7F**). Three additional parameters decreased with increasing growth temperature: photosynthetic capacity (**Figure 7E**), β-carotene levels (**Figure 7G**), and chlorophyll *a*/*b* ratio (**Figure 7H**). In addition to (experimental) growth temperature having a highly significant impact on all of these features, the fraction of vascular cells comprised of tracheary elements was significantly different among ecotypes, and vein density and the minor vein ratio of tracheary to sieve elements exhibited significant ecotype × temperature interactions (**Table 1**).

When evaluated under a common set of experimental conditions, transpiration rate was significantly influenced by the temperature of the plants during growth, and also displayed significant ecotype × temperature interactions (**Table 1**). When combining data for leaves of the three ecotypes grown under either low or high temperature in controlled conditions, transpiration rate was found to increase with increasing vein density (**Figure 8A**), as did several minor vein xylem features, including ratios of either the numbers (**Figure 8C**) or the cross-sectional areas of tracheary to sieve elements (**Figure 8E**), and the number of tracheary elements per minor vein (**Figure 8G**), all of which were significantly influenced by (experimental) growth temperature (**Table 1**). On the other hand, photosynthetic capacity (**Figure 8B**), the fraction of minor veins occupied by phloem (**Figure 8D**), and the cross-sectional area of minor vein phloem (**Figure 8F**) decreased linearly with increasing vein density. One vascular feature that may contribute to the variation in foliar vein density is the level of free-ending veinlets, of which the greatest range of phenotypic plasticity (greatest difference between cool- and hot-grown leaves) was seen in the Col-0 ecotype (**Figure 8H**) and that also varied significantly among ecotypes, with (experimental) growth temperature, and with ecotype × temperature interactions (**Table 1**). One intriguing feature of these relationships is that, for vein density as well as the ratios of tracheary to sieve element number and area (**Figures 8C,E**), the Italian ecotype (circles) exhibited the smallest difference between (experimentally) cool- and hot-grown leaves, the Swedish ecotype (squares) exhibited an intermediate difference, and the Col-0 ecotype (triangles) exhibited the greatest difference.

In contrast to photosynthetic capacity and foliar minor vein phloem features of plants grown under controlled conditions, which varied with latitudinal and temperature gradients between the habitats of origin (**Figures 4–6C,D**), transpiration rate, foliar minor vein density, and the minor vein ratio of tracheary to sieve element numbers and cross-sectional areas of plants grown under controlled conditions did not vary regularly with latitude or average temperatures for the respective sites from which each ecotype originated (relationships not shown). Therefore, these latter parameters were instead evaluated for a possible association with variation in precipitation levels among the three sites of origin. For (experimentally) hot-grown leaves of the three ecotypes, transpiration rate (**Figures 9A,B**), vein density (**Figures 9C,D**), and tracheary to sieve element ratios (**Figures 9E–H**) exhibited trends for decreases with increasing levels of habitat-of-origin precipitation, whereas for (experimentally) cool-grown leaves all three vascular features exhibited trends for increases with increasing levels of habitat-of-origin precipitation level (**Figures 9C–H**). Moreover, the extent of phenotypic plasticity (difference between cool- and hot-grown leaves) of the latter three vascular metrics was *negatively* correlated with average precipitation (annual and monthly during the growing season) in the respective habitats of origin

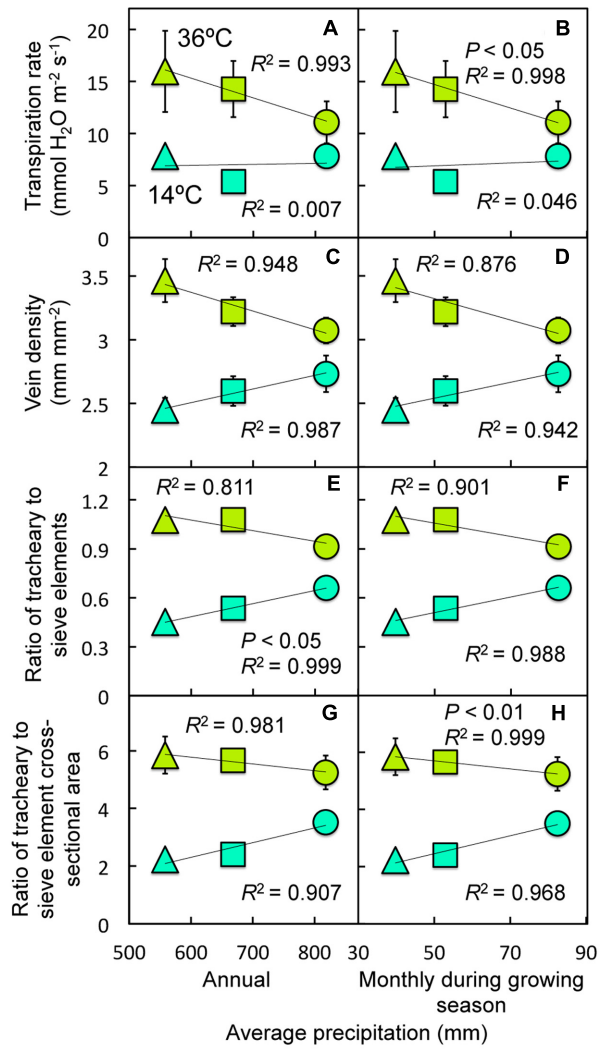

FIGURE 9 | (A,B) Transpiration rate, (C,D) minor vein density, (E,F) the ratio of minor vein tracheary to sieve elements, and (G,H) the ratio of minor vein tracheary to sieve element cross-sectional area for leaves of Italian (circles), Col-0 (triangles), and Swedish (squares) *A. thaliana* ecotypes grown at cool (14°C = blue-green symbols) or hot (36°C = olive-green symbols) leaf temperature as a function of the average (A,C,E,G) annual precipitation and (B,D,F,H) monthly precipitation during the growing season at the site from which each ecotype was obtained.

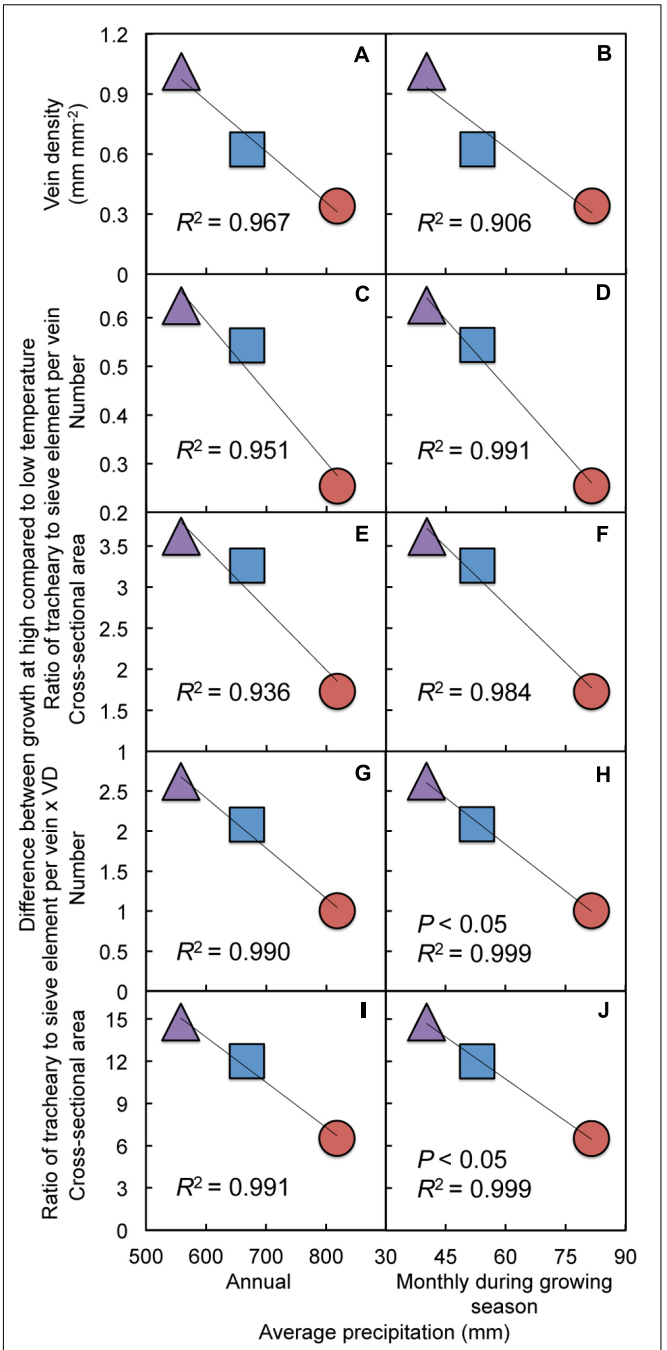

FIGURE 10 | Differences between growth at high (36°C) compared to low (14°C) temperature in (A,B) minor vein density, (C,D) the minor vein ratio of tracheary to sieve elements, (E,F) the minor vein ratio of tracheary to sieve element cross-sectional area, (G,H) the product of the difference in vein density (VD) and the difference in the ratio of tracheary to sieve elements in minor veins, and (I,J) the product of the difference in vein density (VD) and the difference in the ratio of tracheary to sieve element cross-sectional area in minor veins for leaves of Italian (red circles), Col-0 (purple triangles), and Swedish (blue squares) *A. thaliana* ecotypes as a function of the average (A,C,E,G,I) annual precipitation and (B,D,F,H,J) monthly precipitation during the growing season at the site from which each ecotype was obtained.

(**Figures 10A–F**). Intriguingly, when normalized for differences in vein density – by multiplying the difference in vascular cell numbers or cross-sectional area by the difference in vein density (VD) – the correlations between average precipitation in the respective habitats of origin and the extent of the differences in tracheary to sieve element ratios between (experimentally) cool- and hot-grown leaves became stronger yet (**Figures 10G–J**) and significant for average monthly precipitation during the growing season in the habitat of origin (**Figures 10H,J**).

While the capacity for photosynthetic oxygen evolution exhibited *negative* linear relationships with the minor veins' ratio

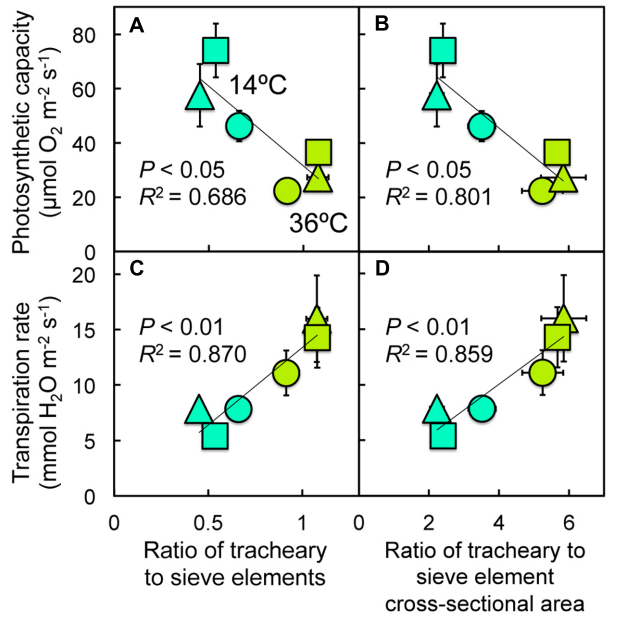

FIGURE 11 | Relationship between (A,B) photosynthetic capacity or (C,D) transpiration rate and the ratio of (A,C) tracheary to sieve elements per minor vein or (B,D) tracheary to sieve element cross-sectional area per minor vein for leaves of Italian (circles), Col-0 (triangles), and Swedish (squares) *A. thaliana* ecotypes grown at cool (14°C = blue-green symbols) or hot (36°C = olive-green symbols) leaf temperature.

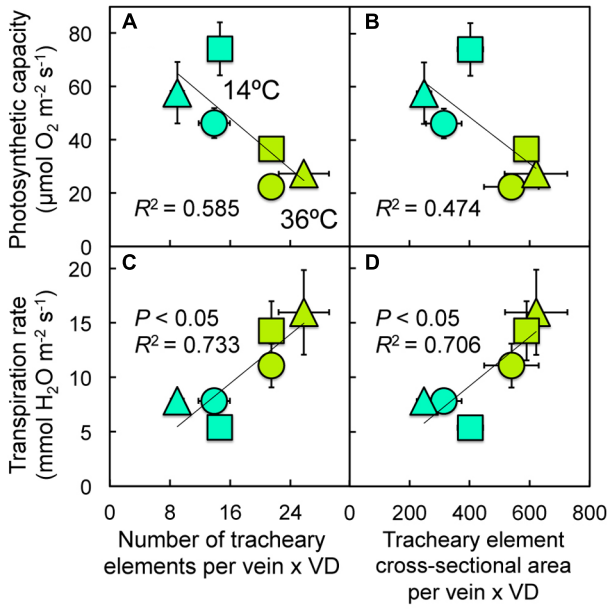

FIGURE 12 | Relationship between (A,B) photosynthetic capacity or (C,D) transpiration rate and the product of minor vein density (VD) and (A,C) the number of tracheary elements per foliar minor vein or (B,D) the cross-sectional area of the minor veins comprised of tracheary elements for leaves of Italian (circles), Col-0 (triangles), and Swedish (squares) *A. thaliana* ecotypes grown at cool (14°C = blue-green symbols) or hot (36°C = olive-green symbols) leaf temperature.

of tracheary to sieve element numbers (**Figure 11A**) and cross-sectional areas (**Figure 11B**) as well as with the product of vein density and (**Figure 12A**) tracheary element number or (**Figure 12B**) tracheary element cross-sectional area of minor veins, transpiration rate was significantly and *positively* correlated with all of these tracheary element features (**Figures 11C,D** and **12C,D**).

DISCUSSION

For an herbaceous annual that overwinters as a rosette and persists through the summer as a seed, an elevated photosynthetic capacity in cool-grown versus warm-grown leaves presumably allows such a species to take full advantage of cool autumn, winter, and spring days for photosynthesis. Consistent with previous studies of *A. thaliana* (Adams et al., 2013a, 2014a; Cohu et al., 2013b, 2014b; Stewart et al., 2016), photosynthetic capacity of plants grown under controlled conditions was closely correlated with a variety of foliar phloem metrics that relate to the loading of sucrose into the phloem and its export from the leaf. A greater cross-sectional area of sieve elements in minor veins of (experimentally) cool-grown leaves of Col-0, and especially of the Swedish ecotype, may aid in the flux of phloem sap that becomes more viscous at the lower temperatures experienced by these ecotypes in their natural habitats (see discussion in Cohu et al., 2013a). On the other hand, the Italian ecotype exhibited no adjustment in minor vein sieve element cross-sectional area in cool-grown leaves, which would be consistent with the assumption that low-temperature-induced increases in viscosity rarely constitute an impediment to foliar sugar export in the Italian ecotype in its warmer natural habitat. Features of the minor vein phloem that increase the cell membrane area available for placement of transport proteins (sucrose efflux proteins in phloem parenchyma cells, ATPases in phloem parenchyma and companion cells, and proton-sucrose co-transporters in companion cells and sieve elements; see Haritatos et al., 2000; Adams et al., 2014a; Duan et al., 2014), including cell number and the level of cell wall ingrowths in phloem parenchyma cells, exhibited a latitudinal trend in cool-grown leaves that paralleled that of photosynthesis across the three ecotypes. Since, among the phloem metrics analyzed, it was only the level of cell wall ingrowth that differed between cool- and hot-grown leaves of the Italian ecotype, one may speculate that cell wall modification may be sufficient to provide the additional level of sucrose feeding and proton pumping into the apoplast to support the modest level of additional photosynthetic activity seen in cool-grown leaves of the Italian versus the Swedish ecotype. For additional discussion of possible cell wall ingrowth functions, see Amiard et al. (2007) and Adams et al. (2014a).

The concomitant upregulation of photosynthetic capacity and greater leaf thickness in response to experimental growth at low compared to elevated temperature under controlled

TABLE 1 | Results of two-way analysis of variance (ANOVA) for the effect of ecotype, growth temperature, and the degree of ecotype response to growth temperature (14°C and 36°C) for all data presented, with statistically significant effects indicated by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; *n.s.*, not significant).

Metric	Ecotype	Temperature	Ecotype × temperature
Photosynthetic capacity (μmol O_2 m^{-2} s^{-1})	***	***	*n.s.*
Leaf thickness (μm)	***	***	*
Sieve element cross-sectional area per minor vein (μm^2)	***	***	***
Loading cell number per minor vein	*n.s.*	***	**
% increase in plasma membrane due to wall ingrowth	*n.s.*	***	*n.s.*
Minor vein cross-sectional area (μm^2)	*	***	*n.s.*
Minor vein phloem cross-sectional area (μm^2)	*n.s.*	***	*
Minor vein xylem cross-sectional area (μm^2)	*	*n.s.*	*n.s.*
Foliar minor vein density (mm mm^{-2})	*n.s.*	***	*
Phloem (% of minor vein cross-sectional area)	*n.s.*	***	*n.s.*
Ratio of tracheary to sieve elements in minor veins	*n.s.*	***	**
Tracheary elements (% of minor vein vascular cells)	*	***	*n.s.*
β-carotene (mmol mol^{-1} Chl $a + b$)	*n.s.*	***	*n.s.*
Chlorophyll *a*/*b*	*n.s.*	***	*n.s.*
Transpiration rate (mmol H_2O m^{-2} s^{-1})	*n.s.*	***	*
Ratio of tracheary to sieve element cross-sectional area	*n.s.*	***	*n.s.*
Number of tracheary elements per minor vein	*n.s.*	***	*n.s.*
Free-ending veinlets (FEVs mm^{-2})	***	***	*
Number of tracheary elements per minor vein × VD	*n.s.*	***	*
Tracheary element cross-sectional area per vein × VD	*n.s.*	***	*n.s.*

VD = vein density.

conditions, as well as the trends for increasing photosynthetic capacity paralleled by increasing leaf thickness with increasing latitude and decreasing temperature for the habitat from which each ecotype originated, are consistent with similar low-temperature induced acclimatory adjustments in leaf thickness and photosynthetic capacity in the winter annual spinach (Dumlao et al., 2012) and the biennial *Verbascum phoeniceum* (Dumlao et al., 2012; Muller et al., 2014b). Increasing the volume of mesophyll tissue per unit leaf area presumably facilitates an increase in the number of chloroplasts that contribute to photosynthesis. The fact that, in addition to multiple phloem metrics, greater levels of foliar β-carotene and higher chlorophyll *a*/*b* ratios paralleled the elevated photosynthetic capacity in cool-grown leaves is consistent with a greater emphasis on reaction centers and electron transport components in cool-grown leaves. Leaves with higher photosynthetic capacities would be expected to feature a greater ratio of reaction centers, where β-carotene and chlorophyll *a* are located, relative to the outer light-harvesting proteins, where chlorophyll *b* is localized (Anderson and Osmond, 1987; Yamamoto and Bassi, 1996).

One can speculate that the trends for vein density to increase with increasing growth temperature (due at least in part to proliferation of free-ending veinlets), and for the number of tracheary elements per vein to increase with increasing vein density, may contribute to an increased capacity to deliver water to the leaves in support of higher levels of transpirational cooling in hot-grown leaves. The absence of a positive association between these xylem features and photosynthesis contrasts with associations observed between foliar hydraulic conductance, vein density, and photosynthesis in studies of other species (Hubbard et al., 2001; Santiago et al., 2004; Brodribb et al., 2005, 2007, 2010; Nardini et al., 2005; Sack and Holbrook, 2006; Maherali et al., 2008; Boyce et al., 2009; Beerling and Franks, 2010; Brodribb and Feild, 2010; McKown et al., 2010; Blonder et al., 2011; Walls, 2011; Zhu et al., 2013). While we previously obtained positive correlations between xylem features and photosynthesis for several summer annual crop species grown under a single controlled growth temperature (Muller et al., 2014a), we obtained only weak, mostly non-significant associations for *A. thaliana* grown under two growth temperatures and two photon flux densities (and only by excluding the data from the Col-0 ecotype; Cohu et al., 2013b). It is possible that such differences may result from differences in the demand for water transport versus photosynthesis in summer versus winter (and in summer versus winter annuals). For a winter annual, such as *A. thaliana*, that is active during the colder months of the year and responds to low temperature with upregulation of photosynthesis, the system for transport and distribution of water to and within the leaves may not be expected to parallel photosynthesis if evaporative demand is relatively low at the cool temperatures. On the other hand, leaves of summer annuals and winter-deciduous perennial species may experience higher temperatures and greater evaporative demand during their seasonal peak periods of photosynthetic activity, and concordance among foliar phloem and xylem metrics, hydraulic conductance, transpiration, and photosynthesis may be common (Muller et al., 2014a).

The different response of vein density and photosynthetic capacity to leaf growth environment in *A. thaliana* may also be

associated with possible differences in response between summer and winter annuals. While foliar vein density and photosynthetic capacity exhibited concomitant upregulation in a summer annual and two biennial species (Amiard et al., 2005; Muller et al., 2014b), winter annuals (including *A. thaliana*) exhibited upregulation of photosynthesis with no difference in vein density in certain controlled environments (Amiard et al., 2005; Cohu et al., 2013b) or an upregulation of vein density in hot-grown leaves associated with a downregulation of photosynthesis (Stewart et al., 2016). Moreover, as shown here, the extent of the adjustment in vein density in response to experimental growth temperature under controlled conditions varied strongly among *A. thaliana* ecotypes: vein density adjustments were least pronounced in the Italian ecotype, followed by the Swedish ecotype (vein densities in cool-and hot-grown leaves bracketing those of the Italian ecotype), with vein density exhibiting the greatest differential response in the Col-0 ecotype (bracketing that of the Swedish ecotype), a pattern that did not follow the latitudinal and temperature gradients of the ecotypes' respective habitats.

Rather than with temperatures at the respective habitats of origin, the extent of vein density adjustments to experimental growth temperature was inversely correlated with average precipitation in the habitat from which each ecotype originated, as was the extent of the adjustment in the ratio of tracheary to sieve elements to experimental growth temperature (particularly when the latter metrics were normalized for adjustments in vein density). Moreover, the number of tracheary elements per minor vein and transpiration rate were both positively correlated with vein density. These findings suggest that (i) water delivery to the leaves is enhanced not only by a greater foliar vein density but also by a greater number of tracheary elements per vein and (ii) the ability to adjust vein density and the number of tracheary elements per vein to growth at hot temperature is particularly important in natural habitats with lower precipitation levels. Under low temperature conditions, the gradient in water potential from leaf to the atmosphere is relatively low, and the needs of the phloem could presumably be met by a lower tracheary to sieve element ratio, whereas a greater gradient in water potential from the leaf to the atmosphere at high growth temperature presumably necessitates a higher tracheary to sieve element ratio. While water delivery to mesophyll and epidermal cells of the leaf is one important function of xylem conduits, another function of tracheary elements is delivery of water to the phloem to facilitate the pressure-driven flow of viscous, sugar-laden sap in sieve elements from leaves to the plant's sinks (Boersma et al., 1991; Patrick et al., 2001; Hölttä et al., 2006; Sevanto et al., 2011; Hölttä and Nikinmaa, 2013; Nikinmaa et al., 2013). For adequate phloem pressurization to drive sugar transport from source leaves to sinks, the water potential gradient from xylem to phloem must be greater than that from xylem to the air when stomates are open (Nikinmaa et al., 2013), and this gradient between xylem and phloem is the primary driver of water flow in the xylem when stomata are closed.

A growing body of evidence supporting a relationship between foliar phloem features that facilitate the export of reduced carbon compounds from the leaf and photosynthesis has been generated over the past decade (Adams et al., 2005, 2007, 2013a, 2014a; Amiard et al., 2005; Ainsworth and Bush, 2011; Cohu et al., 2013a,b, 2014b; Hölttä and Nikinmaa, 2013; Nikinmaa et al., 2013; Muller et al., 2014a,b; Stewart et al., 2016). Given that mesophytic annual and biennial species exhibit an upregulation of photosynthesis in response to growth at low compared to moderate or high temperature (Holaday et al., 1992; Adams et al., 1995, 2001a,b, 2002, 2004, 2013a; Martindale and Leegood, 1997; Strand et al., 1997; Verhoeven et al., 1999; Dumlao et al., 2012), it is perhaps not surprising that the extent of photosynthetic upregulation, as well as the associated capacity of the phloem for sugar transport, follows the temperature cline among the three *A. thaliana* ecotypes in the present study. Such upregulation presumably counteracts the impact of low temperature on the activity of Calvin cycle enzymes, the activity of the membrane-spanning transport proteins, and the viscosity of the phloem sap (Cohu et al., 2013a,b, 2014b), permitting each ecotype to fix CO_2 and export sugars from the leaves despite low temperatures as long as liquid water is available. The finding that the Swedish ecotype, that experiences the lowest temperatures in its native growing season, upregulates photosynthesis and foliar phloem features to the greatest extent in response to (experimental) growth under low temperature, while the Polish ecotype that experiences intermediate temperatures and the Italian ecotype that experiences the relatively highest temperatures during their respective growing seasons exhibit intermediate and lower levels of such upregulation, respectively, are consistent with this interpretation.

Studies that focused on leaf features have revealed relationships that are consistent with the findings of the current study with regard to water availability. Two major overviews showed that leaf vein density (which is often positively associated with leaf hydraulic conductivity; Sack and Scoffoni, 2013) is strongly negatively correlated with precipitation level and positively associated with increasing aridity (Uhl and Mosbrugger, 1999; Sack and Scoffoni, 2013). Moreover, in a study that is most comparable to the current investigation, growth of six Hawaiian species of the herbaceous genus *Plantago* under common conditions (plus a seventh characterized in the field) revealed that species from drier habitats had higher foliar vein densities (Dunbar-Co et al., 2009), consistent with the findings reported here. On the other hand, hydraulic conductivity and/or xylem features associated with hydraulic conductivity have generally been found to be positively associated with increasing levels of precipitation in the stems and trunks of woody shrubs and trees (Olano et al., 2012; Pratt et al., 2012; Abrantes et al., 2013; Gea-Izquierdo et al., 2013; Castagneri et al., 2015; Schreiber et al., 2015; Venegas-González et al., 2015; Pfautsch et al., 2016; but see Barnard et al., 2011). Furthermore, two previous studies on plants from sites differing in temperature and precipitation found opposite relationships to those observed here for the three ecotypes of *A. thaliana*. Creese et al. (2011) grew 26 *Pinus* species under common greenhouse conditions and Zhu et al. (2012) grew seven populations of the oak *Quercus variabilis* (a winter deciduous tree) in a common garden and found that plant hydraulic conductivity was not correlated with precipitation

level but instead with increasing temperature of the habitat from which the species or populations originated (see also Fonti et al., 2013).

Perhaps these seemingly divergent findings can be reconciled by consideration of the habitats in which the different species are found and their diverging strategies for acclimating to the range of conditions to which each species is adapted. Firstly, the habitats to which woody species such as conifers and oaks are adapted may simply extend over a much broader range of aridity (into regions of lower precipitation, lower water availability, and greater evaporative demand) than the habitats in which herbaceous species grow and/or the long-lived nature of woody species may require the latter species to persist through seasonal periods of even lower water availability and greater evaporative demand. Consequently, such woody species may place an emphasis on the prevention of cavitation (through smaller tracheids with lower hydraulic conductivity) with decreasing precipitation level. For herbaceous species, an emphasis may instead be on greater hydraulic conductivity to meet the evapotranspirational demand of the leaves at elevated temperatures. The current findings thus contribute to the debate concerning the trade-off (or lack thereof) between hydraulic efficiency of the xylem versus its susceptibility to cavitation (Maherali et al., 2004; Hacke et al., 2006; Fan et al., 2011; Fichot et al., 2011; Lens et al., 2011; Manzoni et al., 2013; Zhu et al., 2013; Aranda et al., 2015; Kröber et al., 2015; Gleason et al., 2016; Pfautsch et al., 2016). It is also such that woody species tend to utilize mechanisms to ameliorate the heat load on the leaves that also concomitantly reduce water loss (e.g., growth of leaves at angles to reduce light interception at midday, development of thicker, more reflective foliar cuticles; Logan et al., 2014), whereas herbaceous mesophytes often display their leaves to intercept full sunlight at midday to maximize photosynthesis but consequently rely on evaporative cooling to prevent the leaves from overheating. Such a strategy would not, of course, be viable were it not such that the mesophytes occupy sites (in space and/or time) that can provide sufficient water to meet those evaporative requirements. Moreover, the inherent growth rates of long-lived, woody species are typically lower than those of rapidly growing, herbaceous mesophytes (Adams et al., 2014b). Mesophytes thus employ higher rates of photosynthesis (and the concomitant necessity for greater hydraulic conductance to facilitate higher rates of CO_2 fixation through greater stomatal numbers and/or stomata that are open wider and of carbon export from the leaves that requires more water for the greater turgor-pressure driven movement of sugars through the phloem) to meet their greater sink activity compared to woody species with lower sink activity and rates of photosynthesis.

To briefly summarize, photosynthetic capacity and foliar phloem features presumably indicative of foliar sugar-loading and sugar-export capacity varied in a consistent manner with latitude and temperature of the habitat of origin (greatest in the Swedish ecotype from high latitude and lowest prevailing temperature), whereas vein density and water-transporting tracheary elements varied with precipitation level of the habitat of origin (greatest in the Col-0 ecotype from Poland with the lowest level of precipitation). The nature of these relationships depended on conditions experienced by the plants during growth. The phloem and photosynthesis relationships could be observed in the plants grown under low temperature, whereas the xylem and transpiration relationships could be observed in the plants grown under hot temperature. Moreover, the level of responsiveness in the characterized features was most strongly clarified by comparing the range over which the features acclimated to the two extreme experimental growth temperatures utilized in the present study. Growing all three ecotypes under common conditions at an intermediate temperature revealed very little of the ecotypic differentiation that this species harbors. Similarly, growth of the plants at a higher light intensity masked the extent to which the ecotypes respond to temperature (see Cohu et al., 2013b).

Future studies exploring ecotypic differences within a species should thus utilize more than one common experimental growth condition. The straightforwardness of the relationships illuminated in the current study may have been a result of the consistent nature of the three habitats from which the ecotypes were obtained (all being low altitude sites under the influence of maritime conditions along a north–south transect). Were additional ecotypes from more diverse climatic conditions such as higher altitudes, from inland sites further from major bodies of water, or from sites further to the east in latitude (e.g., from Russia, India, China) or west in latitude (e.g., Spain, UK) to be characterized in the same manner, it is possible that their responses may not align closely with those observed in the present study. Moreover, to further understand the relationships evaluated in the current study, additional species from a range of environments should also be characterized. For instance, it is possible that some species responding to declining winter temperatures through shedding of leaves (winter deciduous) or downregulation of photosynthesis (characteristic of many sclerophyllous evergreen species) may exhibit different relationships between xylem features associated with hydraulic conductivity and habitat temperatures and precipitation levels than herbaceous annual and biennial species that respond to low winter temperatures through upregulation of photosynthesis (Adams et al., 2001a, 2002, 2004, 2006, 2013a,b, 2014a,b). Relationships among vein density, foliar phloem and xylem features, hydraulic conductivity, photosynthesis, and transpiration should thus be characterized in the context of growth form (e.g., herbaceous versus woody, mesophytic versus sclerophytic), life span and habit (e.g., annual versus biennial versus perennial, deciduous versus evergreen), major taxonomic groups (e.g., ferns versus gymnosperms versus angiosperms, monocots versus dicots), mode of phloem loading (apoplastic versus symplastic versus non-loading species), and habitat, e.g., across sites that vary in precipitation but not temperature, across sites that vary in temperature but not precipitation, and across sites where temperature and precipitation vary in the same direction or the opposite direction.

AUTHOR CONTRIBUTIONS

BD-A and WA designed the experiments, CC, OM, and JS carried out the experiments and collected the data, JS assembled the climatological data, executed the statistical analyses, and rendered the figures, and WA and BD-A analyzed the data and wrote the manuscript, with input from JS.

FUNDING

Funding for this study was provided by the National Science Foundation (Award Numbers DEB-1022236 and IOS-0841546 to BD-A and WA) and the University of Colorado at Boulder. Publication of this article was funded by the University of Colorado Boulder Libraries Open Access Fund.

ACKNOWLEDGMENTS

We are deeply indebted to our colleagues Profs. Doug Schemske and Jon Ågren for inviting us to participate in the investigation of this *A. thaliana* system and for providing seeds of the Swedish and Italian ecotypes.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 19 June 2017
doi: 10.3389/fpls.2017.01051

Photoprotective Strategies of Mediterranean Plants in Relation to Morphological Traits and Natural Environmental Pressure: A Meta-Analytical Approach

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OPEN ACCESS

Edited by:
Tiina Tosens, Estonian University of Life Sciences, Estonia

Reviewed by:
Bartosz Adamczyk, University of Helsinki, Finland
Linda-Liisa Veromann-Jürgenson, Estonian University of Life Sciences, Estonia

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *12 April 2017*
Accepted: *31 May 2017*
Published: *19 June 2017*

Citation:
Fernández-Marín B, Hernández A, Garcia-Plazaola JI, Esteban R, Míguez F, Artetxe U and Gómez-Sagasti MT (2017) Photoprotective Strategies of Mediterranean Plants in Relation to Morphological Traits and Natural Environmental Pressure: A Meta-Analytical Approach. Front. Plant Sci. 8:1051. doi: 10.3389/fpls.2017.01051

Despite being a small geographic extension, Mediterranean Basin is characterized by an exceptional plant biodiversity. Adaptive responses of this biocoenosis are delineated by an unusual temporal dissociation along the year between optimal temperature for growth and water availability. This fact generates the combination of two environmental stress factors: a period of summer drought, variable in length and intensity, and the occurrence of mild to cold winters. Both abiotic factors, trigger the generation of (photo)oxidative stress and plants orchestrate an arsenal of structural, physiological, biochemical, and molecular mechanisms to withstand such environmental injuries. In the last two decades an important effort has been made to characterize the adaptive morphological and ecophysiological traits behind plant survival strategies with an eye to predict how they will respond to future climatic changes. In the present work, we have compiled data from 89 studies following a meta-analytical approach with the aim of assessing the composition and plasticity of photosynthetic pigments and low-molecular-weight antioxidants (tocopherols, glutathione, and ascorbic acid) of wild Mediterranean plant species. The influence of internal plant and leaf factors on such composition together with the stress responsiveness, were also analyzed. This approach enabled to obtain data from 73 species of the Mediterranean flora, with the genus *Quercus* being the most frequently studied. Main highlights of present analysis are: (i) sort of photoprotective mechanisms do not differ between Mediterranean plants and other floras but they show higher plasticity indexes; (ii) α−tocopherol among the antioxidants and violaxanthin-cycle pigments show the highest responsiveness to environmental factors; (iii) both winter and drought stresses induce overnight retention of de-epoxidised violaxanthin-cycle pigments; (iv) this retention correlates with depressions of Fv/Fm; and (v) contrary to what could be expected, mature leaves showed higher accumulation of hydrophilic antioxidants than young leaves, and sclerophyllous leaves higher biochemical photoprotective demand than membranous leaves. In a global climatic change scenario,

the plasticity of their photoprotective mechanisms will likely benefit Mediterranean species against oceanic ones. Nevertheless, deep research of ecoregions other than the Mediterranean Basin will be needed to fully understand photoprotection strategies of this extremely biodiverse floristic biome: the Mediterranean ecosystem.

Keywords: antioxidant, carotenoid, evergreen, Mediterranean, photoprotection, photosynthetic pigment, sclerophyllous, tocopherol

INTRODUCTION

Since late-90s, Mediterranean biome is recognized as a biodiversity hotspot and a global conservation priority (Cox and Underwood, 2011; Bellard et al., 2014; Matesanz and Valladares, 2014). It should be underlined that the biodiversity maintenance is essential to ecosystem health and resilience and, consequently, the services that they provide (Pereira et al., 2013). Accordingly, important efforts and resources are being invested in the study and preservation of the exceptional diversity of the Mediterranean vascular flora that includes many rare and endemic taxa (Cox and Underwood, 2011; Bellard et al., 2014). Despite these efforts, the loss of biodiversity in the Mediterranean biome does not seem to slow down (Butchart et al., 2010). Anthropogenic activities such as intensive agriculture, fire-regimes, grazing, and urbanization have strongly altered Mediterranean ecosystems, especially in the last decades (Bellard et al., 2014; Matesanz and Valladares, 2014), having negative consequences into native plant communities (Hoekstra et al., 2004; Cox and Underwood, 2011). As the world population continues to grow, the extension of natural habitats in this diminutive biome will probably continue to reduce (Cox and Underwood, 2011).

Moreover, the future holds new threats: Mediterranean regions are expected to be heavily impacted by climate change (Klausmeyer and Shaw, 2009). Climatic scenarios point out not only an increase of aridity but also a higher variability and frequency of extreme climatic events, such as heat waves and droughts, particularly in the Mediterranean areas of Southern and Western Europe (Jacob et al., 2014; Matesanz and Valladares, 2014). The expected ecological effects on vegetation include changes in life cycles (Gordo and Sanz, 2009) and mortality (Gitlin et al., 2006), invasions and/or attacks of pathogens and changes in species composition (Aitken et al., 2008; Hickler et al., 2012; Bussotti et al., 2015), among others. Hence, the study of functional and morphological traits of native Mediterranean species in response to environmental stresses is of paramount importance to delineate future affections and lines of action.

In essence, Mediterranean plant biodiversity is mainly configured by the peculiar climatic drivers of the region (read seasonality) characterized by a temporal dissociation between optimal temperature and availability of water needed for growth. Summers are characterized by a drought period of variable length, intensive sunshine and moderately to extremely high temperatures, whilst winters are mild to cold with variable rainfall. The co-occurrence of light excess with drought in summer and with low temperatures in winter makes both periods the most stressful for vegetation in Mediterranean environments (Mitrakos, 1982). Thus, under these conditions and provoked by the imbalance between light capture and use, plants may suffer photo-oxidative damage of photosystem II (PSII) due to their inability to use the excess of absorbed light (Murata et al., 2007). Photoinhibition, beyond the photodamage of proteins of PSII such as D1 protein, is associated with the impairment of various photoprotective processes that ensure the integrity of the PSII complex, including thermal dissipation of excitation energy (Ort, 2001; Barker et al., 2002). Therefore and depending on the degree of plasticity (i.e., ability to acclimate -and possibly adapt- to different environmental conditions), Mediterranean plants display a suite of morpho-anatomical, physiological and biochemical adjustments to avoid photooxidation and photodamage (Osmond et al., 1997).

Numerous studies have provided evidence of the capacity of Mediterranean plants to adjust their morphology, physiology, phenology and reproduction in response to varying temperature, nutrients, light, and water availability (Domínguez et al., 2012; Matesanz and Valladares, 2014 and references therein). Some of the most common and representative traits of Mediterranean plants include the small size and bushy phenotypes with short internodes, the small and thick leaves: usually sclerophyllous and evergreen (long life span) with high Leaf Mass per Area (LMA) in agreement with leaf economics spectrum (Wright et al., 2004), and the presence of abaxial trichomes. All these traits relate to the generally high water use efficiency (WUE) typical of Mediterranean plants, that is achieved through a complex interaction of mechanisms enabling a reduction of water loss per unit of carbon gain (Matesanz and Valladares, 2014). Yet, an integrated overview of the interplay between these physical traits of Mediterranean plants and their photoprotective mechanisms at metabolic level is still missing.

At biochemical level, is known that plants generally counteract photo-oxidative damage through fine-tuning of a variety of photoprotective mechanisms based on specialized metabolites that keep reactive oxygen species (ROS) at a concentration

Abbreviations: Ant, anteraxanthin; α-Toc, α-tocopherol; AZ/VAZ, de-epoxidation state of the violaxanthin cycle; β-Car, β-carotene; *C, Cistus*; Chl, chlorophyll; D, drought; DW, dry weight; EV, evergreen; Fv/Fm, maximal photochemical efficiency of PSII; FW, fresh weight; g_m, mesophyll conductance; g_s, stomatal conductance; HA, hairy abaxial; HE, herb; HL, high light; HT, high temperature; LMA, leaf mass per area; LT, low temperature; Lut, lutein; Lx, lutein epoxide, Lx-cycle, lutein-epoxide cycle; MA, malacophyllous; ME, membranous; MT, mature; Neo, neoxanthin; NN, none. $^1O_2$, singlet oxygen; PI, plasticity index; PB, pubescent; PSII, photosystem II; *Q*, *Quercus*; ROS, reactive oxygen species; S, seasonality; SC, sclerophyllous; SD, summer deciduous; tAsc, total ascorbic acid; tGSH, total glutathione; VAZ-cycle, violaxanthin cycle; Vio, violaxanthin; Ψ, water potential; WD, winter deciduous; WO, woody; WUE, water use efficiency; WX, Waxy; YG, young; Zea, zeaxanthin.

efficient for signaling (Hadacek and Chobot, 2011). Such mechanisms include: (1) decrease of total chlorophyll (Chl a+b) content and increase of the ratio Chl a/b; (2) activation of an intricate antioxidant network that includes, for instance, the synthesis of non-enzymatic low molecular weight hydrophilic antioxidants such as glutathione (tGSH) and ascorbic acid (Asc), or the accumulation of lipophilic antioxidants such as α-tocopherol (α-Toc) and β-carotene (β-Car); and (3) conversion of the xanthophylls from the violaxanthin (VAZ-cycle) and the lutein-epoxide cycle (LxL-cycle) toward de-poxidised forms: antheraxanthin (Ant), Zeaxanthin (Zea), and Lutein (Lut) (Valladares et al., 2012). Ubiquitous VAZ-cycle, and uncommon LxL-cycle are both involved in the process of thermal dissipation of excess light to PSII through non-photochemical quenching (NPQ) (García-Plazaola et al., 2007; Esteban and García-Plazaola, 2014; Esteban et al., 2015a). Additionally, Zea and β-Car as well as α-Toc operate not only as physical and chemical quenchers of singlet oxygen ($^1O_2$) but they can also modify membrane fluidity (Munné-Bosch et al., 2013; Di Ferdinando et al., 2014), offering a double protection: they prevent the formation of Chl triplet excited state and protect the integrity of chloroplast. Concomitantly, tAsc and tGSH are intimately related to ROS detoxification and, particularly, hydrogen peroxide removal (Szarka et al., 2012). Above-mentioned photoprotective pigments and antioxidants comprise a complex network. It has long been known that Asc and GSH form ascorbate-glutathione cycle, where Asc is regenerated by GSH (Foyer and Noctor, 2011). Ascorbic acid additionally acts as a cofactor for Vio de-epoxidation to Zea in the VAZ-cycle and is also involved in the regeneration of α-Toc (Venkatesh and Park, 2014). Quantitative changes in the profile of previously detailed metabolites, which could be understood as "antioxidant plasticity," frequently reflect deeper structural and functional modifications of the photosynthetic apparatus (Esteban et al., 2015a). Nonetheless, it is important to identify plant functional traits in which plasticity may play a determinant role not just in plant survival under strong seasonal climates such as the Mediterranean climate, but also in predicted novel environmental conditions (Gratani, 2014). Independent studies have characterized photoprotective strategies of Mediterranean species from different natural locations along the last two decades (e.g., García-Plazaola et al., 1999, 2008; Munné-Bosch et al., 2003; Hormaetxe et al., 2007; etc.). Nevertheless, the remarkably high diversity of plants in this ecosystem and the complexity of the photoprotective and antioxidant mechanisms (i.e., accumulation of antioxidants is generally triggered under slight to moderate stress but some are consumed and thus their content decrease because of extenuation under extreme stress) point out the need of a comprehensive compilation of data available that could enhance our understanding of Mediterranean plant photoprotective responses, estimation of their plasticity indexes and prediction of their fitness in a scenario of climate change.

Recently, morpho-physiological traits of Mediterranean plats have been compiled and deeply analyzed (e.g., Domínguez et al., 2012; Niinemets, 2015; etc.). Nevertheless, comparable studies to ascertain the biochemical traits in terms of photoprotective pigments and antioxidants are much scarcer and there is lack of a joined synopsis combining both morphological and biochemical photoprotective traits. Here, we present a literature compilation focused on the photoprotection of wild species from the Mediterranean Basin to add light to this topic. Specially we aimed at (i) providing comprehensive and quantitative synthesis of reference values of photoprotective pigments and antioxidant of native Mediterranean plant species living under non-stressed conditions; (ii) elucidating how these biochemical traits relate to morphological and functional leaf and plant traits; and (iii) assessing how they change under the abiotic stresses associated to Mediterranean climate ("antioxidant plasticity").

MATERIALS AND METHODS

Literature Search

Data were compiled from peer-reviewed journals only, using the "ISI Web of Sciences" source. Only original articles written in English and published in the last 20 years, covering the time laps between January 1996 and May 2016, were considered. Key words used in the search included (i) antioxidant-related terms: "tocopherol OR glutathione OR ascorb* OR zeaxanthin OR lutein OR caroten* OR antioxidant OR *oxid*," (ii) Mediterranean ecosystems-related terms "Mediterranean OR scrub OR chaparral OR shrubland OR woodland OR *Quercus* OR *Arbutus* OR *Cistus* OR *Rosmarinus* OR *Pistacia* OR *Lavandula*)," and (iii) terms related to photosynthetic tissues: "leaf OR leaves OR foliar OR chloroplast*." The search was also filtered by areas of knowledge: "Plant Sciences OR Agriculture OR Environmental Sciences Ecology OR Forestry." A total number of 840 articles fitted to these criteria.

Manuscripts Inclusion Criteria

From the initial search, manuscripts were partitioned according to the following principles: only wild species original from the Mediterranean bioclimatic region were included. Field studies with Mediterranean species conducted out of the Mediterranean bioclimatic region or with aquatic plants (i.e., *Posidonia*), or with crops, were excluded. Works performed under growth chamber or greenhouse conditions were excluded. Finally, only papers using high-performance liquid chromatography for carotenoid and/or tocopherol quantification were included. After all these criteria our final database comprised data from 89 papers, 73 species, and 32 families (Table S1).

Input Data for the Analysis

Numeric data were extracted from text, tables and figures of the 89 selected manuscripts (Table S2). Data from figures were obtained by using the "scale tool" from the software Adobe PhotoShop, CS5 Extended, v 12.1 ×64. Different type of numeric data were obtained from the manuscripts: (1) *foliar content of photosynthetic pigments and small molecular weight antioxidants:* anteraxanthin (Ant), neoxanthin (Neo), violaxanthin (Vio), lutein (Lut), lutein epoxide (Lx), zeaxanthin (Zea), chlorophyll a (Chl a), chorophyll b (Chl b), β-carotene (β-Car), α-tocopherol (α-Toc), total ascorbate (tAsc), total glutathione (tGSH) and/or sum or ratios of these parameters

i.e., (A+Z)/(V+A+Z) (so far referred to as AZ/VAZ), Chl a+b, pigments or antioxidants per Chl, etc.; (2) *parameters indicative of stress:* maximal photochemical efficiency of PSII (Fv/Fm), predawn water potential (Ψ), relative water content (RWC), midday stomatal conductance (g_s); (3) *parameters relating leaf mass and area*: leaf mass per area (LMA), specific leaf area (SLA). In the case of photosynthetic pigments and antioxidants, values expressed per fresh weight (FW), dry weight (DW), leaf area or Chl content were obtained. When any of these data was missing, and whenever possible, they were calculated from the original data from the paper. In some cases, units were converted.

Further details collected from each paper regarded *plant developmental stage* (seedling, adult), *leaf age* (young, mature), *time of the day* (predawn, artificial predawn (> 12 h darkness), morning, midday, evening). Additionally, information about *growth type* (woody: tree, shrub; non-woody: herb), *taxonomic group* (angiosperm, gymnosperm), *leaf texture* (membranous, sclerophyllous, malacophyllous, succulent), *leaf attributes* (none, waxy, pubescent, hairy-abaxial) and *leaf phenology* (evergreen, winter-deciduous, summer-deciduous) were compiled from the available literature.

Data Processing

Every manuscript was first labeled with regard to its main stress or topic (i.e., drought, salinity, climate-comparison, high temperature, low temperature, species comparison, etc.). Secondly, each data-row within the manuscript was labeled as control or treatment. When a treatment was designated as "control" in the study, it was included as such in the database. Nevertheless, there were some manuscripts in which there were not real controls. In those cases, the less stressful condition was considered as control for each study. Apart from this division, and taking into consideration all the manuscripts together, data from non-treated plants, together with real controls, were considered as "non-stress" values (data were excluded from this category if their Fv/Fm values were <0.6, predawn AZ/VAZ > 0.4 and/or predawn Ψ < −2 MPa). Data from "non-stress" category were used for the characterization of photosynthetic pigment and antioxidant composition in Mediterranean species (**Table 1**), and for the assessment of their composition according to plant biological traits (**Figure 1**), and leaf traits (**Figures 2**, **3**, **6**).

To evaluate the influence of the main Mediterranean climatic factors over photosynthetic pigments and antioxidants, manuscripts dealing with drought (D), low temperature (LT), high temperature (HT), and/or seasonality (S) were analyzed in detail (total manuscripts = 43). Within each manuscript, an index of variation with respect to the control was calculated for each of the climatic stressors independently. Hence, all data values were normalized to their respective controls. After data transformation, controls resulted as 1, while the deviation from the value 1 indicated the variation of the parameters analyzed. Reference values (controls) were considered the ones cataloged as "control" in the original papers.

To study the effect of leaf age on photosynthetic pigment and antioxidant content, only those studies where both mature and young leaves were simultaneously reported were included (total articles = 8). The young leaves were taken as reference values and the fold-changes were calculated as follows: Fold-change = (value of mature leave/value of young leave) −1. Thus, reference value was equaled to zero.

Plasticity Index (PI) for each parameter (Fv/Fm, pigments, antioxidants and LMA) was estimated according to Valladares et al. (2006) with the following formula: (Max-Min)/Max (within each paper of origin and parameter). This index generates values between 0 (no plasticity) and 1 (highest plasticity) comparable among parameters. A minimum of three different papers for each parameter was used as source of data for the calculation of average PI values. Only papers with two or more data for at least one species and one parameter where selected.

Statistical Analyses

One-way ANOVA (for normally distributed data) and a Kruskal-Wallis ANOVA (for non-normally distributed data) were applied to test for significant differences among categories within functional groups, growth forms and plant developmental stages (**Figure 1**). Non-parametric Mann Whitney-U test was used to check for significant differences in the content of antioxidants and pigments among different leaf traits (**Figure 2**). Pearson's correlation was used to check for relationship between LMA and photosynthetic pigments or antioxidants (**Figure 3**). Student's *t*-test was performed in order to assess significant differences between control and climatic stresses (**Figure 4**). Student's *t*-test (or single sample Kolmogorov-Smirnov-test, for non-normally distributed data) was performed to assess differences between young and mature leaves (**Figure 6**). The resulting *P*-values were considered to be statistically significant at $\alpha = 0.05$. All statistical analyses were performed with SPSS 24.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Pigment and Antioxidant Composition of Mediterranean Species

Table 1 depicts reference values of photosynthetic pigments and antioxidants from a representative selection of native Mediterranean species living under non-stressful conditions (see Table S3 for an extended data set including parameters with $n < 3$). Average Chl a+b content was remarkably stable, fluctuating between 278 (μmol m^{-2}) in *Rosmarinus officinalis*, and 547 in *Quercus ilex*, with the highest values being found in sclerophyllous species from Fagaceae and Rhamnaceae families. Among Fagaceae, sclerophyllous *Quercus* showed, additionally, the highest Chl a/b ratio. The content of α-Toc was the most variable among lipophilic antioxidants, varying up to two orders of magnitude among species (e.g., *Pistacia lentiscus* vs. *Rosmarinus officinalis*) and tGSH among hydrophilic antioxidants, varying also up to two orders of magnitude (e.g., *Cistus salviifolius* vs. *Buxus sempervirens*, **Table 1**). The intraspecific variability within a species (i.e., maximal amplitude between minimum and maximum values) was the highest for most of the metabolites in the case of *Quercus* species (except for the α-Toc, that appeared as extremely variable in *Pistacia lentiscus).*

TABLE 1 | Composition of photosynthetic pigment and low molecular weight antioxidants in a representation of wild Mediterranean species (covering 9 different families).

	Fam.	**Anacardiaceae**	**Buxaceae**	**Caprifoliaceae**		**Cistaceae**			**Ericaceae**	**Fagaceae**			**Lamiaceae**		**Oleaceae**	**Rhamnaceae**
	Sp.	***Pistacia lentiscus***	***Buxus sempervirens***	***Lonicera implexa***	***Viburnum tinus***	***Cistus albidus***	***Cistus clusii***	***Cistus salviifolius***	***Arbutus unedo***	***Quercus coccifera***	***Quercus ilex***	***Quercus suber***	***Rosmarinus officinalis***	***Salvia officinalis***	***Phyllirea latifolia***	***Rhamnus alaternus***
Chl a+b	Mean	344	321	305	377	303	348	476	407	498	547	521	278		487	564
	Max	379	546	336	395	467	375	625	561	616	791	639	408		583	688
	Min	288	257	262	356	151	311	370	178	223	339	180	117		381	344
	n	5	7	3	3	5	3	4	5	15	18	8	3		3	3
Chl a/b	Mean	2.46	2.22			3.03	2.78			2.81	3.26	3.30	2.96	2.52		
	Max	2.94	3.72			3.73	2.85			3.45	4.07	3.51	3.12	2.60		
	Min	1.79	1.75			2.65	2.67			2.10	1.97	3.10	2.80	2.40		
	n	5	5			3	6			6	17	6	3	3		
Neo	Mean		36							45.8	35.9	33				
	Max		41							62.6	56.3	51				
	Min		32							29.0	25.3	24				
	n		3							6	17	7				
Lut	Mean		159			132	323	118	116	153	138	270	270			
	Max		188			186	543	120	124	210	218	412	412			
	Min		121			93	200	114	106	123	102	196	196			
	n		6			4	3	3	3	7	18	3	3			
VAZ	Mean	86	112	86	106	56		52	85	111	83.1	47	47		114	95
	Max	135	153	98	129	94		57	134	176	197	67	67		182	126
	Min	52	35	66	73	30		47	62	30	36	9	9		89.5	75
	n	3	7	3	3	4		3	4	16	24	3	3		4	3
AZ/VAZ	Mean	0.34	0.18	0.16	0.16	0.19	0.44	0.21	0.32	0.23	0.25	0.21	0.23	0.08	0.19	0.11
	Max	0.87	0.36	0.21	0.21	0.31	0.50	0.53	0.78	0.49	0.86	0.30	0.29	0.12	0.37	0.13
	Min	0.04	0.02	0.11	0.11	0.09	0.33	0.06	0.05	0.09	0.04	0.09	0.13	0.03	0.09	0.08
	n	5	7	3	3	4	4	4	6	17	26	7	3	3	4	4
β-Car	Mean		86			102	185	101	80	110	101	91	136			
	Max		97			121	397	122	100	173	175	161	218			
	Min		68			92	126	87	47	31	53	61	83			
	n		6			3	5	3	3	8	19	7	5			
α-Toc	Mean	1,599	1,534	260	745	259	153	195		469	304		52		288	323
	Max	2,798	3,914	414	1,442	433	344	286		606	815		156		416	496

(Continued)

TABLE 1 | Continued

	Fam.	Anacardiaceae	Buxaceae	Caprifoliaceae		Cistaceae			Ericaceae		Fagaceae		Lamiaceae		Oleaceae	Rhamnaceae
	Sp.	***Pistacia lentiscus***	***Buxus sempervirens***	***Lonicera implexa***	***Viburnum tinus***	***Cistus albidus***	***Cistus clusii***	***Cistus salviifolius***	***Arbutus unedo***	***Quercus coccifera***	***Quercus ilex***	***Quercus suber***	***Rosmarinus officinalis***	***Salvia officinalis***	***Phyllirea latifolia***	***Rhamnus alaternus***
	Min	7	69	63	220	96.4	36	89		212	1		4		109	207
	n	38	5	3	3	4	8	4		3	11		5		3	3
	Mean		680	318	301	134		7	37	30	85				127	323
tGSH	Max		789	326	321	158		9	48	31	141				140	328
	Min		571	310	280	109		5	26	28	34				115	318
	n		2	2	2	2		2	2	2	6				2	2
	Mean		23,339	4,609	8,041	4,056		7,390	13,459	12,090	5,762				8,836	9,084
tAsc	Max		24,025	5,730	9,694	4,801		8,545	14,258	12,102	13,553				12,950	9,336
	Min		22,654	3,488	6,388	3,310		6,236	12,660	12,079	1,335				4,723	8,832
	n		2	2	2	2		2	2	2	7				2	2

Data are mean, maximum and minimum for each species and metabolite. Number of cases is designated as n. Species for which higher number of cases was available were selected: i.e., n ≥ 3 (or n ≥ 2 for hydrophilic antioxidants) for at least two metabolites. Units as in ***Figure 1****.*

Effect of Internal Plant Factors

The internal plant factors considered in the present work were the taxonomic class, the functional group, the growth form and the plant age (**Figure 1**). Our analysis revealed that pigment and antioxidant content in Mediterranean wild plant species under non-stressed conditions was differentially distributed depending on taxonomic class (gymnosperm or angiosperm) and functional group (herbaceous or woody) (**Figure 1**). In this regard, we identified two critical gaps of knowledge that introduce uncertainty to data interpretation: the lack of information about photoprotective features in gymnosperms and in herbs. Even so, when gymnosperms and angiosperms were compared, significantly higher values of AZ/VAZ and α-Toc were observed in the latter (**Figure 1**, left). After subdividing this taxonomic class into growth types, significant differences were found for α-Toc, being much more abundant in trees and lianas than herbs. Total Chl and Chl a/b were also significantly higher in trees and lianas than in the rest of angiosperm groups.

In agreement with this, when the functional group (herbaceous vs. woody) of Mediterranean plants was considered (**Figure 1**, right), levels of Chl a/b and α-Toc were significantly higher in woody species. Within them, we also analyzed the effect of plant age on the antioxidant load. Interestingly, VAZ and AZ/VAZ decreased while α-Toc increased with plant age (**Figure 1**, right).

Effect of Leaf Properties

The influence of different leaf traits (i.e., attributes, morphology, or texture) on the biochemical demand for photoprotection is shown in **Figure 2**. Foliar hairs efficiently decreased the biochemical demand for photoprotection (i.e., lower VAZ, α-Toc, and tAsc) even if they were present at the abaxial side of the leaf only. Higher requirement of biochemical photoprotection was found in evergreen than in deciduous leaves (this was remarkably evident in the higher contents of VAZ, α-Toc, and tAsc) and in summer-deciduous than in winter-deciduous leaves (evidenced in significantly higher Lut and tAsc, and higher, although not significantly, β-Car and AZ/VAZ). Besides having strongly developed structural photoprotective mechanisms, sclerophyllous leaves showed higher values of antioxidants L, VAZ, α-Toc, and tAsc than membranous leaves (**Figure 2**).

Associated to this, the predawn content of carotenoids and antioxidants (expressed in mmol mol^{-1} Chl) increased with leaf mass per area (LMA) (**Figure 3**). This relationship was significant for all the metabolites (including tGSH, which was represented by a low number of cases: $n = 6$), except for α-Toc. At lesser extent, Chl content also followed a similar trend, although the correlation was no significant ($P > 0.05$). No correlation at all was found between Chl a/b ratio and LMA, or between AZ/VAZ and LMA (**Figure 3**).

Response to Mediterranean Climatic Stressors

The most characteristic climatic stresses in Mediterranean areas, and as a consequence, the stresses more deeply studied in the literature were: LT, HT, D, and seasonality. Variation of the

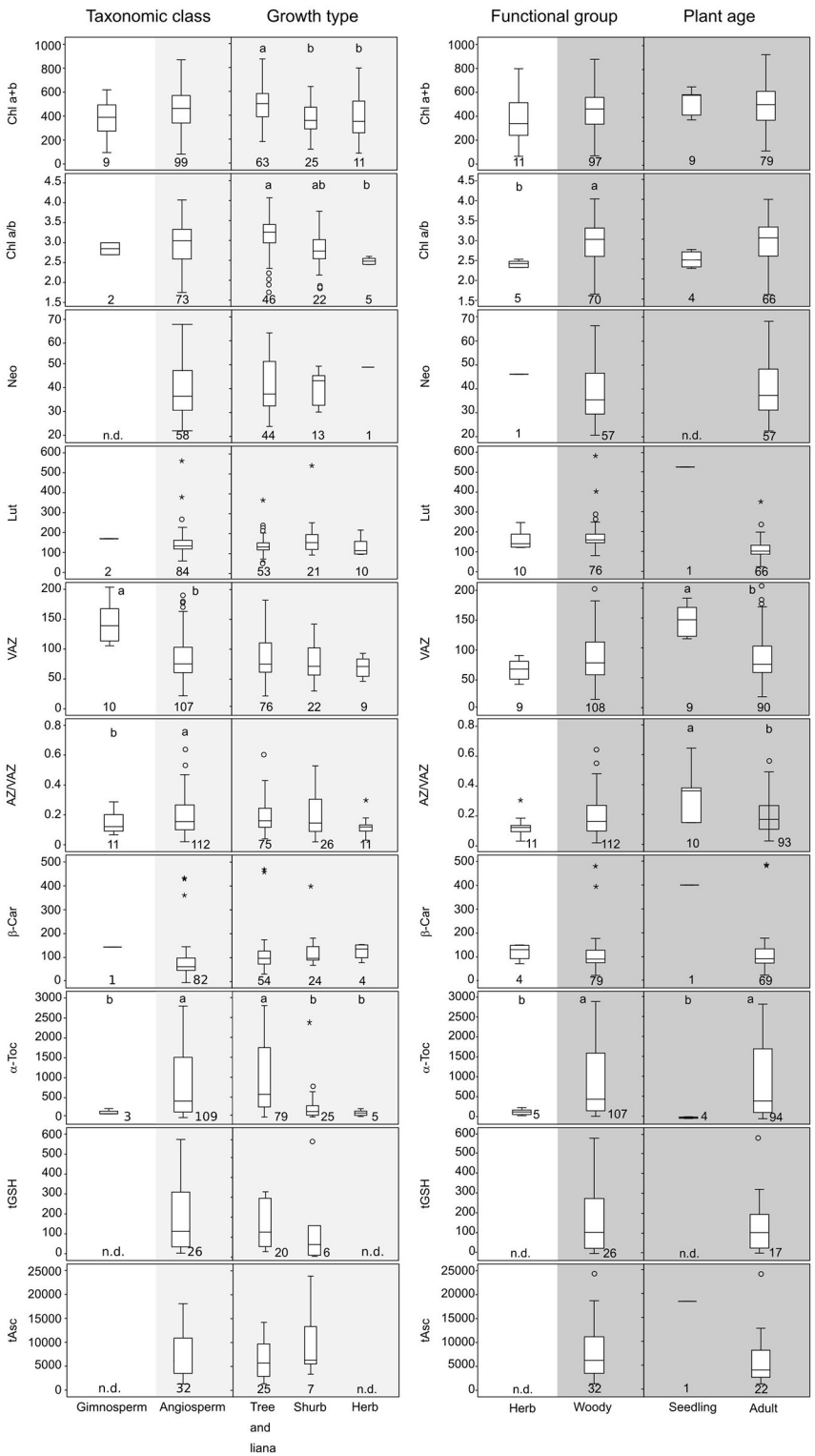

FIGURE 1 | Boxplots showing the concentrations of the main photosynthetic pigments grouped considering different plant traits. **(Left)** Taxonomic class (Gymnosperm vs. Angiosperm) and growth type (Tree and liana, Shrub, and Herb) within angiosperms. **(Right)** Functional group (life style) (Herb vs. Woody) and plant age (Seedling and Adult) within woody. Total chlorophyll (Chl a+b) is expressed in μmol m^{-2}. Carotenoids such as neoxanthin (Neo); lutein (Lut); pool of violaxanthin, anteraxanthin and zexanthin (VAZ); β-carotene (β-Car) and α-tocopherol (α-Toc) are expressed in mmol mol^{-1} of total Chl. Hydrophilic antioxidants such as glutathione (tGSH) and ascorbic acid (tAsc) are also expressed in mmol mol^{-1} of total Chl. De-epoxidation state of violaxanthin cycle (AZ/VAZ) is expressed in mol mol^{-1}; Only

(Continued)

FIGURE 1 | Continued
data for non-stressed plants are included. Boxes cover 50% of the data; central lines represent the medians and whiskers represent the minimum and maximum values among non-atypical data. Open circles and asterisk represent outliers and extreme outliers, respectively. The number of reported data (*n*) is shown below each box. Lowercase and upper case letters above the boxes denote significant differences ($P < 0.05$) among groups analyzed by one-way ANOVA (normally distributed data) or by Kruskal-Wallis ANOVA (non-normally distributed data).

main photosynthetic pigments and antioxidants (by comparison with controls) in response to Mediterranean climatic stressors are shown in **Figure 4**. The results show that these stresses provoked an enhancement in the content of all the parameters, with the exception of Chl a+b under HT (**Figure 4**). Drought significantly enhanced Neo, Lut, β-Car, VAZ, AZ/VAZ and it was the stress that more intensively triggered the accumulation of α-Toc (**Figure 4C**). Low temperature increased the Chl a/b ratio, and Lut, β-Car and α-Toc contents, while HT enhanced the total VAZ pool, AZ/VAZ, Chl a/b, and α-Toc, and induced a significant decrease in Chl a+b. Seasonality provoked an augmentation in the contents of Neo, Chl a/b, β-Car, AZ/VAZ, and α-Toc (**Figure 4**). Interestingly, the total VAZ and the antioxidant α-Toc (**Figures 4B,C**) showed more responsiveness to all the stresses than the other parameters, being the amplitude of the change higher than in the other compounds. The hydrophilic antioxidants tGSH and tASC did not show any significant change under any of the climatic stressors (**Figure 4C**).

Overnight Retention of AZ/VAZ in Mediterranean Species

To tackle the question of whether A+Z can be retained overnight under particular stress conditions in Mediterranean plants, frequencies of predawn values of AZ/VAZ in non-stressed and stressed plants were compared (**Figure 5A**). Predawn AZ/VAZ values higher than 0.4 were reported for 24% of stressed plants while only 1% of the non-stressed plants gave values higher than this threshold. Furthermore, photochemical efficiency (measured as predawn Fv/Fm) decreased with increasing overnight retention of Ant+Zea (measured as AZ/VAZ) (**Figure 5B**). This effect was particularly noticeable in winter, and to a lesser extent in other seasons, and correlated with the size of the VAZ pool (**Figure 5C**). However, apart from winter low temperatures, water stress also triggered the sustained retention of Ant+Zea (**Figure 5D**). Accordingly, data shown in **Figure 5D** evidenced that to attain high values of predawn AZ/VAZ, a low g_s is required and conversely, at high g_s, overnight Ant+Zea is always low. Relationship between water potential (ψ) and predawn AZ/VAZ (**Figure 5E**) showed the same trend but less markedly.

Content-Plasticity of Photosynthetic Pigments and Antioxidants

In order to assess the response capacity of main photosynthetic pigments and antioxidants in Mediterranean plants, a Plasticity Index (PI) was calculated for each of those parameters (**Table 2**). Higher plasticity was found in antioxidants than in pigment contents. Low variability in pigment content was generally found in Chl a+b, Chl a/b, Neo and β-Car. Among pigments, only VAZ and AZ/VAZ showed PI values above 0.5. Among antioxidants α-Toc showed higher PI than tAsc and tGSH suggesting a functional and important role for this metabolite in response to fluctuations in the environment.

Sclerophyllous *Quercus* Species as Model Mediterranean Plants

Within the 73 total species included in this study, higher number of cases concerned sclerophyllous *Quercus,* which is actually one of the most preponderant tree and an important model genus within the Mediterranean Basin ecosystems. The sclerophyllous leaves from oak contain relatively high levels of Chl a+b per area, when compared to other Mediterranean species (**Table 1**). The rest of photosynthetic pigments, (i.e., carotenoids per Chl ratios) however, followed a quite stable stoichiometry, being similar for most of the studied species (**Table 1**). Besides the well-known phenotypic/morphological plasticity of oak leaves, biochemical plasticity appeared also higher than in other species, e.g., higher plasticity indexes were obtained for Chl a/b, AZ/VAZ, α-Toc, and tGSH in *Quercus* than in the other well represented Mediterranean genus *Cistus* (**Table 2**). Regarding leaf age and development, mature leaves of sclerophyllous *Quercus* species had significantly higher content of all photosynthetic pigments and antioxidants (specially, Neo, tGSH, and tAsc) than young leaves (**Figure 6**). This fact, contrasts with the different influence of plant age over the antioxidant pool: i.e., VAZ and AZ/VAZ were lower in older than in younger plants, but higher in mature than in young leaves (**Figure 1** vs. **Figure 6**).

DISCUSSION

Interaction of Morphological Traits and Photoprotection

Mediterranean-type ecosystems are characterized by a period of a summer drought, extreme temperatures, both in winter and summer, and a marked seasonality. This type of climate can be found in all continents, but the largest area is the Mediterranean basin. This biome, one of the richest in terms of plant diversity with 25,000 native species, is recognized as a Global Biodiversity Hotspot (Myers et al., 2000). The succession of these stresses requires the acclimation of plant species living in this ecosystem. In this sense, the plant photoprotective mechanisms are key strategies in the resilience of Mediterranean species to the harsh environment. Such is the case of evergreens with long lived leaves, which compared to deciduous are more likely to suffer and cope with more stressful conditions during their life duration. As expected, the morphological and biochemical characteristics should them adjust along seasons and years. For this reason, evergreens require more rigid mesophyll structure (Onoda et al., 2017) or greater investment in photoprotection (with higher contents of VAZ and α-Toc) than deciduous, as we shown in this study (**Figure 2**). Sclerophyllous leaves, a trait that is closely related to evergreen strategy, also showed the same pattern.

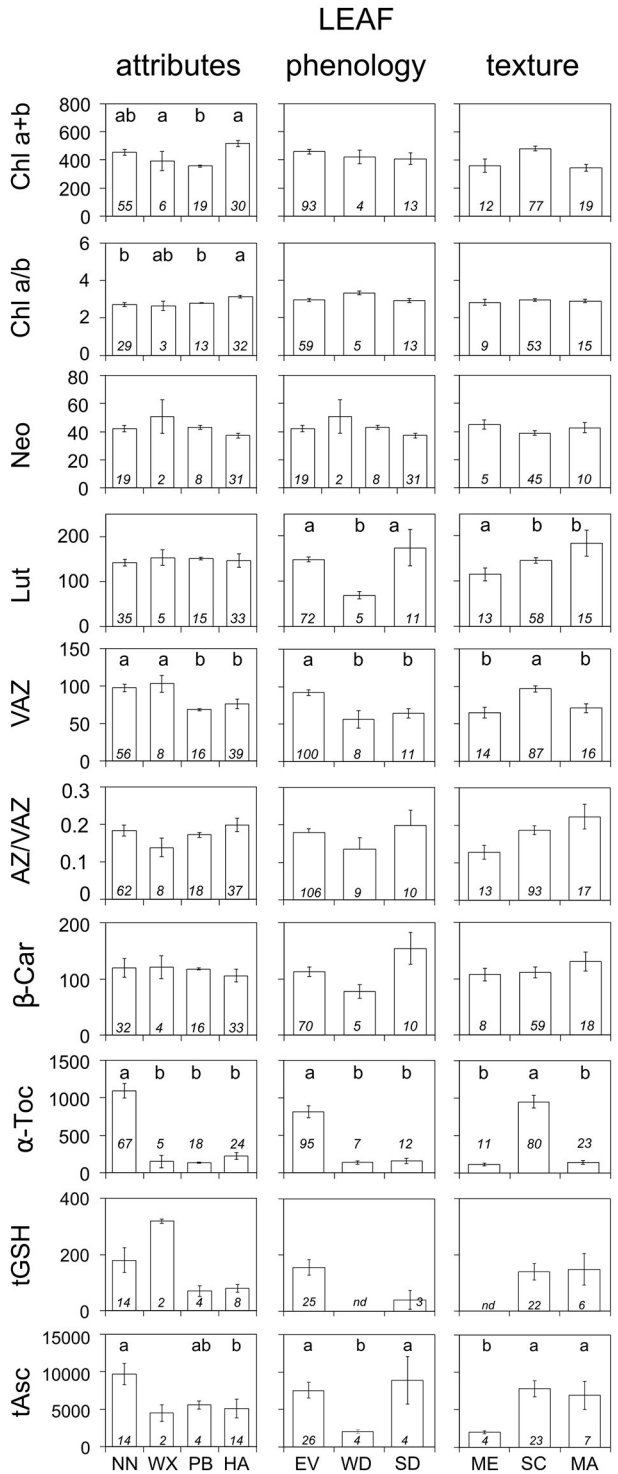

FIGURE 2 | Average composition of photosynthetic pigments and antioxidants according to different leaf traits: attributes, phenology and texture. Units are as in **Figure 1**. Each bar represents the average ± SE of available data (*n*, shown inside the bars). Only data for non-stressed plants were included. When significant, differences in the content of pigments or antioxidants among categories inside each panel are depicted with letters above the bars ($P < 0.05$). "nd" depicts no data available. Abbreviations as in **Table 2**.

Leaf pubescence (presence of trichomes) and glaucescence (waxes) are two of the most conspicuous foliar traits in Mediterranean plants, giving these ecosystems one of their signs of identity. These structures represent an example of evolutionary convergence. They reduce water loss (Peguero-Pina et al., 2017) and they may be highly reflective, limiting the penetration of light to the photosynthetic apparatus and reaching the mesophyll (Agrawal et al., 2009; Camarero et al., 2012). Thereby they reduce the need of photoprotective pigments and antioxidants, supporting the photoprotective role of leaf pubescence in Mediterranean species (Morales et al., 2002). Alternatively, trichomes and waxes can act as protective response since their accumulation is triggered after extenuation of biochemical photoprotection, as has been evidence for the waxes in *Juniperus thurifera*, where their accumulation represented a symptom of species decline (Esteban et al., 2014). Here, we show that biochemical traits (antioxidants and pigments) compensate the absence of morphological protective traits in Mediterranean species (**Figure 2**).

Another characteristic of Mediterranean flora is, compared to other temperate biomes, the higher leaf thickness (Peguero-Pina et al., 2012) and LMA (Traiser et al., 2005). Despite structural filtering of light and efficient protection against water lost of most sclerophyllous leaves, it has been proposed the existence of a trade-off between photosynthesis and LMA in relation with Leaf Economics Spectrum (Wright et al., 2004). It seems that mesophyll conductance (g_s) may be the most limiting factor for carbon assimilation, showing those leaves with highest LMA values, a reduction in CO_2 diffusion to the carboxylation sites (Flexas et al., 2014; Onoda et al., 2017; Peguero-Pina et al., 2017). This implies that plants with high LMA will generally show lower photosynthetic capacity and thus, may require higher levels of photoprotective metabolites. Interestingly, we found a positive relationship between LMA and photoprotective compounds, confirming that thicker leaves had more photoprotective demand (**Figure 3**). The cost associated with greater investment in photoprotection compounds in high LMA leaves underpin longer lifespan in these species (Wright et al., 2004; Díaz et al., 2015), meaning that long-lasting leaves may cope with higher cumulative number of stressful events along their life-span.

Pigments and Photoprotection

Pigment composition in Mediterranean plants does not differ substantially from the standard stoichiometry of higher plants of other biomes. Thus, with a few exceptions, photosynthetic pigment composition of non-stressed Mediterranean plants (**Table 1**) fitted within the 95% percentile of the control values reported by Esteban et al. (2015a) in a wider survey, including more than 800 plant species from diverse biomes. Most of these exceptions were found in the genera *Quercus* and *Cistus*, with the high Chl a/b and β-Car values being singularly frequent. Stress responses diverged from some of the general patterns described by Esteban et al. (2015a) in intensity and direction. In general, most stress factors induced increases on protective carotenoid

TABLE 2 | Plasticity index of main parameters according to relevant factors of variation considered.

		TYPE OF STRESS				MODEL GENERA		WOODY CHARACTER		LEAF PHENOLOGY			LEAF TEXTURE			LEAF AGE		LEAF ATTRIBUTES				
	All (*150*)	D (*55*)	HT (*13*)	LT (*19*)	S (*27*)	Q (*37*)	C (*20*)	WO (*121*)	HE (*29*)	EV (*110*)	SD (*23*)	WD (*16*)	MA (*32*)	ME (*30*)	SC (*85*)	YG (*58*)	MT (*31*)	WX (*7*)	PB (*35*)	HA (*34*)	NN (*74*)	Scale
Fv/Fm	0.13	0.06	0.07	0.23	0.19	0.16	0.15	0.15	0.03	0.15	0.1	0.03	0.14	0.03	0.16	0.06	0.13	0.11	0.12	0.16	0.13	0
Chl a+b	0.26	0.25	0.29	0.17	0.36	0.23	0.21	0.25	0.29	0.26	0.16	0.34	0.25	0.36	0.24	0.22	0.23	0.21	0.33	0.21	0.25	
Chl a/b	0.14	0.14		0.08	0.19	0.14	0.04	0.14	0.1	0.16	0.06	0.13	0.1	0.11	0.16	0.1	0.1	0.22	0.1	0.13	0.17	
Neo	0.17	0.21		0.11	0.2	0.16		0.18		0.17	0.13	0.21	0.27	0.16	0.15	0.19	0.13		0.26	0.17	0.14	
Lut	0.22	0.23		0.19	0.29	0.22	0.2	0.23	0.18	0.23	0.18	0.24	0.32	0.18	0.21	0.22	0.19	0.21	0.26	0.24	0.19	
VAZ	0.38	0.39	0.48	0.28	0.45	0.41	0.26	0.39	0.29	0.39	0.28	0.37	0.42	0.37	0.37	0.33	0.4	0.4	0.4	0.41	0.35	
AZ/VAZ	0.57	0.67	0.48	0.58	0.57	0.6	0.47	0.57	0.64	0.58	0.56	0.53	0.5	0.62	0.58	0.6	0.62	0.7	0.57	0.56	0.57	
β-Car	0.23	0.28		0.18	0.26	0.24	0.16	0.22	0.32	0.23	0.18	0.43	0.24	0.34	0.21	0.3	0.15	0.19	0.23	0.24	0.23	
α-Toc	0.65	0.65	0.84	0.39	0.82	0.61	0.65	0.65	0.62	0.63	0.65	0.77	0.67	0.71	0.61	0.68	0.47	0.61	0.64	0.6	0.67	
tGSH	0.37			0.38		0.5	0.29	0.37		0.32					0.36		0.37		0.63	0.5	0.29	
tAsc	0.44	0.61		0.37		0.5		0.42	0.61	0.45	0.35	0.47	0.37	0.52	0.45	0.6	0.38		0.37	0.53	0.41	
LMA	0.33	0.23			0.46	0.32	0.49	0.33	0.32	0.3	0.55		0.49	0.32	0.28	0.42	0.24		0.37	0.28	0.4	0.9

*Units as in **Figure 1**. Low values appear in green and they get redder as they increase (see scale). Gaps indicate insufficient data to be calculated. D, Drought; HT, high temperature; LT, low temperature; S, seasonal; Q, Quercus; C, Cistus; WO, woody; HE, herb; EV, evergreen; SD, summer deciduous; WD, winter deciduous; MA, malacophyllous; ME, membranous; SC, sclerophyllous; YG, young; MT, mature; WX, Waxy; PB, pubescent, HA, hairy abaxial; NN, none. Number of cases (n) is indicated in brackets.*

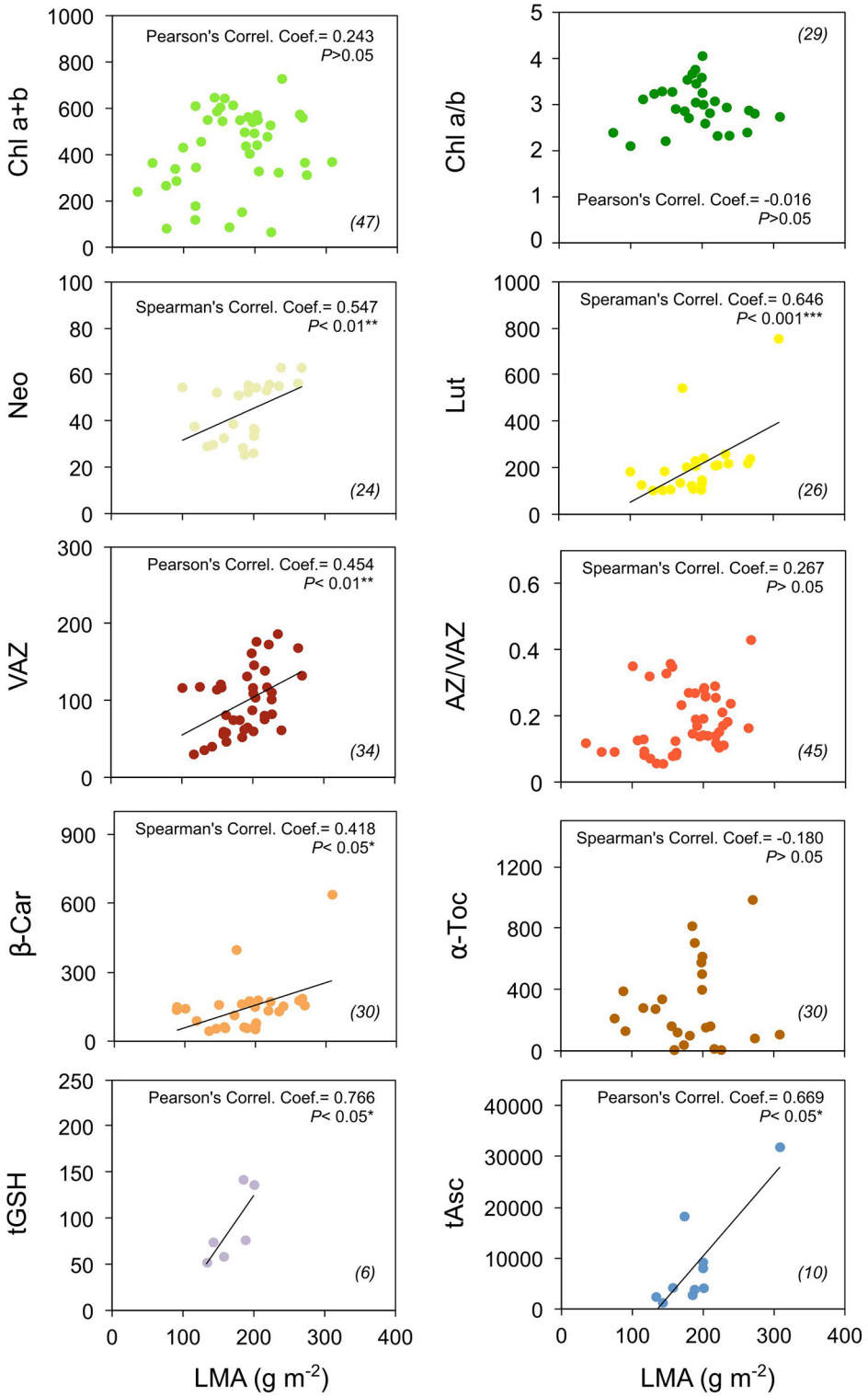

FIGURE 3 | Photosynthetic pigment and antioxidant contents in relation to leaf mass area (LMA) of "non-stressed" Mediterranean plants. Pearson's (or Spearman's for non-normally distributed data) correlation coefficient and significance, and number of data (in brackets) are shown for each panel. Units are as in **Figure 1**. Asterisks denote the extent of significance. Only predawn data were included for this analysis.

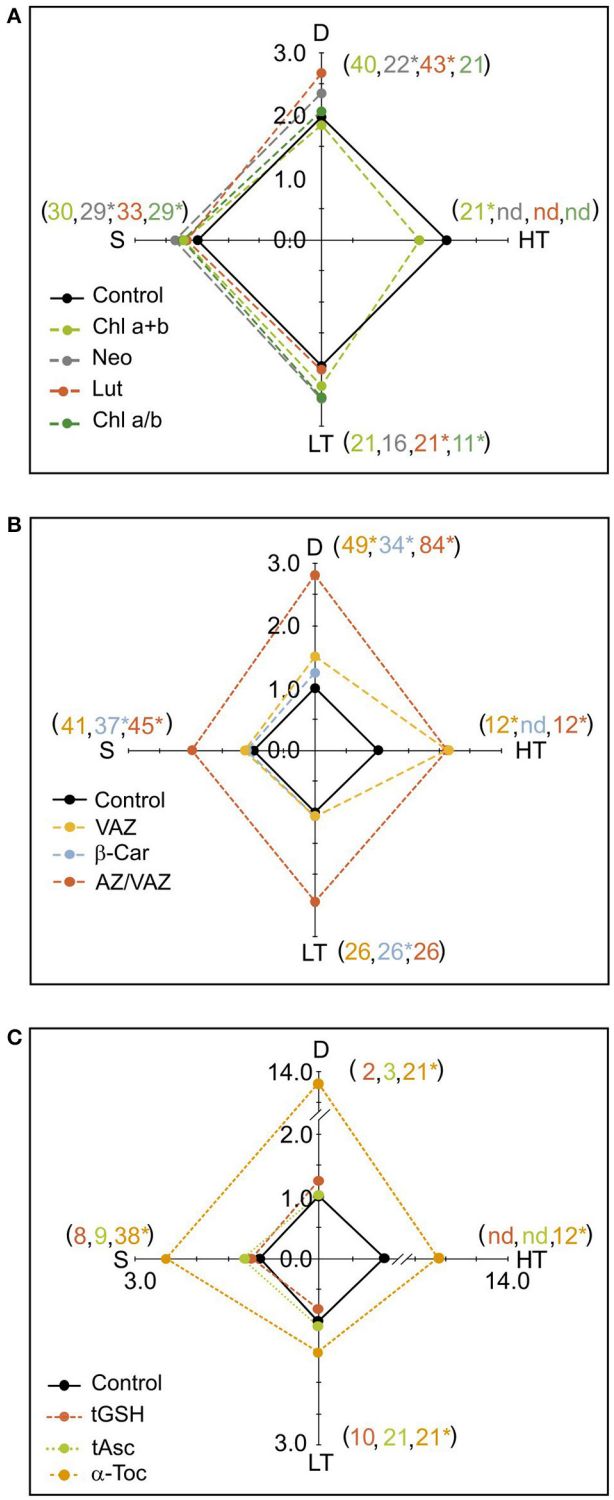

FIGURE 4 | Spiderplots showing the effect of the main stress factors considered in the Mediterranean regions (D, drought; HT, high temperature; LT, low temperature; S; Seasonality) on photosynthetic pigments and antioxidants. **(A)** Photosynthetic pigments: chlorophylls and structural xanthophylls Neo and Lut. **(B)** Photosynthetic pigments: V-cycle xanthophyls and b-Car. **(C)** Free antioxidants: tGSH, tASC and a-Toc. Units are as in **Figure 1**, except for tGSH (here shown in μmol g^{-1} DW). For each stress factor, every data was

(Continued)

FIGURE 4 | Continued

normalized to its respective control within each manuscript of origin (controls were values cataloged as "control" in the original papers). Thus, value 1 indicates the control in the boxplots, while the deviation from 1 indicates the impact of the stress on the parameters analyzed. For more details see material and methods. Shown data are means of several cases. The number of cases used for each factor and compound is shown in brackets. Note that the color of the number indicates the corresponding metabolite. SE is not shown for clarity, but was <6% of the mean in all cases except from α-Toc, for which SE < 10%. Asterisks denote significant differences from control ($P < 0.05$).

pools (**Figures 4A,B**) such as β-Car (Munné-Bosch et al., 2003), VAZ and Lut (Li et al., 2009), but also of structural components such as Neo (**Figure 4A**) that are supposed to be strongly stable (Schmid, 2008). Among the environmental stressors, drought was the factor that affected more strongly pigment composition followed by seasonality and low temperatures. This corresponds with the Mediterranean dual stress model, in which summer drought and, in some places winter low temperatures, determines plant responses and distribution (Mitrakos, 1982). Besides, a high degree of plasticity was observed in all these responses (**Table 2**). This is in agreement with the large phenotypic plasticity as one of the typical strategies of Mediterranean plants to cope with their changing environmental conditions (Hormaetxe et al., 2007).

Under severe stress conditions, specially low temperature, the VAZ cycle does not reach a complete relaxation (Demmig-Adams and Adams, 1996; Demmig-Adams et al., 2006), and AZ/VAZ is typically higher than 0.5 before dawn (Míguez et al., 2015). This process has been characterized in detail in boreal conifers and alpine evergreens (Verhoeven, 2014; Míguez et al., 2017) involving profound change in the organization of photosynthetic apparatus. The overnight retention of AZ/VAZ is usually concurrent with a proportional decrease of photochemical efficiency (measured as Fv/Fm) (Demmig-Adams et al., 2006), also known as "winter photoinhibition." In Mediterranean evergreens, the existence of an inverse relationship between AZ/VAZ and Fv/Fm, notably in winter (**Figure 5B**), suggests that Mediterranean evergreens employ the same mechanism of down-regulation of photochemical efficiency as boreal and alpine evergreens. Furthermore, present analysis suggests that this relationship extends to other stress factors rather than low temperature (**Figure 5B**, white dots), particularly water stress, which is the main environmental driver in Mediterranean ecosystems. Thus, high predawn AZ/VAZ values were observed only in plants with a low stomatal conductance (**Figure 5D**). Drought-stress induction of overnight retention of A+Z has been described in desert and desiccation-tolerant plants (Barker et al., 2002; Fernández-Marín et al., 2011, 2013). Here, we confirm that this mechanism is also activated at milder stress levels, such as that exerted by Mediterranean summer, being more universal than previously expected. Nevertheless, the high dispersion of values shown in **Figure 5** indicates that the retention of A+Z does not always ensure a decrease in photochemical efficiency, likely playing other roles in the responses to stress (Havaux and Niyogi, 1999; Dall'Osto et al., 2010).

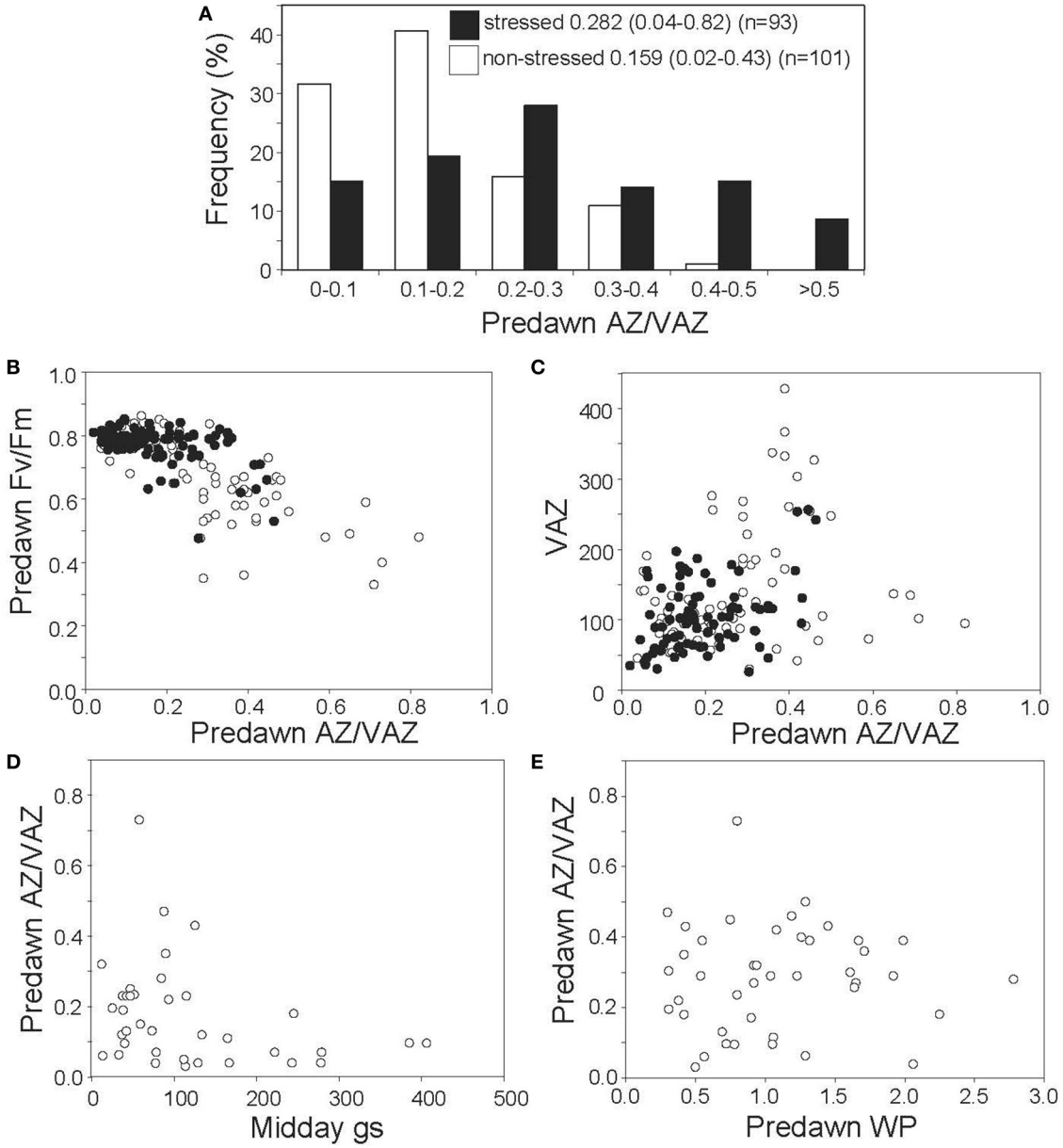

FIGURE 5 | Nocturnal retention of AZ/VAZ in Mediterranean species. **(A)** Frequency histograms of the predawn AZ/VAZ in non-stressed (empty bars) and stressed (black bars) plants. Numbers denote the mean, the minimum and maximum values (in parenthesis) and the number of data. **(B)** Relationship between predawn AZ/VAZ and predawn Fv/Fm in winter (white circles) and other seasons (black circles). **(C)** Relationship between predawn AZ/VAZ (mol mol^{-1}) and predawn VAZ pool (mol mol^{-1}Chl) in winter (white circles) and in other seasons (black circles). **(D)** Relationship between predawn AZ/VAZ and midday stomatal conductance (g_s in mmol H_2O m^{-2} s^{-1}). **(E)** Relationship between predawn AZ/VAZ and predawn water potential (in −MPa).

Role of Lipophilic and Hydrophilic Antioxidants

In contrast to the constricted stoichiometry of pigment composition in chloroplasts caused by the binding of pigments to thylakoid proteins (Antal et al., 2013), antioxidants display a much higher degree of variation, as we show in this study (**Table 1**). Although all species have available the same set of low molecular weight lipophilic (α-Toc, β-Car, and Zea) and hydrophilic antioxidants (Asc and GSH) to cope with oxidative stress, strong differences exist depending on taxonomy, plant growth and leaf characteristics (phenology, texture, age, attributes) (**Figures 1**, **2**) and in response to stressful conditions (**Figure 4**). Thus, such differences can reach several orders of magnitude for α-Toc and tAsc (**Table 1**, **Figures 1**, **4**). Curiously, despite Asc is the most abundant antioxidant in leaves (**Table 1**), neither tAsc nor tGSH was induced by any climatic stressors (**Figure 4**). This result suggests that most photo-oxidative damage undergone by Mediterranean species comes from the chloroplast, where α-Toc and β-Car are embedded in thylakoid membranes (Antal et al., 2013).

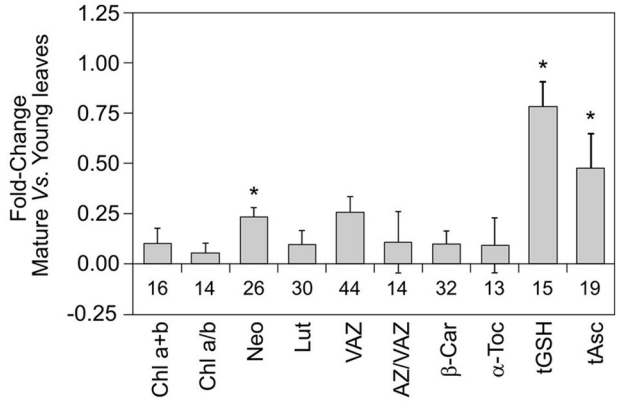

FIGURE 6 | Fold-changes of photosynthetic pigments and antioxidants between mature and young leaves in sclerophyllous *Quercus* (*Q. coccifera*; *Q. ilex*; *Q. suber*). Only articles that studied jointly mature and young leaves were considered (total articles = 8). The data of young leaves were taken as reference values in each unit of the analyzed metabolites. The fold-changes were calculated as follows: Fold-change = (value of mature leave/value of young leave) −1. Thus, reference value was equaled to zero. Bar represents the mean ± SE. The number of reported data (*n*) is shown below each bar. Asterisk represent statistical significance between young and mature leaves ($P < 0.05$).

Interestingly, α-Toc and VAZ contents in angiosperms significantly differed from those found in gymnosperms (**Figure 2**). In angiosperms, there was a higher α-Toc and a lower VAZ contents than in gymnosperm. Within angiosperm, the higher content of α-Toc in woody species suggests an increased demand for α-Toc associated to the woody trait. This result is verified by an increment of α-Toc from seedling to adults, showing at the same time a decrease in VAZ (**Figure 1**). As stated before by Esteban et al. (2009), α-Toc increases progressively in the phylogeny from green algae to gymnosperms and even more to angiosperms, whereas the size of total VAZ pool shows the opposite pattern. This supports the idea that VAZ pigments and α-Toc share some photoprotective functions, which may lead to some degree of inter-compensation (Havaux and García-Plazaola, 2014). The cooperative function of VAZ and α-Toc is accounted because both are lipophilic compounds synthesized in the plastids being able to interact with membranes where they share several functions. Both are antioxidants (Falk and Munne-Bosch, 2010) and quenchers of $^1O_2$ (Triantaphylidès and Havaux, 2009) and carotenoids may act also as membrane stabilizers (Havaux, 1998) and as quenchers of triplet Chl (Esteban et al., 2015b). Consequently, in agreement with Wujeska et al. (2013) VAZ and α-Toc exhibit the highest plasticity among pigments and antioxidants, respectively (**Table 2**). The paramount importance of α-Toc in the photoprotective response of Mediterranean plants is confirmed by the fact that it was the parameter that showed the highest responsiveness under all stresses analyzed in this manuscript (**Figure 4** and **Table 2**). Among stressors, drought induced the highest α-Toc increase, followed by low and high temperatures, highlighting the importance of climatic stressors on the performance of plants in the Mediterranean ecosystems.

Concluding Remarks

Throughout this work several gaps of knowledge related to photoprotection in Mediterranean plants have been identified: (i) herbs have been much less studied than woody species, (ii) and gymnosperms than angiosperms; and (iii) much less data are available about hydrophilic antioxidants than about lipophilic ones. Despite of these limitations in the literature, several conclusive statements have arisen from this analysis. The traits woody and high LMA, were generally related with a high demand for photoprotection. This can be explained by the evergreen strategy of most Mediterranean plants that implies high need of protection because of the long lifespan of their leaves. tGSH and tAsc showed the highest amplitude in their content. Nevertheless, α-Toc and VAZ were more responsive against Mediterranean climatic stressors. Thus, α-Toc responded mainly to drought and also to high temperature, while VAZ responded mainly to low temperature and also to drought. Therefore, the powerful photoprotective system of Mediterranean species pivots on lipophilic antioxidants, in particular Z and α-Toc, which play multiple roles in the plastid. In the context of a global climatic change, in which drought and extreme temperatures events are foreseen to increase their frequency in the Mediterranean area, the plasticity of photoprotective mechanisms to cope with such enhancing stressors will be fundamental. Resilience of Mediterranean species, compared with the template flora will likely benefit from such higher plasticity. To fully address these questions a complete understanding of this issue covering ecoregions other than the Mediterranean Basin (e.g., Western and South Australia, Cape Region, coastal California, and Chile) is needed.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by the Basque Government (UPV/EHU-GV IT-1018-16) and by the Spanish Ministry of Economy and Competitiveness (MINECO) and by the ERDF (FEDER) (CTM2014-53902-C2-2-P). RE and BF received a "Juan de la Cierva-Incorporación" grant (IJCI-2014-21452 and IJCI-2014-22489, respectively). FM received a postdoctoral grant for Recent Ph.D. and MG is recipient of a specialization fellowship for Ph.D. researchers, both awarded by the Research Vice-Rectorate of the UPV/EHU.

ACKNOWLEDGMENTS

We thank Drs. O. Barrutia and J. M. Becerril for valuable contribution to this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01051/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer LLVJ and handling Editor declared their shared affiliation, and the handling Editor states that the process nevertheless met the standards of a fair and objective review

frontiers in Plant Science

PERSPECTIVE
published: 09 November 2016
doi: 10.3389/fpls.2016.01690

Dissecting Long-Term Adjustments of Photoprotective and Photo-Oxidative Stress Acclimation Occurring in Dynamic Light Environments

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Changes in light intensity directly affect the performance of the photosynthetic apparatus. Light energy absorbed in excess of cells' needs leads to production of reactive oxygen species and photo-oxidative damage. Excess light in both constant and dynamic environments induces photoprotective acclimation in plants. Distinct sets of signals and regulatory mechanisms are involved in acclimatory adjustment of photoprotection and photosynthesis under constant and dynamic (fluctuating) light conditions. We are still far away from drawing a comprehensive picture of acclimatory signal transduction pathways, particularly in dynamic environments. In this perspective article, we propose the use of *Arabidopsis* plants that produce H_2O_2 in chloroplasts (GO plants) under atmospheric CO_2 levels as a tool to study the mechanisms of long-term acclimation to photo-oxidative stress. In our opinion there are new avenues to future investigations on acclimatory adjustments and signal transduction occurring in plants under dynamic light environments.

Keywords: acclimation, fluctuating light, photoprotection, reactive oxygen species, retrograde signaling

OPEN ACCESS

Edited by:
Massimiliano Tattini, National Research Council, Italy

Reviewed by:
Or Sperling, Agricultural Research Organization, Volcani Center, Israel
Lucia Guidi, Università di Pisa, Italy

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *12 July 2016*
Accepted: *26 October 2016*
Published: *09 November 2016*

Citation:
Matsubara S, Schneider T and Maurino VG (2016) Dissecting Long-Term Adjustments of Photoprotective and Photo-Oxidative Stress Acclimation Occurring in Dynamic Light Environments. Front. Plant Sci. 7:1690. doi: 10.3389/fpls.2016.01690

ACCLIMATION TO PHOTO-OXIDATIVE STRESS IS INDUCED BY FLUCTUATING LIGHT

Rapid climate changes and transformation of landscapes by extensive agricultural practices impose environmental perturbations. Plants in the affected areas respond to the perturbations through acclimation (within generation) or adaptation (over generations). Light intensity can vary rapidly by a few orders of magnitude as clouds travel in the sky or wind moves outer canopy leaves and taller plants. Especially, wind can briefly expose inner canopy leaves and understory plants to intense sunlight. Upon large and abrupt increase in light intensity, photosynthetic light energy utilization is limited biochemically. This is attributed to low availability of the Calvin-Benson cycle intermediates, low activation state of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), and also low stomatal conductance measured in leaves under low light (LL) conditions (Kirschbaum and Pearcy, 1988; Pearcy, 1990).

When light energy is absorbed by photosynthetic pigments in excess of cells' needs for reducing equivalents and chemical energy (excess light, EL), it can lead to production of reactive

oxygen species (ROS) and photo-oxidative damage in oxygenic photosynthetic organisms. A range of mechanisms have evolved to reduce uncontrolled production of ROS and to protect the photosynthetic apparatus against their detrimental effects (Foyer and Noctor, 2000; Endo and Asada, 2008; Li et al., 2009). These include thermal energy dissipation which is rapidly induced in light-harvesting antenna complexes by a proton concentration gradient (ΔpH) across the thylakoid membrane (termed qE), alternative sinks for excess electrons (e.g., water-water cycle and cyclic electron flows around photosystem I, CEF) which contribute to ΔpH formation especially when liner electron transport rate (ETR) is low, and enzymatic and non-enzymatic antioxidative systems which detoxify ROS (**Figure 1A**).

When EL conditions persist, plants are able to augment their photoprotective capacities via long-term acclimation. In general, multiple mechanisms of photoprotective acclimation (**Figure 1A**) operate in plants under constant as well as dynamic EL environments. In particular, fluctuating light (FL) with short periods of EL (i.e., dynamic EL) induces, primarily or initially, long-term acclimatory changes that are characterized by improved protection against photo-oxidative stress and reduced carbon gain (Alter et al., 2012). For instance, LL-grown *Arabidopsis* plants upregulate photoprotection in highly dynamic EL conditions without developing symptoms of severe photo-oxidative injuries, such as strong photoinhibition or bleaching (Alter et al., 2012). Similar photoprotective responses are also seen during acclimation to high light (HL, i.e., constant EL), although in HL they are often accompanied by enhancement of photosynthesis and thus increased carbon gain (Leakey et al., 2002; Alter et al., 2012). There seems to be an inverse relationship between the maximum photosynthetic capacity, which is developed through photosynthetic acclimation, and the frequency of LL-HL transitions (Retkute et al., 2015). Selective upregulation of photoprotection, but not photosynthesis, in highly dynamic FL suggests that distinct sets of signals and regulatory mechanisms are involved in acclimatory adjustment of photoprotection and photosynthesis, and that signal molecules, which trigger photo-oxidative stress acclimation, are produced in leaves under the FL conditions. In this article we use the term "FL" to refer to highly dynamic EL conditions, while we are aware that FL may not always cause EL and photo-oxidative stress, depending on the amplitude and frequency of light fluctuations (Yin and Johnson, 2000; Alter et al., 2012; Retkute et al., 2015).

CHLOROPLAST RETROGRADE SIGNALING IS INVOLVED IN PHOTO-OXIDATIVE STRESS ACCLIMATION

Today it is widely recognized that ROS not only can damage cellular components but also act as signals to induce abiotic and biotic stress responses (Foyer and Noctor, 2000; Apel and Hirt, 2004; Mittler et al., 2011; Karpinski et al., 2013; Dietz et al., 2016). Multiple ROS can be generated in chloroplasts under photo-oxidative stress, such as singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical (OH), and hydrogen peroxide (H_2O_2) (Asada, 1999; Foyer and Noctor, 2000). Among these, H_2O_2 alone would be able to diffuse into the cytosol because high reactivity and charge of the other species prevent them from diffusing a long distance across chloroplast envelopes. Intracellular H_2O_2 signaling engages both compartment-specific and non-specific pathways. In the case of retrograde signaling, chloroplastic H_2O_2 produced by ectopic overexpression of glycolate oxidase (GO) in chloroplasts (Fahnenstich et al., 2008) (**Figure 1B**) induces transcriptional changes in the nucleus, which partly, but not fully, overlap with the responses to peroxisomal H_2O_2 (Balazadeh et al., 2012; Sewelam et al., 2014).

Though $^1O_2$ may not move far, it can give rise to secondary messengers by reacting with nearby molecules such as β-carotene (Ramel et al., 2013). Oxidation of β-carotene produces β-cyclocitral, a reactive electrophile species that can modify transcription of $^1O_2$-responsive genes in the nucleus (Havaux, 2014). Whilst some β-carotenes are continuously oxidized and degraded in thylakoids during illumination with or without EL (Beisel et al., 2010), elevated production of $^1O_2$ and thus β-cyclocitral under photo-oxidative stress may trigger acclimatory responses that are distinct from $^1O_2$-induced cell death (op den Camp et al., 2003).

The number as well as the variety of agents implicated in chloroplast retrograde signaling have been increasing in the last years. For example, the redox state of the plastoquinone pool, different metabolites (e.g., tetrapyrroles, phosphoadenosine phosphate, and methylerythritol cyclodiphosphate) and hormones (abscisic acid, salicylic acid, and jasmonic acid) are regarded as such signaling agents to trigger long-term acclimatory adjustments (Dietz and Pfannschmidt, 2011; Sun et al., 2011; Estavillo et al., 2012; Xiao et al., 2012; Barajas-Lopez Jde et al., 2013; Karpinski et al., 2013; Dietz, 2015; Laloi and Havaux, 2015). To reconstruct signaling networks from individual components and pathways is a major challenge in understanding time-dependent regulation and interaction of stress response networks in plants (Dietz, 2015). Acclimation to photo-oxidative stress has been studied extensively in the context of HL or constant EL acclimation, in which plants manifest parallel enhancement of photoprotection and photosynthesis alongside other responses related to temperature and/or water stress. Highly dynamic FL, which predominantly elicits photoprotective responses (Alter et al., 2012), offers a complementary approach to investigate signals and pathways that are primarily engaged in photo-oxidative stress acclimation.

Arabidopsis PLANTS THAT PRODUCE H_2O_2 IN CHLOROPLASTS ARE A MODEL TO STUDY PHOTO-OXIDATIVE STRESS ACCLIMATION AND SIGNALING

Arabidopsis plants, in which GO is targeted to chloroplasts, generate H_2O_2 in chloroplasts under ambient CO_2 concentrations (photorespiratory conditions) (Fahnenstich et al., 2008; Strand et al., 2015). Because the GO reaction depends on the substrate

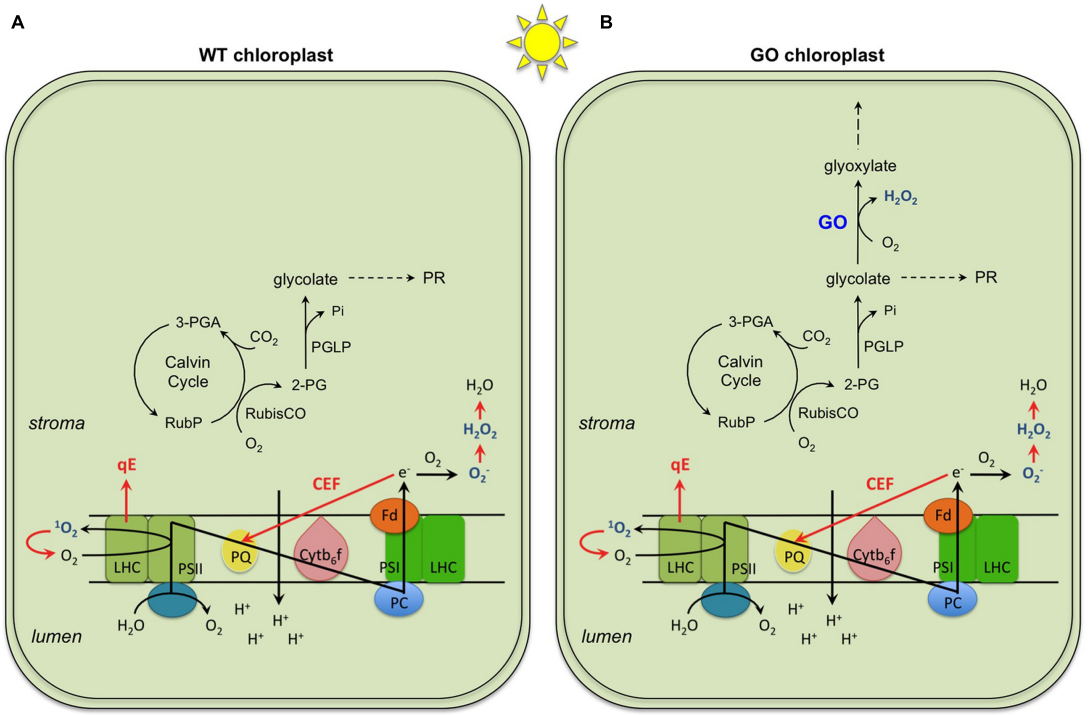

FIGURE 1 | General mechanisms of photoprotection (highlighted in red) occurring in chloroplasts of wild type (WT) (A) and the GO plants **(B)**. Three mechanisms represented are (i) qE, the thermal energy dissipation of excess absorbed light at the light harvesting complex (LHC) of Photosystem II (PSII); (ii) CEF, the cyclic electron flow around Photosystem I (PSI), and (iii) enzymatic and non-enzymatic ROS scavenging through production of O_2, H_2O_2 and H_2O. These mechanisms operate both in FL and CL; the only difference demonstrated so far is a more dramatic effect of CEF in FL than in CL. Shown in or around the thylakoid membrane are the components of photosynthetic electron transport chain: plastoquinone (PQ), cytochrome b_6f complex (Cytb$_6$f), plastocyanin (PC), and ferredoxin (Fd). In addition, the activities of RubisCO as carboxylase and oxygenase in light and under ambient CO_2 and O_2 conditions are shown. In the WT, the 2-phosphoglycolate (2-PG) produced initiates the photorespiratory cycle (PR; not shown in detail in the figure; for details see (Maurino and Peterhansel, 2010)). In the GO plants, 2-PG is delivered to the PR and the glycolate produced in the chloroplasts is also used by glycolate oxidase (GO) generating H_2O_2. 3-PGA, 3-phosphoglycerate; PGLP: 2-phosphoglycolate phosphatase; RubP, ribulose 1-5,bisphosphate.

provided by the oxygenase activity of RubisCO in the light (**Figure 1B**), the level of GO-dependent H_2O_2 production can be controlled by changing the growth conditions. When growing in LL (75 μmol photons m^{-2} s^{-1}) and ambient CO_2 concentration (380 ppm), the GO plants are smaller than the wild-type (WT) plants and present patchy pale-green leaf lamina (**Figure 2A**; constant light, CL) as a result of H_2O_2 production in chloroplasts and overload of the antioxidant machinery (Fahnenstich et al., 2008). Under HL the GO plants develop severe oxidative lesions and ultimately bleach, whereas combinations of very LL (30 μmol photons m^{-2} s^{-1}) and ambient CO_2 or LL and high CO_2 (4,000 ppm) allow them to grow like WT (Fahnenstich et al., 2008; Balazadeh et al., 2012; Sewelam et al., 2014). Also, they become as big and green as WT and recover the WT level of photosynthetic ETR in LL when transferred from ambient to high CO_2 conditions (Fahnenstich et al., 2008). These features make the GO plants a unique, well-established model to study the action of H_2O_2 in chloroplast retrograde signaling (Balazadeh et al., 2012; Sewelam et al., 2014).

Interestingly, FL conditions applied to the GO plants growing in ambient CO_2 —in spite of (or maybe because of) the effect of FL to impose photo-oxidative stress— provoke disappearance of the characteristic patchy pale-green phenotype of these plants (**Figure 2A**). This reversion to normal green leaf lamina under FL allows the GO plants to maintain similar relative growth rates in CL and FL, whereas WT exhibits growth reduction in FL (**Figure 2B**). The FL-induced recovery of leaf color in the GO plants is most probably due to upregulation of H_2O_2 scavenging in the chloroplast, as the patchy pale-green phenotype is a consequence of H_2O_2 accumulation (Fahnenstich et al., 2008). This assumption, i.e., FL-induced acclimatory enhancement of ROS (H_2O_2) scavenging systems in the GO plants, is supported by the observations made in carotenogenic mutants of *Arabidopsis*; despite having reduced capacities for qE and carotenoid-dependent ROS (mainly $^1O_2$) scavenging, these mutants do not suffer from chronic photo-oxidative damage under FL conditions because they can upregulate other photoprotective and ROS scavenging mechanisms by long-term acclimation (Caliandro et al., 2013). The increased activity of superoxide dismutase (SOD) found in leaves of WT following FL acclimation (Alter et al., 2012) also points to an increased detoxification capacity for H_2O_2 which arises from disproportionation of $O_2^{\bullet-}$ catalyzed by SOD.

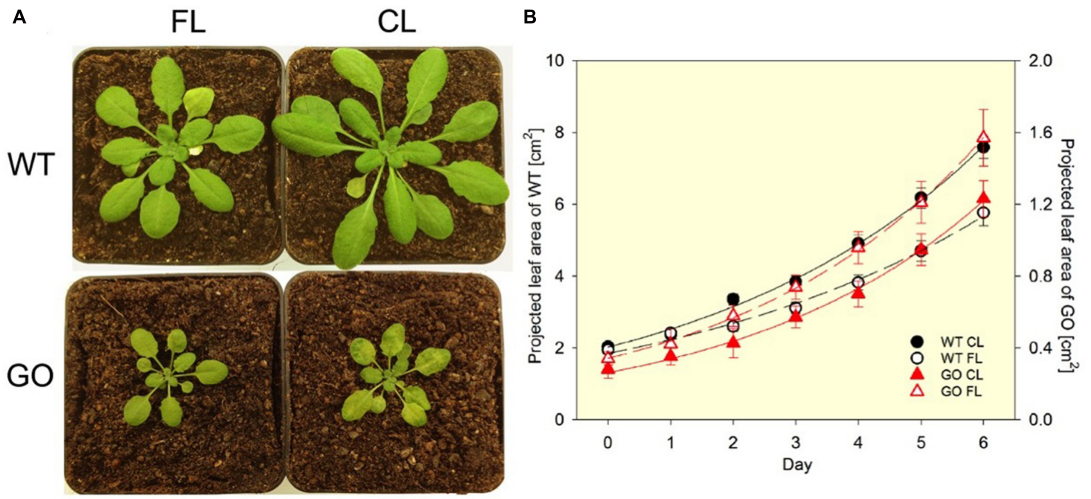

FIGURE 2 | Phenotypes of wild-type (WT) and GO plants grown under fluctuating light (FL) and constant light (CL) conditions in ambient CO_2. **(A)** Under FL (switching between ~50 μmol photons m^{-2} s^{-1} for 280 s and ~1000 μmol photons m^{-2} s^{-1} for 20 s) the GO plants have normal green leaves, while under CL (~75 μmol photons m^{-2} s^{-1}) they develop pale-green patches due to the chloroplastic production of H_2O_2. The pictures were taken after 8-days exposure to FL or CL. **(B)** The FL condition reduces leaf growth of the WT plants (left y-axis). The GO plants (right y-axis) are much smaller than the WT and the exposure to FL does not impair growth in the GO plants compared to the CL condition. Projected leaf area is the leaf area that is visible in a 2D top-view image. Solid and broken lines show exponential regression for the CL and FL data sets, respectively. The R^2 values of the fitting are: 0.996 (WT CL), 0.991 (WT FL), 0.997 (GO CL), and 0.999 (GO FL). Relative growth rates (% d^{-1}) obtained by the regression are: 22.1 ± 0.6 (WT CL), 18.7 ± 0.8 (WT FL), 25.7 ± 0.7 (GO CL), and 25.4 ± 0.3 (GO FL). The difference in relative growth rate between CL and FL is significant for the WT ($P = 0.002$). $n = 16$ (WT) and 9 (GO).

Thus, we hypothesize that long-term acclimatory upregulation of H_2O_2 scavenging occurs in chloroplasts under FL conditions. H_2O_2 reduction in chloroplasts could proceed mainly via peroxidase systems, including glutathione peroxidase (Gpx) and peroxiredoxin (Prx) in the stroma, as well as thylakoid-bound and stromal ascorbate peroxidase (tAPX, sAPX) coupled to monodehydroascorbate reductase (MDHAR) and also dehydroascorbate reductase (DHAR) (Foyer and Noctor, 2011). Gpx and Prx use thiol-based peroxide-detoxification mechanisms that are maintained by glutathione and glutathione reductase (GR) (Dietz and Pfannschmidt, 2011). Regeneration of ascorbate by DHAR is also dependent on glutathione and GR, while MDHAR uses NAD(P)H to regenerate ascorbate. In addition to the removal of H_2O_2, other photoprotective mechanisms such as CEF and carotenoid-dependent reactions could also contribute to the rescuing of the GO phenotype in FL by keeping the level of ROS production under control (**Figure 1**). Indeed, CEF is activated by chloroplastic H_2O_2 produced in HL (Strand et al., 2015) and carotenoid contents (especially xanthophyll-cycle pigments) increase in leaves exposed to FL (Alter et al., 2012; Caliandro et al., 2013).

FUTURE DIRECTIONS

So far it is not known whether, and if yes, which H_2O_2 scavenging pathways are upregulated in chloroplasts during FL acclimation. This could be studied by analyzing antioxidant defense systems in the GO plants during acclimation to CL and FL conditions. An important question that can then be tackled is the long-term acclimation of H_2O_2 scavenging systems. Signal agents, which are generated in FL conditions and lead to acclimatory enhancement of H_2O_2 scavenging, must be different from the signals induced by chloroplastic H_2O_2 produced in the GO plants under CL and ambient CO_2 levels. Close inspections of the GO plants during FL acclimation at different response levels —from gene expression, protein and metabolite accumulation to physiological phenotype— could shed light on components and signal agents involved in upregulation of H_2O_2 scavenging under photo-oxidative stress. Once candidate molecules are identified, the unique feature of the GO plants, which visualizes acclimatory changes in H_2O_2 metabolism under FL, can be exploited again as the genetic background to assess the efficacy of those molecules in upregulating H_2O_2 detoxification. The nature of chloroplast retrograde signaling in FL (dynamic EL), as compared with that in HL (constant EL), inspires further experiments and investigations.

AUTHOR CONTRIBUTIONS

SM and VM contributed equally to writing the manuscript. TS performed the growth analysis shown in **Figure 2**.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 19 July 2017
doi: 10.3389/fpls.2017.01242

Biogeochemical and Ecomorphological Niche Segregation of Mediterranean Woody Species along a Local Gradient

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According with niche theory the species are specialized in different ecological niches, being able to coexist as result of a differential use of resources. In this context, the biogeochemical niche hypothesis proposes that species have an optimal elemental composition which results from the link between the chemical and morphological traits for the optimum plant functioning. Thus, and attending to the limiting similarity concept, different elemental composition and plant structure among co-occurring species may reduce competition, promoting different functional niches. Different functional habits associated with leaf life-span or growth forms are associated with different strategies for resource uptake, which could promote niche partitioning. In the present study, based on the biogeochemical niche concept and the use of resources in different proportions, we have focused on leaf traits (morphological and chemical) associated with resource uptake, and explored the niche partitioning among functional habits: leaf life-span (deciduous, evergreen, and semideciduous) and growth (tree, shrub, and arborescent-shrub). To this end, we have quantified the hypervolume of the leaf functional trait space (both structure and chemical composition) in a sample of 45 Mediterranean woody species from Sierra Morena Mountains (Spain) growing along a local soil resource gradient. Our results show consistent variation in functional space for woody communities distributed along the environmental gradient. Thus, communities dominated by deciduous trees with faster growth and a predominant acquisitive strategy were characteristic of bottom forests and showed highest leaf biogeochemical space. While semideciduous shrubs and evergreen (arborescent, trees) species, characterized by a conservative strategy, dominated ridge forests and showed smaller functional space. In addition, within each topographical zone or environment type, the foliar biogeochemical niche partitioning would underlie the species ability to coexist by diverging on leaf nutrient composition and resource uptake. Lower niche overlap among functional habits were found, which support that different growth forms and leaf life-habits may facilitate the coexistence of the woody species and niche partitioning along and within the gradient.

Keywords: functional ecology, growth forms, hypervolume, carbon isotope ratio, LDMC, leaf habit, nutrients, SLA

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Manuel J. Macía, Universidad Autonoma de Madrid, Spain
Georgina Conti, Instituto Multidisciplinario de Biologia Vegetal, Argentina

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *29 April 2017*
Accepted: *30 June 2017*
Published: *19 July 2017*

Citation:
de la Riva EG, Marañón T, Violle C, Villar R and Pérez-Ramos IM (2017) Biogeochemical and Ecomorphological Niche Segregation of Mediterranean Woody Species along a Local Gradient. Front. Plant Sci. 8:1242. doi: 10.3389/fpls.2017.01242

INTRODUCTION

According to niche theory, species coexistence is promoted by ecological niche differences (MacArthur and Levins, 1967). While this idea is pivotal in community ecology, empirical evidences are contrasted for several reasons. First, theoretically the niche concept is a long and still-debated concept. Second, operationally, quantifying the whole niche of a species remains elusive. As an alternative, it has been proposed to replace niche axes by phenotypic trait axes in community ecology (McGill's et al., 2006; Violle and Jiang, 2009). In plants, a myriad of studies has focused on plant morphological traits to analyze species niche partitioning along environmental gradients as well as niche partitioning within communities (e.g., Mason et al., 2011; Maire et al., 2012; Lamanna et al., 2014). However, the interpretation of these studies is often ambiguous given that a given morphological trait can be involved in several, sometimes opposed, assembly processes (de la Riva et al., 2017). Community species composition can be viewed as the result of a selection process named filtering, which combines species interactions (biotic filtering) and environmental adversity (abiotic filtering) operating simultaneously and favoring a trait convergence (McGill's et al., 2006). At the same time, the process of niche partitioning tends to favor coexistence of species with divergent traits and complementarity in the use of resources (Maire et al., 2012). Therefore, stabilizing niche differences and relative fitness differences are determined by abiotic and biotic environment (HilleRisLambers et al., 2012). In this regard, given that plants all use the same types of resources (light, nutrients, CO_2, water), the biochemical and ecomorphological characterization of plant niches may be a way to more accurately discriminate plants' ecological strategies and niche partitioning along environmental gradients

Any plant to survive must occupy an environment with conditions that they can tolerate (Winemiller et al., 2015). Thus, habitat adaptation is determined primarily by abiotic constraints, which shape the species distribution along variable resource scenarios, and therefore, spatial trait segregation provides a starting point for building a niche scheme and performance measurements to predict how species respond to resource gradients (Winemiller et al., 2015). However, despite this spatial niche partitioning, species coexist within the same environment, because niche partitioning might involve some combination of strategies for resource use. In this context, a proposed mechanism of plant coexistence is based on the 'biogeochemical niche' hypothesis (Peñuelas et al., 2008). According to this hypothesis, species have an optimal elemental composition resulting from the link between the chemical composition and morphological traits for an optimum plant functioning (Peñuelas et al., 2008). Thus, differences in elemental composition and morpho-structure among co-occurring species may reduce competition, promoting different functional niche (Sardans et al., 2015). Within a determined environment type, the co-occurring species compete in the same space by different resources (not only nutrients, also by water and light) with different intensity, allowing the species coexistence in the same biome.

A great challenge is to identify groups associated with functional traits that allow for successful trait syndromes and ecological predictions. To this end, the assimilation and conservation of resources is a fundamental dimension. Wright et al. (2004) proposed a scheme, known as the 'leaf economics spectrum,' that assumes species segregation along environmental resources gradient, according with a plane of trait variation. Hence, this scheme runs from species with a conservative resource-use strategy (i.e., plants of slow growth, with tissues of high-density and low nutrient content) which are more abundant in low-productivity sites, to species from resource-rich sites with the opposite suite of traits, and associated to rapid resource capture (Wright et al., 2001; Villar et al., 2006; Poorter and Garnier, 2007). In this regard, different functional habits associated with leaf life-span (i.e., evergreen or deciduous) or growth forms (i.e., trees or shrubs) are associated with different strategies for resource uptake, which could promote niche partitioning (Mamolos et al., 1995; Niinemets and Kull, 2003; Lusk and Warton, 2007; Sardans et al., 2015; de la Riva et al., 2016a). Nevertheless, there is a large gap in our understanding on the influence of plant traits (leaf morphology and chemistry), leaf habit and growth forms as drivers of species niche partitioning (Sterck et al., 2011).

A variety of multivariate statistical methods have been developed to understand niche partitioning (e.g., Winemiller et al., 2015). However, more powerful tests of niche theories need to move beyond approaches based on species occurrence and instead to focus explicitly on trait-based approaches, recasting the theories in terms of functional space and diversity. The n-dimensional niche space model (Blonder et al., 2014), which is based on the Hutchinson's multidimensional niche concept (Hutchinson, 1957), allows to quantify niche spaces by assessing the functional trait space that characterize the phenotypic volume occupied by a set of species (Violle and Jiang, 2009; Lamanna et al., 2014). A set of n variables, which represent key and independent biologically axes, are used to create a n-dimensional space, which is defined as "hypervolume." The main advantage of this method is that the set of points are projected into a hyperspace, defining a high-dimensional shape which may include holes or complex geometrical features. In addition, this method allows quantifying the proportion of the hypervolumes that overlap in different habitats, i.e., the fraction of them sharing the same functional space (Blonder et al., 2014).

In a previous work, carried out at regional scale and at a whole plant level (i.e., quantifying leaf, root, and stem traits), we demonstrated that soil water scarcity led to lower plant functional space and trait segregation (de la Riva et al., 2017). Thus, communities of woody Mediterranean plants from arid and semi-arid shrublands displayed smaller functional space, with a higher degree of overlap between them. That pattern would reflect the species cluster around adaptive peaks, which are defined by sets of trait combinations associated with a given set of environmental attributes (Winemiller et al., 2015). In the present study, based on the biogeochemical niche concept and the use of resources in different proportions, we have focused on leaf traits (morphological and chemical) associated

with resource uptake, and explored the niche partitioning among functional habits (leaf life-span and growth form). We have quantified the functional space based on leaf functional traits in a set of 45 woody species from Sierra Morena Mountains (South Spain) growing along a broad gradient of soil resources. Assuming that differences in leaf nutrient composition and morpho-structure reflect different ecological strategies, associated with niche partitioning, and so permitting coexistence (McGroddy et al., 2004; Wright et al., 2004; Chen et al., 2011), we hypothesized that: (i) As a consequence of different plant adaptations to maximize their fitness under determined environmental conditions; we expect a segregation in the foliar chemical composition and morphology among the species along the explored soil resource gradient (mainly soil water availability); (ii) the major differences in leaf chemical and morphological traits are found among species with contrasting growth forms and leaf life-spans. This implies that coexisting species would share their niches by using different ranges and proportions of resources, assuming trade-offs in resource allocation.

MATERIALS AND METHODS

Study Area and Sampling Design

The Mediterranean forests studied are located in Sierra Morena Mountains, in the south of Spain (Córdoba province). The area is characterized by a continental-Mediterranean climate with cold, wet winters and dry, warm summers. Mean annual temperature is 17.6°C (with maximum values in summer reaching 40°C) and mean annual precipitation is 536 mm (with a 3-month period in summer without rainfall; data from AEMET for the years 1971–2000[1]). Several shrub and arborescent species, such as *Cistus albidus* and *Quercus coccifera*, are abundant in drier soils, while broad-leaf deciduous trees, such as *Alnus glutinosa* and *Fraxinus angustifolia*, are dominant in moister soils (see Supplementary Table 1 for details of the 45 studied species). One cultivate species (*Cydonia oblonga*) has been included, because is naturalized in the study area and could potentially alter the biogeochemical niche of the coexistent species. Twelve sampling sites distributed over four different south-facing slopes were selected along a topographic gradient (from ridges to valley bottoms; see Supplementary Figure 1) with the aim of spanning a broad range of variations in soil resource availability, mainly of soil water (Supplementary Figures 2, 3 and de la Riva et al., 2016b). The species composition was recorded by measuring the cover of each woody species intercepted by four 20-m transects in each sampling site.

Trait Measurements

For trait measurements, we selected all the species appearing in the sampling transects, excluding only those with a relative abundance below 1% (in these cases it was difficult to find at least six individuals per species in the sampling site). This gave a total of 45 selected species, many of them appearing in more than one sampling site (Supplementary Table 1). We measured different leaf traits associated with resource uptake and conservation: two key functional traits related to morphology and associated with light capture and growth rate [specific leaf area (SLA)] and stress tolerance [leaf dry matter content (LDMC)], all the macronutrients (N, C, P, K, S, Ca, and Mg) and the isotopic ratio of carbon, which is related with gas exchange and water-use efficiency (Pérez-Harguindeguy et al., 2013).

Six samples of leaves from different individuals per species and site were collected in spring. Specific leaf area (leaf area per unit dry leaf mass; $m^2\ kg^{-1}$) and LDMC (dry mass per unit of water-saturated fresh mass; $g\ g^{-1}$) were measured according to the methods recommended by Pérez-Harguindeguy et al. (2013). Chemical composition was determined for a mixture of leaves, from six different individuals per species and sampling site (except N and C which were measured in each individual). N and C concentrations were measured using an elemental analyser (Eurovector EA, 3000; EuroVector SpA, Milan, Italy). The macronutrients P, K, S, Ca, and Mg were extracted by wet oxidation with concentrated HNO_3 under pressure in a microwave digester, and analyzed by ICP-OES (Domínguez et al., 2012). Carbon isotope ratio ($\delta^{13}C$; ‰) was measured by combustion at 1020°C using a continuous flow isotope-ratio mass spectrometry system by means of Flash HT *Plus* elemental analyzer coupled to a Delta-V Advantage isotope ratio mass spectrometer via a CONFLO IV interface (Thermo Fisher Scientific, Bremen, Germany). The analytical measurement errors were ±0.1‰ for $\delta^{13}C$.

Data Analysis

For all the statistical analyses, plant species were sorted into different groups according to (I) their topographical position (environment): Ridge Forest (hereafter ***RF***), Middle-slope Forest (hereafter ***MF***) and Riparian Forest (hereafter ***RiF***); (II) leaf habit: Evergreen (hereafter ***Ev***), Deciduous (hereafter ***De***) and Semideciduous (hereafter ***Sd***); and (III) growth form: Shrubs (hereafter ***Sr***), Trees (hereafter ***T***), Arborescent-Shrubs (hereafter ***ST***), and Climber (***C***) sensu lato. Semideciduous species are considered those that have the capacity to drop their leaves under severe drought conditions (Zunzunegui et al., 2005; Ciccarelli et al., 2016), and therefore they have functional differences with respect evergreens.

A general principal components analysis (PCA) was performed with the whole set of leaf structural and chemical traits (10 leaf variables) for the 100 observations (measurements) made of woody plants (of 45 species, as some species appear in more than one sampling site), to study the degree of leaf trait variation among them. We used linear mixed models (considering species as a random variable) to assess whether the PCA scores of the first, second and third components (see details in Supplementary Table 2) differed among all possible combinations of the following fixed explanatory factors: environment, leaf habit, and growth form.

The total niche space of the community was calculated by the estimation of the n-dimensional hypervolume (Blonder et al., 2014), from the trait space occupied by the total species group

[1]http://www.aemet.es/es/serviciosclimaticos/datosclimatologicos

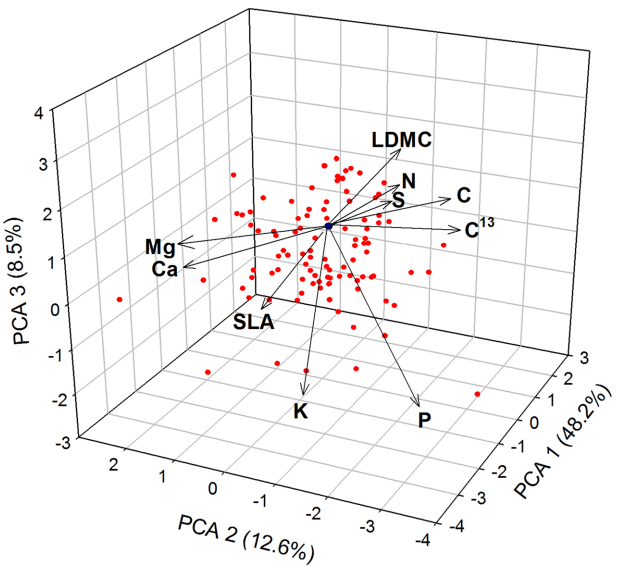

FIGURE 1 | Principal components analysis (PCA) for the 10 leaf traits – morphological (LDMC and SLA), macronutrients (C, N, P, K, Mg, Ca, S) and carbon isotope ratio (C^{13}) – and 45 woody species.

in the different categories (environment, leaf habit, and growth form; the number of observations for climbers was not enough to calculate the hypervolume). In order to reduce the number of dimensions (which is recommended for this analysis), we used the first three PCA axes to calculate the hypervolume for each group, using a multidimensional kernel density estimation (KDE) procedure (see Blonder et al., 2014 for mathematical details). The units of the hypervolumes are reported as the standard deviations of centered and scaled log-transformed trait values, raised to the power of the number of trait dimensions used (sd^{number} of dimensions). We also calculated the overlap between the hypervolumes of each group with the correlation analysis of the "hypervolume" package, which compares the similarity between different hypervolumes using the Sørensen index (see Blonder et al., 2015). A rarefaction analysis was performed to control for the effects of species richness on the hypervolume. Thus, for each environment type, leaf habit and growth form, we built 100 randomized communities composed of species drawn (nine observations) from the species pool of that group. Then, we calculated the hypervolume of each sample and performed a one-way ANOVA to compare the hypervolumes of the groups, independently of species richness. In addition, to assess the functional trait overlap within and among groups (leaf habit and growth form) within each environment type, we calculated the hypervolumes and the Sørensen index between each pair of the most representative groups along the gradient.

All these analyses were conducted in the R 2·10·0 statistical platform (R Development Core Team, 2011), using the packages "vegan" (Oksanen et al., 2013), "nlme" (Pinheiro et al., 2015), and "hypervolume" (Blonder et al., 2014).

RESULTS

Species Variation

The overall PCA showed a clear separation among the 45 woody species in the volume defined by PCA_1 (accounting for 48.2% variance), PCA_2 (12.6% variance), and PCA_3 (8.5% variance) (**Figure 1**). The PCA_1 mostly reflected a gradient of increasing LDMC and C, and decreasing SLA, K and Mg; the PCA_2 was mostly related in one extreme (negative values) with high N, P, and S and at the opposite extreme (positive values) with high values of Ca and Mg; the PCA_3 was represented mainly by variations in N, S, P, and K (see variables scores in Supplementary Table 2).

The woody species inhabiting the ridge forest (***RF***) had different trait values (well-separated according the first and the third PCA axes) from those living at the Riparian forest (***RiF***) (**Figure 2**). There was also a separation in the trait PCA between species grouped by leaf habit: Deciduous (***De***) were different from ***Sd*** and ***Ev***. With regards to growth form, there were differences among the arborescent shrubs (for PCA_1), and trees (for PCA_3) with arborescent shrubs and climbers (**Figure 2**); while no significant differences were found for PCA_2.

Functional Space

The results from the *n*-dimensional hypervolume approach showed that the functional space was greatest for the riparian communities from riparian forest (**Figure 3A**), the group of

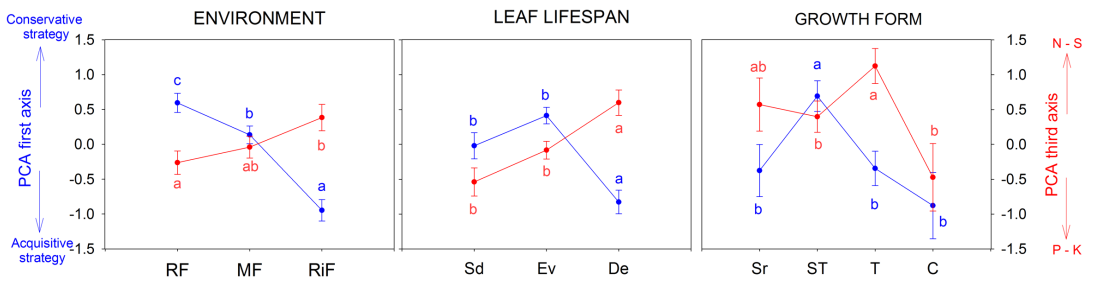

FIGURE 2 | Differences among the scores of the first (leaf economic spectrum) and third (macronutrients) PCA axes, for species grouped according with environment (RF-Ridge forest, MF-Medium-slope, and RiF-Riparian forest), leaf life-span (Sd, semideciduous; Ev, evergreen; and De, deciduous) and growth forms (Sr, shrubs; ST, arborescent-shrubs; T, trees; and C, climbers). All the results were significant; Linear mixed models, $P < 0.05$ (Different letters means significant differences).

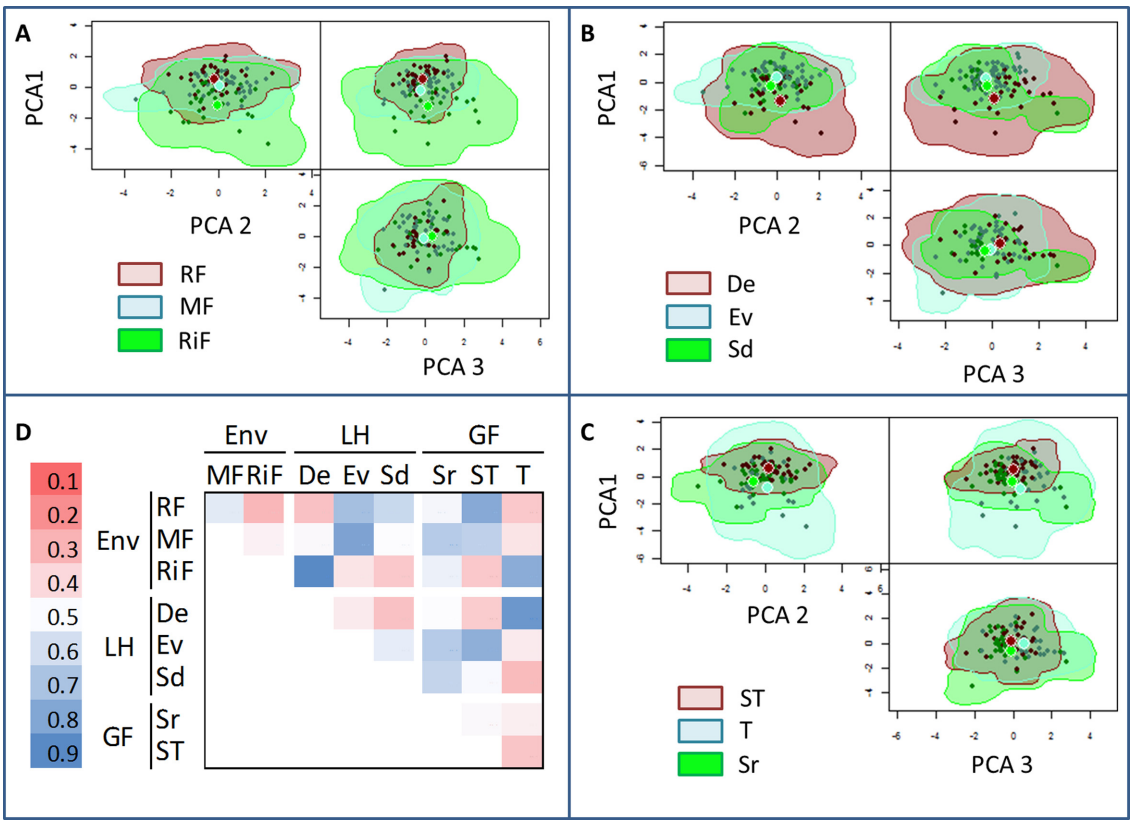

FIGURE 3 | Estimated three-dimensional hypervolumes for: **(A)** Environment (RF, Ridge forest; MF, Medium-slope; and RiF, Riparian forest), **(B)** Leaf life-span (Sd, semideciduous; Ev, evergreen; and De, deciduous), **(C)** Growth forms (Sr, shrubs; ST, arborescent-shrubs; T, trees). **(D)** Sørensen similarity index among hypervolumes. Each plant dimension was based in each of the first three PCA axes (**Figure 1**).

deciduous species (**Figure 3B**), and the growth form "trees" (**Figure 3C**), in the three plant dimensions (PCA axes 1, 2, and 3). In addition, after standardizing for species richness, the functional space showed significant variation along the topographic gradient: the hypervolume was significantly greater at the riparian forest ($P < 0.001$; **Figure 4**). Semideciduous species showed the lowest hypervolume, while deciduous species had the highest. Among the growth form types, trees had the highest hypervolume (**Figure 4**).

Overlapping Niches

The degree of overlapping among the hypervolumes of the different communities was variable, ranging from 28 to 90% (**Figure 3D**). Attending to the environment, the overlap degree of the ridge forest ***RF*** was greater with middle ***MF*** than with Riparian forest ***RiF***. Semideciduous type showed the highest overlap with evergreen type, and the lowest overlap with the deciduous group. Among the growth form types, the lowest degree of overlapping was found among the hypervolumes of trees, and arborescent-shrubs. In general, the degree of hypervolume-overlapping among different growth forms was relatively low, which indicate a trait space occupation more variable for growth forms than for environment or leaf habit.

Differences within the same groups were also remarkable (**Figure 5**). Only evergreen type was represented at the three environment types; it showed low functional niche space overlap (<0.3) with semideciduous (at the ridge) and (<0.2) with deciduous (at the riparian). The functional space was also very different among growth forms within the same environments (overlap < 0.4); with exception of trees and arborescent-shrubs in ridge forests, which had the highest overlap (**Figure 5**).

DISCUSSION

Environment and Niche Partitioning

We have documented a consistent variation in functional space (based on leaf morphology and chemical composition) for woody communities distributed along the topographical gradient. Thus, communities dominated by deciduous trees with faster growth and a predominant acquisitive strategy (i.e., with high values of SLA and leaf nutrient concentration) were characteristic of riparian forests, while semideciduous shrubs and evergreen (arborescent, trees) species, characterized by a conservative strategy (i.e., with high-density leaves and a predominance of carbon-based structural defenses), dominated

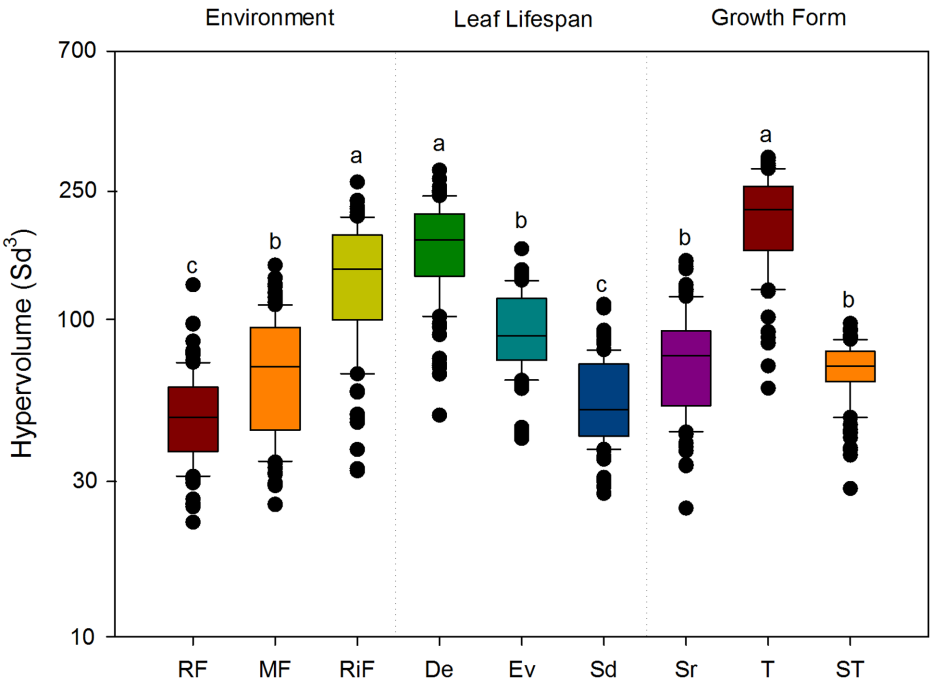

FIGURE 4 | Size of the hypervolume variations for each environment, leaf life-span and growth form. Different letters indicate significant differences ($P < 0.05$), according to *post hoc* multiple pairwise comparisons (Tukey's test). The ANOVA results were significant in all cases ($P < 0.001$).

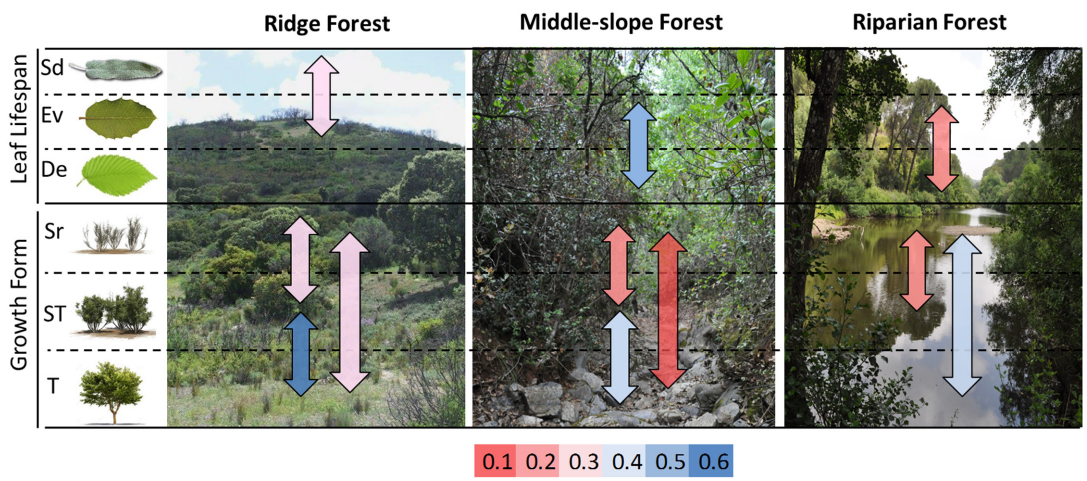

FIGURE 5 | Sørensen similarity index for the functional niche space among species, grouped by leaf life-span and growth form, within the same habitat. Values are indicated by color according to the rank in the bottom of the figure.

ridge forests. Hence, the first strong mechanism of species segregation and niche partitioning was related to habitat suitability. Along that local gradient of stress-productivity, plant establishment might be limited physiologically at one end (by stress tolerance) and by competition at the other end. Overall, leaf trait variation followed the patterns commonly associated to leaf economics spectrum (Grime, 1979; Wright et al., 2004).

This general niche differentiation began with the evolutionary accentuation of trait differences during the expansion of deciduous species on Cretaceous (Axelrod, 1966). The evolutionary and environmental drivers have determined this leaf chemical and morphological composition to improve plant functioning (Reich et al., 2003). However, we detected another trait dimension related with leaf concentration in some macronutrients (third PCA axis) that was not clearly

aligned with the leaf economics spectrum. Other factors, like site differences or evolutionary history, might explain the lower values of leaf P and K associated to deciduous trees (Watanabe et al., 2007; Auger and Shipley, 2013). In a previous study on leaf chemical traits in 98 Mediterranean woody species, we also observed that leaf P and K were highly conditioned by the environment and phylogeny (de la Riva et al., accepted). These results suggest that leaf trait variation is not aligned along a single acquisition-conservation axis, and highlights the necessity of considering plant traits from other potentially independent leading dimensions such as those related with leaf chemical composition.

Biogeochemical Niches

Within each topographical zone or environment type, the leaf biogeochemical niche partitioning would underlie the species ability to coexist by diverging on leaf nutrient composition and resource uptake. Thus, riparian communities from valley bottom showed the highest leaf biogeochemical space, which supports the existence of a greater number of functionally different species, as far as the acquisition of resources is concerned. In a regional study, the higher functional diversity (at whole plant level) was also found in wetter environments along the aridity gradient (de la Riva et al., 2017). On the contrary, the lowest diversity could be due to resource scarcity limiting the establishment of species that are not physiologically able to tolerate such abiotic constraints; as a consequence, the range of functional traits is reduced within the range of viable traits that allow plants to persist in that arid environment, in detriment of the functional space (de la Riva et al., 2016c). The more productive environments, however, are able to increase resource heterogeneity, and promote the coexistence of the species with different demand of them (de la Riva et al., 2017).

Niche partitioning within the same growth form or leaf life-span may also facilitate the coexistence among woody species. Riparian forests were dominated by deciduous tree species, which in fact was the life-span type with higher biogeochemical and morphological niche functional space. The dense shade created by the trees in this type of environment likely may induce a high among-species competition for light. However, woody species may also exhibit some degree of plasticity in nutrient uptake to respond to this competition. Plant species adapted to these productive environments, where nutrients have usually intermittent availability, might respond better to these temporal changes showing higher capacities for taking up resources and higher nutrient flexibility (Aerts, 1999; Sardans et al., 2015). Thus, our results suggest that differences in niche functional space (implying different strategies of resource capturing) among coexisting species would potentially allow to buffer competition in these resource-rich environments.

On the contrary, we detected smaller functional space in more stressful environments (i.e., ridge forests). This result could be explained because species growing in poor environments comprise traits which lead to nutrient retention and higher nutrient-use efficiency, which seem to confer a lower capacity to change their functioning in response to environmental changes (Aerts, 1999). In addition, plant size seems to be related with root expansion and nutrient uptake. Thus smaller species (more frequent in resource-poor environments) tend to show lower values of leaf nutrients than trees as a result of their lower capacity to maintain larger root systems and explore larger soil volumes to uptake nutrients (Niinemets and Kull, 2003 and references therein). As a consequence, shrub and arborescent-shrub species had lower niche space than trees. The combined effects of the slow population dynamics in poor environments (Wright, 2002), and their lower capacity to alter their elemental composition independently of the nutrient pulses (Sardans et al., 2015) would facilitate the coexistence of ecologically equivalent species.

Growth Forms and Niche Partitioning

Differences in growth form and leaf life-span may facilitate the coexistence of woody species and their niche partitioning, as showed by the lower functional overlap among them. In spite of the general patterns found along the topographical gradient (e.g., dominance of deciduous trees at riparian forests, and evergreen arborescent-shrubs at ridge forests), strong divergence were also found between their functional spaces within the same habitat type. Thus, the low degree of overlap between the functional space of deciduous trees and evergreen arborescent-shrubs at the riparian forest reflects a niche partitioning, probably promoted by light competition and resource partitioning. The dense-shaded forest understory is a relatively stressful habitat for plants (in terms of light availability), which promotes a resource gradient also related with leaf economics spectrum (Lusk and Warton, 2007). On the one hand, deciduous trees (such as *Alnus glutinosa* and *Fraxinus angustifolia*) are effective competitors, since they are able to rapidly acquire nutrients and water, that allow them to grow faster and compete more efficiently for light interception (Lambers and Poorter, 1992; Wright et al., 2001); on the other hand, evergreen shrub species (such as *Rubus ulmifolius*) had higher LMA, lower nutrient concentration, and are associated with shade tolerance (Williams et al., 1989; Lusk, 2002).

In the opposite extreme of the environmental gradient – at the ridge forests – woody species with semideciduous life-span seem to display more acquisitive patterns than evergreens. Shorter leaf life-span is related to higher relative nutrient requirement and lower resistance to physical hazards (Ryser, 1996; Pérez-Harguindeguy et al., 2013). In this sense, the habit of semideciduous leaf could be considered a facultative strategy, typical of shrubs in dry Mediterranean conditions (Axelrod, 1966; Zunzunegui et al., 2005). That strategy allows them to be effective competitors during favorable conditions, while they shed partly or completely their leaves during summer, so reducing water loss by transpiration (Zunzunegui et al., 2005; Ciccarelli et al., 2016; Bongers et al., 2017). Therefore, the shrub species might be segregated along the resource gradient through a trade-off between growth rate and survival. In summary, changes in plant structure such as growth form or leaf life-span,

might reduce competition by differences in the ability to capture and use resources, supporting that limiting similarity was operating within the communities (Stubbs and Wilson, 2004).

AUTHOR CONTRIBUTIONS

EdlR designed the study, harvested samples, collected the data, analyzed the data, wrote the first draft, and prepared the manuscript. TM, CV, RV, and IP-R designed the study and prepared the manuscript.

FUNDING

This study was funded by the Spanish MEC projects DIVERBOS (CGL2011-30285-C02-01 and C02-02), ECO-MEDIT (CGL2014-53236-R), and RESTECO (CGL2014-52858-R), and European FEDER funds. CV was supported by the European Research Council (ERC) Starting Grant Project "Ecophysiological and biophysical constraints on domestication in crop plants" (Grant ERC-StG-2014-639706-CONSTRAINTS).

ACKNOWLEDGMENTS

Thanks are due to C. Navarro and M. Olmo for help during field sampling and trait measurements. Leaf multielemental analysis was carried out at the IRNAS, CSIC, isotopic analysis at the LIE, EBD-CSIC, and elemental C and N in the SCAI of the University of Córdoba.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01242/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 November 2016
doi: 10.3389/fpls.2016.01774

Plant Physiological, Morphological and Yield-Related Responses to Night Temperature Changes across Different Species and Plant Functional Types

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Land surface temperature over the past decades has shown a faster warming trend during the night than during the day. Extremely low night temperatures have occurred frequently due to the influence of land-sea thermal difference, topography and climate change. This asymmetric night temperature change is expected to affect plant ecophysiology and growth, as the plant carbon consumption processes could be affected more than the assimilation processes because photosynthesis in most plants occurs during the daytime whereas plant respiration occurs throughout the day. The effects of high night temperature (HNT) and low night temperature (LNT) on plant ecophysiological and growing processes and how the effects vary among different plant functional types (PFTs) have not been analyzed extensively. In this meta-analysis, we examined the effect of HNT and LNT on plant physiology and growth across different PFTs and experimental settings. Plant species were grouped according to their photosynthetic pathways (C_3, C_4, and CAM), growth forms (herbaceous, woody), and economic purposes (crop, non-crop). We found that HNT and LNT both had a negative effect on plant yield, but the effect of HNT on plant yield was primarily related to a reduction in biomass allocation to reproduction organs and the effect of LNT on plant yield was more related to a negative effect on total biomass. Leaf growth was stimulated at HNT and suppressed at LNT. HNT accelerated plants ecophysiological processes, including photosynthesis and dark respiration, while LNT slowed these processes. Overall, the results showed that the effects of night temperature on plant physiology and growth varied between HNT and LNT, among the response variables and PFTs, and depended on the magnitude of temperature change and experimental design. These findings suggest complexities and challenges in seeking general patterns of terrestrial plant growth in HNT and LNT. The PFT specific responses of plants are critical for obtaining credible predictions of the changes in crop production, plant community structure, vegetation dynamics, biodiversity, and ecosystem functioning of terrestrial biomes when asymmetric night temperature change continues.

Keywords: high night temperature, low night temperature, photosynthesis, respiration, biomass, plant functional types

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
M. Teresa Sanchez-Ballesta, Spanish National Research Council, Spain
Nobuhiro Suzuki, Sophia University, Japan

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *14 September 2016*
Accepted: *10 November 2016*
Published: *24 November 2016*

Citation:
Jing P, Wang D, Zhu C and Chen J (2016) Plant Physiological, Morphological and Yield-Related Responses to Night Temperature Changes across Different Species and Plant Functional Types. Front. Plant Sci. 7:1774. doi: 10.3389/fpls.2016.01774

INTRODUCTION

The increased intensity of human activities has been magnifying the climate change and its consequences in recent decades (IPCC, 2013). A remarkable feature of climate change is global warming, caused by anthropogenic emissions of key greenhouse gasses that absorb infrared radiation, such as CO_2, CH_4, and N_2O, deforestation and urbanization. The global temperature is forecasted to continuously increase 1–3.7°C by the end of the 21st century (IPCC, 2013). Compared with day temperature, night temperature has increased faster at local (Peng et al., 2004), country (Zhou et al., 2004; Rao et al., 2014), and global scales (Vose et al., 2005). On average, the lowest land nighttime temperature increased about 0.2°C per decade between 1950 and 1993, which is double the increased highest daytime temperature (IPCC, 2001). It is probably due to the incremental cloudiness, which leads to less radiant heat loss (Alward et al., 1999). Night temperature increased 1.13°C in the Philippines from 1979 to 2003 (Peng et al., 2004), whereas night temperature in Lybia over a period of 45 years (1950–1995) increased at a rate of 0.18°C per decades (Jones et al., 1999). Based on the prediction of multi-model ensembles, asymmetric warming between day and night is going to continue in the future (Christensen et al., 2007; Sillmann et al., 2013). Therefore, plants in the future will be exposed to warmer nights, which could greatly influence crop yield and vegetation dynamics as well as ecosystem biodiversity, structure and productivity.

Due to the influence of land-sea thermal differences, topography and climate change, extremely low temperatures have also occurred frequently around the world (Yang et al., 2006). Low temperature is one of the major environmental factors impacting plant growth, development and ecological distribution (Allen and Ort, 2001). A variety of crops from tropical and sub-tropical regions, such as maize, tomato, cucumber, and mango, are sensitive to cold when cultivated in temperate environments (Jones and Ort, 1998; Allen and Ort, 2001; Meng et al., 2008). As people have begun introducing plants from warm climates into cool climates, it has become important to understand the effects of LNT stress, which needs substantially more research.

Studies on plant response mechanisms to warming or chilling temperatures serve a great purpose in understanding agriculture and natural ecosystems. Increased research efforts have used manipulated field experiments across the world to investigate the potential impacts of climate warming on terrestrial plants and ecosystems (Rustad, 2008). However, the majority of these previous studies have focused on the increase of daily or monthly mean temperature, assuming no difference in the impact of day versus night temperature (Peng et al., 2004). Rustad et al. (2001) conducted a meta-analysis of experimental data from ecosystem warming studies and found that elevated temperatures significantly increased above ground productivity by 19%. In fact, the effects of night temperature are different from that of day temperature (Xia et al., 2014) and produced a relatively greater challenge in estimating global change impact on crop yield and ecosystem functions (Jagadish et al., 2015). Previous studies on night temperatures have focused either on the effects of HNT and LNT alone (Friend, 1981; Seddigh and Jolliff, 1984a,b,c; Koscielniak, 1993; Bertamini et al., 2005) or the mixed effects of night temperatures and CO_2 concentration (Mortensen and Moe, 1992; Volder et al., 2004; Cheng et al., 2008, 2009, 2010), light period (Gimenez and Rumi, 1988; Turner and Ewing, 1988; Lee et al., 1991; Verheul et al., 2007), intensity (Bunce, 1985; Mortensen, 1994; Rapacz, 1998; Flexas and Osmond, 1999; Davies et al., 2002) as well as other environmental factors (Schoppach and Sadok, 2013) and growth regulators (Shah et al., 2011; Mohammed et al., 2013; Zhang et al., 2014). These experiments had been conducted on pineapple (Neales et al., 1980), peanut (Bagnall et al., 1988; Wang, 2007; Lin et al., 2011) and shrub-grass ecosystems (Beier et al., 2004). Although the interest in the influence of night temperatures on many aspects of plants is growing, studies are scattered and there lacks a synthetic study on how and to what extent night temperature change impacts terrestrial plant growth and biomass accumulation. To accurately predict the effects of climatic change and develop sound adaptive agricultural systems and land management practices, it is imperative to understand how night temperature affects photosynthetic carbon gain, loss and allocation through a comprehensive analysis of HNT and LNT studies.

Night temperature has both direct and indirect effects on plant physiology, morphology, growth and yield. HNT and LNT impact plant physiology in many aspects, of which photosynthesis is the most severely affected process (Berry and Bjorkman, 1980; Damian and Donald, 2001; Yu et al., 2002; Liu et al., 2010, 2011). There was a consistent suppression on A_{net} (net CO_2 assimilation rate) at LNT for both C_3 (Flexas and Osmond, 1999; Bange and Milroy, 2004; Zhang et al., 2010; Sao et al., 2013b) and C_4 species (Sao et al., 2013a), but a stimulation for CAM species (Chen et al., 2008; Pollet et al., 2011). HNT had a positive (Seddigh and Jolliff, 1984c; Prieto et al., 2009; Darnell et al., 2013), negative (Teragishi et al., 2001; Mohammed et al., 2013; Narayanan et al., 2015; Peraudeau et al., 2015), or no effect (Veatch et al., 2007; Ibrahim et al., 2010; Cheesman and Klaus, 2013) on A_{net} for C_3 species and a negative (Prasad and Djanaguiraman, 2011) effect for C_4 species. The effect of HNT and LNT on photosynthesis was related to leaf chlorophyll content (Prasad and Djanaguiraman, 2011), fluorescence parameters including photochemical efficiency of PSII (F_v/F_m), PSII quantum yield (Φ_{PSII}) and ETR (Liu et al., 2011, 2012; Zhang et al., 2014), nitrogen (N) concentration (Mohammed and Tarpley, 2009a), g_s (stomatal conductance) (Farquhar and Sharkey, 1982) and enzyme activities related to carbon fixation (Noctor and Foyer, 1998). Among different PFTs, a positive correlation between HNT and plant height was reported (Patterson, 1990; Papadopoulos and Hao, 2000; Cheng et al., 2009; Lucidos et al., 2013). However, LNT had

Abbreviation: ANT, ambient night temperature; A_{net}, net CO_2 assimilation rate ($\mu mol\ m^{-2}\ s^{-1}$); C, carbon; C_i, intercellular CO_2 concentration ($\mu mol\ mol^{-1}$); ETR, electron transport rate ($\mu mol\ m^{-2}\ s^{-1}$); F_v/F_m, Photosystem II (PSII) efficiency; g_s, stomatal conductance ($mol\ m^{-2}\ s^{-1}$); HNT, high night temperature; LNT, low night temperature; LAI, leaf area index; LAR, leaf area ratio ($cm^2\ g^{-1}$); N, nitrogen; J_{max}, maximum electron transport rate ($\mu mol\ m^{-2}\ s^{-1}$); PFTs, plant functional types; Φ_{PSII}, PSII quantum yield; R_d, dark respiration rate ($\mu mol\ m^{-2}\ s^{-1}$); RuBP, ribulose-1,5-bisphosphate carboxylase; SLA, specific leaf area ($cm^2\ g^{-1}$); TNC, total non-structural carbohydrate ($mg\ g^{-1}$); T_r, transpiration rate ($mmol\ m^{-2}\ s^{-1}$); V_{cmax}, maximum carboxylation rate ($\mu mol\ m^{-2}\ s^{-1}$).

a negative effect on plant height for C_3 (Zieslin et al., 1986; Pressman et al., 2006; Kjær et al., 2008) and C_4 species (Uehara et al., 2009), but a positive effect for CAM species (Serra and Carrai, 1988). The responses of biomass accumulation to different night temperature conditions were not identical among different species. HNT had a positive effect on stem dry weight for woody plants (Malek et al., 1992; Cheesman and Klaus, 2013), a positive (Cheng et al., 2008, 2009; Darnell et al., 2013) or negative (Seddigh and Jolliff, 1984a; Lee and Myeongwhan, 2011) effect for herbaceous plants. However, LNT had a positive (Lepage et al., 1984), negative (Kjær et al., 2008; Uehara et al., 2009; Rehmani et al., 2014) or no effect (Dejong and Smeets, 1982) for herbaceous plants. Both HNT and LNT resulted in a reduction in crop yield, such as rice (Ziska and Manalo, 1996; Kanno and Makino, 2010; Mohammed and Tarpley, 2010; Shi et al., 2013), winter wheat (Zhang Y. H. et al., 2013; Narayanan et al., 2015), sorghum (Prasad and Djanaguiraman, 2011) and tomato (Khayat et al., 1985; Zhang et al., 2010; Qi et al., 2011; Zhang Y. et al., 2013). Clearly, lessons from previous studies are not all consistent and sometimes, contradictory. It is essential to conduct a comprehensive review on the responses of different plant functional groups to different night temperatures.

In addition to species functional groups and night temperature treatments, experimental design (e.g., treatment duration and growth facility) may also matter in understanding plant responses to night temperature change. A significant reduction in rice yield at HNT was associated with the reduction of N and non-structural content translocation after flowering in a field experiment (Shi et al., 2013). In a pot-growing experiment, yield loss was concerned with decreased dry matter allocation to grain due to reduced spikelet fertility during the reproductive stage (Cheng et al., 2009). The variation of A_{net} deduction due to HNT and LNT was dependent on experimental durations (Teragishi et al., 2001; Zhu et al., 2005; Ibrahim et al., 2010; Sao et al., 2010; Prasad and Djanaguiraman, 2011). However, the difference between responses to different treatment durations or to growing facilities is ambiguous. Confirming the effects of experimental methodology is of key theoretical and practical significance to help agriculture to choose the right cultivation practice to mitigate adverse effects caused by HNT or LNT.

A meta-analysis of plant responses to temperature indicated that CO_2 elevation affected plant ecophysiology and growth, with different magnitudes at different temperature treatments (Wang et al., 2012). Not only daily temperature, but also the magnitude of night temperature variation caused different impacts. Elevated night temperature by 5°C had a negative effect on A_{net} and g_s but no effect on intercellular CO_2 concentration (C_i) (Mohammed et al., 2013), while elevated night temperature by 8°C significantly increased A_{net}, g_s, and C_i of bell peppers (Darnell et al., 2013). With more night temperature reduction, the decrease in total dry weight, number of leaves and leaf area for goatsrue were increased gradually (Patterson, 1993). Although the effects of different magnitude of night temperature variation on plant physiology and growth varied significantly, the comparisons between these effects are missing and a quantitative review would reveal the optimal night temperature for different ecophysiological processes and growth.

The primary objective of this study was to investigate the effects of high and LNTs on various aspects of plant responses, including physiological, morphological, and growth characteristics. Specifically, our objectives were to: (1) assess the difference and magnitude of HNT and LNT effects on plant physiology, morphology and yield-related responses. The physiological characters included A_{net}, PSII function, g_s, dark respiration (R_d), maximum carboxylation rate (V_{cmax}), maximum ETR (J_{max}), tissue N and TNC. The morphology responses included plant height and leaf characteristics (number of leaves, LAI, SLA and LAR). Yield-related parameters included dry biomass, number of reproductive organs and yield; (2) detect differences among different PFTs based on photosynthetic pathways, growth forms and economic value; (3) investigate the effect of the magnitude of the night temperature changes on different responses; (4) tease apart the effect of growth facilities or treatment durations on affecting these responses. Accordingly, the specific hypotheses were proposed as: (1) HNT and LNT would have similar negative effects on plant physiological performance and growth; (2) LNT would have a stronger negative effect on C_4 species than on C_3 species; HNT would have a stronger negative effect on C_3 than on C_4 species. To test these hypotheses, we conducted a comprehensive meta-analysis of night-temperature manipulated studies published from 1980 through 2015, using the response ratio lnr as an estimate of the effect size of night-temperature relative to control plots.

MATERIALS AND METHODS

Data Selection

Peer-reviewed journal publications were searched with the key word "night temperature" on the Web of Science to build a comprehensive database. The list of papers were then cross-checked with a list of references cited in review articles that were relevant to night temperature effects in order to assure that all articles available for this meta-analysis were included. Any article published in English from 1980 to 2015 that met the following two criteria were included: (1) plants were treated at ANT as a control group, and HNT or LNT as treatment groups; (2) measurements on physiology, morphology, and yield were carried out on both control and treatment groups. The following two criteria were applied to exclude studies: (1) day and night temperatures were treated at the same time; (2) studies focused on extreme temperature values, which resulted in the death of plants. Eventually, 112 papers were selected in this study (Supplementary Material S1). Data were extracted directly from the tables in the articles or were obtained by using the software GetData Graph Digitizer when presented in graphical formats. In these studies, night temperature was 1–20°C above or below ANT, with only four studies more than 20°C above or below ANT (Supplementary Figure S2). Response variables extracted from these articles contained physiological characters including net photosynthesis (A_{net}), PSII efficiency (F_v/F_m), stomatal conductance (g_s), dark respiration (R_d), non-structural carbohydrate content (TNC), tissue nitrogen (N) content (i.e.,

stem, leaf, panicle, spike, grain, shoot, root, and total N) and tissue carbon (C) content (i.e., stem, leaf, shoot, root, and total C), morphological features (i.e., plant height, stem diameter, internode length, number of leaves, SLA, LAI, LAR) and yield-related parameters (i.e., dry weight, number of reproductive organs, days to flowering and yield). For multi-year studies on annual species, results from different seasons were considered independent and all observations were included in this analysis. To ensure the independent nature of the data, we excluded duplicate results found in different publications. However, our analyses were not completely independent because individual papers often provided data with more than one treatment (e.g., different HNT or LNT magnitudes) and/or different response variables. To examine the influence of non-independence of data, we first averaged those data from the same published study by PFTs so that only one comparison was used from a published study for each PFT. Nonetheless, we found that most of the response patterns were unchanged; therefore, all data were used in our study.

Categorization of the Studies

Night temperature was categorized into three levels: ANT, HNT, and LNT. In addition to the response variables and night temperature categories described above, plant species, sample sizes, growth facilities and treatment durations under each temperature treatment were also collected. Following the categorization of Wang et al. (2012), plant species were classified based on photosynthetic pathways (C_3, C_4, or CAM), growth forms (herbaceous or woody) and economic values (crop or non-crop). We listed the species, PFTs and associated references used in this study (Supplementary Table S3). The experiments conducted in these studies were either indoors (growth chamber and greenhouse) or field studies. Due to relatively less data in the field studies, growth facilities used in these experiments were categorized as two levels of pot size: <10L and >10L. Because the treatment duration varied from hours to years, we grouped then into two levels: short-term (hours–days) and long-term (months–years).

Meta-Analysis Methods

We employed a similar method from Hedges et al. (1999). To avoid the adverse effects of different units, we used the response ratio $r = X_t/X_c$ to estimate the effect size of night temperature treatments, where X_t is the treatment mean and X_c is the control mean. In order to compare expediently, we calculated the natural logarithm of the response ratio (lnr). In addition to the mean value, standard deviation (SD) and sample size (n) for each individual observation were also collected to calculate the variance of effect size. Using METAWIN software 2.1 (Sinauer Associates, Inc. Sunderland, MA, USA), we calculated the effect size of the targeted variables and used a weighted, fixed-effects model to evaluate the categorical effects on night temperature treatments, plant species, pot sizes and treatment durations. If the 95% confidence interval (CI) of lnr generated by the fixed-effects model overlapped 0, the temperature treatment was considered to have no significant impact on the response variables. If the upper bound of the 95% CI was smaller than 0, the response was considered significantly negative. Conversely, it indicated that the treatment had a significantly positive effect on variables if the lower bound of the 95% CI was greater than 0. Although total difference among groups was divided into within-group and between group difference, the significance level of the latter revealed whether the response was different among groups (Hedges et al., 1999). The response of plants was considered significantly disparate between HNT and LNT overall or for different species, pot size or treatment duration if their 95% CIs did not overlap. Significance was established at $p < 0.05$ unless otherwise noted.

Publication bias of the effect size (lnr) in this meta-analysis was determined with METAWIN software 2.1 (Sinauer Associates, Inc. Sunderland, MA, USA). We calculated Spearman's rank-order correlation (r_s) which indicates the relationship between the effect size (lnr) and the sample size (Begg and Mazumdar, 1994), and Rosenthal's fail-safe number which represents the number of additional studies with a mean effect size of zero needed to eliminate the significance of a significant effect (Rosenthal, 1979). Publication bias was significant if p-value of r_s was smaller than 0.05. However, the publication bias may be safely ignored if the fail-safe number is larger than a critical value of 5n+10 where n is the number of studies (Rosenberg, 2005).

Statistical Analysis

Original data collected from these studies were arranged into a database in which the value of response variables was lnr. The relationship between lnr of all the variables and the magnitude of night temperature treatments were evaluated by a second-degree polynomial or linear regression analysis with the R statistical programming language (R 3.2.2 for Windows GUI front-end).

RESULTS

Significance of HNT and LNT

Across all of the studies, HNT increased A_{net}, g_s, R_d, and tissue N content on average by 2.56, 11.37, 27.02, and 26.87%, respectively, decreased F_v/Fm, chlorophyll content, starch, sucrose and TNC content by 0.98, 8.08, 22.26, 13.77, and 13.97%, but unaffected T_r (transpiration rate), C_i, PSII quantum yield, ETR and tissue C content (**Figure 1A**). LNT had negative effects on most physiological response variables by different magnitudes, but increased chlorophyll (4.81%), C (1.11%), starch (5.73%), sucrose (4.71%) and TNC content (3.32%). HNT decreased stem diameter and internode length by 1.61%, and 15.97%, which were unchanged by LNT (**Figure 1B**). HNT and LNT had an opposite effect on plant height, number of leaves, SLA, LAI, and LAR (**Figure 1B**). HNT had positive effects on total dry weight and number of productive tillers, negative effects on leaf, stem, and fruit dry weight, number of reproductive organs, flowering time and yield, and no effects on above-ground, below-ground dry weight and fruit size (**Figure 1C**). LNT decreased leaf (13.69%), fruit (15.18%), above-ground (6.7%), below-ground (23.8%), and total dry

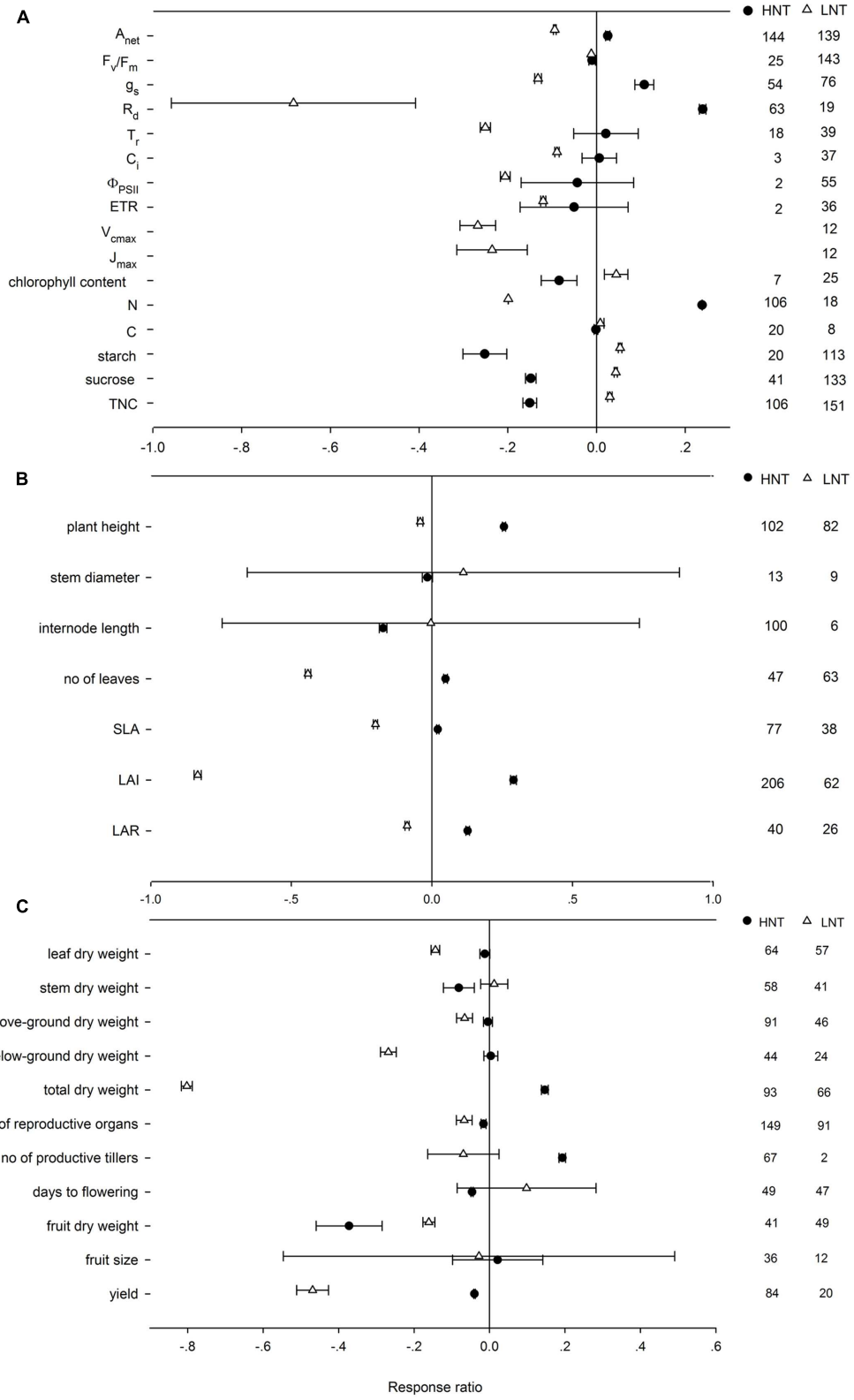

FIGURE 1 | Plant (A) physiological, **(B)** morphological, and **(C)** yield-related responses to HNT (filled circles) and LNT (open triangle). Each data point represents the mean ± 95% confidence interval (CI). The number of observations for each variable is given on the right of the graph. Note that N is tissue nitrogen content including stem, leaf, panicle, spike, grain, shoot, root, and total N. C is tissue carbon content including stem, leaf, shoot, root, and total C.

weight (55.33%), reproductive organs number (6.82%) and yield (37.66%), respectively, but had no effects on stem dry weight, number of productive tillers, anthesis and fruit size (**Figure 1C**). Among all the variables, there was publication bias for chlorophyll content ($r_s = -0.399$, $p = 0.02$), leaf ($r_s = 0.346$, $p = 0.002$), stem ($r_s = 0.339$, $p = 0.0006$), above-ground ($r_s = 0.235$, $p = 0.006$), and below-ground dry weight ($r_s = 0.22$, $p = 0.07$), which could not be ignored based on Rosenthal's value.

Variable Responses among Plant Functional Types (PFTs)

HNT stimulated A_{net} by 3.43% for C_3 species, but suppressed it by 35.57% for CAM species (**Figure 2**). Note that there were not enough publications for a summary on C4 species. LNT suppressed A_{net} more for C_4 species than for C_3 and CAM species. HNT increased plant height differently for C_3 and C_4 species by 6.41 and 150%, respectively. For woody species, A_{net}, R_d and biomass (stem and below-ground) responded more positively, while g_s and plant height responded less positively to HNT than for herbaceous species (**Figure 3**). The LNT effect on woody and herbaceous species was significant for A_{net}, g_s, T_r, stem dry weight and plant height. LNT had a less negative effect on A_{net}, T_r, and g_s but a larger negative effect on stem dry weight and plant height in herbaceous species than in woody species.

High night temperature had a greater positive effect for g_s and R_d in crops and A_{net} in non-crops (**Figure 4**). Positive effects of LNT on g_s and TNC were significantly greater for non-crops than for crops. Positive effects of HNT on plant height, number of leaves and LAR were greater in crops, but the effects on SLA and LAI were greater in non-crops (**Figure 5**). LNT decreased plant height and number of leaves more in crops, but decreased SLA and LAI more in non-crops. HNT had positive effects on leaf, stem and total dry weight for crops but negative effects on non-crops (**Figure 6**). For below ground, dry weight and number of reproductive organs, non-crops responded more negatively to HNT than crops. LNT had positive effects on above-ground and total dry weight for crops and negative effects for non-crops, while stem dry weight in crops and non-crops responded to LNT oppositely. LNT had a greater negative effect on leaf dry weight and anthesis for crops than for non-crops.

Magnitude-Introduced Uncertainty

Most ecophysiological and growth parameters formed a quadratic relationship, except for R_d, which responded linearly, to night temperature treatment (**Figures 7–9**). A_{net}, g_s, and tissue N were the highest when NT was 0.675, 5.43, and 2.1°C above ambient temperature, respectively (**Figure 7**). Morphological parameters, including number of leaves, LAI, SLA, and LAR, formed downward-opening parabola relationships with night temperature change, while plant height, on the other hand, formed an upward-opening parabola relationship with night temperature change (**Figure 8**). Yield-related parameters including leaf, stem, above-ground and below-ground dry biomass as well as the number of reproductive organs, days to flowering, fruit size and fruit weight had downward-opening quadratic relationships with night temperature change (**Figure 9**).

Uncertainty Related to the Experimental Design

Pot size involved in the experiments was a significant factor influencing the effects on A_{net}, tissue N, TNC, total dry weight, number of reproductive organs, and plant height's responses to HNT (**Figure 10**). Plants in smaller pots (<10L) responded more positively for A_{net}, N, w_t, and plant height to HNT. TNC, however, responded more negatively at HNT in larger pots. N, w_t, number of reproductive organs and plant height were reduced more in smaller pots at LNT. A_{net} was decreased more and TNC was increased more in larger pots with LNT.

Experimental duration also played an important role in affecting plant responses to HNT and LNT. HNT increased respiration less in short-term treatments than that in long-term treatments, while LNT duration had insignificant effects (**Figure 11**). Stomatal conductance was significantly increased at short-term HNT but decreased at long-term HNT. Compared with short-term duration, long-term LNT caused more reduction on A_{net} and g_s. Experimental duration had no effects on the responses of SLA to HNT or to LNT, but generated different effects on plant height and LAI at both HNT and LNT. Long-term HNT increased plant height and LAI more, whereas short-term LNT reduced plant height more and LAI less. For total dry weight, long-term HNT and LNT had greater effects than short-term. Yield was decreased more at short-term HNT and different durations had no significant effects in affecting yield responses to LNT.

DISCUSSION

Asymmetric night warming and chilling have long been acknowledged as a universal phenomenon in recent years and caused great agricultural, economic and ecological consequences (Xia et al., 2014). At the leaf and organism level, however, comprehensive quantitative analysis of the effect of HNT and LNT on plants ecophysiology and growth remain unclear. In this study, we collected data from night temperature manipulative studies and analyzed the changes in ecophysiological and whole-plant responses due to changes in night temperatures. Overall, we found that: (1) the significance and degree of the effect of HNT and LNT and the causes of yield reduction at HNT and LNT were different; (2) there existed significant variations among different PFTs in responding HNT and LNT; (3) there was an optimal night temperature for important processes of plants physiology and growth; (4) the responses to HNT and LNT appeared dependent of the experimental designs.

Plant Responses to HNT and LNT

Consistent with our hypothesis, both HNT and LNT had a negative effect on plants yield, with a greater negative effect at LNT, probably due to a greater night temperature reduction for LNT treatments (Supplementary Figure S2). HNT and LNT

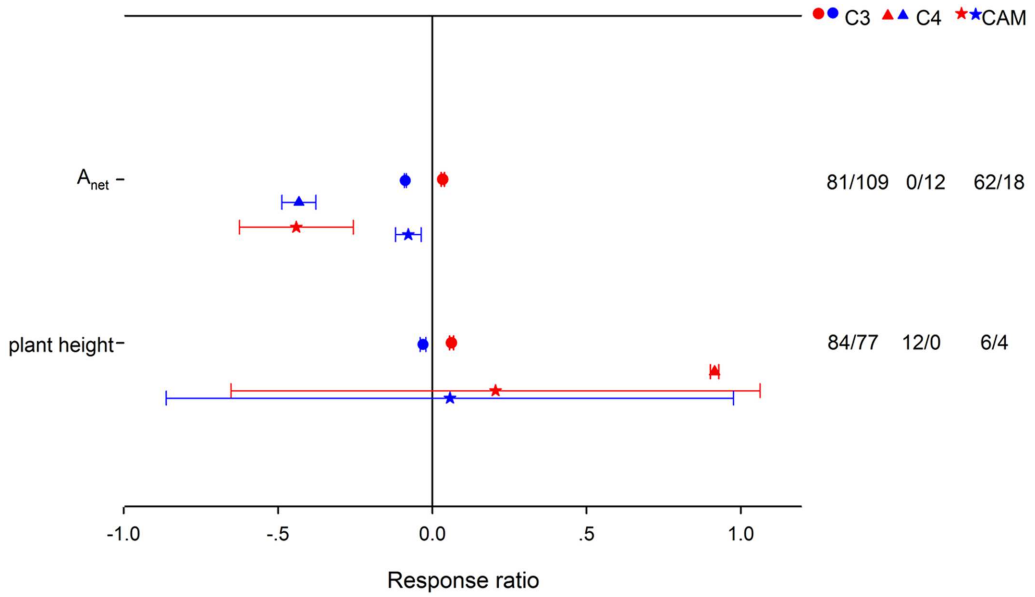

FIGURE 2 | Photosynthetic rate (A_{net}) and plant height responses to HNT (red) and LNT (blue) in C_3 (circles), C_4 (triangles), and CAM (stars) species. Each data point represents the mean ± 95% CI. The number of observations for each variable is given on the right of the graph.

are considered great threats to plants production, especially for crops (Hall, 2000; Maali and Heidarvand, 2010). The impacts of temperature change on plant yield have been assessed directly through greenhouse (Cheng et al., 2009; Mohammed and Tarpley, 2009a,b; Kanno and Makino, 2010; Zhang et al., 2010; Qi et al., 2011) and field experiments (Peng et al., 2004; Nagarajan et al., 2010; Shi et al., 2013; Zhang Y. H. et al., 2013). The long-term effect of climate change on crops was also estimated through crop-growth models, such as CERES (Ritchie and Otter, 1985; Jones et al., 1986), ORYZA2000 (Bouman and Laar, 2006), and CropSyst (Stockle et al., 1994). Warming stress triggered a significant loss of crop yield worldwide, particularly in nations such as China (Li et al., 2004), Japan (Hasegawa et al., 2009), Philippines (Peng et al., 2004), as well as nations in south and southeast Asia (Welch et al., 2010). The deduction of yield was often associated with the decrease in the number of panicles (Peng et al., 2004), grain maturity (Suzuki and Moroyu, 1962; Ziska and Manalo, 1996) and final grain weight (Morita,

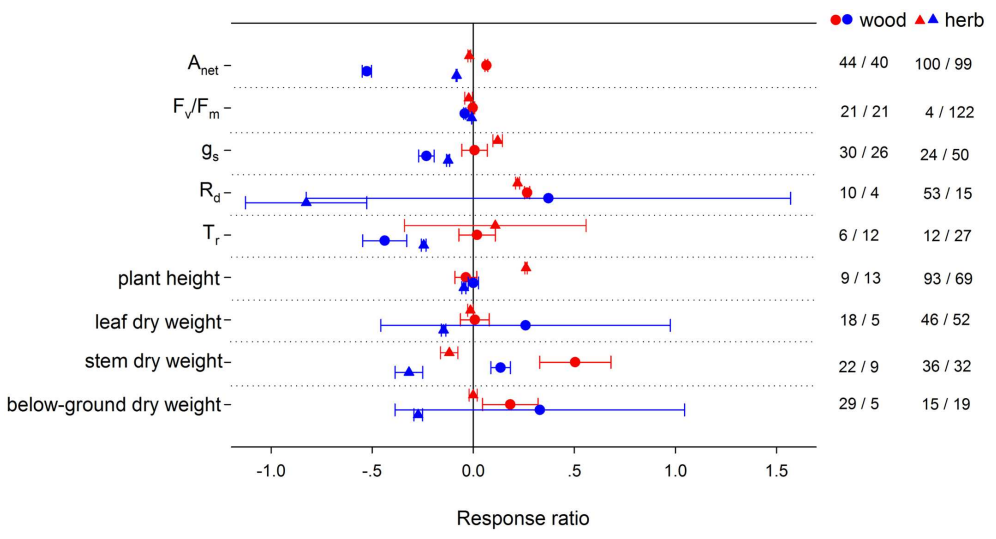

FIGURE 3 | Plant physiological, morphological and yield-related responses to HNT (red) and LNT (blue) in woody (circles) and herbaceous (triangles) species. Each data point represents the mean ± 95% CI. The number of observations for each variable is given on the right of the graph.

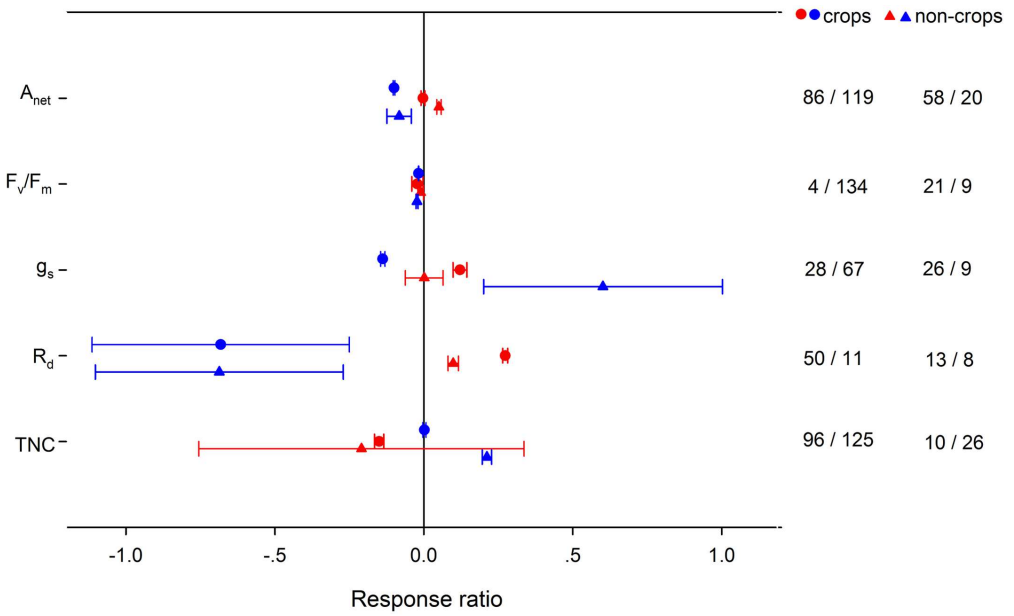

FIGURE 4 | Plant physiological responses to HNT (red) and LNT (blue) in crops (circles) and non-crops (triangles) species. Each data point represents the mean ± 95% CI. The number of observations for each variable is given on the right of the graph.

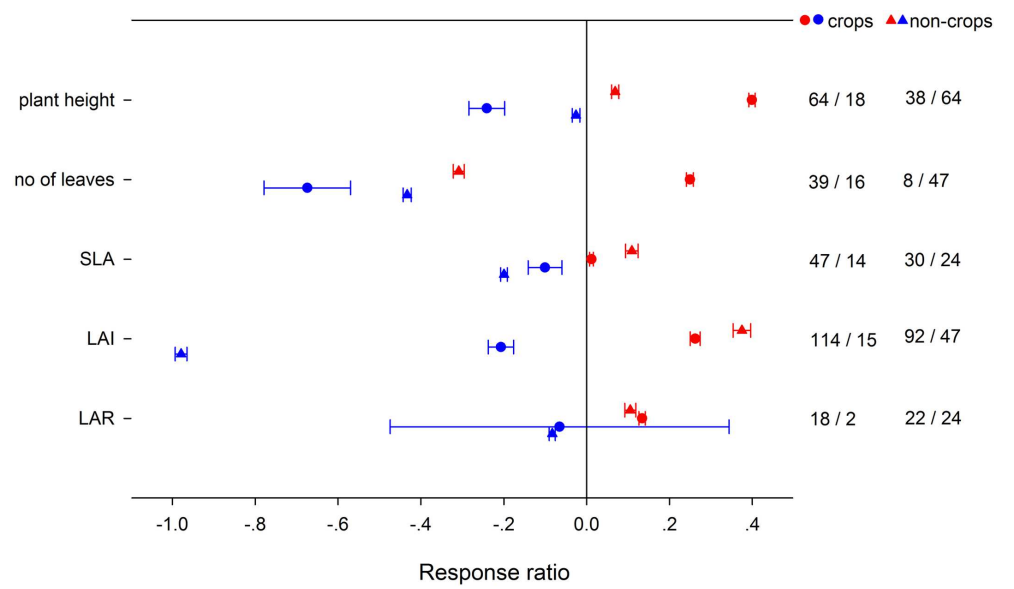

FIGURE 5 | Plant morphological responses to HNT (red) and LNT (blue) in crops (circles) and non-crops (triangles) species. Each data point represents the mean ± 95% CI. The number of observations for each variable is given on the right of the graph.

2005; Kanno and Makino, 2010), spikelet number per plant (Morita et al., 2002, 2004; Peng et al., 2004) and spikelet fertility (Cheng et al., 2009; Mohammed and Tarpley, 2009a, 2010), accelerative respiration rates (Mohammed and Tarpley, 2010), grave membrane leakage (Mohammed and Tarpley, 2009b), lower pollen germination (Mohammed and Tarpley, 2009a), and poor assimilates and *N* translocation to grains (Morita, 2005; Cheng et al., 2009; Kanno and Makino, 2010; Shi et al., 2013).

We found that the negative effect of HNT on yield was associated with a reduction in number of reproductive organs, fruit dry weight, and time for flowering. The reproductive process was regarded as most susceptible to heat stress (Prasad et al., 2006; Jagadish et al., 2007, 2008, 2010), with limited pollen viability as the major cause of yield reduction (Yoshida et al., 1981; Ziska and Manalo, 1996; Jagadish et al., 2010; Zhang et al., 2010; Zhang Y. H. et al., 2013; Fang et al., 2013; Rehmani et al., 2014).

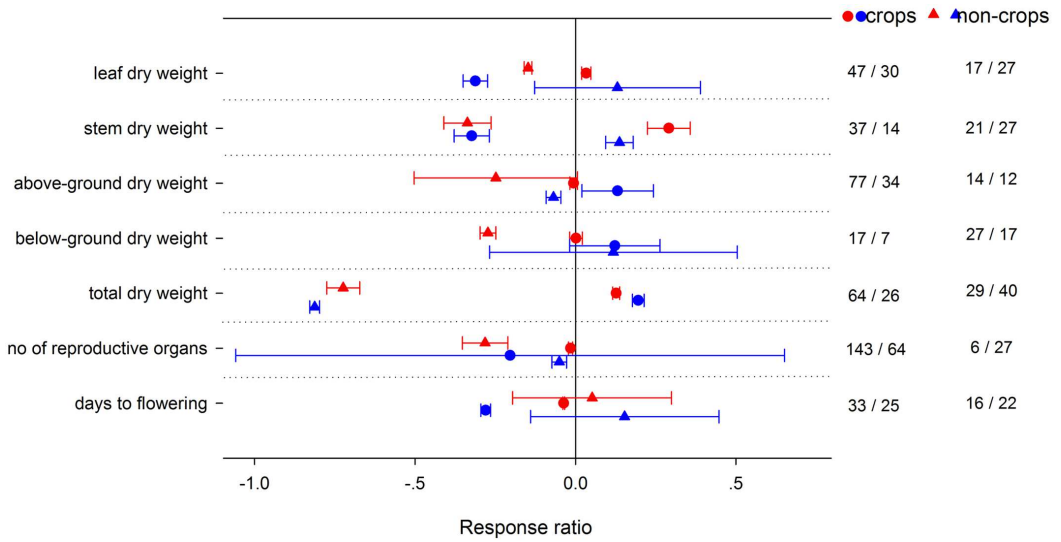

FIGURE 6 | Plant yield-related responses to HNT (red) and LNT (blue) in crops (circles) and non-crops (triangles) species. Each data point represents the mean ± 95% CI. The number of observations for each variable is given on the right of the graph.

Decreased seed yield and lower seed-set under HNT were also reported in wheat (Prasad et al., 2008), rice (Mohammed and Tarpley, 2009b), cowpea (Ahmed et al., 1993), and tomato (Peet and Bartholemew, 1996). Low temperature is one of the most important abiotic stresses for plant growth, development, energy distribution (Xiong et al., 2002; Oufir et al., 2008), and yield (Kasuga et al., 1999; Lang et al., 2005). The negative effect of LNT on yield in this study appeared to be associated with a decline in the number of reproductive organs, fruit dry weight, as well as above-ground, below-ground, and total dry weight. Clearly, the mechanisms for the negative effect of HNT and LNT on plant yield were different (**Figure 1**). The negative effect of LNT on plant yield was primarily related to the negative effect of LNT on the total biomass accumulation, but the negative effect of HNT on plant yield was caused mostly by the reduced allocation of biomass to reproductive organs, as the total biomass was even stimulated by HNT. HNT had been shown to cause no change, or indeed an increase, in total biomass accumulation in crop plants such as rice (Ziska and Manalo, 1996; Cheng et al., 2009), sorghum and sunflower (Manunta and Kirkham, 1996), tobacco (Camus and Went, 1952), and cotton (Königer and Winter, 1993).

The balance between photosynthesis and respiration controls plant growth. Several recent meta-analyses of plant responses to increasing temperature had highlighted how plants may be particularly vulnerable to increases in both HNT and LNT (Lin et al., 2010; Way and Oren, 2010). In contrast to the prediction that A_{net} is constrained at both LNT and HNT (Berry and Bjorkman, 1980), HNT increased A_{net} by 2.56% and LNT decreased A_{net} by 8.73% among the plants included in this study (**Figure 1A**). The positive effect of HNT on A_{net} could be associated with the increment of g_s and tissue N, instead of PSII quantum yield and ETR (**Figure 1A**). HNT increased g_s of two rice genotypes (Shah et al., 2011) and wheat (Lu et al., 1998). HNT enhanced nitrogen soil mineralization (Sardans et al., 2008; Patil et al., 2010) and therefore increased leaf N concentrations (Rustad et al., 2001), which was closely related to photosynthetic capacity (Mae, 1997; Llorens et al., 2003). The loss of chlorophyll owing to HNT has been reported in many crops (Reynolds et al., 1994; Guo et al., 2006). The negative effect of LNT on A_{net} was associated with the negative effect on g_s, PSII function (Φ_{PSII}, ETR, and F_v/F_m), ribulose-1,5-bisphosphate carboxylase (RuBP) inactivation and leaf N (**Figure 1A**), consistent with other studies (Allen and Ort, 2001; Huang and Guo, 2005; Bertamini et al., 2007). Photosystem II has been regarded as the most sensitive to LNT (Huang et al., 2010), as LNT induced PSII photoinhibition and caused reversible inhibition of photosynthetic capacity (Dongsansuk et al., 2013; Zhang et al., 2014).

Our meta-analysis indicated that dark respiration (R_d) was increased with HNT but was decreased with LNT. Increased dark respiration in HNT was reported in rice (Mohammed and Tarpley, 2009b) and *Stipa krylovii* Roshev (Chi et al., 2013a,b) at leaf scale and in rice (Cheng et al., 2009), lettuce, tomato, soybean (Frantz et al., 2004), and cotton (Loka and Oosterhuis, 2010) at organism scale. Different from the hypothesis proposed by Peng et al. (2004) that HNT increased biomass loss by enhancing respiration, our study concluded that HNT increased total biomass but LNT decreased the total, above-ground and below-ground biomass. Total biomass was stimulated by HNT in rice (Cheng et al., 2008, 2009), *Ficus insipida* and *Ochroma pyramidale* (Cheesman and Klaus, 2013), *Rosebay rhododendron* (Starrett et al., 1993), panicum (Patterson, 1990) and soybean (Hewitt et al., 1985). Studies in natural systems have seen impacts on plant phenological development. For example, the large herbaceous perennial, *Phytolacca americana* (Phytolaccaceae) demonstrated no difference in biomass accumulation but had flower set faster as a result of HNT (Wolfe-Bellin et al., 2006). Studies in temperate North America demonstrated that HNT in urban environments

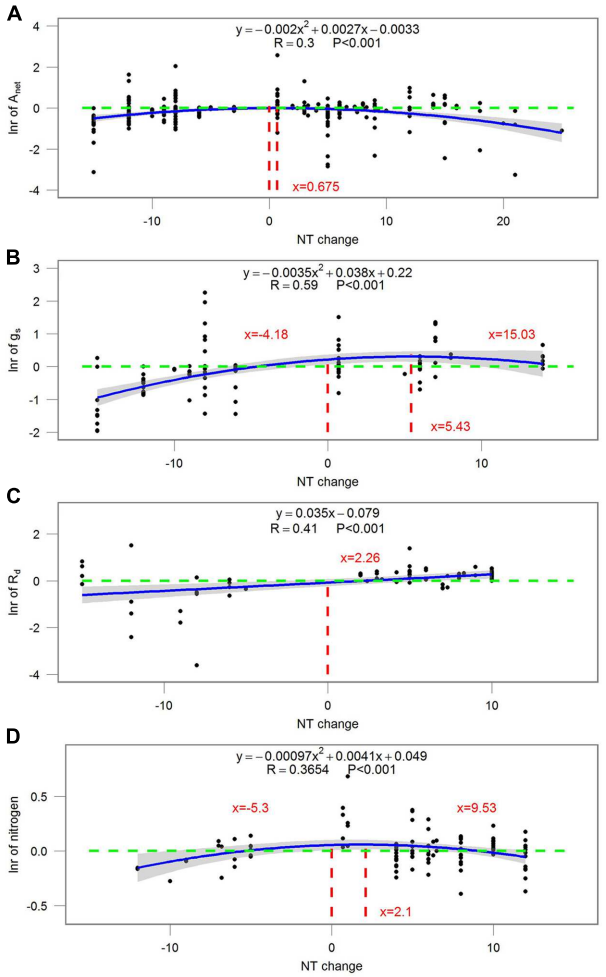

FIGURE 7 | Correlations between the magnitude of NT treatment and the response ratio of (A) net photosynthetic rate (A_{net}); **(B)** stomatal conductance (g_s); **(C)** dark respiration rate (R_d) and **(D)** tissue nitrogen content (N). Each point represents response ratio (lnr) to HNT or LNT. Regression function, variation coefficient and *p*-value are presented in the middle of each graph. Different lines indicate $X = 0$ (red line), *x*-value when *y* is the maximum, crossing points of $y = 0$ (green line) and regression relationships. Note that N is tissue nitrogen content including stem, leaf, panicle, spike, grain, shoot, root, and total N.

increased growth rates in seedlings of *Quercus rubra* (Searle et al., 2012). Similarly, changes in R_d of non-photosynthetic tissues (Saveyn et al., 2008), or a change in carbon-use efficiency (Hansen et al., 2009), could contribute to increased growth rate under HNT.

The balance between photosynthesis and respiration also controls carbohydrate accumulation, which is essential for plant growth and development (Azcón-Bieto and Osmond, 1983; Guy et al., 1992). The processes of photosynthesis and respiration responded independently to temperature and are linked by leaf carbon status (Turnbull et al., 2002). TNCs including starch and sucrose responded differently to HNT and LNT. Although HNT stimulated both photosynthesis and respiration, carbohydrate content was significantly decreased, probably due to the imbalance of the stimulation of HNT on R_d and A_{net}. Leaf carbohydrates synthesized during the daytime were observed to be consumed more quickly during warmer nights because of enhanced leaf respiration, which depletes foliar carbohydrates and may produce a rebound effect of compensatory stimulated photosynthesis during the following day. Evidence supporting this hypothesis has been reported in both greenhouse and field experiments (Beier et al., 2004; Lin et al., 2010). Turnbull et al. (2002) found that leaf starch concentration, soluble sugar and total non-structural carbohydrate declined significantly with the increase of nocturnal temperature. However, no compensatory effect was found between respiration and photosynthesis under nocturnal warming in Mediterranean grassland (Xia et al., 2014). Further investigation is required to discover whether the compensatory effect between respiration and photosynthesis under night warming is related to other environmental conditions, such as water and nitrogen availability. LNT increased carbohydrate content via a lesser negative effect on R_d than on A_{net}, which was approved in many studies (Siminovitch and Briggs, 1953; Steponkus and Lanphear, 1968; Guy et al., 1980; Kaurin et al., 1981; Miao et al., 2009). The increased carbohydrate content posed a negative effect on the day-time photosynthesis at LNT.

Plants adapt to climate stresses via multiple strategies, such as the adjustments of phenology and morphology (Wahid et al., 2007). Leaf morphology was particularly sensitive to HNT as leaf expansion often reached its peak during the night (Schurr et al., 2000; Pantin et al., 2011). HNT had positive effects on SLA, LAR, number of leaves and LAI. When exposed to HNT, the expansion of leaf area and plant height benefited capturing more light for photosynthesis (Seddigh and Jolliff, 1984c; Darnell et al., 2013). Elevated water temperature, in addition to air temperature, can also stimulate leaf expansion and elongation (Tsunoda, 1964; Cutler et al., 1980). Kanno et al. (2009) attributed the positive effect of HNT on plant biomass to an increase in leaf area in rice plants, which was found in tomatoes and *Galega officinalis* (Hussey, 1965; Patterson, 1993) as well. LNT, on the other hand, suppressed plant height, number of leaves, LAI, SLA, and LAR, corresponding to the previous studies (Cockshull, 1979; Dejong and Smeets, 1982; Patterson, 1993; Kjær et al., 2007, 2008; Kjaer et al., 2010).

Variable Responses among Plant Functional Types (PFTs)

Previous studies indicated that increased daytime temperature had stronger effects on A_{net} of C_3 species than C_4 species (Wahid et al., 2007). No consistent results for HNT effect on A_{net} were found due to insufficient data for C_4 species. However, we did find that LNT decreased A_{net} more in C_4 species than in C_3 species, which was closely associated with relatively more reduction of g_s, ETR, V_{cmax}, and J_{max} for C_4 species (**Figure 2**; Supplementary Figure S4). Low temperature effects on C_4 photosynthesis have been frequently examined (Long, 1998). C_4 photosynthesis has been suggested to be inherently sensitive to chilling because of the cold lability of the C_4 cycle enzymes (Long, 1983; Sage, 2002; Sage and Kubien, 2007).

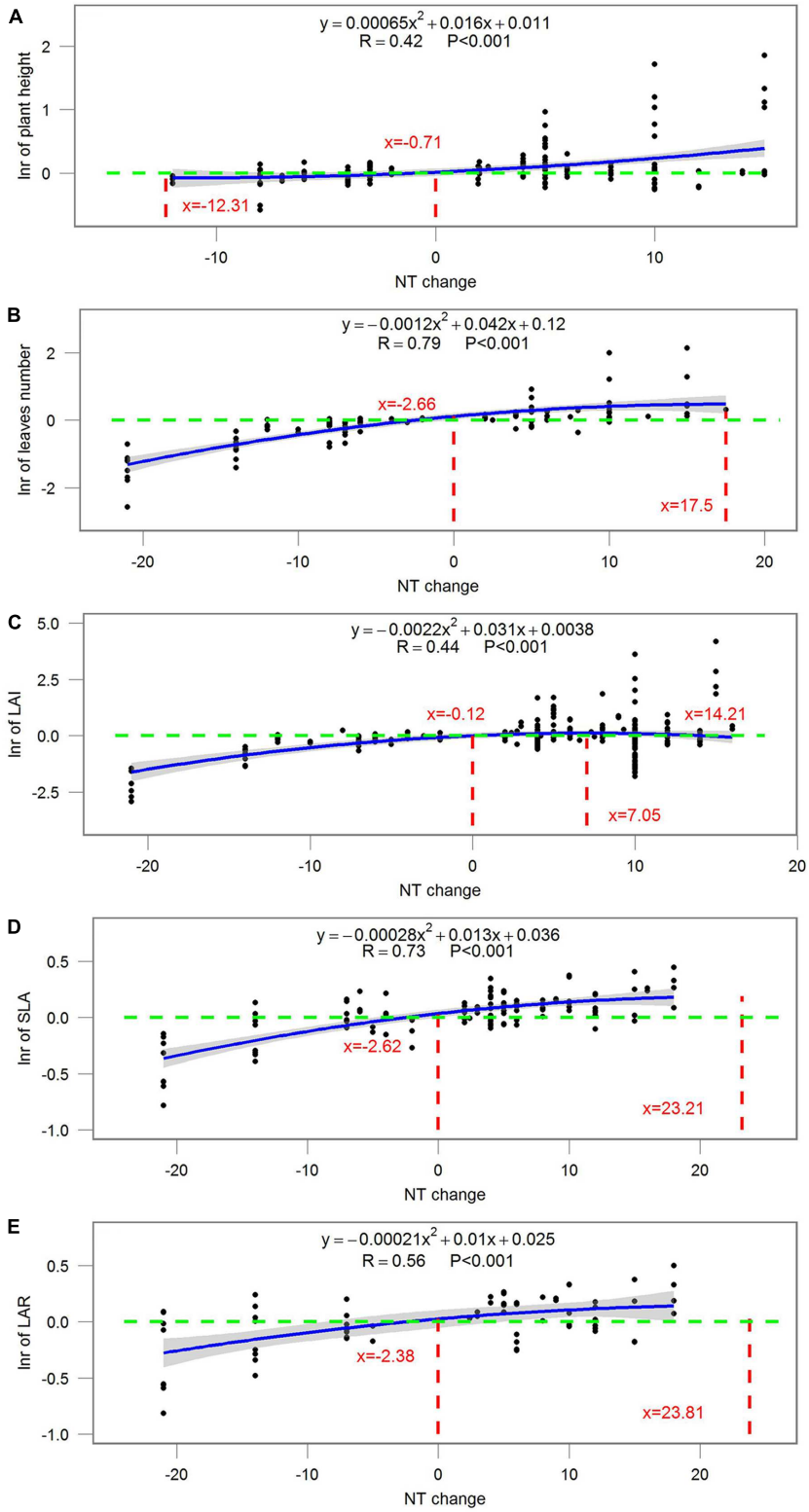

FIGURE 8 | Correlations between the magnitude of NT treatment and the response ratio of (A) plant height; **(B)** number of leaves; **(C)** leaf area index (LAI); **(D)** specific leaf area (SLA); **(E)** leaf area ratio (LAR). Each point represents response ratio (lnr) to HNT or LNT. Regression function, variation coefficient and *p*-value are presented in the middle of each graph. Different lines indicate $X = 0$ (red line), *x*-value when *y* is the maximum, crossing points of $y = 0$ (green line) and regression relationships.

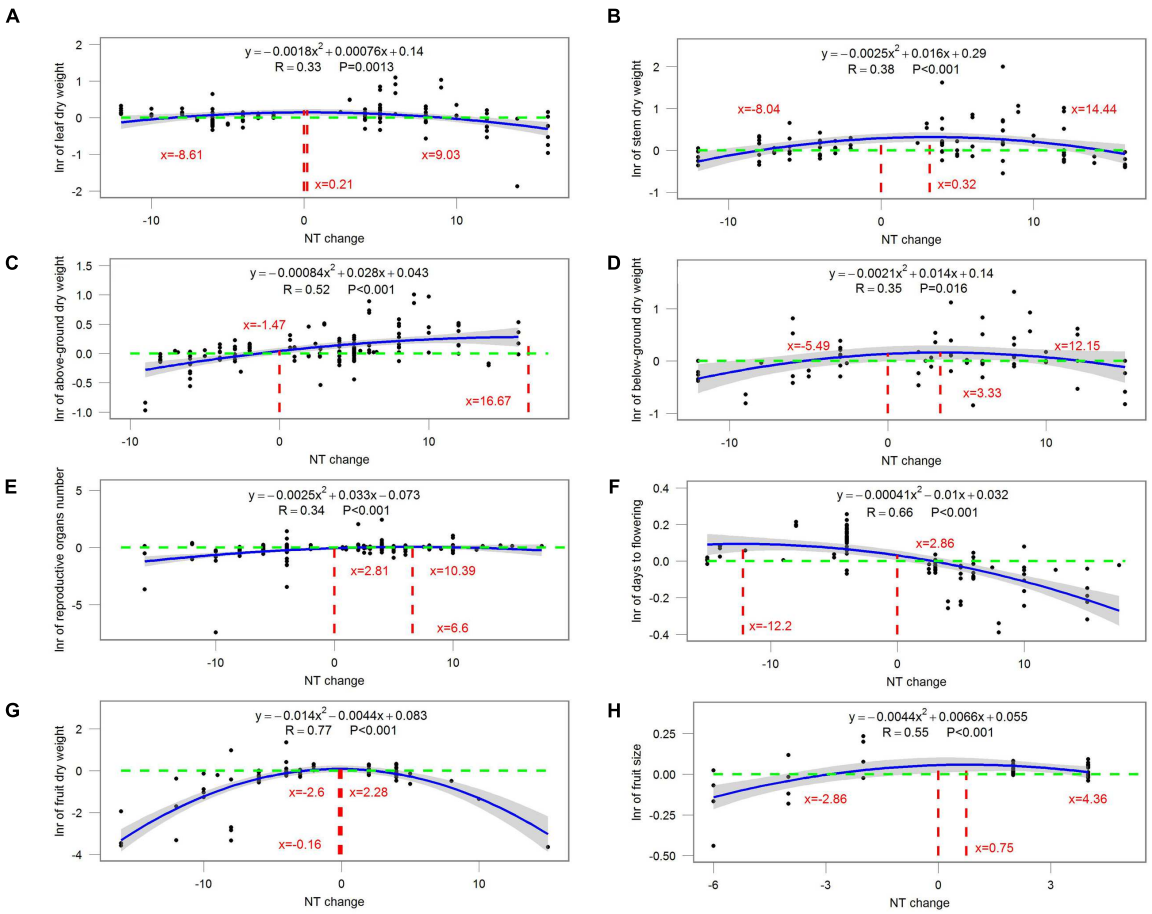

FIGURE 9 | Correlations between the magnitude of NT treatment and the response ratio of (A) leaf dry weight; **(B)** stem dry weight; **(C)** above-ground dry weight; **(D)** below-ground dry weight; **(E)** number of reproductive organs; **(F)** days to flowering; **(G)** fruit dry weight and **(H)** fruit size. Each point represents response ratio (lnr) to HNT or LNT. Regression function, variation coefficient and *p*-value are presented in the middle of each graph. Different lines indicate $X = 0$ (red line), *x*-value when *y* is the maximum, crossing points of $y = 0$ (green line) and regression relationships.

High night temperature stimulated A_{net} in woody plants but suppressed it in herbaceous plants. The same pattern was found in stem biomass. Though studies have reported a greater warming-induced stimulation in woody biomass than in herbaceous biomass (Lin et al., 2010), the result that woody plants were more favored under night warming than herbaceous plants has not been reported. The stimulation of HNT on stem biomass is greater than that on below-ground biomass for woody species, however, for herbaceous species, HNT had no effect on leaf- or below-ground biomass, yet suppressed stem biomass significantly. Our results imply that more resources will be allocated to aboveground growth, and therefore above-ground competition for resources, such as light, will be more important for woody species under night warming (Suding et al., 2005; Lin et al., 2010). In ecosystems where herbaceous and woody plants coexist, a greater biomass stimulation of woody than of herbaceous species may increasingly suppress growth, especially above-ground growth, of herbaceous species via a shading effect (Castro and Freitas, 2009). LNT also had a positive effect on woody stem biomass, which might be related to a stronger suppression in R_d than A_{net}. The negative effect of HNT on A_{net} for herbaceous plant was caused primarily by damage to PSII efficiency (F_v/F_m) (**Figure 3**). LNT decreased A_{net} more in woody plants than in herbaceous plants, along with a greater decrease of F_v/F_m and g_s in woody plants, which was consistent with previous studies (Sao et al., 2010, 2013b; Liu et al., 2011). It is important, however, to note that more research is needed on the effect of night temperature on plants biomass allocation, since the publication bias on this effect could not be ignored in this meta-analysis. Low sample sizes for some functional groups used in this analysis (e.g., C_4 and woody species) require that some results in this analysis be interpreted with caution. Nevertheless, results from these under-represented groups demonstrate that further study of these groups is critical for this untested hypothesis in the future.

Teasing apart the variations of the responses among crops and non-crops is important to guide future research in agricultural practice and genetic engineering. HNT had a positive effect on A_{net} for non-crops but had no effect for crops, while HNT had a positive effect on R_d for both crops and non-crops. Due

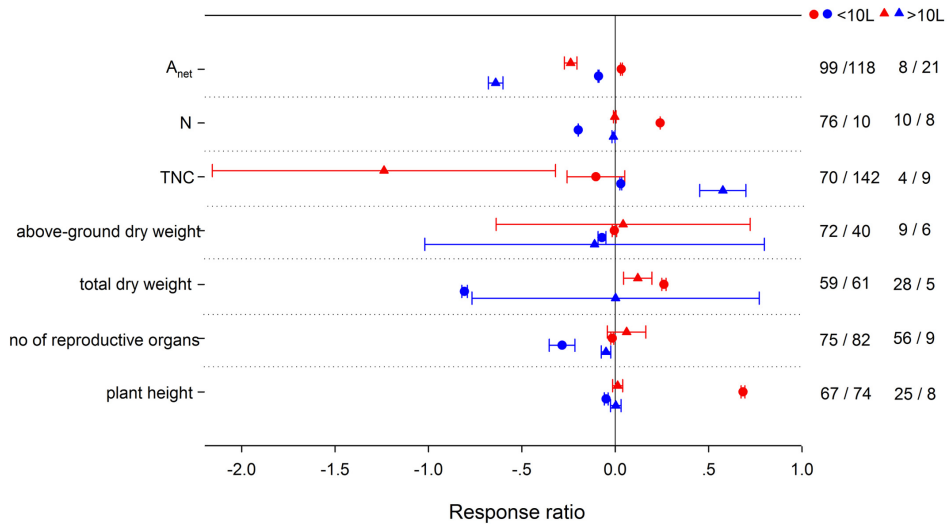

FIGURE 10 | Plant physiological, morphological and yield-related responses to HNT (red) and LNT (blue) grown in <10 L pots (circles) or >10L pots (triangles). Each data point represents the mean ± 95% CI. The number of observations for each variable is given on the right of the graph. Note that N is tissue nitrogen content including stem, leaf, panicle, spike, grain, shoot, root, and total N.

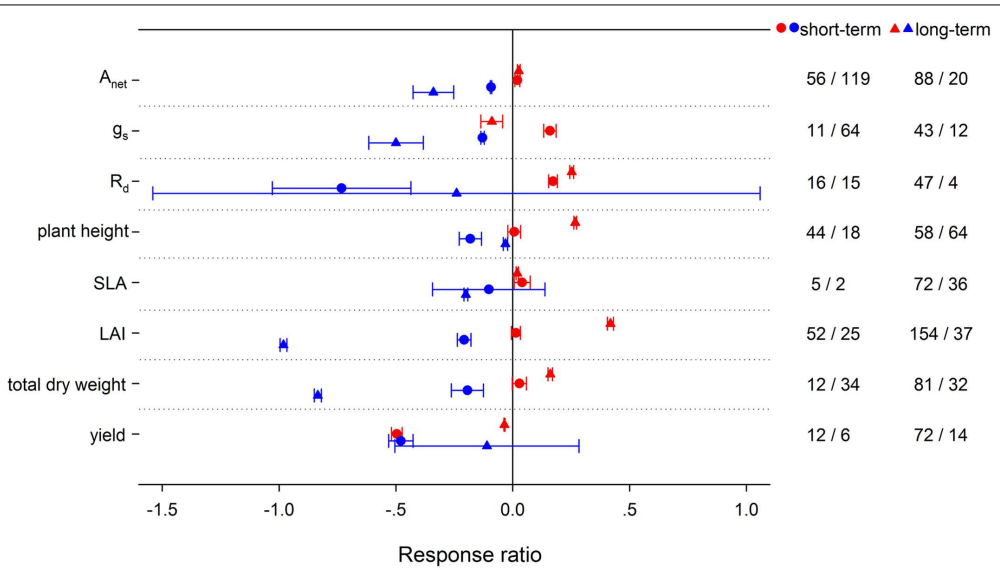

FIGURE 11 | Plant physiological, morphological and yield-related responses to HNT (red) and LNT (blue) for different treatment durations (circles, short-term; triangles, long-term). Each data point represents the mean ± 95% CI. The number of observations for each variable is given on the right of the graph.

to different responses of A_{net} and R_d to HNT between crops and non-crops, TNC was decreased in crops but unchanged in non-crops. Accordingly, HNT stimulated leaf-, stem-, total dry biomass for crops but decreased leaf-, stem-, below-ground, total dry biomass for non-crops. Similarly, crops better adapted to LNT than non-crops, as LNT had a positive effect on total dry biomass for crops but negative for non-crops. The fact that crops coped with HNT and LNT better than non-crops could imply an improved stress-tolerant ability for improved crops through breeding, genetic engineering, and management practices. Changes in HNT and LNT could influence vegetation dynamics and ecosystem structure through shifting competitive interactions among different functional groups in natural or agricultural systems.

Uncertainties

The magnitude of night temperature treatment did have an impact on most of the parameters that were investigated in the study. The negative effects of HNT and LNT on plants ecophysiological parameters (A_{net}, g_s, and tissue N), morphological parameters (SLA, LAI, and LAR), and yield related parameters (above-ground and below-ground biomass,

fruit size and dry weight) were more evident with the increasing magnitude of HNT and LNT treatment (**Figures 7–9**). Consistent with the results discussed before, plant peak physiology and growth mostly occurred at night temperatures higher than the ambient, especially for leaf growth. Plant height, on the other hand, was even stimulated by HNT and not much affected by LNT. Whether plants at HNT tend to be thinner and taller requires further investigation. In all cases, photosynthesis shows an optimum temperature that roughly corresponds to the middle of the non-harmful range and drops off with an increased slope as temperatures rise above the thermal optimum.

It is essential that potential confounding factors be considered in a meta-analysis, which synthesizes results from a large number of studies conducted under a variety of growing conditions on different plant species. In our analysis, studies in plant responses to other environmental stresses (e.g., drought, low nutrients, light deficiency, or elevated ozone) were excluded. In addition to the variation caused by PFTs, different experimental design can be also responsible for the inconsistent responses (Mohammed and Tarpley, 2009a,b; Nagarajan et al., 2010; Cheesman and Klaus, 2013; Rehmani et al., 2014). Here we focused on the effects of pot size (<10L vs. >10L) and treatment duration (short vs. long term) on plant responses. Responses of plant growth under HNT and LNT may vary with time because thermal sensitivity of plants may differ among growth stages. Long-term LNT treatment had a stronger negative effect on A_{net} than short-term treatment, whereas HNT treatment of different durations had no significant effect on A_{net}. On the contrary, R_d was increased greater in long-term HNT but not different between different LNT durations. Several studies had reported the long-term acclimatization of dark respiration on tropical trees (Atkin and Tjoelker, 2003; Cheesman and Klaus, 2013). Total biomass responded differently between different treatment durations of HNT and LNT, though no significant experiment duration effect was found in above- and below-ground dry matter. Plant growth and yield were decreased less at long-term HNT, which might be related to plant acclimation ability (Hare et al., 1998; Wahid et al., 2007). Pot size significantly altered the responses of A_{net}, non-structural carbohydrates and total biomass to HNT and LNT. Small pots constrained below-ground growth along with more limitation on above-ground growth (Thomas and Strain, 1991; Loh et al., 2003; Climent et al., 2011), given that a skimpy supply of nutrients and water which might induce strong nutrient or water inhibition (Walters and Reich, 1989).

CONCLUSION

We found that both HNT and LNT had a negative effect on plants yield, with the HNT effect primarily related to reduced biomass allocation to reproductive organs, flower development and seed maturation and the LNT effect more related to a negative effect on total biomass. HNT accelerated plants ecophysiological processes, including photosynthesis and dark respiration, while LNT slowed these processes. The responses to LNT and HNT varied significantly among different PFTs. HNT stimulated photosynthesis in C_3, woody and non-crop species, but inhibited it in herbaceous, and had no effect in crops. LNT caused more reduction of A_{net} in woody than in herbaceous species but decreased it for both crops and non-crops with no significant difference. Both experimental settings and durations had significant effects in plants responses to night temperature change. Long-term HNT led to a relatively smaller loss of yield while the response of yield to LNT was unchanged by different treatment durations. The magnitude of night temperature did have an impact on most of the parameters that were investigated in the study. Plants peak physiology and growth mostly occurred at night temperatures higher than the ambient, especially for leaf growth. Our results suggest that the diurnal variations in vegetation responses to night temperature changes are important for understanding the changes in vegetation photosynthetic activity and growth in future climates. Such diurnal variations, however, have rarely been incorporated into current modeling studies on vegetation responses to global warming—which are overwhelmingly based on daily or growing season mean air temperature and will not capture the response of vegetation to asymmetric diurnal temperature changes. New field experiments with different elevated temperatures during day vs. night, and across different seasons, are urgently needed for different plant functional groups. Such experiments will shed new light on the ecophysiological effects of night-time temperature change during different seasons, and can be used to improve the performance of current land surface models. The functional type specific response patterns of plants to night temperature changes are critical for obtaining credible predictions of the changes in food production, carbon sequestration and climate regulation.

AUTHOR CONTRIBUTIONS

DW proposed the research idea. PJ collected and analyzed the data and then wrote the paper. DW, CZ, and JC help modify the paper.

FUNDING

Funding for this research was provided by Nanjing University of Information Science and Technology, the Jiangsu Natural Science Foundation (BK20150894), The National Natural Science Foundation of China (31500503), and the International S&T Cooperation Program of China (2012DFA60830) through DW.

ACKNOWLEDGMENT

We thank Gabriela Shirkey of Michigan State University for editing the language.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2016.01774/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 December 2016
doi: 10.3389/fpls.2016.01871

Functional and Integrative Analysis of the Proteomic Profile of Radish Root under Pb Exposure

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Lead (Pb) is one of the most abundant heavy metal (HM) pollutants, which can penetrate the plant through the root and then enter the food chain causing potential health risks for human beings. Radish is an important root vegetable crop worldwide. To investigate the mechanism underlying plant response to Pb stress in radish, the protein profile changes of radish roots respectively upon $Pb(NO_3)_2$ at 500 mg L^{-1} (Pb500) and 1000 mg L^{-1} (Pb1000), were comprehensively analyzed using iTRAQ (Isobaric Tag for Relative and Absolute Quantification). A total of 3898 protein species were successfully detected and 2141 were quantified. Among them, a subset of 721 protein species were differentially accumulated upon at least one Pb treatment, and 135 ones showed significantly abundance changes under both two Pb-stressed conditions. Many critical protein species related to protein translation, processing, and degradation, reactive oxygen species (ROS) scavenging, photosynthesis, and respiration and carbon metabolism were successfully identified. Gene Ontology (GO) and pathway enrichment analysis of the 135 differential abundance protein species (DAPS) revealed that the overrepresented GO terms included "cell wall," "apoplast," "response to metal ion," "vacuole," and "peroxidase activity," and the critical enriched pathways were involved in "citric acid (TCA) cycle and respiratory electron transport," "pyruvate metabolism," "phenylalanine metabolism," "phenylpropanoid biosynthesis," and "carbon metabolism." Furthermore, the integrative analysis of transcriptomic, miRNA, degradome, metabolomics and proteomic data provided a strengthened understanding of radish response to Pb stress at multiple levels. Under Pb stress, many key enzymes (i.e., ATP citrate lyase, Isocitrate dehydrogenase, fumarate hydratase and malate dehydrogenase) involved in the glycolysis and TCA cycle were severely affected, which ultimately cause alteration of some metabolites including glucose, citrate and malate. Meanwhile, a series of other defense responses including ascorbate (ASA)–glutathione (GSH) cycle for ROS scavenging and Pb-defense protein species (glutaredoxin, aldose 1-epimerase malate dehydrogenase and thioredoxin), were triggered to cope with Pb-induced injuries. These results would be helpful for further dissecting molecular mechanism underlying plant response to HM stresses, and facilitate effective management of HM contamination in vegetable crops by genetic manipulation.

Keywords: proteomics, radish, iTRAQ, heavy metal, lead stress

OPEN ACCESS

Edited by:
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University of Florence, Italy

Reviewed by:
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Josselin Noirel,
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *12 August 2016*
Accepted: *28 November 2016*
Published: *15 December 2016*

Citation:
Wang Y, Xu L, Tang M, Jiang H, Chen W, Zhang W, Wang R and Liu L (2016) Functional and Integrative Analysis of the Proteomic Profile of Radish Root under Pb Exposure. Front. Plant Sci. 7:1871. doi: 10.3389/fpls.2016.01871

INTRODUCTION

Heavy metal (HM) contamination from natural, agricultural, and industrial sources has become a worldwide public health concern, which could seriously deteriorate the environment and cause adverse impacts on human health through the food chain (Pourrut et al., 2013; Singh et al., 2016). Lead (Pb) is one of the most abundant HM pollutants with no physiological function, mostly penetrating the plant through the roots and accumulating in different parts (Gupta et al., 2013; Pourrut et al., 2013). Pb can influence various morphological, physiological and biochemical processes leading to a restriction of plant growth, inducing membrane cell damages and, ultimately, to cell death (Sharma and Dubey, 2005; Gupta et al., 2011). Accordingly, plants also have developed diverse defense mechanisms to prevent the toxic effect of heavy metals including generating signal sensing and transduction proteins, compartmenting into the vacuoles and induction of higher levels of metal chelates like a protein complex, organic and inorganic complexes (Gupta et al., 2011, 2013; Thapa et al., 2012).

Radish (*Raphanus sativus* L.), a major member of the Brassicaceae family, is an important annual or biennial root vegetable crop worldwide (Wang and He, 2005). Because the root is considered as the vulnerable part which is easily affected by HM (Wang et al., 2015a), it has become of vital importance to investigate the HM-response mechanisms and explore the molecular regulatory network of tolerance and homeostasis in radish. The identification of the HM-responsive genes or proteins is a fundamental step in understanding the molecular mechanism underlying response to HM stress (Ahsan et al., 2009). In our previous study, NGS-based transcriptome, miRNA and degradome analysis were employed to investigate the expression patterns of genes and miRNAs in radish exposure to Pb stress. A lot of Pb-responsive transcripts, miRNA and its targets were detected, which were predominately involved in stress-related signal sensing and transduction, specific metal uptake and homeostasis, glutathione metabolism-related processes and carbohydrate metabolism-related pathways (Wang et al., 2013, 2015b).

Although transcriptomics provides a useful tool for unraveling gene expression networks at the mRNA level which enhanced our understanding the response of radish under Pb stress, proteomics can offer a new platform for investigating complex biological functions involving large numbers of proteins and provide further insight into posttranscriptional modifications thereby complementing genomics analysis (Ralhan et al., 2008; Ahsan et al., 2009; Wang et al., 2014). In the past two decades, classical two-dimensional electrophoresis (2-DE) technology has been widely employed for protein identification and analysis. However, there were some limitations in its applications such as poor reproducibility, weak sensitivity and low automation (Sazuka et al., 2004). Isobaric tags for relative and absolute quantification (iTRAQ) is a robust mass spectrometry of protein quantitative technology, which can perform relative and absolute quantification in up to eight samples in parallel (Bindschedler and Cramer, 2011; Glibert et al., 2014). Recently, iTRAQ has been widely used for large-scale quantitative plant proteomic studies in exploration of various metabolic processes at the post-transcriptional level (Kambiranda et al., 2013; Martínez-Esteso et al., 2013) in response to various stresses (Yang et al., 2014; Li et al., 2015; Fu et al., 2016). Additionally, iTRAQ-based proteomics has been proven as a powerful method for unraveling the molecular regulatory networks involved in interactions between heavy metals and plant species, and a set of HM-responsive candidate proteins have been successfully identified. Alvarez et al. (2009) reported that exposure of *Brassica juncea* roots to cadmium (Cd) could activated several protein species involved in sulfur assimilation, redox homeostasis and xenobiotic detoxification, and depressed multiple proteins involved in protein synthesis and processing by two quantitative proteomics approaches including fluorescence two-dimensional differential gel electrophoresis (DIGE) and iTRAQ technology. More recently, the protein abundance changes in rice roots in response to Aluminum (Al) at an early phase were conducted with iTRAQ, and a total of 700 distinct protein species with >95% confidence were identified and 106 protein species were differentially accumulated upon Al toxicity in sensitive and tolerant cultivars (Wang et al., 2014). However, investigation of the dynamically protein abundance changes in response to Pb stress in radish has not been reported.

In this study, an iTRAQ-based quantitative proteomics approach was firstly employed to detect the effects of Pb responses at the protein abundance levels in radish roots. The differential abundance protein species (DAPS) involved in Pb-response of radish were quantified, and the enriched networks for regulating Pb stress at the protein level were acquired. Furthermore, to deeply reveal the integrative molecular network of radish plant response to Pb stress, the proteomic data were integrated with our previous transcriptomic, miRNA, degradome and metabolomic data, which provided a more global view of the molecular and cellular changes elicited by Pb stress in radish. This work would provide valuable information for further functional analyses of the critical Pb-responsive protein species in radish, which will be helpful for effectively facilitating the management of Pb and other HM contaminations in vegetable crops by genetic manipulation.

MATERIALS AND METHODS

Plant Material

The variety of high-Pb-accumulation "NAU-RG" was selected for exploring the molecular regulation mechanisms in radish roots responding to the Pb stress. This genotype is an advanced inbred line with a medium size root in globular shape, white skin and flesh. According to the related evaluation methods and previous studies, different concentrations of Pb $(NO_3)_2$ (100, 200, 400, 500, 1000, and 1500 $mg{\cdot}L^{-1}$) were set to investigate the changes of visible physiological symptoms with different temporal durations in the preliminary experiment. Interestingly, no special obvious morphologic differences were found among individuals when exposed to low dose of heavy metal treatment, while the plants were seriously hampered and grew abnormally when exposed to 1500 $mg{\cdot}L^{-1}$ Pb $(NO_3)_2$. Therefore, the concentrations of Pb

$(NO_3)_2$ at 500 mg·L^{-1} (Pb500) and 1000 mg·L^{-1} (Pb1000) were selected for the comparative proteomic analysis. Additionally, a control group was defined using non-treated seedlings (CK).The growth conditions of radish plants were conducted according to the reported descriptions (Wang et al., 2013). Plants were collected after 72 h with three different concentration treatments including an untreated control (CK) and two Pb-stressed conditions (Pb500 and Pb1000), based on the previous reported study (Wang et al., 2013). Each treatment consisted of three biological replicates. Equal amount of radish taproot samples (2.5 g) from three randomly selected individual plants of each replicate were pooled and cut into small pieces, which were rapidly frozen in liquid nitrogen and stored at −80°C for protein extraction.

Protein Extraction

Total protein was extracted from the radish taproots using a phenol (Phe) extraction procedure according to the reported methods with some modifications (Saravanan and Rose, 2004; Yang et al., 2013). In brief, about 2.5 g frozen taproots were finely powdered in liquid nitrogen adding 0.5 polyvinylpolypyrrolidone (PVPP) and then suspended in 20 mL of precooled extraction buffer containing 500 mM Tris-HCl pH 7.5, 50 mM ethylenediaminetetraacetic acid (EDTA), 100 mM KCl, 2 mM dithiothreitol (DTT), 2 mM phenylmethylsulfonyl (PMSF) and 30% sucrose (w/v). The mixture was extensively homogenized on ice for 2 min, and then an equal volume of Tris-HCl pH 7.5-saturated Phe was added. The mixture was thoroughly vortexed and proteins were collected by centrifuging at 12,000 g for 15 min at 4°C. The upper Phe phase was removed and re-extracted two or three times with extraction buffer. Proteins were precipitated from the final Phe phase with five volumes of saturated ammonium acetate in methanol at −20°C overnight. After centrifuging at 12,000 g for 15 min at 4°C, the pellets were washed twice with 20 mL of 0.1 mol·L^{-1} cooled acetone, and then dried by lyophilization and finally stored at −80°C until use. The protein was quantified by a Bradford protein assay kit (Sangon Biotech, China), and an equal amount of protein from three replicates of each treatment (CK, Pb500, and Pb1000) was respectively pooled for iTRAQ analysis.

iTRAQ Labeling and Strong Cation Exchange (SCX) Chromatography

The protein samples were dissolved in 100 mM triethylammonium bicarbonate (TEAB, pH 8.5) containing 1% SDS (w/v), reduced with 10 mM DTT at 56°C for 1 h, and followed by alkylation with 55 mM iodoacetamide (IAM) for 45 min at room temperature in the dark. Trypsin was then added to a final enzyme/substrate ratio of 1:20 (w/w) for protein digestion, which was incubated at 37°C for 12 h. The resulting tryptic peptides were vacuum-concentrated and then labeled with iTRAQ reagents (Applied Biosysterms, USA) according to manufacturer's instruction. The control sample (CK) was labeled with 118 iTRAQ reagent, and Pb-stressed samples treated with 500 and 1000 mg·L^{-1} Pb $(NO_3)_2$ were labeled with 119 and 114, respectively. The labeling reactions were incubated for 2 h at room temperature. Subsequently, all the peptides from three groups were combined and further fractionated using SCX chromatography on a Ultremex SCX column (250 × 4.6 mm, 5 μm particle size, 200 Å pore size, Phenomenex, USA) by high performance liquid chromatography (HPLC) system (Shimadzu LC-20AB, Japan). The HPLC gradient consisted of buffer A (25 mM NaH_2PO4, 25% ACN, pH 2.7) for 10 min, 5−35% buffer B (25 mM NaH_2PO_4, 25% ACN, 1M KCL, pH 2.7) for 11 min and 35−80% buffer B for 1 min, which were eluted at a flow rate of 1 mL·min^{-1}. The chromatograms were recorded at 214 nm and 20 constituents were obtained. The collected fractions were desalted with StrataX (Phenomenex, USA) and concentrated to dryness using a vacuum centrifuge.

Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS)

The mass spectroscopy analysis was performed using an ABSCIE X TripleTOF 5600 mass spectrometer (AB SCIE X, Framingham, MA, USA), coupled with an online flow HPLC nanoAcquity system (Waters, USA), which comprised two parts of columns including Symmetry C18 column (5 μm particles, 180 um × 20 mm) and BEH130 C18 column specification (1.7 μm particles, 100 μm × 100 mm). Symmetry C18 column was used for adsorption and desalination of peptides, while BEH130 for the separation of peptides. The correction liquid (Thermo Fisher Scientific) was respectively added to liquid A (water:acetonitrile:formic acid = 98:2:0.1) and B (water:acetonitrile:formic acid = 2:98:0.1) with a certain proportion. A 2.25 μg aliquot of supernatant (9 μl) was transferred, and then the samples were eluted with liquid A at 2 μl·min^{-1} for 15 min for the purpose of adsorption and desalination of peptides. After that, the mobile phase which contain 5% liquid B was used to elute the supernatant (300 nl·min^{-1}, 1 min). Next, the concentration of liquid B was raised from 5% to 35% in 40 min, from 35% to 80% in 5 min using linear gradients, and then the samples were eluted for 5 min with 80% liquid B. The separation was performed at a constant flow rate of 20 μl·min^{-1}.

Data Analysis and Protein Identification

Raw data files were converted into MGF files using Proteome Discoverer 1.2. Proteins identification were performed by using Mascot search engine (Matrix Science, London, UK; version 2.3.02) against our sequenced radish root transcriptome database (SRX316199 and http://www.ncbi.nlm.nih.gov/sra/; Wang et al., 2013).The search parameters were set according to the published studies (Qiao et al., 2012; Yang et al., 2013; Wang et al., 2014). For protein identification, a mass tolerance of 0.05 Da was permitted for intact peptide masses and 0.1 Da for fragmented ions, with allowance for one missed cleavages in the trypsin digests. The methionine oxidation (M), protein N-terminal acetylation, deamidation (N, Q), and iTRAQ (Y) were selected as variable modifications, and carbamidomethyl modification of cysteines (C), iTRAQ (Nterminal), iTRAQ (K) were as fixed modifications. The search results were passed through additional filters before exporting the data. For protein identification, the filters were set as follows: significance threshold *P*, 0.05 (with 95% confidence)

and ion score or expected cutoff <0.05 (with 95% confidence). For protein quantitation, it was required that a protein contains at least two unique spectra. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. The DAPS were defined: Showing a fold-change of greater than 1.2 or less than 0.83 using a greater statistically significant value $P < 0.05$.

Functional Analysis

The online software BLAST2GO (http://www.blast2go.com/b2ghome) was used to automatically assign protein description and obtain annotations from homologous sequences of public databases (Conesa et al., 2005; Conesa and Götz, 2008). A metabolic pathway analysis was undertaken based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database. The Gene Ontology (GO) and metabolic pathway enrichment analysis of the DAPS were conducted through two publically available tools (webservers), namely, DAVID 6.7 and KOBAS 2.0. Furthermore, an integrated expression analysis of Pb-responsive miRNAs, mRNAs, metabolites and proteins were conducted based on our previous studies (Wang et al., 2013, 2015a,b). The same gene annotations were used to link expression levels across the four technologies.

RESULTS

Protein Identification and Quantification of Radish Root in Response to Pb Stress

A total of 241,107 spectra were obtained from the iTRAQ LC−MS/MS proteomic analysis of these three group samples including an untreated control (CK), Pb500 and Pb1000. After data filtering to eliminate low-scoring spectra, a total of 17,579 unique spectra were matched to 10,896 peptides representing 9725 unique peptides. The criteria that the peptides with ≥ distinct unique peptide were used for protein identification, and a total of 3898 protein species were successfully detected at a 95% confidence limit. The relative molecular mass distribution of the identified protein species showed that the most abundant sequences ranged from 10 to 80 kDa.

Only the proteins with at least two unique identified peptides were retained for further quantification analysis. In total, 2141 protein species were permitted the quantification of their abundance. Among them, 345 protein species were identified as differentially accumulated between Pb500 and CK groups including 177 (51%) up-accumulated, and 168 (49%) down-accumulated (**Table S1**). Among the 509 DAPS responding to the Pb1000 treatment, 48% (244) showed increased abundance, while 52% (265) showed decreased (**Table S2**). The proportion of common DAPS were calculated in response to the two Pb stress conditions. In total, 721 species were differentially accumulated under Pb treatments with 19% (135) common DAPS in both Pb treatments and 81% (586) distinct DAPS, among which 211 unique to Pb500-stress and 375 unique to Pb1000-stress (**Figure 1**).

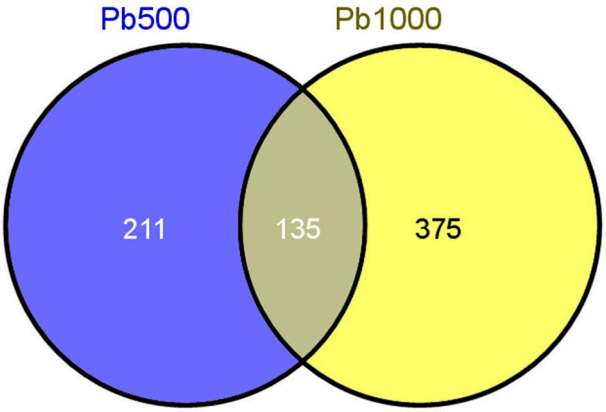

FIGURE 1 | The Venn diagram of differential abundance protein species (DAPS) involved in radish root under Pb500 and Pb1000 exposure.

Functional Classification and Enrichment Analysis of the DAPS

All of the 721 non-redundant DAPS sequences were functionally annotated using BLAST2GO (Conesa et al., 2005; Conesa and Götz, 2008). GO terms were assigned to query sequences and cataloged groups were produced basing on biological processes, molecular functions and cellular components. In total, 709 DAPS were assigned with 1687 GO terms and could be classified into 43 functional groups at the second level 2 (**Figure 2**). Among them, biological processes represented the largest category, containing 22 groups with metabolic (78.0%) and cellular process (75.3%) as the two most frequent terms. Within the molecular function category, the predominant groups were assigned to catalytic activity (56.7%) and binding (55.1%). For cellular components, all the DAPS were mostly located in cell (84.2%) and organelle (67.7%).

To systematically understand its biological functions in terms of networks, the 721 DAPS were mapped into the KEGG pathway database. A total of 75 pathways were assigned, which were largely involved in carbohydrate metabolism (such as starch and sucrose metabolism, pyruvate metabolism, glycolysis/gluconeogenesis, pentose phosphate pathway, and amino sugar and nucleotide sugar metabolism), amino acid metabolism (phenylalanine metabolism, alanine, aspartate, and glutamate metabolism, cysteine and methionine metabolism and arginine and proline metabolism) and energy metabolism (carbon fixation in photosynthetic organisms, carbon fixation pathways in prokaryotes, methane metabolism, oxidative phosphorylation and sulfur metabolism) (**Table S3**). The pathways with the DAPS number larger than five were shown in **Figure 3**.

Furthermore, through functional enrichment analysis of the 135 common DAPS (**Table 1**), it was shown that "cell wall" (GO: 0005618), "apoplast" (GO: 0048046), "response to metal ion" (GO: 0010038), "vacuole" (GO: 0005773), and "peroxidase

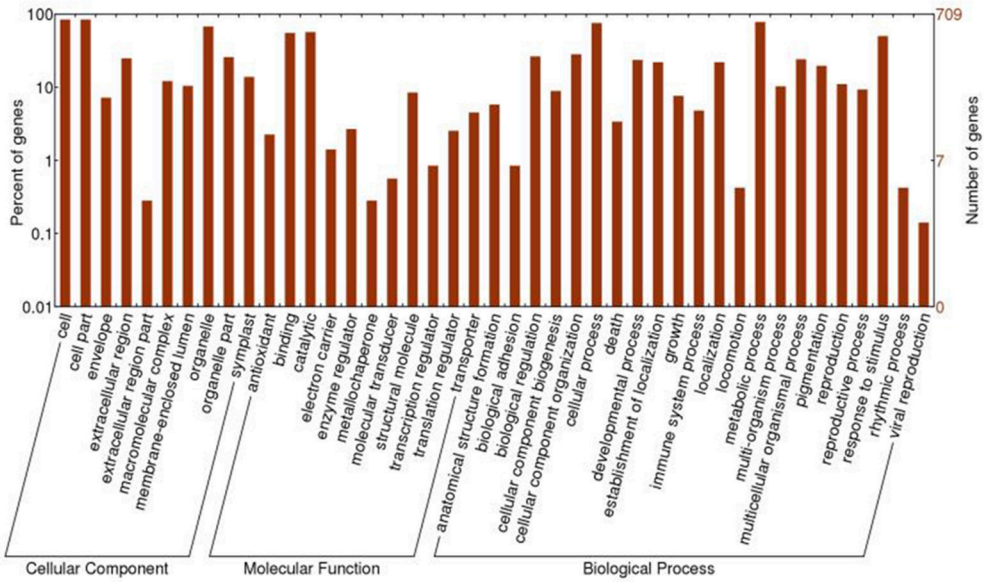

FIGURE 2 | GO classification of the DAPS of radish root in response to Pb stress.

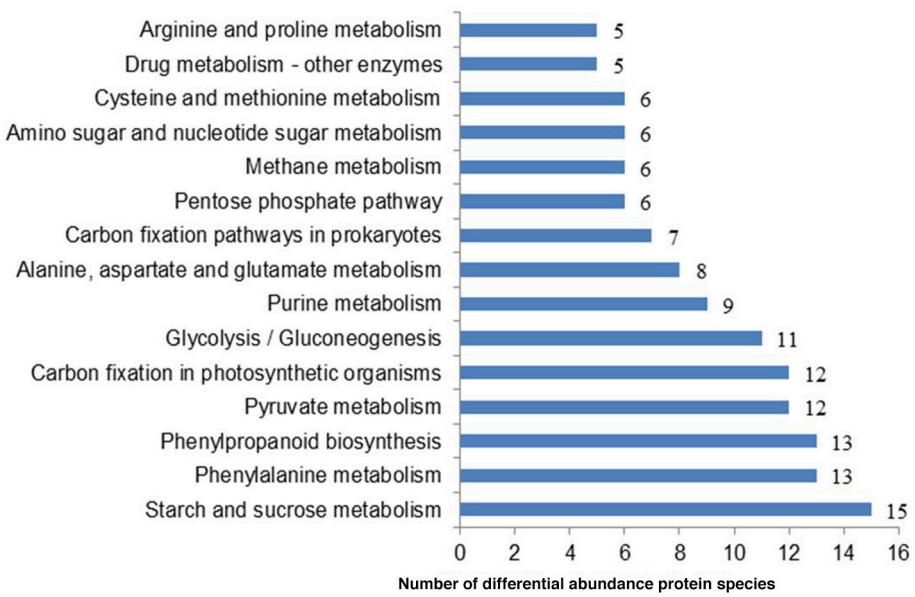

FIGURE 3 | The representative pathways of the DAPS involved in radish root response to Pb exposure.

activity" (GO: 0004601) were the most overrepresented GO terms (**Table 2**). KOBAS 2.0 webserver was employed to identify statistically enriched pathways from pathway databases involved in KEGG, BioCyc, Reactome, Pathway Interaction and Panther. The critical enriched pathways included "citric acid (TCA) cycle and respiratory electron transport," "pyruvate metabolism," "phenylalanine metabolism," "phenylpropanoid biosynthesis," and "carbon metabolism" (**Table 3**).

Characterization of the Critical Pb Stress-Responsive Proteins in Radish

Protein modification, the balance between synthesis and degradation, is a critical form of regulation that is coordinated to achieve a unified cellular under the stress of environmental stimuli (Hinkson and Elias, 2011). In this study, 27 protein species implicated in protein translation, processing and degradation were identified to be co-differentially accumulated

TABLE 1 | The common DAPS involved in radish root under Pb500 and Pb1000 exposure.

Accession	Protein ID	Description	Pb500/CK	Pb1000/CK
PROTEIN METABOLISM				
1	CL10682.Contig1	20s proteasome subunit	1.508	0.729
2	CL6704.Contig2	40s ribosomal protein	0.758	0.781
3	CL8114.Contig2	60s acidic ribosomal protein	1.452	1.850
4	Unigene19731	60s acidic ribosomal protein	1.599	1.448
5	CL2397.Contig1	60s ribosomal	1.267	1.708
6	Unigene1307	60s ribosomal protein	0.749	0.616
7	CL9354.Contig1	60s ribosomal protein	0.625	1.255
8	CL10582.Contig2	60s ribosomal protein	2.084	3.621
9	CL2279.Contig3	60s ribosomal protein	1.404	3.372
10	CL5126.Contig2	60s ribosomal protein	1.377	2.232
11	Unigene2385	Cyclase family protein	0.675	0.555
12	Unigene21215	Cyclophilin 1	1.430	0.413
13	CL714.Contig1	d-3-phosphoglycerate chloroplastic-like	0.654	1.524
14	CL89.Contig1	Eukaryotic translation initiation factor	1.214	1.258
15	Unigene29764	RNA binding protein	1.298	1.715
16	CL1044.Contig6	RNA helicase drh1	0.735	0.632
17	CL7993.Contig7	RNA-binding protein	1.568	2.112
18	CL3437.Contig4	Root hair defective 3	0.820	1.512
19	Unigene19929	Rotamase cyclophilin 2	1.203	0.485
20	CL14184.Contig1	Sar DNA-binding protein	1.555	3.115
21	CL9725.Contig1	Small nuclear ribonucleoprotein g	0.789	0.805
22	Unigene926	Translation initiation factor	1.842	1.386
23	Unigene26860	Translation initiation family protein	1.496	1.660
24	CL4590.Contig3	Ubiquitin-conjugating enzyme e2 5	1.502	0.606
25	CL83.Contig2	Ubiquitin-conjugating enzyme e2 variant 1d	1.204	0.772
26	Unigene282	Ubiquitin-conjugating enzyme e2-17 kda	1.292	0.717
27	CL1707.Contig1	protein Disulfide isomerase	1.254	1.339
STRESS AND DEFENSE				
28	CL14023.Contig1	Profilin 1	1.487	0.731
29	Unigene29769	Leucine-rich repeat family protein	0.509	0.539
30	CL9570.Contig1	Leucine-rich repeat receptor-like protein	0.759	0.423
31	CL7251.Contig1	Temperature-induced lipocalin	0.477	0.710
32	Unigene29834	Thaumatin-like protein	0.112	0.782
33	Unigene8320	Disease resistance response	0.652	0.410
34	Unigene29734	Early nodulin-93-like	0.535	0.548
35	CL6279.Contig2	Early nodulin-like protein 13	2.053	1.928
36	CL752.Contig1	Blue copper-binding 15k	1.315	0.446
37	CL77.Contig1	Chitinase family protein	0.762	0.561
38	Unigene16718	Lectin-like protein	0.606	0.562
39	CL7174.Contig1	Peroxidase	0.743	0.438
40	CL1027.Contig4	Peroxidase	0.567	0.542
41	Unigene8343	Peroxidase	1.318	0.602
42	Unigene21004	Peroxidase 12	0.520	0.548
43	Unigene21694	Peroxidase atp3a homolog	1.228	0.331
44	CL3864.Contig1	Peroxidase atp8a	0.791	0.679
45	Unigene27310	atpca atprx33 prx33	0.374	0.377
46	CL959.Contig2	Bacterial-induced peroxidase	0.831	0.656
47	CL13300.Contig1	Thioredoxin 1	0.714	0.764
48	CL2689.Contig1	12-oxophytodienoate reductase 1	1.545	1.274
49	CL5307.Contig5	12-oxophytodienoate reductase 3	0.765	0.566
50	CL10604.Contig2	Glutaredoxin-like protein	1.547	1.752
51	CL4589.Contig1	Anamorsin homolog	0.788	0.416
52	CL11999.Contig2	Monodehydroascorbate reductase	0.823	1.456
53	CL2553.Contig1	Adenine nucleotide alpha hydrolases-like superfamily protein	0.682	1.376
54	CL11402.Contig1	Reversibly glycosylated polypeptide-3	1.417	1.784
55	CL2995.Contig1	Selenium-binding protein	0.663	0.661
56	CL8292.Contig1	Serpin family protein	0.716	0.625
57	CL214.Contig1	Strictosidine synthase	0.668	0.658
58	CL2053.Contig1	Stromal cell-derived factor 2-like protein	1.383	1.618
59	Unigene15048	Subtilase family protein	0.653	0.568
60	Unigene6675	Subtilisin-like protease-like	0.662	0.445
61	CL7173.Contig1	Thiol protease aleurain	1.940	1.247
62	CL603.Contig2	tpx2 (targeting protein for xklp2)-like protein	2.121	1.589
SIGNAL TRANSDUCTION AND TRANSPORT				
63	CL14365.Contig1	Elongation factor 1-alpha	1.519	2.972
64	CL11865.Contig1	Receptor kinase	0.664	0.650
65	CL292.Contig1	Signal recognition particle 72 kda	0.677	1.252
66	Unigene8449	cdp-diacylglycerol–glycerol-3-phosphate 3-partial	0.792	0.584
67	CL2540.Contig2	rae1-like protein at1g80670-like	1.566	0.644
68	CL1217.Contig1	Clathrin light chain protein	1.860	1.231
69	CL10110.Contig2	Calreticulin-3-like isoform x1	0.648	2.147
CARBOHYDRATE AND ENERGY METABOLISM				
70	CL3177.Contig3	Aldose 1-epimerase family protein	0.650	0.485
71	CL12533.Contig1	ATP citrate lyase	1.214	1.424
72	Unigene22436	Beta-fructofuranosidase 5	1.625	0.726
73	Unigene24884	Beta-glucosidase 21	1.993	2.132
74	CL13638.Contig1	Electron transfer flavoprotein subunit mitochondrial-like	1.278	0.615
75	Unigene27210	Fumarate hydratase 1	0.771	0.679

(Continued)

TABLE 1 | Continued

Accession	Protein ID	Description	Pb500/CK	Pb1000/CK
76	Unigene20278	Fumarate hydratase mitochondrial-like	0.671	0.569
77	CL10499.Contig1	Glucan endo--beta-glucosidase 4	1.252	0.441
78	Unigene25466	Glucan endo--beta-glucosidase 6-like	1.268	0.659
79	CL4038.Contig1	Isocitrate dehydrogenase	0.803	0.743
80	Unigene23018	Malate dehydrogenase	0.778	0.735
81	CL8917.Contig1	Myosin heavy chain kinase	1.319	1.551
82	Unigene28617	NADH-cytochrome b5 reductase-like	1.244	0.666
83	CL6783.Contig1	Pyruvate dehydrogenase family protein	0.724	0.819
84	CL6151.Contig2	Protein weak chloroplast movement under blue light 1-like	1.657	2.037
85	CL5706.Contig6	Transketolase	1.426	5.285
86	CL859.Contig3	Transaldolase-like protein	0.808	1.288
87	CL5075.Contig1	Succinate-semialdehyde mitochondrial-like isoform x1	0.790	0.811
88	CL3677.Contig1	Aconitase c-terminal domain-containing protein	1.266	0.805
89	CL452.Contig1	Beta-xylosidase alpha-l-arabinofuranosidase 2-like	0.524	0.526
90	CL452.Contig4	Beta-xylosidase alpha-l-arabinofuranosidase 2-like	0.754	0.451
91	CL7567.Contig2	Dihydrolipoyl dehydrogenase 2	0.705	1.526
AMINO ACID AND LIPIDS METABOLISM				
92	Unigene10784	Macrophage migration inhibitory factor family protein	1.567	0.560
93	Unigene24925	gdsl esterase lipase at1g54790-like	2.379	0.683
94	Unigene8067	gdsl esterase lipase at3g26430-like	0.540	0.450
95	Unigene22679	Glycine dehydrogenase	0.787	0.768
96	Unigene25700	Glycosyl hydrolase family 38 protein	0.738	0.572
97	Unigene24148	Xylem cysteine proteinase 1-like	0.811	0.566
98	CL9552.Contig2	Methyltransferase pmt24	1.336	1.571
99	Unigene2767	Myosin-related family protein	1.327	1.358
100	CL12773.Contig2	Nascent polypeptide-associated complex subunit alpha-like	1.602	0.562
101	CL2541.Contig2	Serine carboxypeptidase-like 29	3.161	1.950
102	Unigene2790	Aminomethyltransferase	0.601	0.585
103	CL783.Contig2	Peptidyl-prolyl cis-trans isomerase cyp20-3	0.566	0.629
104	CL2368.Contig1	Proteasome subunit alpha type-2-b	1.292	0.574
105	Unigene289	s9 Tyrosyl aminopeptidase	1.606	2.699
106	CL6065.Contig3	Aspartic proteinase	0.511	0.612
107	CL9279.Contig1	Aspartyl protease family protein	0.503	0.179
108	CL5981.Contig1	Dimethylmenaquinone methyltransferase family protein	1.301	0.408
109	CL330.Contig1	Cupin family protein	0.714	0.522
110	CL2774.Contig2	Dehydrin erd14	1.678	1.539
CELL WALL AND CYTOSKELETON				
111	CL2803.Contig4	Polygalacturonase inhibiting protein	0.383	0.491
112	CL12269.Contig1	Polygalacturonase-like protein	0.703	0.502
113	CL1120.Contig1	GTP-binding protein sar1a-like	1.252	1.646
114	CL741.Contig1	Nuclear RNA binding	1.847	1.539
115	CL14456.Contig1	Neurofilament protein	3.295	1.200
116	CL5177.Contig3	Nuclear protein	1.503	2.413
117	CL319.Contig1	Nucleic acid binding isoform partial	1.319	1.582
118	CL12221.Contig1	Nucleolin family protein	1.464	1.674
119	CL1396.Contig2	Nucleolin like 1	1.550	1.814
120	CL4932.Contig1	Pectinacetylesterase family protein	1.306	0.638
121	Unigene6860	Pectinesterase inhibitor	0.630	0.563
122	CL2232.Contig2	Probable pectinesterase pectinesterase inhibitor 51-like	0.823	0.627
123	CL1396.Contig2	Nucleolin like 1	1.550	1.814
124	Unigene21122	GPI-anchored protein	0.695	0.420
125	CL2657.Contig1	Lipid-associated family protein	1.459	0.475
126	Unigene25152	Vesicle associated protein	1.618	1.678
127	Unigene22796	Organellar DNA-binding protein 1	0.756	0.544
128	CL8050.Contig1	Partial	1.295	0.681
129	Unigene2988	O-glycosyl hydrolases family 17 protein	1.529	0.726
130	Unigene29720	Endochitinase isolog	0.619	0.651
131	Unigene29711	Adenine nucleotide alpha hydrolases-like protein	0.698	0.472
132	CL3373.Contig1	Luminal binding protein	1.210	1.573
UNCHARACTERIZED PROTEIN				
133	CL12123.Contig1	Unknown	3.464	1.979
134	CL8554.Contig2	Uncharacterized protein	1.310	0.677
135	CL14500.Contig1	Uncharacterized protein	1.643	0.763

during Pb500 and Pb1000 exposures. Among them, nine ribosomal protein species were up-accumulated under either one or both Pb treatments, while only one ribosomal component was both down-accumulated during the two Pb-stressed conditions.

TABLE 2 | The dominant enriched GO terms for the 135 common DAPS under Pb500 and Pb1000 exposures.

Go term	ID	Input number	Background number	*P*-Value	Corrected *P*-Value
Cell wall	GO:0005618	24	480	4.69E-12	2.89E-09
Apoplast	GO:0048046	18	267	2.21E-11	9.07E-09
Response to metal ion	GO:0010038	17	425	1.25E-07	2.77E-05
Vacuole	GO:0005773	21	650	1.42E-07	2.77E-05
Peroxidase activity	GO:0004601	8	68	1.62E-07	2.77E-05
Plasmodesma	GO:0009506	20	645	5.16E-07	5.43E-05
Organelle lumen	GO:0043233	15	373	6.29E-07	5.54E-05
Membrane-enclosed lumen	GO:0031974	15	376	6.93E-07	5.69E-05
Antioxidant activity	GO:0016209	8	86	8.46E-07	6.51E-05
Response to inorganic substance	GO:0010035	25	1085	4.04E-06	0.000293
Cytosol	GO:0005829	27	1270	7.07E-06	0.000484
Nucleolus	GO:0005730	10	208	1.04E-05	0.000677
Extracellular region	GO:0005576	40	2386	1.48E-05	0.00091
Plant-type cell wall	GO:0009505	10	240	3.37E-05	0.001442
Positive regulation by symbiont of host innate immune response	GO:0052166	3	6	3.86E-05	0.001442

TABLE 3 | The dominant pathways for the 135 common DAPS under Pb500 and Pb1000 exposure.

Term	Database	Input number	Background number	*P*-Value	Corrected *P*-Value
The citric acid (TCA) cycle and respiratory electron transport	Reactome	5	26	2.30E-05	0.001287
Citrate cycle (TCA cycle)	KEGG PATHWAY	7	60	0.000112	0.002884
Phenylalanine metabolism	KEGG PATHWAY	9	106	0.000117	0.002933
Pyruvate metabolism and Citric Acid (TCA) cycle	Reactome	4	22	0.000192	0.004371
Phenylpropanoid biosynthesis	KEGG PATHWAY	10	145	0.000243	0.005078
Citric acid cycle (TCA cycle)	Reactome	3	10	0.000369	0.007341
Carbon metabolism	KEGG PATHWAY	11	217	0.001399	0.022388

The abundance levels for three translation initiation family proteins (CL89.Contig1, Unigene926 and Unigene26860), two RNA binding proteins (Unigene29764 and CL7993.Contig7) and one protein disulfide isomerase (CL1707.Contig1) were observed to be increased under these two Pb exposures. However, one RNA helicase (CL1044.Contig6) was identified to be decreased responding to these two Pb treatments. All three identified ubiquitin-conjugating enzymes (CL4590.Contig3, CL83.Contig2, and Unigene282) and one cyclase family protein (Unigene2385) were coordinately increased at the Pb500 exposure while decreased at the Pb1000 (**Table 1**).

It is known that the antioxidant enzymes could ensure cellular protection from the reactive oxygen species (ROS) mediated damage in plant responding to various environmental stresses. In the present study, the abundance changes for many antioxidant enzymes were detected including two 12-oxophytodienoate reductases (CL2689.Contig1 and CL5307.Contig5), one glutaredoxin-like protein (CL10604.Contig2), one thioredoxin (CL13300.Contig1) and eight peroxidases (CL7174.Contig1, CL1027.Contig4, Unigene8343, Unigene21004, Unigene21694, CL3864.Contig1, Unigene27310, and CL959.Contig2). Among these identified DAPS for antioxidant enzymes, most of them were decreased in radish under the exposure of Pb except one OPR (CL2689.Contig1) and one GR (CL10604.Contig2) up-accumulated at both Pb treatment, and two peroxidases (Unigene 8343 and Unigene 21694) up-accumulated under the Pb500 exposure (**Table 1**).

Additionally, a lot of protein species involved in carbohydrate and energy metabolism-related pathways (i.e., "TCA cycle and respiratory electron transport," "pyruvate metabolism," and "carbon metabolism") were shown to exhibit abundance changes under Pb exposure. The activities of most metabolic enzymes involved in these pathways were both repressed in the two concentrations of Pb treatments, including malate dehydrogenase (Unigene23018), endochitinase (Unigene29720), lectin-like protein (Unigene16718), polygalacturonase-like protein (CL12269.Contig1), aldose 1-epimerase family protein (CL3177.Contig3), beta-xylosidase alpha-l-arabinofuranosidase 2-like (CL452.Contig1), chitinase family protein (CL77.Contig1), and glycosyl hydrolase family 38 protein (Unigene25700). However, other important enzymes showed differentially accumulated during the two Pb stresses. For example, the expression levels of the acid invertase beta-fructofuranosidase 5 (Unigene22436), a beta-glucosidase glucan endo-beta-glucosidase 4 (CL10499.Contig1), and a hydrolases o-glycosyl hydrolases family 17 protein (Unigene2988) were increased in Pb500 exposure, but decreased in Pb1000 stress (**Table 1**).

Associated Analysis of Pb-Responsive Genes through Proteomic in Combination with Other–Omic Techniques of Radish in Response to Pb Stress

In order to investigate whether the protein level is correlated with the corresponding mRNA level alterations, the proteomic data was compared with our previous transcriptome data between CK vs. Pb1000 (Wang et al., 2013). Proteins were considered to be correlated when quantified proteins have expression information at the transcriptome level, and a total of 1667 protein species (77.9% of all the quantified proteins) were detected from the transcriptome data (**Table S4**). Out of the 510 protein species found to be significantly accumulated under Pb1000 stress of radish, there were only 57 DAPS could be matched with their cognate differentially expressed genes (DEGs) including 25 regulated in the same trends and 32 found in the opposite direction (**Table S5**). In addition, there were 343 DAPS exhibiting no change in mRNA expression and 144 DEGs without altered expression at protein level (**Table S5**). These results indicated that the accumulation of transcripts and proteins occur independently. A generally low congruency of proteomic and transcriptional profiles has been reported in other previous studies (Lan et al., 2011; Zhuang et al., 2013).

Increasing evidences have revealed that miRNA-mediated gene regulation play significant roles in plant response to HM stress, and 19 Pb-responsive miRNAs and their corresponding target mRNAs were identified in radish by siRNA sequencing and degradome analysis (Wang et al., 2015a). By exploring an integrated expression analysis of miRNA, mRNAs and proteins, a total of seven miRNA-mRNA pairs and matching proteins were identified in radish response to Pb stress, which mainly included two miRNA families, miR156 and miR396. As shown in **Table 4**, almost all Pb-responsive miRNAs and their corresponding mRNA targets had an anti-relationship at protein level. For example, two members of miR156 family (miR156b and miR156c) were found to be up-regulated in response to Pb exposure, while the expressions of their targets (glutaredoxin, aldose 1-epimerase and malate dehydrogenase) were all repressed at protein levels. Two corresponding protein species for the down-regulated miR396b were identified during Pb stress of radish, one (Translation initiation factor eIF-3) was up-accumulated, while the other one (thioredoxin-like 1-2, chloroplastic) was shown to be down-accumulated.

For deeply dissecting the molecular mechanism underlying Pb tolerance and homeostasis in radish, we further performed an association analysis of the seven Pb-responsive miRNA-mRNA-proteins pairs with the differentially regulated metabolites identified in radish roots during Pb stress (Wang et al., 2015b). Based on the intersected pathway analysis, three Pb-responsive metabolites including glucose, citrate and malate were found to be linked with the miRNA-mRNA-proteins, which may play a significant role in radish response to Pb stress (**Table S6**).

DISCUSSION

Proteomic technique provides a powerful tool for the analysis of molecular mechanism of plant response against stresses, and a path for linking gene expression to cell metabolism in the rapidly processing post-genome era (Ahsan et al., 2009; Liang et al., 2013). Increasing evidences have revealed that proteomic studies are playing important roles in the post-genomic era for characterizing the molecular mechanisms underlying plant responses to HM stresses (Fukao et al., 2011; Wang et al., 2013, 2014; Sebastiani et al., 2014). Radish is an important root vegetables worldwide, which has been considered as one of the most significant root species for dissecting the molecular regulatory network of HM stress in Brassicaceae crops (Xu et al., 2012; Wang et al., 2014, 2015a; Liu et al., 2015; Xie et al., 2015). In the current study, comparative proteomics analysis by iTRAQ together with other-omics techniques reveals complex regulatory network and provides insights into the response of radish root under Pb exposure. To the best of our knowledge, this is the first study to systematically investigate and characterize the protein abundance changes under Pb stress exposure in radish root with iTRAQ-based proteomics analysis.

Proteins of Signal Sensing Mechanisms Involved in Radish Root Response to Pb Stress

It was known that the cell wall could activate a variety of specific stress-responsive signaling proteins when perceive outer stress conditions (Jamet et al., 2006; Day et al., 2013). In previous

TABLE 4 | Association analysis of miRNAs, genes, and proteins responsive to Pb stress in radish root.

miRNA	LogFC[a]	Target mRNA annotation	Protein ID	LogFC[b]	LogFC[c]
miR156b	1.1813	Glutaredoxin	CL10604.Contig2	−0.12	−0.247
miR156b	1.1813	Aldose 1-epimerase	CL3177.Contig3	−0.19	−0.314
miR156c	1.0788	Malate dehydrogenase	Unigene23018	−0.11	−0.134
miR396b	−0.5131	Thioredoxin	CL13300.Contig1	−0.15	−0.117
miR396b	−0.5131	Translation initiation factor	CL89.Contig1	0.084	0.0997
miR396b	−0.5131	Translation initiation factor	Unigene926	0.265	0.1418
miR396b	−0.5131	Translation initiation factor	Unigene26860	0.175	0.2201

[a] *miRNA fold change under Pb500.*
[b] *Protein fold change under Pb500.*
[c] *Protein fold change under Pb1000.*

studies, a set of cell wall-related protein species exhibited dynamic changes in response to HM stress, such as Al exposure in rice and cadmium (Cd) exposure in flax (Hradilová et al., 2010; Wang et al., 2013, 2014). Currently, "cell wall" (GO: 0005618) was identified as the most enriched GO term for all the 135 common DAPS involved in the Pb500 and Pb1000 stress, which indicated that cell wall may play a vital role in response to Pb stress of radish. Two critical inhibiting protein species including a pectinesterase inhibitor (CL2232.Contig2) and a polygalacturonase inhibitor (CL2803.Contig4) were both down-accumulated during the stresses of Pb500 and Pb1000 in radish. The two protein species were verified to modulate the activities of pectinesterases (PE) and polygalacturonases (PG), which could help the plant to fine-tune cell wall remodeling processes when exposure stress conditions (Pelloux et al., 2007; Ferrari et al., 2012). The down-accumulation of the inhibiting proteins maybe result in up- accumulation of PE and PG, indicating their positive role to the cell wall remodeling when encounter the Pb stress of radish.

Calreticulin (CRT) is an abundant endo reticulum Ca^{2+} binding protein, and plays a critical role in Ca^{2+} homeostasis and signaling sensing network (Corbett and Michalak, 2000; Jia et al., 2009). In this study, one CRT (calreticulin-3-like isoform x1, CL10110.Contig2) was found to be induced by the Pb1000, indicating that the altered expression of CRT maybe function as signaling molecular in modulating the radish plant to adapt to the Pb-stressed environments. Additionally, jasmonate (JA) is known to be a vital signaling molecule which can be activated in response to a wide range of environmental cues including heavy metals (Maksymiec et al., 2005; Schommer et al., 2008; Liu et al., 2009). In this study, the expression level of 12-oxo-phytodienoic acid reductase (OPR), a key enzyme involved in the JA biosynthesis pathway, showed differential abundance levels during the Pb stress. The OPR-1 (CL2689.Contig1) was up-accumulated in radish roots when subjected to the either Pb500 or Pb1000 stress, and a similar result was found at the proteome level in rice root exposure to As stress (Ahsan et al., 2008; Srivastava et al., 2009). However, the expression level of OPR-3 (CL5307.Contig5) was decreased in radish roots under the conditions of Pb500 and Pb1000 exposure, indicating that different members of the OPR were differentially accumulated under the Pb exposure in plant.

Proteins of Carbohydrate and Energy Metabolism Involved in Radish Root Response to Pb Stress

The accumulation of HM in plants can severely affect the photosynthetic pathway and thus lead to symptoms of toxicity, such as chlorosis and growth reduction (Pourrut et al., 2013). To maintain the normal growth and development, or at least to protect the cells against excess damages, plants need to activate the carbohydrate and energy metabolism related metabolic pathways (Sarry et al., 2006; Thapa et al., 2012). In this study, "citric acid (TCA) cycle and respiratory electron transport" was identified as the most enriched pathway, and a lot of critical metabolic enzymes were shown to exhibit abundance changes under Pb exposure. Beta-glucosidase catalyzes the hydrolysis of the glycosidic and release of glucose, which then entry into the glycolysis process. ATP-citrate lyase (ACL, EC4.1.3.8) is a key enzyme involved in TCA cycle, which catalyzes the cleavage of citrate to yield acetyl CoA, oxaloacetate, ADP, and orthophosphate, and the ACL gene was found to be induced under various stresses including low temperature and drought stimulus (Hu et al., 2015). Pyruvate dehydrogenase could exert a key role in linking the glycolysis to the TCA cycle by catalyzing the formation of an acetyl-CoA from pyruvate (Vuoristo et al., 2015), which is a vital rate-limiting step reaction that determines the rate and efficiency of TCA cycle. In this study, a beta-glucosidase 21 (Unigene24884), ATP citrate lyase (ACL12533.Contig1), and a pyruvate dehydrogenase (CL6783.Contig1) were observed to be both up-accumulated during the Pb500 and Pb1000 stress of radish root, which revealed their positive potential role in producing more reducing power to compensate high-energy demand response to Pb stress. However, the abundances of the some other metabolic enzymes including Isocitrate dehydrogenase (CL4038.Contig1), fumarate hydratase (Unigene27210, Unigene20278), and malate dehydrogenase (Unigene23018) were decreased under the Pb-stresses, indicating their activity suffered repression. These decreased proteins may influence the biosynthesis and accumulation of organic acids such as citrate and malate, which play critical roles in HM tolerance (Ma et al., 2001; Wang et al., 2015b).

Proteins of Antioxidative Defense and Detoxification Involved in Radish Root Response to Pb Stress

The presence of excess metal ions causes ROS in plants, which can irreversibly damage the cells and attack macromolecules (Ahsan et al., 2009). However, the ROS can be scavenged by plant antioxidant defense system consisting of antioxidant compounds and enzymes (Apel and Hirt, 2004; Suzuki et al., 2012). The ascorbate (ASA)–glutathione (GSH) cycle is one of the main antioxidant systems in plants to keep ROS under control being, which involves a lot of critical antioxidant enzymes in a series of cyclic reactions to detoxify H_2O_2 (de Sousa et al., 2016; Noshi et al., 2016). One of the antioxidant enzymes is peroxidase (POD), which can detoxify H_2O_2 by oxidizing ascorbate. It was reported that different members of the peroxidase gene family were differentially accumulated under various HM exposures in Arabidopsis (Kumari et al., 2008), wheat (Houde and Diallo, 2008) and aspen (Grisel et al., 2010). In this study, the term of "peroxidase activity" (GO: 0004601) was identified as one of the most over-represented GO terms and six POD protein species were identified as DAPS, indicating the POD may be the critical ROS-scavenging protein in radish root response to Pb stress. The abundance of another antioxidant enzyme involved in ASA–GSH cycle, monodehydroascorbate reductase (MDHAR, CL11999.Contig2) was also found to be altered in response to Pb stress. The main function of MDHAR is responsible for ascorbate regeneration in plant tissues, and the expression level changed during the HM stress in *B. juncea* root

(Alvarez et al., 2009) and *A. Halleri* shoot (Farinati et al., 2009). Additionally, many thiol-containing antioxidants, peroxiredoxin (PRX33, unigene27310), glutaredoxin-like protein (GRX, CL10604.Contig2) and thioredoxin (TRX, CL13300.Contig1), were also found to be altered during the Pb-stressed condition of radish root. The Prx is a thiol peroxidase with multiple functions, which was found to be induced under various HM stresses such as Cd (Hossain et al., 2013) and As (Pandey et al., 2012). Glutaredoxin (GRX) and thioredoxin (TRX) could be oxidized by peroxides and regenerate peroxiredoxins, which were verified to play direct roles in the antioxidative system by regenerating peroxiredoxins oxidized by peroxides (Hossain and Komatsu, 2013).

The Genetic Regulatory Network of Radish Root Response to Pb Stress

The mechanism of HM response is a complex process that a variety of genes and response components involved in plants (Fukao et al., 2011; Sebastiani et al., 2014). In the present study, to reveal the molecular mechanism underlying Pb stress response in radish root, a putative schematic network was put forward based on proteomic information of this study in conjunction with integrated analysis of miRNA, transcriptomic and metabolomic data (**Figure 4**). Once Pb enters the cell of radish plants, the cell wall firstly perceive stress signals and then activate specific stress-responsive signaling molecules including fine-tuning cell wall remodeling processes through special proteins like PE and PG, triggering the HM stress responsive hormones levels such as JA as well as modulating calcium-signaling molecules. With the aid of signaling molecules, the stress signals were transmitted and ultimately give rise to the alterations in gene expression and protein levels. A direct consequence of HM stress was the disturbance in the balance of protein synthesis and degradation, which is essential to both cellular homeostasis and dynamics because almost all biological processes need the involvement of enzymes. Under Pb stress, many key enzymes (i.e., ATP citrate lyase, Isocitrate dehydrogenase, fumarate hydratase and malate dehydrogenase) involved in the glycolysis and TCA cycle were severely affected, which ultimately cause alteration of some metabolites including glucose, citrate and malate. The glucose could act as the osmoprotectants protecting the cell constituents, and the organic acids (such as citrate and malate) played critical roles in chelating toxic HM ions. Meanwhile, a series of other defense responses were triggered to cope with Pb-induced injuries. For example, the ASA–GSH cycle was the main antioxidant systems for scavenging the accumulated ROS to alleviate oxidative damages. Moreover, several Pb-defense protein species (glutaredoxin, aldose 1-epimerase malate dehydrogenase and thioredoxin) encoding genes targeted by miRNA156 and miR396, which were found to play critical

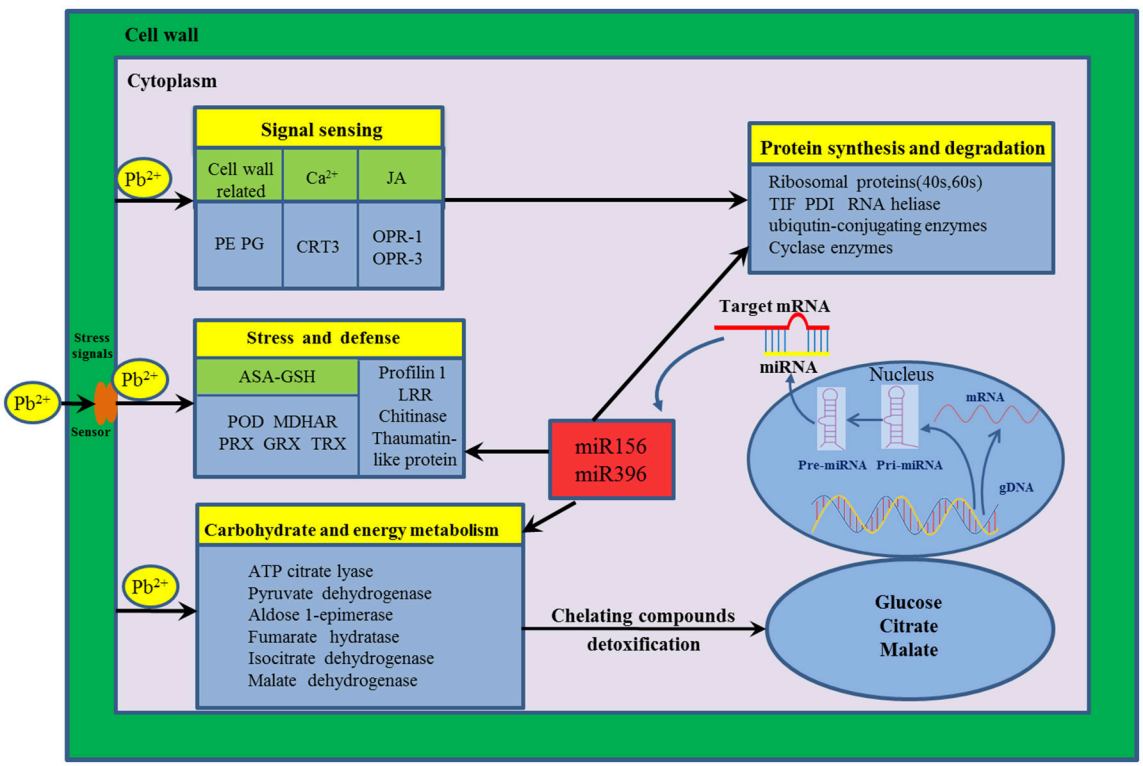

FIGURE 4 | The putative genetic regulatory network of Pb stress response in radish root. 12-oxo-phytodienoic acid reductase-1 (OPR-1), 12-oxo-phytodienoic acid reductase-3 (OPR-3), Ascorbate (ASA)–Glutathione (GSH), Calreticulin-3-like isoform x1 (CRT3), Glutaredoxin (GRX), thioredoxin (TRX), Leucine-rich repeat family protein (LRR), Monodehydroascorbate reductase (MDHAR), Pectinesterases (PE), Peroxidase (POD), Peroxiredoxin (PRX), Polygalacturonases (PG), Protein disulfide isomerase (PDI), Translation initiation factor (TIF).

roles in defense system when radish root responds to Pb stress.

In summary, this is the first report on systematically investigating and characterizing the protein abundance changes under Pb stress in radish root with the iTRAQ technique. A total of 3898 protein species were successfully detected and 2141 were quantified. A subset of 721 protein species were differentially accumulated upon at least one Pb exposure, and 135 ones showed common abundance alterations under two Pb-stressed conditions. GO and pathway enrichment analysis revealed that these 135 common DAPS were strongly enriched in the categories of cell structure, carbohydrate and energy metabolism-related pathways and antioxidative defense. Furthermore, the integrative analysis of transcriptomic, miRNA, degradome and metabolomic with proteomic data provided a strengthened understanding of radish root response to Pb stress, and a schematic genetic regulatory network was put forward. The genes associated with signal sensing, protein synthesis and degradation, carbohydrate and energy metabolism and ASA–GSH cycle for ROS scavenging as well as several key miRNAs and metabolites were crucially responsible for radish in response to Pb stress. Overall, the results of this study would be beneficial for further dissecting molecular mechanisms underlying other plant responses to HM stresses, and facilitate genetically effective management of HM contamination in root vegetable crops.

AUTHOR CONTRIBUTIONS

YW designed the experiments and drafted the manuscript. LX, MT and WZ participated in the design of the study and performed the data analysis. MT, RW and HJ planted radish seedlings and collected samples. LX and WC reviewed and edited the manuscript. LL conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was in part supported by grants from the NSFC (31601766, 31501759), NSF of Jiangsu Province (BK20140706), China Postdoctoral Science Foundation (2016T90472, 2015M570458, 2016M590466) and the National Key Technology Research and Development Program of China (2016YFD0100204-25).

SUPPLEMENTARY MATERIAL

Table S1 | The DAPS involved in radish root response under Pb500 exposure.

Table S2 | The DAPS in radish root response under Pb1000 exposure.

Table S3 | KEGG pathways of the DAPS involved in radish root response under Pb500 and Pb 1000 exposure.

Table S4 | The quantitative proteins detected in the transcriptomic analysis of radish under Pb exposure.

Table S5 | The correlated DAPS and DEGs involved in Pb stress response of radish.

Table S6 | Association analysis of miRNAs, genes, metabolites and proteins responsive to Pb stress in radish root.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 20 July 2016
doi: 10.3389/fpls.2016.01064

Alternative Growth and Defensive Strategies Reveal Potential and Gender Specific Trade-Offs in Dioecious Plants *Salix paraplesia* to Nutrient Availability

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Population sex ratios of many dioecious plants in nature are biased. This may be attributed to sexually different resource demands and adaptive capacity. In male-biased*Populus*, males often display stronger physiological adaptation than females. Interestingly, *Populus* and *Salix*, belonging to Salicaceae, display an opposite biased sex ratio, especially in nutrient-poor environmental conditions. Do female willows have a greater tolerance to nutrient deficiency than males? In this study, we investigated the growth and defensive strategies of *Salix paraplesia* cuttings, which were grown with high and low soil fertility for about 140 days over one growing season. Results suggest that different strategies for biomass allocation may result in sexually different defense capacities and trade-offs between growth and defense. Females are likely to adopt radical strategies, overdrawing on available resources to satisfy both growth and defense, which seems to be more like a gamble compared with males. It is also suggested that females may have an extra mechanism to compensate for the investment in growth under nutrient-poor conditions. In summary, the results may help focus restoration efforts on sex selection such that a moderate increase in female willow quantity could increase the resistance and resilience of willow populations to early sporadic desertification.

Keywords: desertification, dioecy, low soil fertility, sex differences, willow

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *17 May 2016*
Accepted: *06 July 2016*
Published: *20 July 2016*

Citation:
Jiang H, Zhang S, Lei Y, Xu G and Zhang D (2016) Alternative Growth and Defensive Strategies Reveal Potential and Gender Specific Trade-Offs in Dioecious Plants Salix paraplesia to Nutrient Availability. Front. Plant Sci. 7:1064. doi: 10.3389/fpls.2016.01064

INTRODUCTION

Dioecy is found in 175 flowering plant families and in 7% of flowering plant genera (Renner, 2014). Classic sex ratio theory suggests that when reproductive costs to produce a female vs. a male offspring are equal, natural selection will act to balance the sex ratio of the population (Fisher, 1930). However, numerous investigations have revealed that the population sex ratios of many dioecious plants are biased (Barrett et al., 2010; Sinclair et al., 2012; Munné-Bosch, 2015). Barrett et al. (2010) reported that most plant species exhibit equal or male-biased sex ratios, whereas female-biased sex ratios occur less frequently. Many researchers speculate that this phenomenon is most likely to be associated with the fundamental biological processes of gender dimorphism (Tognetti, 2012). However, we cannot ignore the fact that females have a higher

resource requirement for reproduction than males. Obeso (2002) suggested that females invest more resources in reproduction, which would leave them with a smaller amount of resources available for defense. Fujita et al. (2014) suggested that if plants in phosphorus-limited communities invest little in sexual reproduction, plants would be in danger. In addition, sex-specific physiological responses, such as nitrogen uptake and use, would have a great effect on plant stress tolerance. For example, *Juniperus thurifera* females may use a long-term strategy by increasing N storage to compensate for massive reproductive masting events, whereas males prefer to use current nutrients for promoting gas exchange capacity (Montesinos et al., 2012). If plants grow in nutrient-poor conditions, especially under N shortage, the contrasting responses of males and females may have a direct impact on plant survival.

In *Populus*, a genus with male-biased sex ratios, females usually experience greater negative effects than do males under environmental stresses. To interpret this important case, a series of studies were carried out. The results suggested that different-sex individuals display distinct morphological and physiological adaptations to environmental stresses (Juvany and Munné-Bosch, 2015). For example, in a water-limited scenario, drought stress could limit poplars' photosynthetic capacity more in females than in males (Xu et al., 2008a). In addition, when the two sexes grow in a combined stress condition, such as elevated temperature (Xu et al., 2008b) or salinity (Chen et al., 2010), some sex-specific reactions will differ from single-stress responses. For instance, the combination of drought and salinity induced greater sex-specific differences in the levels of total chlorophyll, carotenoid, H_2O_2 and Cl^- in the leaves than did single stresses (Zhang et al., 2011). Furthermore, when poplars were subjected to low-temperature conditions, males exhibited improved chloroplast structure and more intact plasma membranes than did females, suggesting a better self-protective capacity (Zhang et al., 2011). *Populus* and *Salix*, belonging to Salicaceae, display different biased sex ratios that female-biased sex ratios are of approximately 2:1 in many willow species (Alliende and Harper, 1989; Dudley, 2006; Myers-Smith and Hik, 2012). Do female willows have a greater tolerance than males and thus survive multiply stressful conditions? So far, few studies have been performed to confirm the sex-specific mechanisms that produced female-biased sex ratios of willows in nature from an ecophysiological perspective (Jones et al., 1999; Dudley, 2006; Randriamanana et al., 2015). Moreover, previous studies on sexual differences in plant growth were merely based on a single time point, which may miss important information and could not reflect the temporal variation because sexes differ in their timing of development (Glynn et al., 2007; Hultine et al., 2013).

In this study, we employed *Salix paraplesia* individuals, which were grown with high and low soil fertility for about 140 days over one growing season. Briefly, our study monitored plants' growth parameters at three important time points, which respectively represent the initial-growth stage, rapid-growth stage and late-growth stage according to our observations for many years. The experiment was designed not only for research on how *S. paraplesia* males and females respond to nutrient limitation but also to provide support for research on willows' tolerance to low nutrients in nature. The latter is important because sporadic desertification has been observed in many of their natural habitats. Temporal variation in the relative growth rate (RGR), net assimilation rate (NAR), stable isotope compositions, non-structural carbohydrates (NSCs), and condensed tannins (CTs) were measured. It is hypothesized that there is a gender-specific trade-off between growth and defense, whereby females have a stronger capacity to overdraw on available resources to satisfy growth requirements (for example, greater biomass accumulation and higher RGR) or defense (for example, higher CT concentrations) than males under nutrient-poor conditions.

MATERIALS AND METHODS

Plant Material and Experimental Design

In this study, 60 male and 60 female cuttings were produced from ten individuals of each population (6 populations in total) in their natural habitat in northern Sichuan province, China (**Table 1**). Briefly, each stem was cut into similar sections (1-cm diameter × 15-cm long) that were planted in 10-L plastic pots (one plant per pot), filled with 8 kg of homogenized soil on April 20. The two soil levels represented topsoil and deep soil. Topsoil was sieved from surface humus (0–15 cm), and deep soil was sieved from a depth of 20–40 cm at the site of origin. Topsoil represented nutrient-rich soil, and deep soil represented nutrient-poor soil. The properties of the nutrient-rich soil (nutrient-poor soil) used in this study were as follows (based on kg^{-1} dry soil): total carbon 30.33 g (17.86 g), organic carbon 28.51 g (16.08 g), total N 3.11 g (1.94 g), nitrate-N 26.06 mg (11.28 mg), and ammonium-N 21.86 mg (12.78 mg).

TABLE 1 | Willow population location and climate characteristics.

Population site name	Location	Elevation (m)	MAT (°C)	January MAT (°C)	July MAT (°C)	MAP (mm)
Baxi	33°36′ N, 103°13′ E	3140	0.7	−10.6	10.8	656.8
Hongxing	34°05′ N, 102°44′ E	3150	1.1	−8.9	12.2	648.5
Rangtang	32°16′ N, 100°58′ E	3390	4.8	−2.7	14.1	763.0
Aba	32°54′ N, 101°42′ E	3400	3.3	−7.9	11.7	712.0
Hongyuan	32°47′ N, 102°32′ E	3485	1.1	−10.3	10.9	753.0
Dazhasi*	33°34′ N, 102°57′ E	3439	1.7	−9.4	11.5	660.0

Dazhasi, study site. MAT, mean annual temperature; MAP, mean annual precipitation.

The experimental layout was completely randomized with two factors (sex and nutrients). Local soil has high fertility; therefore, in the present study, topsoil with high fertility was used as the control soil. There were finally four treatments: (i) males with high soil fertility (control); (ii) females with high soil fertility (control); (iii) males with low soil fertility; and (iv) females with low soil fertility. Thirty plants of each sex were exposed to each treatment. To monitor the ontogenetic variation in plants responding to relative high and low soil fertility, plants were destructively harvested at 100 (representing initial-growth stage, on July 29), 120 (representing rapid-growth stage, on August 18), and 140 days (representing late-growth stage, on September 7) after plants were planted.

RGR, NAR, and Biomass Allocation

Parameters related to plant growth were measured according to Glynn et al. (2007) with a minor modification. Briefly, leaf area was measured from the whole fully expanded leaves from each plant using a scanner with leaf area analysis software (WINFOLIA; Regent Instruments Inc. Quebec, Canada), after which leaves were dried at 60°C for 48 h and weighed. Leaf mass per unit area (LMA, g m^{-2}) was then calculated as the quotient of the mass and area of the leaf sample. At harvest, plants were partitioned into leaf, stem and root fractions, respectively. Each plant fraction was dried at 60 °C for 48 h and weighed. Indices of plant growth and allocation were then calculated from dry mass and total leaf area measurements according to the following equations:

Total plant mass (g) = total leaf mass + total stem mass + total root mass;
RGR (g g^{-1} d^{-1}) = [ln(final total mass) − ln(initial total mass)]/time;
Total leaf area (m^2) = total leaf mass/LMA;
LAR (cm^2 g^{-1}) = total leaf area/total plant mass (LAR, leaf area ratio);
NAR (g m^{-2} d^{-1}) = RGR/LAR;
SWR (g g^{-1}) = stem mass/total plant mass (SWR, stem weight ratio);
RWR (g g^{-1}) = root mass/total plant mass (RWR, root weight ratio).

Carbon, Nitrogen Concentrations, and Stable Isotope Signatures

The samples of shoots and roots were ground and passed through a 20-mesh screen after being dried (60°C, 48 h) to constant weight. The carbon and nitrogen concentrations were determined by the semi-micro Kjeldahl method as described by Kost and Boerner (1985), respectively. The stable carbon and nitrogen isotope abundance in the combusted samples was measured with a mass spectrometer (Finnegan MAT Delta-E, Bremen, Germany). The overall precision of the δ-values was better than 0.1‰, as determined from repeated samples.

Condensed Tannins

Condensed tannins (CTs) were analyzed using standard techniques (Orians, 1995; Hagerman and Butler, 1998; Hunter and Forkner, 1999). Briefly, leaves were removed from the trees and immediately put into a cooler, transported to the lab, and immediately vacuum dried. Once dry, leaves were ground with a mortar and pestle. Approximately 10 mg of leaf powder was weighed into 2-ml microfuge vials and washed with 500 µl diethyl ether. Following centrifugation (4 min at 3700 rpm), the diethyl ether was discarded. Tannins were extracted four times with 200 µl of a 7:3 acetone: water combination with 1-mM ascorbate. After each addition, samples were sonicated for 10 min at 5°C and centrifuged at 3700 rpm for 4 min. The supernatant was decanted into another microfuge vial. The acetone in the final supernatant was removed by evaporation. Water was added to attain the final volume of 500 µl. Some *S. paraplesia* tannins were prepared as standards in a similar manner, which generated by multiple sequential washes of samples in diethyl ether, followed by acetone extraction. *S. paraplesia* tannin standards were purified by Sephadex LH20 column chromatography. Samples were then analyzed using the butanol-HCl assay (Porter et al., 1985). Finally, tannin concentration (mg g^{-1} dry weight) was calculated.

Non-structural Carbohydrates

The NSC was determined as described by O'Brien et al. (2014). Five cuttings of each sex from each treatment were used to qualify the changes in stored NSC concentrations in the leaves, stems and roots, respectively. Plant materials were ground, and 15 mg of each sample was used for NSC analysis. Soluble sugars were extracted with a shaking bath of 80% ethanol at 27°C for one night, followed by two additional 2-h baths (Marquis et al., 1997; Myers and Kitajima, 2007). The remaining starch was digested with amyloglucosidase. The concentrations of soluble sugars and starch (measured as glucose equivalents) were measured at 487 nm by spectrophotometry after a phenol–sulphuric acid reaction. Mean NSC concentration was calculated by multiplying the extracted concentration for each organ by organ biomass, which provides total NSC per organ. The NSC values were summed and divided by total plant mass. Therefore, the NSC concentration in each organ represents the percentage of NSC relative to the whole cutting, and the sum of each organ percentage represents the total NSC concentration in the cutting.

Statistical Analyses

Experimental data were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). Two-way analyses of variance were performed to evaluate the interaction effect of sex and variable nutrient conditions. Sexual differences were analyzed using a model with nutrient and sex as fixed effects. Significant individual differences among means of different treatments were determined by Tukey's multiple range tests after conducting tests of homogeneity for variances. Differences were considered as statistically significant at the $P < 0.05$ level.

RESULTS

Plant Growth

The treatments of nutrient availability had different effects on the plant growth of male and female *S. paraplesia* individuals

(**Tables 2**, **3**). Distinct sexual differences were detected in both total plant mass and total leaf area throughout the growing season. Briefly, nutrient limitation significantly decreased the accumulation of total plant mass of both sexes, and importantly, females consistently had relatively higher total plant mass than males. The effects of both sex and nutrient were significant for total plant mass on all three harvest dates. Similar results were observed for total leaf area with one exception: the nutrient factor had no effect for plants grown in the first harvest intervals (Days 1–100). However, sexually different LMA of both sexes were not detected on any of the three harvest dates, as confirmed by the statistical analyses (**Table 3**). Moreover, nutrient limitation had no effect on the LAR of either sex at the first harvest time (Day 100), but it did have obvious effects for males during the last two harvest intervals (Days 101–120, 121–140). Statistical analyses revealed a significant effect on LAR by nutrient. In addition, we found effects on SWR for sex, nutrient and their combination. These effects were significant when plants grown during the first (Days 1–100) and the last (Days 121–140) harvest intervals. However, there was no effect on SWR (except for the nutrient effect) for plants grown in the middle harvest interval (Days 101–120). There was an effect on RWR of nutrient for plants grown in the first two harvest dates, but this had no effect during the last harvest interval (Days 121–140). Interestingly, the effect of sex exhibited completely opposite changes, which could be associated with variation of the combined effects by sex × nutrient. Nevertheless, it should be noted that little interactive effect on plant growth by sex × nutrient was detected in most harvest intervals (**Tables 2**, **3**). Although the interaction effect by sex × nutrient had no effect on RGR or NAR of males and females on any of the three harvest dates, distinct sexual differences existed for plants grown during the first two harvest intervals (**Table 4**). Over this time period, nutrient limitation significantly decreased cuttings' RGR and NAR when compared with those of cuttings grown under nutrient-rich conditions. Furthermore, females maintained relatively higher RGR and NAR than males under both conditions during the first harvest interval (Days 1–100). Statistical analyses suggested a significant effect on RGR and NAR by sex, which played an important role mainly during the first harvest interval (Days 1–100).

Carbon, Nitrogen Concentrations and Carbon, Nitrogen Isotope Signatures

Multiple effects (sex, nutrient, and their combination) were generally not significant for C concentration, N concentration, C/N ratio or $\delta^{13}C$ in the shoots and roots of *S. paraplesia* male and female cuttings (**Table 5**). Nutrient limitation significantly increased $\delta^{15}N$ in shoots, and females had more $\delta^{15}N$ than did males. Statistical analyses suggested a significant effect on the $\delta^{15}N$ in shoots by sex and by nutrient, but the interaction effect by sex × nutrient was not significant. In addition, nutrient limitation increased $\delta^{15}N$ in roots, but the changes were only detected in males (**Table 5**). Statistical analyses indicated that the effect on $\delta^{15}N$ by nutrient played a key role in roots, but the effect by sex as well as the interaction effect by sex × nutrient was not significant (**Table 5**).

TABLE 2 | Statistical significance of single and interactive effects of sex and nutrient on parameters related to plant biomass accumulation based on two-way ANOVA over three harvest intervals (day 1–100, 101–120, and 121–140, respectively) after cuttings planted (April 20, 2014).

Conditions		Total plant mass (g)			Stem weight ratio (SWR; g g^{-1})			Root weight ratio (RWR; g g^{-1})		
		Day 100	Day 120	Day 140	Day 100	Day 120	Day 140	Day 100	Day 120	Day 140
Nutrient-rich	Male	1.97 ± 0.03b	6.22 ± 0.32b	14.62 ± 0.49*b*	0.37 ± 0.008c	0.55 ± 0.012a	0.52 ± 0.007ab	0.18 ± 0.006a	0.17 ± 0.006a	0.25 ± 0.001b
	Female	2.80 ± 0.08a	7.25 ± 0.07a	16.02 ± 0.19*a*	0.40 ± 0.006b	0.54 ± 0.009a	0.53 ± 0.006b	0.17 ± 0.005 b	0.17 ± 0.003a	0.26 ± 0.001b
Nutrient-poor	Male	1.59 ± 0.08c	3.08 ± 0.23c	6.58 ± 0.21*d*	0.40 ± 0.005b	0.42 ± 0.021b	0.39 ± 0.016c	0.15 ± 0.008b	0.19 ± 0.008a	0.30 ± 0.006a
	Female	2.26 ± 0.1b	3.89 ± 0.10c	8.08 ± 0.25*c*	0.52 ± 0.003a	0.40 ± 0.005b	0.58 ± 0.009a	0.15 ± 0.007b	0.19 ± 0.009a	0.19 ± 0.001c
	P: F_S	***	**	**	***	ns	***	ns	ns	***
	P: F_N	***	***	***	***	***	**	**	*	ns
	P: $F_S \times F_N$	ns	ns	ns	***	ns	***	ns	ns	***

*Different letters represent statistical significances between treatments (mean ± SE, n = 5) at P < 0.05 according to Tukey's multiple range tests. F_S, sex effect; F_N, nutrient effect; $F_S \times F_N$, the interactive effect of sex and nutrient. Significance values of the factorial analysis (ANOVA) for the effects are denoted as follows: ns, non-significant; *P < 0.05; **P < 0.01; ***P < 0.001.*

TABLE 3 | Statistical significance of single and interactive effects of sex and nutrient on leaf traits based on two-way ANOVA over three harvest intervals (day 1–100, 101–120, and 121–140, respectively) after cuttings planted (April 20, 2014).

Conditions		Total leaf area (LA; cm^2 $plant^{-1}$)			Leaf mass per unit area (LMA; g cm^{-2})			Leaf area ratio (LAR; cm^2 g^{-1})		
		Day 100	Day 120	Day 140	Day 100	Day 120	Day 140	Day 100	Day 120	Day 140
Nutrient-rich	Male	104.28 ± 14.87ab	234.73 ± 21.12ab	372.15 ± 36.51a	83.79 ± 4.21a	74.46 ± 0.23a	89.89 ± 8.33a	52.79 ± 6.95a	37.57 ± 1.41c	25.42 ± 2.20b
	Female	145.78 ± 9.07a	289.11 ± 24.43a	347.67 ± 38.68a	83.56 ± 2.59a	73.16 ± 3.42a	101.77 ± 9.35a	51.96 ± 1.89a	39.83 ± 3.06bc	21.69 ± 2.36b
Nutrient-poor	Male	96.92 ± 4.16b	171.56 ± 12.58b	240.61 ± 12.53a	73.06 ± 2.88a	69.06 ± 3.58a	84.78 ± 1.17a	61.26 ± 2.68a	56.02 ± 4.64a	36.62 ± 2.01a
	Female	111.41 ± 11.71ab	204.46 ± 7.19b	242.51 ± 26.59a	67.97 ± 7.59a	78.09 ± 1.61a	80.58 ± 5.01a	49.21 ± 3.69a	52.58 ± 1.41ab	29.96 ± 2.71ab
	P: F_S	*	*	ns	ns	ns	ns	ns	ns	ns
	P: F_N	ns	**	**	*	ns	ns	ns	**	**
	P: $F_S \times F_N$	ns	ns	ns	ns	ns	ns	ns	ns	ns

*Different letters represent statistical significances between treatments (mean ± SE, n = 5) at $P < 0.05$ according to Tukey's multiple range tests. F_S, sex effect; F_N, nutrient effect; $F_S \times F_N$, the interactive effect of sex and nutrient. Significance values of the factorial analysis (ANOVA) for the effects are denoted as follows: ns, non-significant; *$P < 0.05$; **$P < 0.01$.*

Non-structural Carbohydrates and Condensed Tannins

Low soil fertility increased plants' NSC levels, but the increases were different in males and females. Briefly, females had more NSC than did males (**Figure 1**). Statistical analyses suggested an effect on the NSC by sex and by nutrient, but the interaction effect by sex × nutrient was not significant. In addition, there was a positive relationship between total plant mass and NSC. Females not only accumulated more NSC but also had greater total plant mass than males. **Table 6** revealed that nutrient limitation caused plants to produce more CTs among females than males on Day 100. This effect gradually diminished toward the end of the growing season. An effect on the CTs by nutrient was found throughout the plant growing season, while the effect by sex was only detected on Day 100. Females possessed more CTs than did males under nutrient-poor conditions on Day 100, but this pattern was completely changed when plants were subjected to nutrient-rich conditions. However, the sexual differences in CT concentration between males and females detected on Day 120 or 140 were not significant (except plants under nutrient-poor conditions on Day 140).

DISCUSSION

Male and female plants play different roles in productive biology and thus have sexually different resource demands imposed upon them. Any decision about resource utilization made by males or females may have different consequences, even contributing to spatial segregation of the sexes (Fujita et al., 2014). Many studies have implied that the population sex ratio of certain species would become female-biased if dioecious plants were placed under high-quality environments. The same reports maintain that the sex ratio would become male biased under stressful or resource-poor habitats, because reproduction is very costly for females (Sanchez-Vilas et al., 2012). However, there are exceptions. *Salix* often exhibits female-biased population sex ratios in naturally stressful environments (Dudley, 2006; Myers-Smith and Hik, 2012). Thus, we have attempted to find possible explanations that gender-specific trade-offs between growth and defense may be relevant to willow's female-biased sex ratios. In this study, *S. paraplesia* male and female cuttings were employed. A critical subject is posed: once plants suffer from nutrient limitation, how do males and females make their own choices: growing fast enough to compete or maintaining physiological adaptations necessary for survival?

Estimating growth parameters can help to elucidate whether plants suit the current environment or not. In the present study, female cuttings are likely to accumulate greater biomass than males under either nutrient-rich or nutrient-poor conditions. Statistical analyses have indicated that both sex and nutrients are important factors. Hermans et al. (2006) indicated that plants often allocate biomass to the root system when mineral elements are scarce. However, in our study, nutrient limitation significantly decreased biomass allocation below ground (see RWR) in males, while few effects were observed in females compared with those under nutrient-rich conditions during the

TABLE 4 | Statistical significance of single and interactive effects of sex and nutrient on growth parameters based on two-way ANOVA over three harvest intervals (day 1–100, 101–120, and 121–140, respectively) after cuttings planted (April 20, 2014).

Conditions		Relative growth rate (RGR; g g^{-1} d^{-1})			Net assimilation rate (NAR; g cm^{-2} d^{-1})		
		Day 1–100	Day 101–120	Day 121–140	Day 1–100	Day 101–120	Day 121–140
Nutrient-rich	Male	0.007 ± 0.0002b	0.057 ± 0.0019a	0.043 ± 0.0035a	1.327 ± 0.169b	15.296 ± 0.104a	17.174 ± 2.317a
	Female	0.01 ± 0.0003a	0.048 ± 0.0019a	0.04 ± 0.001a	1.982 ± 0.023a	12.065 ± 0.833b	18.781 ± 2.392a
Nutrient-poor	Male	0.005 ± 0.0005c	0.033 ± 0.0017b	0.038 ± 0.0046a	0.756 ± 0.105c	5.999 ± 0.626c	10.594 ± 1.767a
	Female	0.008 ± 0.0005b	0.027 ± 0.0036b	0.037 ± 0.0027a	1.662 ± 0.113ab	5.207 ± 0.723c	12.302 ± 0.958a
	P: F_S	***	ns	ns	***	*	ns
	P: F_N	***	***	ns	**	***	*
	P: $F_S \times F_N$	ns	ns	ns	ns	ns	ns

*Different letters represent statistical significances between treatments (mean ± SE, n = 5) at P < 0.05 according to Tukey's multiple range tests. F_S, sex effect; F_N, nutrient effect; $F_S \times F_N$, the interactive effect of sex and nutrient. Significance values of the factorial analysis (ANOVA) for the effects are denoted as follows: ns, non-significant; *P < 0.05; **P < 0.01; ***P < 0.001.*

initial-growth stage. This suggests that nutrient-limitation did not cause severe effects on the root growth of female cuttings but rather was fatal to males. This may ultimately reduce male cuttings' survival rate and might explain the segregation of the sexes. The largest value of RWR was observed in males under nutrient-poor conditions during the late-growth stage, suggesting that males indeed sensed and experienced greater environmental stress than did females. In other words, nutrient limitation may cause more severe impacts on plant growth in males than in females. In addition to RWR, nutrient limitation increased resource output to the stem in both sexes, and females constantly had higher SWR than males. *S. paraplesia* females gave priority to vegetative investment at the cost of stem growth during the initial- and late-growth stages, which may have enhanced stem functions including water transport and mechanical stability (Albrectsen et al., 2004; Taneda and Tateno, 2004). Relatively higher levels of nutrient storage in the female cuttings used for propagation may partially explain why female cuttings exhibited stronger capacity than male cuttings during the initial-growth stage.

In addition to plant growth, two compounds' NSC and CTs were investigated. NSC stores are assumed to be an important trait for plant survival under stress (O'Brien et al., 2014), particularly in maintaining basic metabolic functions to optimize growth and defense (Dietze et al., 2014). In general, most carbohydrates are produced in foliage leaves, and some are synthesized in flowers and fruits (Kozlowski, 1992). However, trees in the juvenile stage of development do not flower and, therefore, only have vegetative tissues as carbohydrate sinks. In our study, we analyzed the total NSC contents of entire cuttings (**Figure 1**). As expected, low soil fertility increased plants' NSC content, conducive to enhancing plant tolerance capacity (Chapin et al., 1990). Interestingly, females had higher NSC levels than males, and statistical analyses indicated a role for sex. This suggests that female cuttings with greater NSC storage were more likely to survive nutrient-poor conditions (Canham et al., 1999). In addition, there was a positive relationship between total plant mass and NSC, suggesting a good balance between photosynthesis and respiration, which ultimately influences carbon availability for growth (Chapin et al., 1990; Dietze et al., 2014).

Recent advances in plant metabolism studies indicate that plants are dependent on the deployment of secondary metabolites for their response to abiotic and biotic stresses (Mumm and Hilker, 2006; Neilson et al., 2013), and a variety of secondary compounds are produced from carbohydrates, amino acids and lipids. For example, *Populus tremuloides* seedlings increased CT concentrations under conditions of low fertility with competition (Donaldson et al., 2006). In the present study, comparative analysis showed that females produced more CTs than males under nutrient-poor conditions, while producing fewer CTs than males when plants are subjected to nutrient-rich conditions during the initial-growth stage. This suggests that females are likely more sensitive than males to nutrient availability, with regard to chemical defense. Our studies indicate that CTs may play a different role, sexually, in the initial- and late-growth stages rather than the rapid-growth stage (**Table 6**), which could provide females with greater defense capacity than enjoyed by males. In contrast, CTs are traditionally thought to play a key role in plant defense against herbivorous insects (Agrawal, 2007; Gols, 2014; Agrawal and Weber, 2015). In our study, temporal variation and sex-based differences in the regulatory strategies of CTs were observed in males and females. This may suggest sex-based differences between willows and herbivorous insects and sex-based differences in competition- and resource-mediated trade-offs between growth and defense (Donaldson et al., 2006).

According to the growth-differentiation balance hypothesis (Herms and Mattson, 1992; Glynn et al., 2007), if levels of defense remain stable while resources decrease, fewer resources will be available for growth or reproduction. Stamp (2003) suggested that resources allocated to the synthesis, storage, and regulation of plant secondary metabolites would come at the expense of those allocated for growth and reproduction. In accordance with our hypotheses, the results presented here suggest sex-specific trade-offs between the growth and defense of willows facing nutrient limitation. In the initial-growth stage, male and

TABLE 5 | Statistical significance of single and interactive effects of sex and nutrient on carbon (C), nitrogen (N) concentrations, C/N ratios, carbon and nitrogen isotope composition of shoots and roots based on two-way ANOVA at the last harvest time point (Day 140).

Conditions		C (g kg^{-1})		N (g kg^{-1})		C/N ratio		δ^{13}C (‰)		δ^{15}N (‰)	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Nutrient-rich	Male	473.82 ± 4.44a	407.1 ± 8.81a	26.46 ± 0.84a	20.65 ± 0.85a	17.95 ± 0.73a	19.75 ± 0.44a	−26.33 ± 0.2a	−25.69 ± 0.16a	1.8 ± 0.11c	3.86 ± 0.26b
	Female	472.56 ± 0.91a	419.49 ± 6.88a	28.62 ± 1.36a	19.11 ± 0.36a	16.59 ± 0.79	21.98 ± 0.72a	−26.43 ± 0.14a	−25.83 ± 0.09a	2.32 ± 0.07b	3.83 ± 0.1b
Nutrient-poor	Male	463.14 ± 1.38a	397.38 ± 10.63a	28.52 ± 0.73a	20.23 ± 0.46a	16.26 ± 0.41a	19.68 ± 0.92a	−26.76 ± 0.27a	−25.45 ± 0.31a	2.36 ± 0.11b	4.93 ± 0.27a
	Female	473.44 ± 2.83a	410.72 ± 7.77a	27.74 ± 0.24a	20.94 ± 0.57a	17.07 ± 0.24a	19.63 ± 0.45a	−26.53 ± 0.12a	−25.68 ± 0.16a	2.9 ± 0.13a	4.33 ± 0.11ab
	P: F_S	ns	ns	ns	ns	ns	ns	ns	ns	**	ns
	P: F_N	ns	ns	ns	ns	ns	ns	ns	ns	**	**
	P: $F_S \times F_N$	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*Different letters represent statistical significances between treatments (mean ± SE, n = 5) at P < 0.05 according to Tukey's multiple range tests. F_S, sex effect; F_N, nutrient effect; $F_S \times F_N$, the interactive effect of sex and nutrient. Significance values of the factorial analysis (ANOVA) for the effects are denoted as follows: ns, non-significant; **P < 0.01.*

FIGURE 1 | Effects of nutrient availability on non-structural carbohydrates (mean ± SE, *n* = 5) in *Salix paraplesia* males and females. Nutrient-poor condition: black; nutrient-rich condition: white. Different letters above the bars represent statistically significant differences between treatments at $P < 0.05$ according to Tukey's multiple range tests. Significance values of the factorial analysis (ANOVA) are denoted as follows: sex, sex effect; nutrient, nutrient effect; sex × nutrient, sex × nutrient interaction effects.

TABLE 6 | Statistical significance of single and interactive effects of sex and nutrient on condensed tannins (CTs) based on two-way ANOVA over three harvest intervals (day 1–100, 101–120, and 121–140, respectively) after cuttings planted (April 20, 2014).

Conditions		Condensed tannins (CTs, mg g^{-1} dry weight)		
		Day 100	Day 120	Day 140
Nutrient-rich	Male	2.309 ± 0.06c	0.535 ± 0.06b	0.261 ± 0.03b
	Female	1.507 ± 0.05d	0.52 ± 0.03b	0.222 ± 0.03b
Nutrient-poor	Male	2.993 ± 0.09b	1.399 ± 0.1a	0.357 ± 0.02b
	Female	3.37 ± 0.07a	1.11 ± 0.06a	0.533 ± 0.05a
	P: F_S	*	ns	Ns
	P: F_N	***	***	***
	P: $F_S \times F_N$	***	ns	*

*Different letters represent statistical significances between treatments (mean ± SE, n = 5) at P < 0.05 according to Tukey's multiple range tests. F_S, sex effect; F_N, nutrient effect; $F_S \times F_N$, the interactive effect of sex and nutrient. Significance values of the factorial analysis (ANOVA) for the effects are denoted as follows: ns, non-significant; *P < 0.05; ***P < 0.001.*

female strategies when confronting challenges related to nutrient availability differ substantially. Under nutrient-rich conditions, males invested more resources in defense than females (as reflected in the synthesis of more condensed tannins than females, see **Table 6**), which could be defined as a conservative adaptive strategy. This could impact male cuttings' growth (**Table 4**). Although high resource availability may diminish allocation costs and allow for growth and defense (Siemens et al., 2002), once plants face limited resources, females' positive regulatory mechanisms seem to be more efficient than those of males. It was found that females synthesized more CTs to enhance defense; levels were greater than those found in males (**Table 6**). As predicted, nutrient limitation significantly

decreased plants' RGR and total plant mass accumulation, but females still had higher RGR and greater total plant mass than males (**Table 4**). Most explanations of plant stress tolerance indicate an evolutionary trade-off such that plant species with high chemical defense would lower their RGR to maintain relative equilibrium (Herms and Mattson, 1992; Stamp, 2003; Fine et al., 2006; Glynn et al., 2007). However, in our study, females were likely to adopt radical strategies, overdrawing on available resources to satisfy both growth and defense, suggesting sex-based strategy differences. The results also indicated that females may have an extra mechanism to compensate for the investment in growth under nutrient-poor conditions. For example, female *Salix* may have higher photosynthetic rate than males, or increase allocation to photosynthetic organs, which would enable them to compensate for their higher resource demands (Dudley and Galen, 2007; Ueno et al., 2007). Similar relationships between RGR and CTs were observed when males and females were in the late-growth stage. These results suggest that such aggressive mechanisms could help female individuals win from the start when willows face intersexual competition for limited resources. In the rapid-growth stage, both males and females maintained a high level of growth, and CT levels were significantly decreased when compared with those measured in the initial-growth stage, suggesting an obvious trade-off between growth and defense (Stamp, 2003). The total biomass of females was found to be significantly higher than that of males under nutrient-rich conditions, but nutrient shortages could alter this relationship (the differences between sexes were not substantial). Previous studies have suggested that biomass allocation plays a key role in the plant response to nutrient shortage (Hermans et al., 2006; Agrawal, 2007), and we speculate that different strategies for biomass allocation may result in sex-based differences in defense capacity and trade-offs between growth and defense.

Recent studies have suggested various inter-sexual competition patterns in a male-biased dioecious plant *Populus* with a sex ratio, in terms of resource utilization within given environments (Chen et al., 2014, 2015). Our study further provided new insights into *S. paraplesia*, a female-biased sex ratio dioecious plant, in response to nutrient availability. In particular, we demonstrated sex-specific trade-offs between growth and defense. Females are likely to adopt radical strategies, overdrawing on available resources to satisfy both growth and defense, which seems to be more like a gamble compared to the strategies pursued by males. Females may have an extra mechanism to compensate for the investment in growth under nutrient-poor conditions.

AUTHOR CONTRIBUTIONS

HJ designed the experiment and measured the dynamic changes of plant biomass; he was also responsible for the statistical analysis and manuscript writing. SZ contributed largely to language improvement, and provided much useful suggestions and comments for the revision. YL and GX carried out much of the field work and chemical analyses. DZ had the initial research idea and was also doing some of the writing of the paper during initial submission and revisions.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 31300512, 31370607), and the Excellent Young Scientist Program of the National Natural Science Foundation of China (NO. 31322014).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 28 July 2016
doi: 10.3389/fpls.2016.01116

Seasonal, Sex- and Plant Size-Related Effects on Photoinhibition and Photoprotection in the Dioecious Mediterranean Dwarf Palm, *Chamaerops humilis*

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In Mediterranean-type ecosystems plants are exposed to several adverse environmental conditions throughout the year, ranging from drought stress during the warm and dry summers to chilling stress due to the typical drop in temperatures during winters. Here we evaluated the ecophysiological response, in terms of photoinhibition and photoprotection, of the dioecious Mediterranean palm, *Chamaerops humilis* to seasonal variations in environmental conditions. Furthermore, we considered as well the influence of plant size, maturity, and sexual dimorphism. Results showed evidence of winter photoinhibition, with a marked decrease of the F_v/F_m ratio below 0.7 between January and March, which was coincident with the lowest temperatures. During this period, the de-epoxidation state of the xanthophyll cycle and zeaxanthin levels increased, which might serve as a photoprotection mechanism, owing the full recovery from winter photoinhibition during spring. Furthermore, mature plants showed lower chlorophyll levels and higher β-carotene levels per unit of chlorophyll than juvenile plants, and females displayed lower leaf water contents and higher photoinhibition than males during summer, probably due to increased reproductive effort of females. However, neither low temperatures during winter nor reproductive events in females during the summer led to irreversible damage to the photosynthetic apparatus. We conclude that (i) the Mediterranean dwarf palm, *C. humilis*, suffers from photoinhibition during winter, but this is transient and does not lead to irreversible damage, and (ii) females from this plant species are more sensitive than males to photoinhibition during reproductive events.

Keywords: dioecy, environmental stress, mediterranean, photoprotection, seasonal dynamics, winter photoinhibition

OPEN ACCESS

Edited by:
Raquel Esteban, Consejo Superior de Investigaciones Científicas, Spain

Reviewed by:
Bartosz Adamczyk, University of Helsinki, Finland
Chunyang Li, Zhejiang A & F University, China

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *21 June 2016*
Accepted: *13 July 2016*
Published: *28 July 2016*

Citation:
Morales M, Pintó-Marijuan M and Munné-Bosch S (2016) Seasonal, Sex- and Plant Size-Related Effects on Photoinhibition and Photoprotection in the Dioecious Mediterranean Dwarf Palm, Chamaerops humilis. Front. Plant Sci. 7:1116. doi: 10.3389/fpls.2016.01116

INTRODUCTION

Different regions of the world are characterized by the so-called Mediterranean-type ecosystems, with generally warm and dry summers, and wet and mild winters, which determines a great diversity in vegetation that is specifically known as "chaparral" in California, "fynbos" in the Cape Province of South Africa, "matorral" in Chile, "malle" in Australia, and "macchia" (or "maquis")

in the Mediterranean basin (Cody and Mooney, 1978). Plant species distribution in Mediterranean macchias appear to be mainly limited by drought stress, that is a combination of water deficit, high temperatures and high solar radiation during the summer, but it has been suggested that low temperatures in winter may also play a role in plant adaptation and fitness (Mitrakos, 1982). However, most of the ecophysiological research on this macchia vegetation has been focused on the effects of drought stress during summer, and very few studies have investigated thus far the response and adaptation of Mediterranean plants to low-temperature winter stress (Martínez-Ferri et al., 2004; Verhoeven, 2014; Esteban et al., 2015; Míguez et al., 2015).

Although Mediterranean ecosystems are generally considered to be characterized by hot and dry summers, and wet, mild winters, previous studies have already shown sustained decreases in the maximum efficiency of photosystem II (PSII) photochemistry (F_v/F_m ratio) not only during summer drought, but also during winter, the so-called "winter photoinhibition", in some evergreen species (Kyparissis et al., 1995; García-Plazaola et al., 1999b; Oliveira and Peñuelas, 2000; Martínez-Ferri et al., 2004; Valladares et al., 2005). Low temperatures during winter may lead to an impairment of the photosynthetic apparatus leading to reductions in PSII efficiency (Berry and Bjorkman, 1980; Adams et al., 1994; Demmig-Adams and Adams, 2000; Yamori et al., 2014), since chilling temperatures strongly reduce photosynthetic activity (Huner et al., 1998; Du et al., 1999; Allen and Ort, 2001; Foyer et al., 2002; D'Ambrosio et al., 2006; Grennan and Ort, 2007; Mohanty et al., 2007), an effect that may be exacerbated on bright days (Öquist and Huner, 1993; Huner et al., 1998). Despite solar radiation decreases considerably during winter (approximately by one-half compared to yearly maxima during late June), Mediterranean plants can still absorb more solar energy that they can use for photosynthesis, particularly when low temperatures are interacting with other stressors, such as drought or abrasive damage due to wind that can strongly enhance foliage cuticular water loss, increasing potential desiccation stress (Grace, 1977; Larcher, 2003). Indeed, drought events are becoming more and more unpredictable and are increasingly occurring during winters in Mediterranean-type ecosystems in the frame of global change (Intergovernmental Panel on Climate Change [IPCC], 2014).

Aside from seasonal variations in environmental conditions that determine plant performance in the Mediterranean macchia, it is also important to consider that some of these plant species show sexual dimorphism. It is generally thought, that females, due to a higher investments in reproductive structures may suffer more photoinhibition and photo-oxidative stress than males under environmental stresses, particularly drought stress (Li et al., 2004, 2007; Rozas et al., 2009; Simancas et al., 2016), but also low temperatures (Xu et al., 2008b; Zhang et al., 2011). However, some exceptions exist, in which no differences between sexes can be observed or even that females outperform males in their tolerance to abiotic stress, so that more studies are required to better understand secondary sexual dimorphism in plants (Barrett and Hough, 2013; Juvany and Munné-Bosch, 2015; Munné-Bosch, 2015).

Mediterranean plants have evolved complex mechanisms of photoprotection to prevent photoinhibition and irreversible damage to the photosynthetic apparatus. When excited states of chlorophyll (^{1}Chl*) are not readily processed by photochemistry (photosynthesis), they are converted to triplet excited states (^{3}Chl*) and the energy may be transferred to oxygen producing singlet oxygen ($^1O_2$). This reactive oxygen species may eventually cause photo-oxidative stress, thus leading, among other processes, to the inactivation of PSII and/or an increased peroxidation of membrane lipids (Pintó-Marijuan and Munné-Bosch, 2014). To prevent ^{3}Chl* and the subsequent singlet oxygen ($^1O_2$) formation, a carotenoid (xanthophyll cycle)-dependent dissipating pathway is activated to safely return ^{3}Chl* to its ground state. The excess excitation energy is thereby dissipated as heat, directly within the carotenoid protein complexes, bound to the light-collecting chlorophylls (Demmig-Adams and Adams, 2000). Since low temperatures decrease the rate of photosynthesis and increase the excitation energy in chloroplasts; xanthophyll cycle-dependent energy dissipation, which operates in the antenna complexes of PSII, is considered one of the most efficient mechanisms to protect the photosynthetic apparatus (Adams et al., 1994; Ottander et al., 1995; Demmig-Adams and Adams, 2000). Therefore, the role of carotenoids, particularly those of the xanthophyll cycle and zeaxanthin in photoprotection, are considered to be essential in excess energy dissipation and are generally associated with reversible photoinhibition (García-Plazaola et al., 1997, 1999b; Kyparissis et al., 2000).

Singlet oxygen is formed under adverse climatic conditions when the xanthophyll cycle-dependent energy dissipation system is not activated or when it cannot dissipate more excess energy in chloroplasts. $^1O_2$ can then be eliminated both physically and chemically (the so-called "quenching" and "scavenging" of $^1O_2$) by the action of antioxidants, which react more quickly than lipids with $^1O_2$. Carotenoids, and most particularly β-carotene and zeaxanthin, and α-tocopherol (which belongs to the vitamin E group of compounds) eliminate $^1O_2$. α-Tocopherol, in turn, inhibits the propagation of lipid peroxidation in thylakoid membranes by reacting with lipid peroxyl radicals and therefore preventing the oxidation of poly-unsaturated fatty acids in cascade (Munné-Bosch and Alegre, 2002). Indeed, previous studies have shown that β-carotene and α-tocopherol act synergistically to protect PSII efficiency from $^1O_2$-induced damage (Trebst, 2003).

The Mediterranean dwarf palm (*Chamaerops humilis* L.) is one of the only two native palms in Europe and the only one in the Iberian Peninsula, where it is autochthonous. Distinctively, this palm is also one of the very few palms originally from a temperate zone and not from the tropics, where most palms are abundant and grow naturally, being able to reach latitudes of up to 44°N (Merlo et al., 1993). This palm is native from continental Europe being mainly found in the western Mediterranean basin, all over the Mediterranean coast of Spain and Portugal, central and southern Italy, some parts of the southern coast of France, islands of the western Mediterranean and northwest Africa (from Morocco to Libya, Global Biodiversity Information Facility [GBIF], 2016). *C. humilis* appears in all phases of the succession

of the degradation of Mediterranean ecosystems due to its tolerance to disturbances, such as deforestation, fires, or grazing (Santiesteban et al., 1992; Götzenberger et al., 2003; Alados et al., 2004). Furthermore, it is generally used in gardening, in preference to other palms of alien origin.

Here, we hypothesized that *C. humilis* is very well adapted to the Mediterranean climate and will therefore not suffer photoinhibition and photo-oxidative stress due to the activation of efficient photoprotection mechanisms neither during summer drought nor during winter-associated low-temperature stress. An emphasis was put on the study of photoinhibition and photoprotection mechanisms throughout seasonal variations and the possible influence of plant size, maturity and sexual dimorphism during reproductive events. Our final goal was to improve our knowledge about the ecophysiological response of this unique, native palm from Europe, which is autochthonous in the Iberian Peninsula and is considered a protected species in certain parts of its distribution area (European Food Safety Health [EFSA], 2014).

MATERIALS AND METHODS

Studied Species, Site Description, and Sampling

The native Mediterranean dwarf palm *C. humilis* L. (*Arecaceae*) is a phanerophyte typical of the thermophilic vegetation found in the western Mediterranean basin. From a physiognomic point of view, it is one of the most important determinants of the natural landscape of the coastal macchias. This palm abounds in Mediterranean regions with an accumulated rain above 400 mm and it is more commonly found as part of thickets and spiny shrublands, not just because these are drier areas, but also because of the current deterioration of the Mediterranean vegetation caused by the action of human activities. *C. humilis* is a multi-stemmed shrub with short trunks under natural growth conditions (usually 1.5 m tall maximum), hence its name "dwarf palm." However, under more optimal growth conditions like gardens, it can reach heights of 4 or even 6 m. The leaves, which emerge in a terminal tuft, have long woody stalks armed with thorns and fan-shaped blades which fold along the midribs (Merlo et al., 1993). The flowering period is in spring, typically from April to May. The flowers appear in dense, short inflorescences at the tops of the stems. The plants are dioecious with male and female flowers on separate plants. The fruit is a globular reddish-brown drupe, oblong or ovoid, measuring 1-4 cm. Unripe fruits are bright green, turning from dull yellow to brown as they ripen during later summer and autumn (September–November).

The present study was carried out on the Garraf Natural Park, one of the 12 natural areas of the Network of Natural Parks of the Barcelona Provincial Council, located near Barcelona (41° 16.443′ N, 1° 55.120′ E; north–east Spain) at 345 m a.s.l. Climatological conditions during samplings were registered in a meteorological station in the same Natural Park at 161 m a.s.l. (41° 15.0′N, 01° 46.0′E). Mean monthly precipitation during the experiments was 40.3 mm (from January 2014 to January 2015, see **Figure 1**), but most rain accumulated during autumn, while the other seasons were quite dry. The hottest month was June, with a mean maximum monthly temperature of 28.4°C, while the coldest month was January, with a mean minimum monthly temperature of 5.0°C (**Figure 1**).

To study seasonal effects on photoinhibition and photoprotection, 12 randomly-selected *C. humilis* individuals were sampled every 2 months at midday (at maximum daily incident solar radiation) on sunny, clear days from January 2014 to January 2015. To study size, maturity and sex-related effects, an additional sampling was performed in 35 juvenile, 35 male, and 35 female randomly-selected plants. Individuals, with a height ranging between 30 cm to 170 cm and georeferenced in the study area (by using Google Earth Pro software, **Figure 2**) were sampled on a sunny, clear day during June 2014. Plant height was measured in every individual to estimate plant size. Fully expanded, mature leaves with no visual damage were collected for measurements, frozen *in situ* in liquid nitrogen and immediately transported to the laboratory, where they were stored at – 80°C and later used for biochemical analyses.

Leaf Water Content, LMA and *Fv*/*Fm* Ratio

Leaves were collected, transported to the laboratory in thermal bags at about 4°C in darkness, and weighed to estimate fresh weight (FW). Then, leaves were immersed in distilled water for 24 h at 4°C and weighed for turgid weight (TW). Thereafter, leaves were dried at 80°C until constant weight to determine dry weight (DW). Relative leaf water content (RWC) was calculated as $100 \times (FW - DW)/(TW - DW)$ and, in the same leaves, leaf mass area (LMA) was calculated by measuring its DW and leaf area (g DW/m^2).

The F_v/F_m ratio was estimated following Van Kooten and Snel (1990). For this purpose, we used chlorophyll fluorescence data obtained with a portable fluorimeter (Mini-PAM; Walz, Effeltrich, Germany) in leaves maintained for at least 1 h in darkness.

Photosynthetic Pigments and Photoprotection

The levels of photosynthetic pigments (including chlorophylls and carotenoids) and tocopherols were measured by high performance liquid chromatography (HPLC). In brief, leaf samples were ground in liquid nitrogen and extracted with cold methanol containing 0.01% butylated hydroxyltoluene using ultrasonication. After centrifuging at 12000 rpm for 10 min at 4°C, the supernatant was collected and the pellet re-extracted with the same solvent until it was colorless; then, supernatants were pooled and filtered. Chlorophylls and carotenoids were separated on a binary-solvent gradient using reverse-phase HPLC system and quantified with a diode array detector as described by Munné-Bosch and Alegre (2000). Shortly, pigments were separated on a non-endcapped Zorbax ODS-5 mm column (250 mm long, 4.6 mm i.d., 20% Carbon, Teknokroma, St. Cugat, Spain) at 30°C for 38 min at a flow rate of 1 $mL \cdot min^{-1}$ and the injection volume of 80 μL. The solvent mixture for the

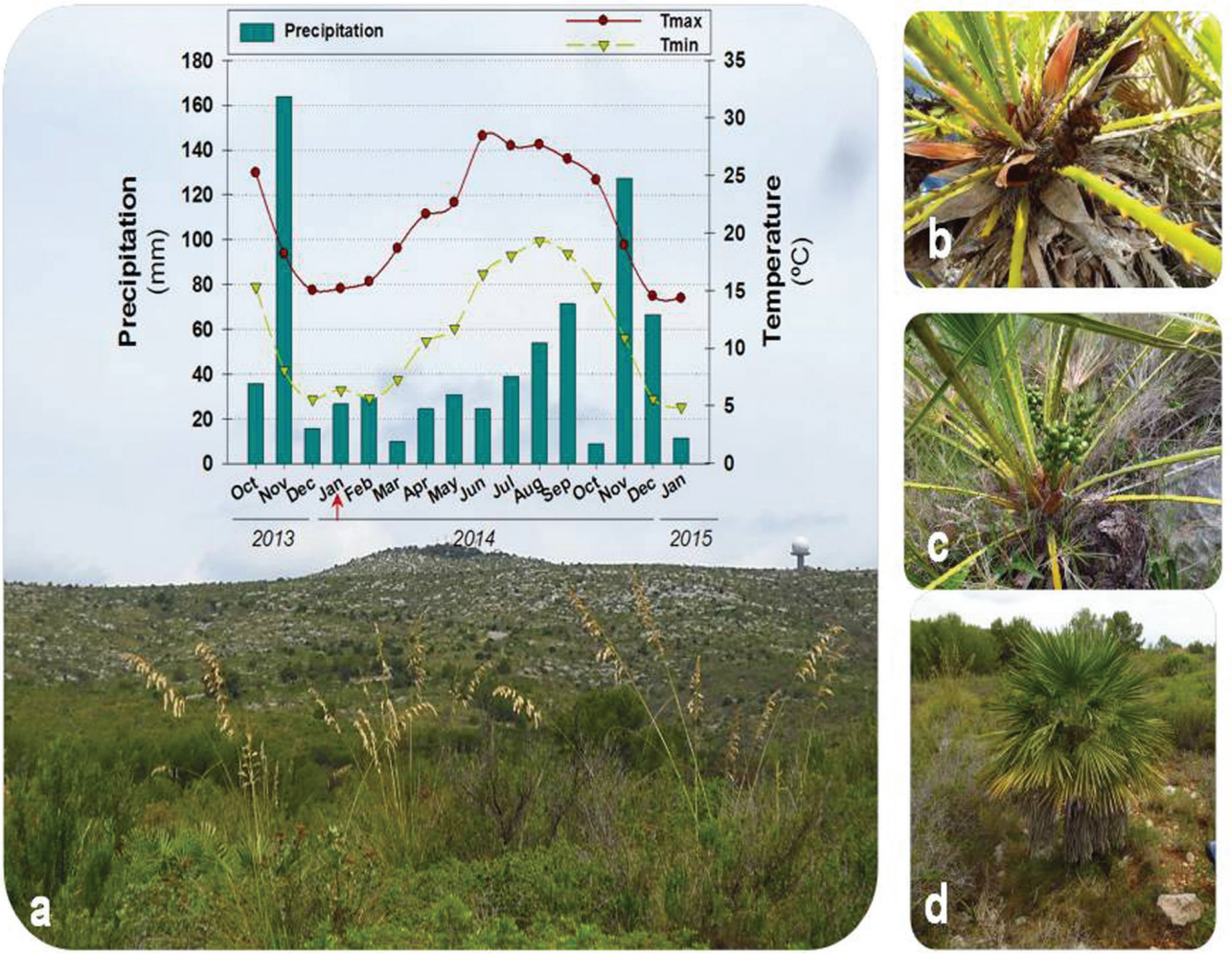

FIGURE 1 | Seasonal dynamics in environmental conditions during samplings and detail of plants. Variations in monthly mean maximum and minimum temperatures and precipitation during the period of samplings (January 2014 to January 2015, including 3 months prior to experiments) at the Garraf Natural Park **(a)**, with a detail of a male **(b)**, a female **(c)**, and the tallest plants sampled **(d)**. The red arrow indicates the start of experiments (January 2014).

gradient consisted on (A) acetonitrile:methanol (85:15, v/v) and (B) methanol:ethylacetate (68:32, v/v). The gradient used was: 0–14 min 100% A, 0% B; 14–16 min decreasing to 0% A, 100% B; 16–28 min 0% A, 100% B; 28–30 min increasing to 100% A, 0% B; and 30–38 min 100% A, 0% B. Detection was carried out at 445 nm and compounds were identified and quantified as described previously (Munné-Bosch and Alegre, 2000). On the other hand, tocopherols were separated isocratically on a normal-phase HPLC system and quantified with a fluorescent detector as described by Amaral et al. (2005). The HPLC equipment consisted on an integrated system with a Jasco PU-2089 Plus pump, a Jasco AS-2055 Plus auto-sampler and a FP-1520 fluorescence detector (Jasco, Tokyo, Japan). Tocopherols were separated on an Inertsil 100 A (5 μm, 30 × 250 mm, GL, Sciences Inc, Tokyo, Japan) normal-phase column, operating at room temperature. The flow rate was 0.7 mL·min^{-1} and the injection volume was 10 μL. The mobile phase was a mixture of n-hexane and p-dioxane (95.5:4.5, v/v). Detection was carried out at an excitation of 295 nm and emission at 330 nm. Quantification was based on the results obtained from the fluorescence signal and compared to that of a calibration curve made with authentic standard (Sigma–Aldrich, Steinheim, Germany). α-Tocopherol was the only tocopherol homologue present in *C. humilis* leaves.

Lipid Peroxidation

The extent of lipid peroxidation was estimated from the amount of malondialdehyde acid (MDA) in leaves, following the method described by Hodges et al. (1999), which takes into account the possible influence of interfering compounds in the thiobarbituric acid-reactive substances (TBARS) assay.

Statistical Analyses

Seasonal variation effects were tested by one-factorial analyses of variance (ANOVA) using Duncan's *post hoc* tests to identify differences over time. To determine the effect of maturity and sex during reproduction (June), mean values were

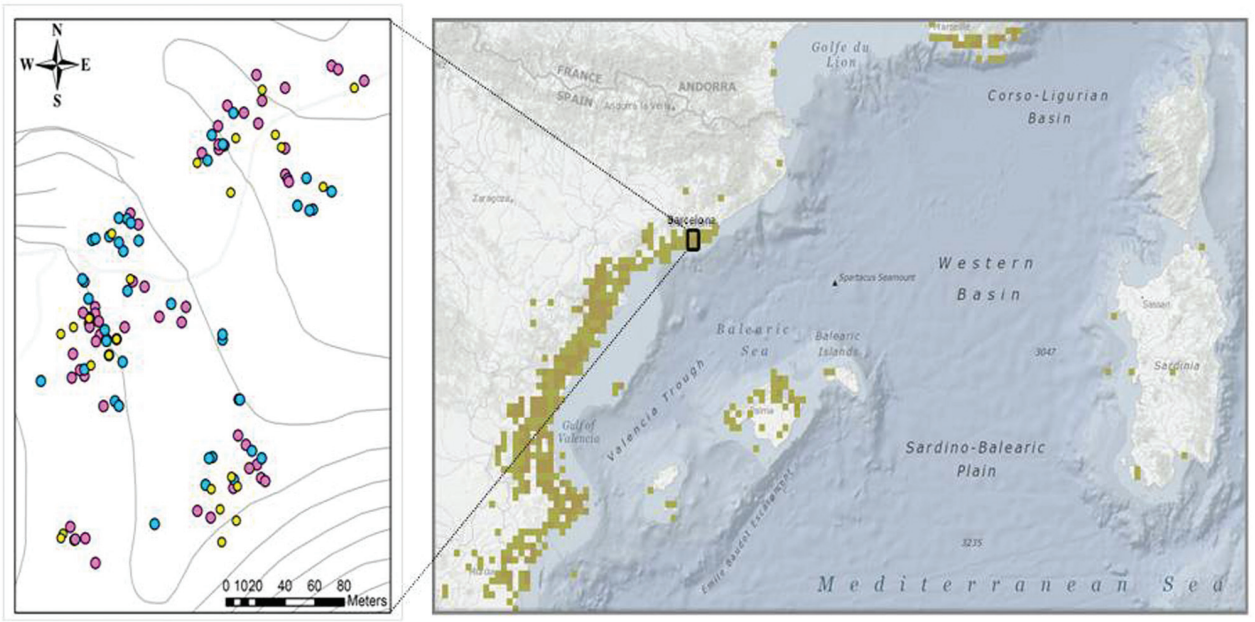

FIGURE 2 | Absence of spatial segregation in dwarf palms n the Garraf Natural Park. Distribution of plants sampled from January 2014 to January 2015 at the field site, indicating their exact GPS location **(left)** of juveniles (yellow spots), males (blue spots), and females (pink spots) and location of the population within a detail of macchia Mediterranean distribution **(right)**.

compared between mature and juvenile plants, and between male and female plants, by using a Student's *t*-test. Spearman rank's correlation analyses were performed between plant size (estimated as plant height) and all studied parameters. In all cases, effects were considered significant at a probability level of $P < 0.05$. All statistical tests were performed using the SPSS package (Chicago, IL, USA).

RESULTS

Seasonal Dynamics in Photoinhibition

Seasonal variations in environmental conditions during the study were typical of the Mediterranean climate with a warm, dry summer and a wet, mild – relatively cold – winter, with most precipitation concentred in autumn, particularly during November (**Figure 1**). Mean monthly maximum temperatures above 25°C and low precipitation occurred during the summer months, although rainfall was progressively increasing from June to September. In contrast, winter months were characterized by mean monthly minimum temperatures below 10°C. The start of experiments (January 2014) coincided with a cold period (with mean monthly minimum temperatures below 7.5°C during the preceding 2 months), and low water availability. Although rainfall accumulated 162 mm during November, December was unexpectedly very dry (17 mm, **Figure 1**). Then, monthly rainfall increased progressively but still was low up to late summer and next November (**Figure 1**). Although the RWC was always kept above 80% throughout the study, it showed a significant increase from 81 to 92% from January to November 2014. The LMA also increased slightly (up to 15%) but significantly throughout the study, with minimum values obtained in January 2014 and maximum values obtained during 2015 (**Figure 3**).

Winter photoinhibition occurred in *C. humilis* leaves. The lowest F_v/F_m ratios were observed during winter (from January to March 2014, and during January 2015, with values ranging 0.63–0.64 and 0.70, respectively, **Figure 3**). Full recovery of the F_v/F_m ratio was observed just after the winter 2014, with values ranging between 0.77 and 0.81 during May and November 2014, with mean values around 0.8 during spring, summer, and autumn (**Figure 3**).

In summary, winter photoinhibition (with F_v/F_m ratios below 0.75) occurred in *C. humilis* leaves (**Figure 3**), but this photoinhibition was transient and occurred in parallel with a drop of temperatures during winter (**Figure 1**), being more evident on drier winters (both the F_v/F_m ratio and precipitation were lower during winter of 2014 than January 2015, **Figures 1** and **3**).

Seasonal Dynamics in Photoprotection

While total chlorophyll levels increased throughout the study (**Figure 4**), in parallel with increases in the RWC (**Figure 3**), the Chl a/b decreased during the summer months, reaching minimum values during July and September 2014 (**Figure 4**). The ratio of total carotenoids (Car) per unit of Chl decreased also during the summer in parallel with reductions in the Chl a/b ratio to recover later (**Figure 4**). Malondialdehyde (MDA) levels showed significant seasonal variations, with maximum levels attained during May 2014 and January 2015 (**Figure 4**).

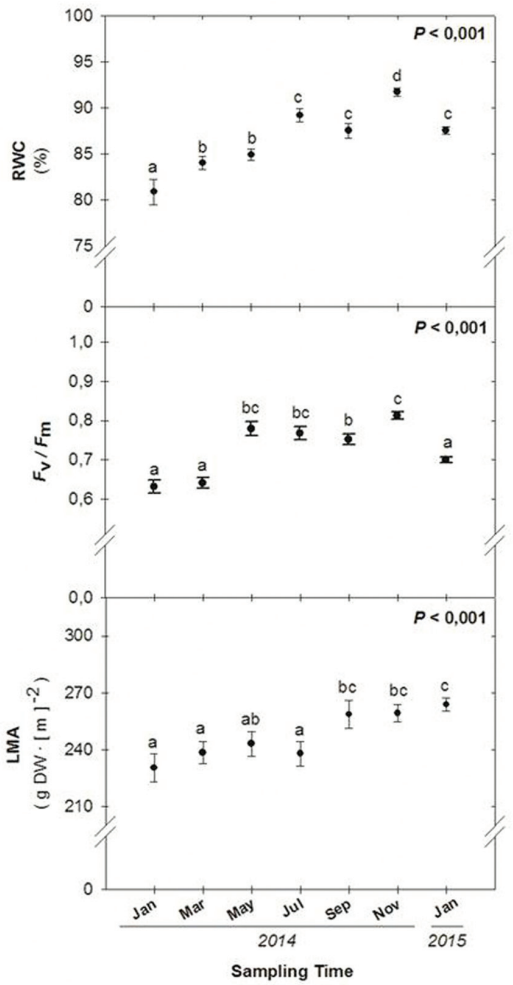

FIGURE 3 | Seasonal variations in the relative leaf water content (RWC), the maximum efficiency of PSII phochemistry (F_v/F_m ratio) and leaf mass per area ratio (LMA) in leaves of *Chamaerops humilis*. Seasonal differences were tested by one-factorial analyses of variance (ANOVA). Different letters indicate significant differences between months using Duncan's *post hoc* tests. Data represent the mean ± SE of $n = 12$ individuals. Significant *P* values (<0.05) are bold.

As the Car/Chl ratio increased during winter photoinhibition (**Figure 4**), the levels of specific carotenoids were determined by HPLC, including the levels of xanthophylls (lutein, the xanthophyll cycle pool – VZA – and zeaxanthin), the de-epoxidation state of the xanthophyll cycle (DPS) and β-carotene (**Figure 5**). Maximum levels of VZA, zeaxanthin and the DPS were attained during winter 2014 (January and March), summer 2014 (July and September) and again during winter 2015 (January). In contrast, maximum levels of β-carotene per unit of chlorophyll were attained during the winters 2014 and 2015 (January in both cases), and maximum levels of α-tocopherol per chlorophyll unit were attained during January 2014, decreasing progressively throughout the experiment (**Figure 5**), inversely with the RWC (**Figure 3**).

In summary, the DPS increased both during winter and summer, while β-carotene per unit of chlorophyll increased during winter, and α-tocopherol per chlorophyll unit inversely correlated with the RWC.

Influence of Maturity, Dioecy, and Size

The influence of maturity, sex, and size on all measured parameters was specifically evaluated during the reproductive stage, particularly during June 2014. Mature plants showed higher LMA, lower chlorophyll levels, and higher chlorophyll a/b ratio and β-carotene per unit of chlorophyll than juvenile plants (**Table 1**). These differences were observed both in males and females, since these parameters (LMA, chlorophylls and β-carotene) did not differ between sexes. In contrast, sexual dimorphism was evidenced on the leaf water contents and the F_v/F_m ratio, females showing slightly lower leaf water contents and higher photoinhibition than males during the summer (**Table 1**).

Plant size (estimated as plant height) positively correlated with the LMA, chlorophyll a/b and carotenoid/chlorophyll ratios, particularly in the case of lutein and β-carotene, and negatively correlated with total chlorophyll levels (**Table 2**). The strongest correlation was observed for the LMA ($r = 0.371$, $P < 0.001$). In all other cases, correlations were weaker, with r values below 0.3 in all cases (**Table 2**). Indeed, if Bonferroni correction was applied to the data, the *P* value would decrease to 0.003, correlations being therefore significant for the LMA and total chlorophyll levels only. In other words, the largest plants investigated (with a height of up to 1.7 m) tended to have thicker leaves (as indicated by the LMA) and reduced chlorophyll contents compared to smaller individuals.

In summary, females from this plant species are more sensitive than males to photoinhibition during reproductive events. Furthermore, both increased plant size and maturity led to structural changes in leaves (as indicated by increases in the LMA) and changes in the accumulation of photosynthetic pigments (as indicated by the reductions in chlorophyll levels).

DISCUSSION

In their post-embryonary stage, plants are sessile and cannot escape from adverse environmental conditions, so they have evolved a number of complex, interconnected mechanisms of adaptation to allow their survival. Plants living in Mediterranean-type ecosystems are subjected to marked seasonal variations throughout the year and, consequently, exposed to multiple stresses. Here, it is shown that *C. humilis* can withstand both summer droughts and low temperatures during winter, despite a transient photoinhibition was observed during the winter months. Previous studies have shown that there are two mechanisms to counteract the potential damaging effects of excess solar radiation: (i) prevent or avoid photoinhibition, which is found in species that are able to maintain a sustained PSII photochemical efficiency, and (ii) tolerate photoinhibition, which

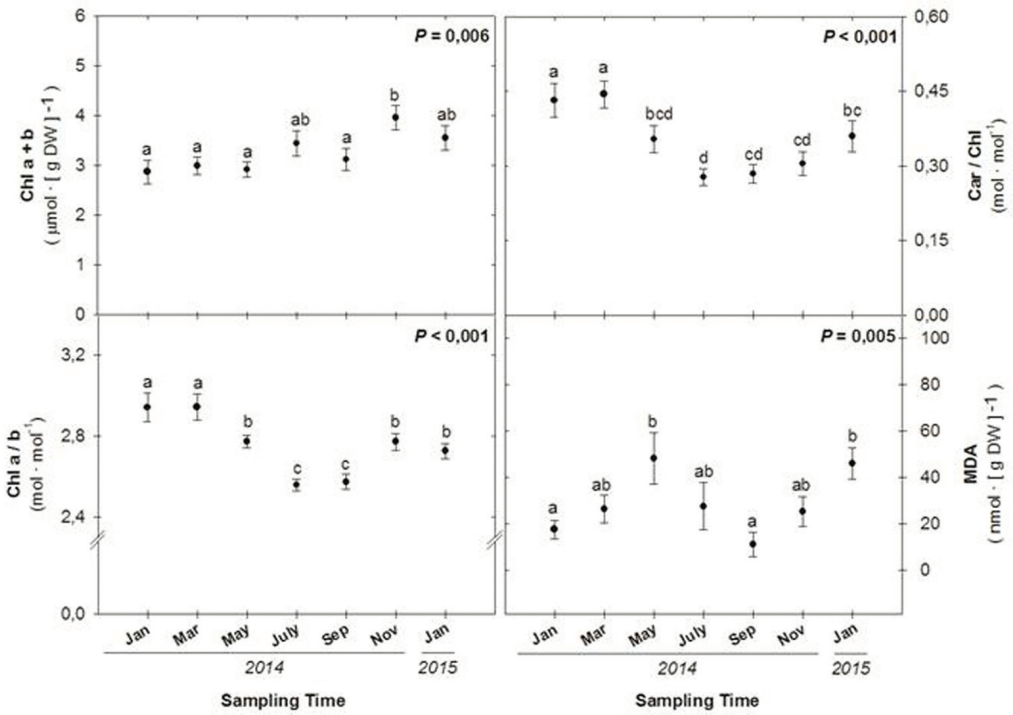

FIGURE 4 | Seasonal variations in the amounts of photosynthetic pigments (chlorophylls and carotenoids), anthocyanins and malondialdehyde acid (MDA) in leaves of *C. humilis*. Seasonal differences were tested by one-factorial ANOVA. Different letters indicate significant differences between months using Duncan's *post hoc* tests. Data represent the mean ± SE of $n = 12$ individuals. Significant P values (<0.05) are bold.

is observed in species displaying dynamic photoinhibition, which is in turn associated with fast reversible mechanisms (Demmig-Adams and Adams, 1992; Long et al., 1994). The latter include activation of the synthesis of non-epoxy xanthophylls (typically zeaxanthin, although some species additionally accumulate lutein from lutein epoxide), leading to the development of non-photochemical quenching (Martínez-Ferri et al., 2000, 2004; Jahns and Holzwarth, 2012).

Reversible, transient photoinhibition during the winter was manifested by F_v/F_m ratios below 0.75 during the cold temperatures of January–March in the present study, which was followed by a full recovery to optimal values (F_v/F_m ratios of 0.8) during spring. *C. humilis* is described here, to our knowledge for the first time, as a photoinhibition-tolerant palm. Furthermore, we show that this tolerance is achieved through the activation of xanthophyll cycle de-epoxidation, leading to an enhanced accumulation of zeaxanthin, as it occurs in other evergreen species from Mediterranean-type ecosystems (García-Plazaola et al., 1999a,b, 2003) and other habitats (Baker, 1995; Verhoeven et al., 1996). In these species, low predawn F_v/F_m values during winter have been associated with the overnight retention of high amounts of zeaxanthin, which has been attributed to the inhibitory effect of chilling temperatures on the enzymatic conversion of zeaxanthin from violaxanthin in the xanthophyll cycle (Adams and Demmig-Adams, 1994; Adams et al., 1994; Leipner et al., 2000). Thereby, the winter photoinhibition observed in *C. humilis* in the present study can be interpreted as an adaptive photoprotection mechanism of the photosynthetic apparatus, as it occurs in some evergreen conifers, which are adapted to extreme environments, showing the greatest degree of photoinhibition during the winter months (Adams et al., 2004; Demmig-Adams and Adams, 2006).

In an attempt to get some insights into the possible causes and consequences of winter photoinhibition, we correlated the F_v/F_m ratio with all other measured parameters (**Table 3**). It turned out that the F_v/F_m ratio not only negatively correlated with the DPS, VZA and zeaxanthin levels, but also with the levels of lutein and β-carotene per chlorophyll unit, and it positively correlated with the leaf water content (see "Seasonal" column, **Table 3**). This indicates that water deficit had some influence on the development of the transient photoinhibition during winter months, which has important biological implications in the framework of global change, in which drought events are becoming more unpredictable. Furthermore, it is interesting to note that this transient photoinhibition was achieved with parallel decreases in the content of chlorophylls, particularly that of chlorophyll b (as indicated by the negative correlation with the chlorophyll a/b ratio, **Table 3**), but increases in the levels of lutein and β-carotene per unit of chlorophyll. Both lutein and β-carotene protect the photosynthetic apparatus from $^1O_2$-induced damage (Telfer, 2005; Dall'Osto et al., 2006; Triantaphylidès and Havaux, 2009;

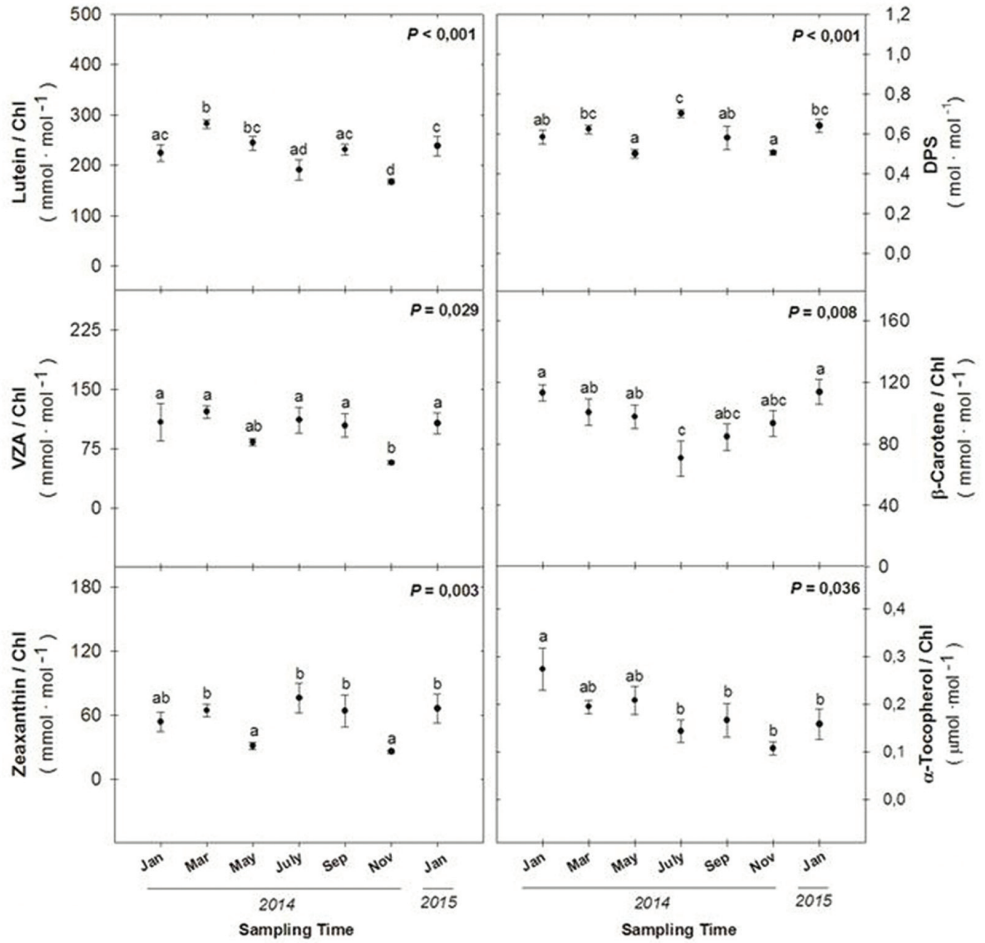

FIGURE 5 | Seasonal variations in the levels of lutein, xanthophyll cycle pool (VZA), zeaxanthin (Z), de-epoxidation state of the xanthophyll cycle (DPS), β-carotene, and α-tocopherol per unit of chlorophyll in leaves of *C. humilis*. Seasonal differences were tested by one-factorial ANOVA. Different letters indicate significant differences between months using Duncan's *post hoc* tests. Data represent the mean ± SE of $n = 12$ individuals. Significant *P* values (<0.05) are bold.

Ramel et al., 2012, 2013), thus suggesting photoprotection to PSII is achieved by decreasing the amount of photons that enter the photosynthetic electron transport (by reducing chlorophyll levels, particularly those found in the antenna), and by increasing the amount of photoprotective molecules that protect from $^1O_2$-induced damage. It appears, therefore, that xanthophyll cycle-dependent energy dissipation operates in parallel with mechanisms that increase the elimination of $^1O_2$. Prevention and elimination operate therefore simultaneously to avoid chronic photoinhibition of the photosynthetic apparatus in *C. humilis*.

The dwarf palm also showed strategies for overcoming severe drought conditions, such as keeping leaf water content always above 80% and the activation of xanthophyll cycle-dependent excess energy dissipation during the summer. It has already been shown that this palm is well adapted to the Mediterranean summer conditions. In a comparative study between dominant species of a coastal Mediterranean macchia ecosystem, the dwarf palm showed greater water potential values, thus indicating a better tolerance to water deficit (Rotondi et al., 2003). As July might be a critical period for this species due to high temperatures, high solar radiation and drought, it is the month with maximum activation of xanthophyll cycle-dependent excess energy dissipation, which acts as a photoprotection mechanism helping to tolerate summer drought as in other typically Mediterranean species (Míguez et al., 2015). These results emphasize the importance of understanding adaptive mechanisms to drought tolerance in these habitats (Bermúdez and Retuerto, 2014; Catoni and Gratani, 2014; Gratani et al., 2014; Lloret et al., 2016). In other palms, it has also been shown enhanced drought tolerance related to greater efficiency in preventing oxidative damage by activating antioxidant mechanisms (e.g., see Gomes and Prado, 2007 for the coconut palm *Cocus nucifera*; Al-Khayri and Ibraheem, 2014 and

TABLE 1 | Influence of maturity and sex on all measured parameters in the Mediterranean palm, *Chamaerops humilis*, under field conditions during their reproductive stage (June 2014).

	Maturity		Sex	
	Juvenile	**Mature**	**Male**	**Female**
RWC (%)	82.4 ± 0.66	84.1 ± 0.73	85.70 ± 0.96	82.60 ± 1.00*
LMA (g·DW·m^{-2})	242.86 ± 5.94	276.76 ± 3.11*	279.71 ± 4.77	273.75 ± 4.00
F_v/F_m	0.65 ± 0.02	0.64 ± 0.01	0.67 ± 0.02	0.61 ± 0.02*
Chl a+b (μmol·g DW^{-1})	2.96 ± 0.15	2.52 ± 0.08*	2.52 ± 0.10	2.51 ± 0.12
Chl a/b	2.64 ± 0.03	2.78 ± 0.02*	2.78 ± 0.03	2.76 ± 0.02
Car/Chl (mol·mol^{-1})	0.35 ± 0.02	0.35 ± 0.01	0.34 ± 0.02	0.36 ± 0.02
MDA (nmol·g DW^{-1})	52.90 ± 9.30	50.03 ± 3.98	57.28 ± 6.64	43.00 ± 3.98
Lut/Chl (mmol·mol^{-1})	257.7 ± 11.6	255.3 ± 7.54	262.71 ± 83	248.02 ± 1.02
VZA/Chl (mmol·mol^{-1})	92.28 ± 6.94	90.93 ± 5.15	88.47 ± 8.38	93.31 ± 6.17
Z/Chl (mmol·mol^{-1})	58.91 ± 6.33	55.73 ± 4.68	51.62 ± 7.39	59.71 ± 5.83
DPS (mol·mol^{-1})	0.60 ± 0.03	0.56 ± 0.03	0.52 ± 0.04	0.61 ± 0.03
β-Car/Chl (mmol·mol^{-1})	95.34 ± 9.86	112.05 ± 3.63*	113.35 ± 3.72	110.79 ± 4.59
α-Toc/Chl (μmol·mol^{-1})	0.47 ± 0.05	0.41 ± 0.03	0.39 ± 0.04	0.42 ± 0.04

Data correspond to the mean ± SE n = 35 juvenile and n = 70 mature plants, n = 35 male and n = 35 female plants. An asterisk indicates differences between mature and juvenile plants; male and female plants (Student's t-test, P < 0.05). RWC, relative water content; LMA, leaf mass per area ratio; F_v/F_m, maximum efficiency of photosystem II photochemistry; Chl, chlorophyll; Car, carotenoids; MDA, malondialdehyde acid; Lut, lutein; VZA, xanthophyll cycle pool; Z, zeaxanthin; DPS, de-epoxidation state of the xanthophyll cycle; β-Car, β –carotene; α-Toc, α-tocopherol.

TABLE 2 | Coefficient (*r*) and *P*-values (shown in parentheses) of Spearman's rank correlation between the plant size (height) and all parameters analyzed during the reproductive stage (June 2014, *n* = 105).

RWC	0.226 (0.010)
LMA	**0.371 (<0.001)**
F_v/F_m	−0.066 (0.252)
Chl a+b	**−0.278 (0.002)**
Chl a/b	**0.277 (0.011)**
Car/Chl	**0.174 (0.040)**
MDA	−0.064 (0.261)
Lut/Chl	**0.232 (0.010)**
VZA/Chl	−0.115 (0.125)
Z/Chl	−0.097 (0.168)
DPS	−0.081 (0.210)
β-Car/Chl	**0.227 (0.011)**
α-Toc/Chl	−0.046 (0.322)

*The P values below 0.05 are bold. Abbreviations used as in **Table 1**.*

TABLE 3 | Coefficient (r) and *P*-values (shown in parentheses) of Spearman's rank correlation between the maximum efficiency of PS II photochemistry (F_v/F_m ratio) and all other measured parameters.

	All data	Seasonal	Sex
RWC	**0.281 (<0.001)**	**0.397 (<0.001)**	0.025 (0.400)
LMA	−0.033 (0.326)	0.041 (0.345)	−0.014 (0.444)
Chl a+b	**0.319 (<0.001)**	**0.301 (0.003)**	**0.207 (0.018)**
Chl a/b	**−0.127 (0.042)**	**−0.374 (<0.001)**	0.087 (0.191)
Car/Chl	**−0.352 (<0.001)**	**−0.489 (<0.001)**	**−0.258 (0.004)**
MDA	−0.077 (0.146)	0.003 (0.488)	0.037 (0.354)
Lut/Chl	**−0.330 (<0.001)**	**−0.450 (<0.001)**	−0.117 (0.122)
VZA/Chl	**−0.375 (<0.001)**	**−0.479 (<0.001)**	**−0.305 (0.001)**
Z/Chl	**−0.304 (<0.001)**	**−0.458 (<0.001)**	**−0.316 (0.001)**
DPS	**−0.339 (<0.001)**	**−0.352 (0.001)**	**−0.319 (0.001)**
β-Car/Chl	**−0.324 (<0.001)**	**−0.401 (<0.001)**	**−0.191 (0.028)**
α-Toc/Chl	**0.144 (0.024)**	−0.148 (0.090)	−0.143 (0.074)

*Correlations have been performed for all pooled data (n = 189), and separately for seasonal variations data (n = 84) and the reproductive stage (June 2014, n = 105). The P-values below 0.05 are bold. Abbreviations used as in **Table 1**.*

Arab et al., 2016 for the date palm *Phoenix datilifera;* Silva et al., 2016 for the oil palms *Elaeis guineensis* and *Elaeis oleifera*).

Sexual dimorphism can lead to profound differences in secondary sexual characters, such as vegetative growth and plant response to both biotic and abiotic stresses; but the extent to which these stresses can affect photoinhibition, photoprotection, and photo-oxidative stress in dimorphic plants is still very poorly understood. Previous studies have shown that females are generally more sensitive to photoinhibition that males when subjected to either drought stress (Li et al., 2004, 2007; Xu et al., 2008a,b; Rozas et al., 2009; Chen et al., 2010, 2014; Zhang et al., 2010, 2014; Kumar et al., 2016) or low temperatures (Li et al., 2007; Zhang et al., 2011; Juvany et al., 2014), although some exceptions exist (e.g., see Oñate et al., 2012; Morales et al., 2013; He et al., 2016). In the present study, it was found that females are more sensitive than males to photoinhibition only during the summer. It is interesting to note that sex-related differences disappeared during winter, when plants were not reproducing, thus indicating photoinhibition in *C. humilis* is a clear secondary sexual character associated to an increased reproductive effort in females. This may be associated with sex-related differences in trade-offs in life-history traits, so that plants may allocate resources on growth, reproduction or maintenance (metabolism or defense) (Yang et al., 2014). Since females allocate a greater investment in reproduction, generally showing distinct nutrient requirements (Zhao et al., 2012; Zhang et al., 2014; Chen et al., 2015; Simancas et al., 2016), they decrease resources for other functions like defenses, and this will be the reason why females are more sensitive to photoinhibition that males when they are subjected to drought stress. Despite this, females are able to recover from photoinhibition and sex differences disappear after

the reproductive period. This is particularly interesting since it suggests that females simply respond differently than males, showing compensatory mechanisms to overcome enhanced nutrient requirements during reproductive events (Obeso, 2002; Barrett and Hough, 2013; Juvany and Munné-Bosch, 2015). Previous studies have shown sex-related differences in flowering response to photoperiod (Zhao et al., 2009) and source-to-sink transitions (Zhao et al., 2012), which may indeed reflect complex sex-related differences in the physiology of dioecious plants (Ashman, 2006). If environmental stress is mild and/or transient, such compensatory mehanisms may be effective to overcome higher nutrient requirements during reproductive events in females than in males, but when stress is too severe or persists in time, more damage occurs in females than males. Our results suggest that transient photoinhibition during reproductive events in summer in females of *C. humilis* is indeed an adaptive mechanism to prevent irreversible damage. In this respect, it is also noteworthy that *C. humilis* did not show spatial segregation between sexes, as shown in **Figure 2**. Previous studies indicate lower ability for competition in females than males, which is associated with increased nutrient requirements in the former (Chen et al., 2014, 2015). In *C. humilis*, at least in our field site in the Garraf Natural Park, sex competition may occur and play a role determining sexual differences in physiological traits, but competition with other plant species, particularly some invasive ones, such as *Cortaderia selloana*, may be indeed even more important. This aspect requires, however, further investigations.

CONCLUSION

The present study demonstrates the photoprotective capacity of the unique native Mediterranean palm during winter photoinhibition, in which plants are able to overcome the harmful effects of chilling stress combined with high light. Furthermore, it is shown here that females are more sensitive than males to photoinhibition during reproductive events, although these effects are transient and reversible. Due to the ecological importance of the Mediterranean dwarf palm, the high capacity of photoprotection described here supports the use of this species in reforestation programs, both after fires and to recover degraded soils.

AUTHOR CONTRIBUTIONS

MM, MP-M, and SM-B designed the experiments; MM and MP-M performed experiments; SM-B contributed materials and reagents; MM and SM-B wrote the manuscript.

FUNDING

This work was funded by the Generalitat de Catalunya (ICREA Academia award given to SM-B.). MPM was supported by a postdoctoral fellowship from Generalitat de Catalunya (2013 BP-B 00235).

ACKNOWLEDGMENTS

We wish to thank Laura Siles, Marta Juvany, Xavier Miret, and Bárbara Simancas for their helpful assistance during field sampling. Also we are indebted to Alba Cotado and Erola Fenollosa for their invaluable help taking individual georeferentiation, and Erola for her help doing georeferenced maps. The authors are also indebted to Serveis Científico-Tècnics from the University of Barcelona for their technical support with HPLC analyses. Also we appreciate to Diputació de Barcelona for granting permissions.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 16 May 2017
doi: 10.3389/fpls.2017.00423

Climate Influences the Content and Chemical Composition of Foliar Tannins in Green and Senesced Tissues of *Quercus rubra*

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: 03 December 2016
Accepted: 13 March 2017
Published: 16 May 2017

Citation:
Top SM, Preston CM, Dukes JS and Tharayil N (2017) Climate Influences the Content and Chemical Composition of Foliar Tannins in Green and Senesced Tissues of Quercus rubra. Front. Plant Sci. 8:423. doi: 10.3389/fpls.2017.00423

Environmental stresses not only influence production of plant metabolites but could also modify their resorption during leaf senescence. The production-resorption dynamics of polyphenolic tannins, a class of defense compound whose ecological role extends beyond tissue senescence, could amplify the influence of climate on ecosystem processes. We studied the quantity, chemical composition, and tissue-association of tannins in green and freshly-senesced leaves of *Quercus rubra* exposed to different temperature (*Warming* and *No Warming*) and precipitation treatments (*Dry, Ambient, Wet*) at the Boston-Area Climate Experiment (BACE) in Massachusetts, USA. Climate influenced not only the quantity of tannins, but also their molecular composition and cell-wall associations. Irrespective of climatic treatments, tannin composition in *Q. rubra* was dominated by condensed tannins (CTs, proanthocyanidins). When exposed to *Dry* and *Ambient***Warm* conditions, *Q. rubra* produced higher quantities of tannins that were less polymerized. In contrast, under favorable conditions (*Wet*), tannins were produced in lower quantities, but the CTs were more polymerized. Further, even as the overall tissue tannin content declined, the content of hydrolysable tannins (HTs) increased under *Wet* treatments. The molecular composition of tannins influenced their content in senesced litter. Compared to the green leaves, the content of HTs decreased in senesced leaves across treatments, whereas the CT content was similar between green and senesced leaves in *Wet* treatments that produced more polymerized tannins. The content of total tannins in senesced leaves was higher in *Warming* treatments under both *dry* and *ambient* precipitation treatments. Our results suggest that, though climate directly influenced the production of tannins in green tissues (and similar patterns were observed in the senesced tissue), the influence of climate on tannin content of senesced tissue was partly mediated by the effect on the chemical composition of tannins. These different climatic impacts on leaves over the course of a growing season may alter forest dynamics, not only in decomposition and nutrient cycling dynamics, but also in herbivory dynamics.

Keywords: hydrolysable tannins, condensed tannins, drought, warming, Proanthocyanidins, *Quercus rubra*

INTRODUCTION

Global changes, through their widespread influence on all biological processes, can significantly impact plant life cycles and functions (Norby and Luo, 2004). Elevated levels of CO_2 and ozone, increased temperatures, as well as higher drought frequency, all alter the content and composition of plant metabolites by disrupting the physiological stoichiometry in plants (Kuokkanen et al., 2001; Xu and Zhou, 2006; Prior et al., 2011; Xu et al., 2014, 2015), thus influencing plant survival and distribution. Furthermore, by influencing the chemical composition of senesced litter that fuels the metabolism of soil heterotrophs, the climate-induced physiological alterations in plants could also influence soil nutrient cycling and ecosystem productivity. For example, climate-induced changes in the composition of senescing leaves (Top and Filley, 2014; Suseela et al., 2015) could affect the subsequent detritivory (Currano et al., 2008; Couture et al., 2012) that regulates the cycling of soil carbon and mineral nutrients (Aerts, 1997; Liu et al., 2009; Suseela et al., 2013), which in turn could influence ecosystem productivity. These protracted influences of environmental stress on ecosystem performance could be mediated in part by climatic regulation of defense compounds that retain their biological-inhibitory properties even after tissue senescence.

Polyphenols, specifically tannins, are ecologically relevant, multifaceted carbon-based secondary metabolites that are common in most plant species (Kraus et al., 2003a). These compounds perform multiple protective functions and facilitate the interactions of plants with their biotic and abiotic environments during the active growth of tissues and after tissue senescence. In green tissues, as protective compounds, tannins play a critical role in plant—herbivore and plant-to-plant interactions (Salminen and Lempa, 2002; Kraus et al., 2003a; Barbehenn and Constabel, 2011), and also serve as a photo-protectant (Close and Mcarthur, 2002). Tannins in senescent leaves can account for up to 25% leaf dry weight (Kraus et al., 2003b) and thus can be a major form of carbon reaching the belowground ecosystems. Tannins in senescent tissues also retain their ability to complex with proteins (Hagerman et al., 1998) and inactivate soil enzymes (Triebwasser et al., 2012), which could hinder soil N mineralization (Hättenschwiler and Vitousek, 2000; Kraus et al., 2003a; Adamczyk et al., 2013; Tharayil et al., 2013). Thus, environmental conditions that exist during plant growth, by regulating the production of tannins in green tissues, can influence carbon and nitrogen cycling in soils.

Tannins can be broadly classified into condensed tannins (CTs) and hydrolysable tannins (HTs; Kraus et al., 2003a), with gymnosperms and monocots producing primarily CTs, and dicots able to produce either HTs or CTs alone or as mixtures (Bate-Smith, 1977; Haslam, 1988; Kraus et al., 2003a; Triebwasser et al., 2012). Condensed tannins, also known as proanthocyanidins, are polymers of flavan-3-ols such as catechin and gallocatechin (Xie and Dixon, 2005). Hydrolysable tannins are composed of gallic acid units linked via an ester linkage to a glucose functional group, and they can be further classified as gallotannins and ellagitannis by the respective presence or absence of C–C linked galloyl groups (Hartzfeld et al., 2002). In plants, tannin production is genetically (Scioneaux et al., 2011), as well as environmentally, controlled (Tharayil et al., 2011). Environmental conditions that affect the production of tannins include photoperiod, soil pH, moisture and nutrient availability, herbivory, and atmospheric CO_2 and O_3 (Herms and Mattson, 1992; Bussotti et al., 1998; Kraus et al., 2003a; Cohen and Kennedy, 2010; Jaakola and Hohtola, 2010; Lindroth, 2010; Malisch et al., 2016). Along with their total quantity, the biological reactivity of tannins is closely regulated by the chemistry of the polymer, *viz.* identity of monomers, substitution pattern of the phenolic ring, and the degree of polymerization (Kraus et al., 2003b). Although tannin quantities have been shown to respond to environmental changes, little is known about the influence of climate on the chemical composition of tannins. Further, less is known about the changes in the composition of tannins between green and senesced tissues, which are primarily influenced by the overall resorption metabolism during tissue senescence.

We investigated the influence of warming and altered precipitation on tannin production in *Quercus rubra*, an important species in many forested ecosystems of eastern North America (Prasad et al., 2007-ongoing). Specifically, we sought to determine the interactive effects of temperature and precipitation on (1) the content and molecular composition of tannins, (2) the association of tannins within different tissue fractions (operationally defined as extractable and non-extractable tannins) and (3) whether climatic effects on content and chemical composition of tannins differ between green and senescent leaves. We hypothesized that the climate stresses we imposed (drought, increased temperature) would induce the production of tannins that are quantitatively and compositionally different, and that the climate-induced changes in composition of tannins in green leaves would also influence the content of tannins that are retained in the senescent leaves.

METHODS AND MATERIALS

Site Description

Samples were collected from the Boston-Area Climate Experiment (BACE), located in Waltham, Massachusetts, USA. The experiment is located in an old-field ecosystem, and the soil is a Mesic Typic Dystrudept (Haven series) with loamy topsoil (0–30 cm; sand:silt:clay ratios of 45:46:9) over a gravelly sandy loam subsoil. The BACE has four temperature treatments and three precipitation treatments and is divided into three replicate blocks with 12 plots (2×2 m, with 1 m spacing between plots) within each block. Lateral movement of water is prevented by lining each plot with polyethylene sheets to a depth of 0.6 m. Each block has ambient (i.e., control, unmanipulated), wet (150% of ambient rainfall during the growing season) and dry (50% of ambient rainfall year-round) precipitation zones, which are administered through a passive removal and active redistribution system mounted above the plots on otherwise open greenhouse frames. *Dry* plots are located under portions of the greenhouse frames that are covered in evenly spaced, clear, six-inch polycarbonate slats (15 cm slats arranged 15 cm

apart) that exclude 50% of incoming precipitation. Frames over the *Ambient* and *Wet* treatments are covered in deer netting that provides similar shading, reducing photosynthetically active radiation by ∼6% but letting all precipitation in. The precipitation collected from the dry plots was immediately reapplied to the *Wet* plots via electric pumps and an overhead sprinkler system (Hoeppner and Dukes, 2012). Within each precipitation zone are plots designated for one of four levels of warming: no warming, low (+c. 1.0°C), medium (+c. 2.7°C) and high (+c. 4.0°C) (Tharayil et al., 2011; Hoeppner and Dukes, 2012; Suseela and Dukes, 2013). The warming treatments were applied, starting in 2008, with infrared heaters that were mounted 1 m above each corner of the plot. Temperature at the middle of the plots was measured every 10 s. The difference in temperature between the plant canopies of the warmest and ambient plots within each group of four plots was used to achieve feedback control (Hoeppner and Dukes, 2012). Three bare-root plants (30–45 cm) of different tree species such as *Q. rubra*, *Betula lenta*, *Ulmus americanus*, and *Betula populifolia* were planted along the margins of each plot during April 2012. For this study, mature, non-shaded, leaves were collected from three *Q. rubra* trees per plot in 2013 during the first week of September (green leaves) and in the second week of October (freshly senesced leaves). Leaves of similar size were collected from 2 to 3 apical whorls per tree during each harvest, resulting in at least 12 leaves per plot per harvest. Leaves from trees within a plot were pooled together to obtain a composite sample. Leaves were flash frozen between slabs of dry ice immediately after harvest, and the samples were maintained below −20°C during all analyses. The *Q. rubra* leaves used for this study were collected from the unwarmed (*No Warm*) and high temperature (*Warm*) treatments exposed to *Dry*, *Ambient*, and *Wet* precipitation treatments. Three treatment replicates were maintained for each treatment in all analyses.

Extraction of Tissues

Leaf petioles were removed, and frozen leaf samples were finely ground in dry ice using mortar and pestle. Approximately 100 mg of the ground tissue was extracted twice with 10 ml of 100% MeOH. The methanol extracted tissues were further extracted with 10 ml of 75% acetone, three more times. During each extraction step the tissues were sonicated for 2 min, and then shaken for 2 h. The supernatant from the five extractions was pooled to obtain a composite extractable-fraction. The extracts were stored at −20°C and the residual litter (non-extractable) was washed with 2 ml of 80% methanol and dried overnight at 40°C.

Condensed Tannin Quantity

The total content of condensed tannins (CTs) in the pooled extracts and in the litter residue was quantified using the acid-butanol assay modified from Porter et al. (1986). For the extractable tannins, subsamples (2 ml) of the pooled extract were dried down under N_2 gas at 40°C before adding 6 ml of the butanol:HCl (95:5) reagent. For the analysis of the non-extractable tannins, approximately 20 mg of the residual litter was weighed into glass tubes and combined with 6 ml of the butanol:HCl reagent. Samples were then placed in a water bath at 90–95°C for an hour and then cooled on ice. The amount of depolymerized anthocyanidin in the samples was quantified spectrophotometrically by measuring the absorbance at 550 nm (Jasco V-550 UV/VIS spectrophotometer, Jasco, Analytical Instruments, Easton, MD, USA) with the amount of tannins quantified from a standard curve derived from purified tannins of *Q. rubra* collected from the same experimental plot as described in Tharayil et al. (2011). Since *Quercus* tannins are a mixture of CT and HTs, the weight percent of CTs in the purified tannins were determined during the depolymerizing of the purified tannins in the presence of excess phloroglucinol as described below. The CT concentration in the standard curve was adjusted accordingly to avoid the potential overestimation of tannins.

Mean Degree of Polymerization and Monomer Identity of Condensed Tannin

Mass spectrometric analysis of intact polymeric tannins is challenging, especially when using electrospray as the ionization interface. This is because as the degree of polymerization increases the ionization efficiency of proanthocyanidins decreases (Karonen et al., 2006; Mouls et al., 2011). Also, stability of deprotonated proanthocyanidins and their fragments decreases with the increase in degree of polymerization of the molecule (Gu et al., 2003). Combined, this would result in an under sampling of the polymers with a higher chain-length, thus underestimating the degree of polymerization of proanthocyanidins in a sample. In order to further elucidate CT chemistry, subunit composition and mean degree of polymerization were determined by depolymerizing CTs in the presence of excess phloroglucinol (Kennedy and Jones, 2001; Karonen et al., 2006). Phloroglucinol reagent was prepared by dissolving 2.5 g phloroglucinol in 55 ml methanol containing 400 µl of concentrated HCl. The reagent was sparged with argon for ∼30 min until the methanol volume was reduced to 50 ml. Two milliliters of the reagent were added to glass tubes containing ∼150 mg of finely ground leaf tissues that were not subjected to prior solvent extraction. Samples in the tubes were then sparged with argon for another 2 min, immediately closed with Teflon-lined caps and incubated on a heating block at 50°C for 130 min. The tubes were then cooled on ice, centrifuged (1,000 *g*, 5 min), and 1.5 ml supernatant was transferred to centrifuge tubes. Saturated $MgSO_4$ solution (∼45 g in 100 ml water) was added to the supernatant and samples were cooled on ice again to facilitate the subsequent phase separation. For liquid-liquid extraction, 1.5 ml of ethyl ether was added and the tubes were shaken end to end on a rotatory shaker for 5 min. The tubes were centrifuged (1000 *g*, 5 min) and 40 µl of the top ethyl ether fraction was transferred to 100 µl glass inserts and completely dried under N_2 gas. The samples were reconstituted in 50% methanol and analyzed using a liquid chromatograph coupled to a triple quadrupole mass spectrometer. Detailed chromatography and mass spectrometry parameters are given in supporting information.

Peak identities were determined based on authentic standards of catechin and gallocatechin as well as literature values reported

for phloroglucinol adducts and procyanidin fragments (**Table 1**). Mass spectral patterns relative to un-adducted parent monomers were also compared. Concentrations of extractable catechin monomers were subtracted from the result of the phloroglucinol depolymerization assay. Relative mass responses (Kennedy and Jones, 2001) were used to normalize the difference in the ESI responses among the terminal units and phloroglucinol adducts. Procyanidin content was determined using the catechin and epicatechin monomers as well as fragments. Prodelphinidin content was defined as the gallocatechin/epi-gallocatechin pair (**Table 1**). Mean degree of polymerization of proanthocyanidins, was determined by dividing the sum of the normalized peak areas of extender units (phloroglucinol adducts of catechin, gallocatechin) by the sum of the peak areas of the terminal units (catechin and gallocatechin).

Hydrolysable Tannin Analysis

For the quantification of hydrolysable tannins (HTs), methanolysis was carried out with a subsample of the methanol extract or 40 mg of methanol-extracted residual litter in methanolic H_2SO_4 at 85°C as described by Hartzfeld et al. (2002). The concentrations of methyl gallate were then quantified using high-pressure liquid chromatography (HPLC; see supporting information). Ellagic acid (ellagitannins), and methyl gallate (gallotannins), were identified and quantified in samples using authentic standards. Efficiency of methylation was monitored by measuring percent methylation of gallic acid standard under similar conditions as described above.

Calculations and Statistical Analysis

The total concentration of tannins in the leaf tissue was calculated by summing CT and HT concentrations. A subsample of leaves collected at the same time was used for determining specific leaf area, which was similar between the green and freshly-senesced for all treatments meaning that trends observed on a mass basis would be similar if based on area (See Figure S1 in Suseela et al., 2015).

To test the main and interactive effects of warming and altered precipitation on CTs and HTs, a mixed model restricted maximum likelihood estimation (REML) with time of sampling (green or senesced) as repeated measures was used in SAS 9.2 (SAS Institute, Inc., Cary, North Carolina, USA). Degrees of freedom were calculated using the Kenward-Rogers method. Warming and precipitation treatments were fixed effects and blocks were treated as the random effects. Tukey's HSD multiple comparison test was used to identify differences among treatments. Significance was set at $\alpha = 0.05$.

TABLE 1 | Identification and categorization of condensed tannin monomers.

Condensed Tannin Monomers	Categorization	$[M–H]^-$
(+)-Catechin	*Procyanidin*	289
(−)-Epicatechin	*Procyanidin*	289
(−)-Epigallocatechin	*Procyanidin*	305
(+)-Catechin with phloroglucinol adduct	*Procyanidin*	413
(−)-Epi-catechin with phloroglucinol adduct	*Procyanidin*	413
Epi-gallocatechin with phloroglucinol adduct	*Prodelphinidin*	429

$[M-H]^-$: deprotonated molecular mass.

RESULTS

Type of Tannins

The total tannin concentration in green leaves varied with temperature and precipitation treatments (**Figures 1A,B**; Table S1). The green leaves formed in the *Dry*Warm* and *Ambient*Warm* treatment had the highest concentrations of total tannins (121.2 and 128.8 mg g^{-1} tissue, respectively; **Figure 1A**), while the *Ambient*No Warm* treatment had the lowest total tannin concentration (73.5 mg g^{-1} tissue; **Figure 1A**). Only the *Ambient* treatment exhibited an increase in green leaf tannin concentrations with an increase in temperature ($P < 0.001$). This pattern was also observed in senesced tissues, where the tannin concentration of senesced leaves also increased with higher temperature in both *Dry* (34% increase; $P < 0.005$) and *Ambient* (53% increase; $P < 0.004$) treatments (**Figure 1B**, Table S1). Within the *Warm* treatment, the senesced leaves in the drier treatments had more tannins ($P < 0.05$; **Figure 1B**). *Dry*Warm* had the highest concentration (110.5 mg g^{-1} tissue) and *Wet*Warm* had the lowest concentration (62.1 mg g^{-1} tissue) of total tannins (**Figure 1B**). The same trend was evident for senesced leaves exposed to *Dry*, *Ambient*, and *Wet* conditions in the *NoWarm* treatments. Decreases between the green and senesced tissue were observed in total tannins for the *Wet* treatments ($P < 0.05$) and the *Ambient*No Warm* treatment ($P < 0.05$).

When separating total tannins into CTs and HTs, CTs made up between 29 and 89% of the green leaf tissue and 53–88% of the senesced leaf tissue. In the green tissue of the *Wet*No Warm* treatment, HTs made up the larger percentage of total tannins (∼69%) (**Table 2**). In green leaf tissue, warming did not alter HT concentrations in the *Dry*, *Ambient* ($P = 0.06$), or the *Wet* ($P = 0.12$) treatments (**Table 2**). The *Wet* precipitation treatment had the highest percentages of HTs in green leaf tissue for both the *No Warm* (69.1%) and the *Warm* (50.0%) treatments (**Table 2**). In senesced leaf tissue, warming increased ($P < 0.001$) the percent of HTs in the *Ambient* treatment, but decreased HTs ($P < 0.001$) in the *Wet* treatment (**Table 2**). In unwarmed plots, leaves in the *Ambient* treatment had the lowest percentage of HTs and those in the *Wet* treatment had the highest percentage (**Table 2**). Senesced leaf tissue had lower ($P < 0.05$) in HT percentages than green tissue in the all the *Wet* treatments and the *Ambient*Warm* treatment.

The responses of CT to climate treatments were similar to those of total tannins (**Figures 2A,B**). *Warm* treatments did not increase the total CTs in the green leaves in any of the precipitation treatments *Dry* ($P = 0.41$), *Ambient* ($P = 0.14$), or *Wet* ($P = 0.42$; **Figure 2A**). *Warm* treatments increased the total CTs in the senesced leaves for the *Dry* ($P < 0.02$), but not for the *Ambient* ($P = 0.15$) or the *Wet* ($P = 0.082$; **Figure 2B**). While total content of CTs did not change between the green and senesced leaf tissue, the content that CTs represented of the total

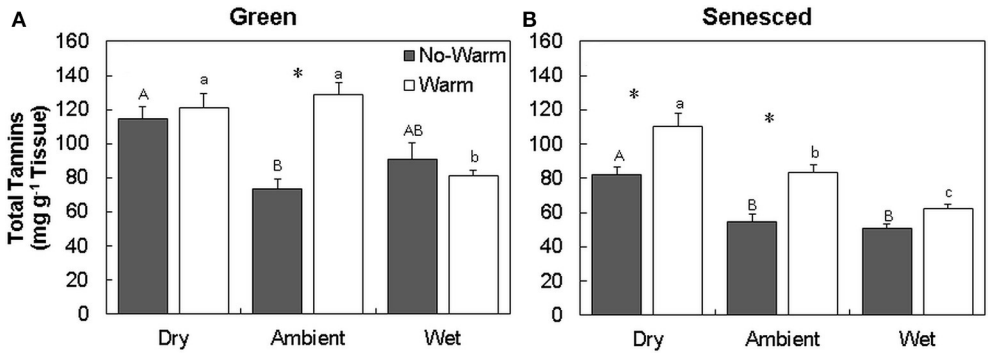

FIGURE 1 | Total average (±SE) tannin content for green (A) and senesced **(B)** leaf tissue. Asterisks (*) above a set of columns indicate significant ($P < 0.05$) differences between *No Warm* and *Warm* treatments within each precipitation treatment. Uppercase letters indicate significant differences ($P < 0.05$) between precipitation treatments within the *No Warm* temperature regime. Lowercase letters indicate significant differences ($P < 0.05$) between precipitation treatments within the *Warm* temperature regime.

TABLE 2 | Percentages of hydrolysable tannins calculated from total tannins (±SE) for both green and senesced leaf tissue.

Treatments		Green	Senesced
Precipitation	**Temperature**	**% HT**	**% HT**
Dry	No warm	30.7 ± 4.0^{A}	21.2 ± 0.4^{A}
Dry	Warm	24.4 ± 1.3^{a}	20.6 ± 0.9^{a}
Ambient	No warm	13.5 ± 1.4^{A}	$12.7 \pm 0.6^{*B}$
Ambient	Warm	36.1 ± 0.9^{ab}	26.0 ± 1.2^{a}
Wet	No warm	69.1 ± 0.9^{B}	$42.2 \pm 2.2^{*C}$
Wet	Warm	50.0 ± 2.0^{b}	26.3 ± 1.2^{a}

Upper- and lower-case letters indicate significant ($P < 0.05$, Tukey's HSD) differences between precipitation treatments within the No Warm and Warm temperatures, respectively.
Asterisk (*) indicates difference between No Warm and Warm within a specific precipitation treatment.

proportion of tannins significantly ($P < 0.05$) increased for the *Wet*No Warm* and the *Ambient*Warm* and *Wet*Warm*.

Climatic treatments influenced the chemistry of CTs in leaf tissues (**Table 3**). The *Wet* treatment had CTs with longer subunit chains than in the *Dry* and *Ambient* treatments (**Table 3**). The proportion of epi/catechin to epi/gallocatechin or procyanidin (PC) to prodelphinidin (PD) differed only by precipitation treatment; warming did not change the percentage of CT monomers that were PD or PC. The *Ambient* treatment had the highest percentage of PDs (∼27%) and *Wet* had the lowest, at <6.8%, with the *Dry* treatment intermediate (∼10–14%; **Table 3**).

The total content of HTs in green tissues increased with warming under *Ambient* ($P < 0.01$) precipitation, but decreased with warming in the *Wet* treatment ($P < 0.02$; **Figure 2C**). In senesced tissue, HTs generally made up a lower percentage of the total tannin concentration than they did in green tissue (**Figure 2D**). Similar trends between *Warm* and *No Warm* treatments also appeared in the senesced tissue, except in the senesced leaf tissue the differences between *Warm* and *No Warm* were significant for all the precipitation treatments. Warming increased HTs in the *Dry* ($P < 0.01$) and the *Ambient* ($P < 0.001$) treatments, but decreased them in the *Wet* treatment ($P < 0.005$; **Figure 2C**). Total HT content significantly decreased from green to senesced leaf tissue in the *Wet*No Warm* treatment and all the *Warm* treatments.

The HTs can be further differentiated into two HT structural compounds in the *Q. rubra* leaves: ellagitannins and gallotannins (**Tables 2**, **4**). Both green and senesced tissues generally had higher proportions of ellagitannins, although total contents were higher in the green tissue compared to the senesced tissue (**Table 4**). In the green tissue both gallotannins and ellagitannins displayed the same relationship, where the *Ambient*No Warm* treatment had the least ($P < 0.05$; **Table 4**). A similar relationship also existed in the senesced leaf tissue (**Table 4**). Over the course of the growing season, ellagitannin content decreased in green to senesced leaf tissue, but only in the *Wet* treatments (*No Warm*, $P < 0.05$; *Warm*, $P < 0.001$). Total gallotannin content also decreased ($P < 0.05$) between green and senesced leaf tissue in all the treatments except the *Ambient*No Warm* control.

Tannin Fractions

Total tannins, when separated into extractable and non-extractable fractions, showed similar patterns in both the green and senesced leaf tissue (**Table 5**). For the extractable fractions, the lowest concentration of tannins was in the *Wet* precipitation treatment, with no warming effect (**Table 5**). Warming increased the non-extractable fraction in green leaf tissue ($P < 0.05$) in the *Ambient* treatment (**Table 5**), and did not affect the non-extractable fraction in senesced tissue. In both the green and senesced leaf tissues, the lowest non-extractable tannin concentration existed in the *Ambient*No Warm* treatment (**Table 5**). Between seasons (green and senesced) there was a decrease (5.2%) in the *Ambient*No Warm* control and an increase (13.2%) in the *Wet*Warm* treatment in the percentage of tannins that were extractable.

The majority of CTs were located in the extractable fraction (**Figures 2A,B**). Warming did not affect the total content of CTs in each fraction in the green leaf tissue (**Figure 2**). The percentage of non-extractable CTs in the green tissue was higher in the

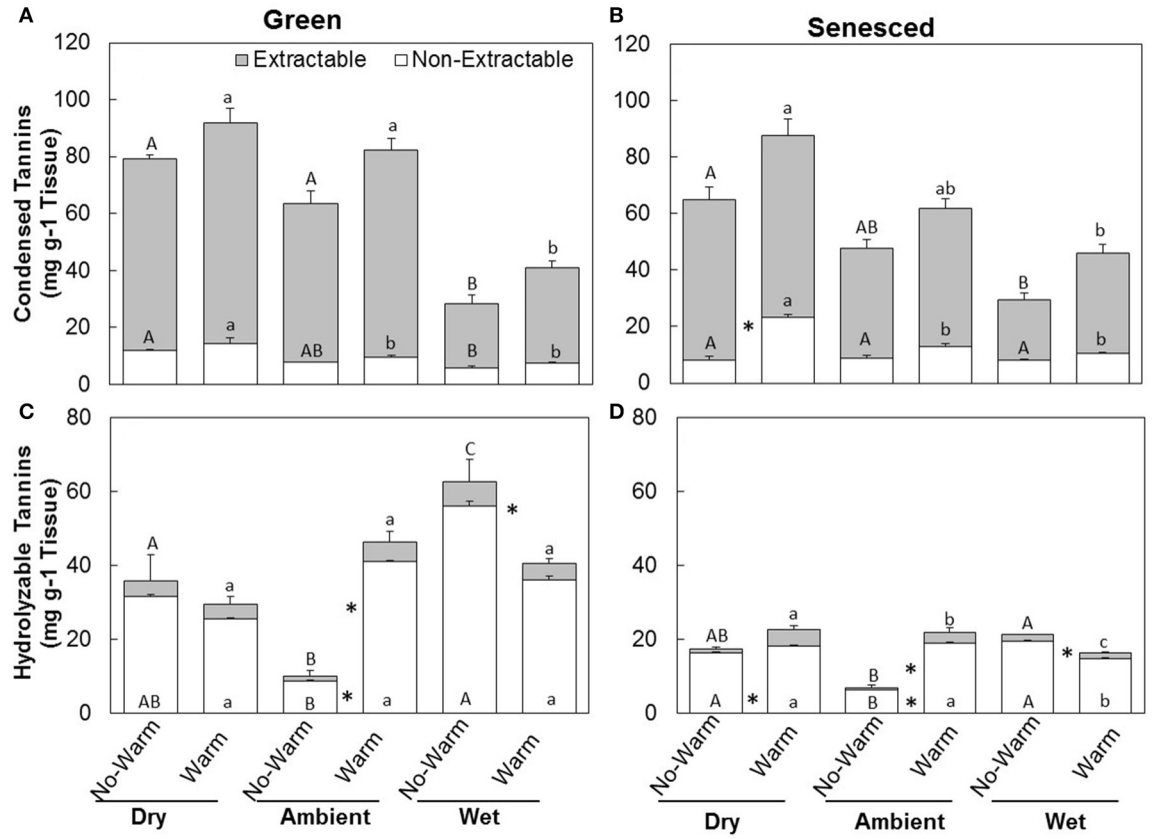

FIGURE 2 | Total average (±SE) condensed (A,B) and hydrolysable **(C,D)** tannin content for the extractable and non-extractable portions of both green **(A,C)** and senesced **(B,D)** leaf tissue. Upper asterisks (*) indicate significant differences ($P < 0.05$) between the *No Warm* and *Warm* treatments within each precipitation treatment for extractable condensed tannins and lower asterisks indicate significant effects of warming within each precipitation treatment for non-extractable condensed tannins. Different upper case letters indicate significant differences ($P < 0.05$) between precipitation treatments within the *No Warm* temperature regime. Different lowercase letters indicate significant differences between precipitation treatments within the *Warm* treatment.

TABLE 3 | Percentage (±SE) of type of condensed tannins and average (±SE) chain length of condensed tannin polymers.

Treatments		PD %	Chain length (mean degree of polymerization)
Precipitation	**Temperature**		
Dry	No warm	13.8 ± 1.5^{a}	6.2 ± 0.5^{b}
Dry	Warm	10.2 ± 1.7^{a}	7.4 ± 0.6^{b}
Ambient	No warm	27.7 ± 1.9^{b}	6.1 ± 1.5^{b}
Ambient	Warm	27.7 ± 1.3^{b}	6.9 ± 0.5^{b}
Wet	No warm	5.2 ± 0.3^{c}	8.9 ± 0.8^{a}
Wet	Warm	6.8 ± 0.5^{c}	9.6 ± 1.4^{a}

Different letters indicate significant ($P < 0.05$, Tukey's HSD) differences between treatments.
*PD refers to prodelphinidin, see **Table 1**.*

*Wet*No Warm* ($P < 0.005$), but not the *Wet*Warm* ($P = 0.09$; **Figure 4A**). The *Ambient* treatments had the lowest percentage of non-extractable CTs (*No Warm* = 12.2%, *Warm* = 11.7%; **Figure 4A**). In the senesced tissue, warming had an effect only in the *Dry* treatment, where it increased ($P < 0.001$) the content of CTs in the non-extractable fraction (**Figure 2B**). The percentage of non-extractable CTs tended to increase with warming only in the *Dry* treatment, and then only marginally ($P < 0.1$; **Figure 3B**). Within the *No Warm* temperature treatment, the *Wet* had the highest percentage (28.4%) of non-extractable CTs (**Figure 3B**). The percentage of non-extractable CTs significantly increased (by 8.5, 11.4, and 9.3%) from green leaves to senesced leaves for the *Wet*No Warm*, *Dry*Warm*, and *Ambient*Warm*, respectively.

The relative majority of HTs were located in the non-extractable fraction (**Figures 2C,D**) for both the green and senesced tissue. In green leaf tissue, warming increased the proportions of HTs in both the extractable ($P < 0.03$) and non-extractable ($P < 0.005$) fractions of the *Ambient* treatment (**Figure 2C**). Warming also decreased ($P < 0.03$) HTs in the non-extractable fraction in the *Wet* treatment (**Figure 2C**). Similar patterns occurred in the senesced tissue as well (**Figure 2D**), where HTs also increased with warming in the *Dry* treatment (**Figure 2D**).

For individual HTs, the percent of gallotannins increased the most in the *Ambient* treatment, with *Ambient*No Warm*

TABLE 4 | Average concentrations, mg g^{-1} tissue, (±SE) of structural types of hydrolysable tannins for both green and senesced leaf tissue.

Treatments		Green		Senesced	
Precipitation	**Temperature**	**Gallotannins**	**Ellagitannins**	**Gallotannins**	**Ellagitannins**
Dry	No warm	15.4 ± 2.0^{A}	20.4 ± 4.8^{A}	8.7 ± 0.5^{A}	$8.7 \pm 0.1^{*A}$
Dry	Warm	12.6 ± 0.6^{a}	18.8 ± 1.3^{a}	10.0 ± 0.7^{a}	12.6 ± 0.04^{a}
Ambient	No warm	$2.3 \pm 0.2^{*B}$	$7.8 \pm 1.8^{*B}$	$1.6 \pm 0.2^{*B}$	$5.3 \pm 0.6^{*B}$
Ambient	Warm	20.3 ± 1.4^{a}	26.2 ± 4.1^{a}	9.9 ± 0.4^{a}	11.8 ± 0.9^{ab}
Wet	No warm	$24.8 \pm 2.2^{*A}$	37.9 ± 3.6^{A}	10.9 ± 0.1^{A}	10.3 ± 0.2^{A}
Wet	Warm	15.2 ± 0.5^{a}	25.4 ± 0.3^{a}	7.3 ± 0.2^{a}	9.0 ± 0.2^{b}

Upper- and lower-case letters indicate significant ($P < 0.05$, Tukey's HSD) differences between precipitation treatments within the No Warm and Warm temperatures, respectively. Asterisk (*) indicates difference between No Warm and Warm within a specific precipitation treatment.

TABLE 5 | Average concentrations, mg g^{-1} tissue, (±SE) of total extractable and non-extractable tannins for both green and senesced leaf tissue.

Treatments		Green		Senesced	
Precipitation	**Temperature**	**Extractable**	**Non-extractable**	**Extractable**	**Non-extractable**
Dry	No warm	71.6 ± 2.1^{A}	43.3 ± 7.6^{A}	57.8 ± 4.7^{A}	24.4 ± 1.0^{A}
Dry	Warm	81.2 ± 5.1^{a}	40.0 ± 3.6^{a}	68.9 ± 5.8^{a}	41.5 ± 1.6^{a}
Ambient	No warm	57.2 ± 4.5^{A}	$16.4 \pm 1.6^{*B}$	39.5 ± 3.2^{AB}	15.0 ± 1.5^{B}
Ambient	Warm	78.1 ± 4.0^{a}	50.6 ± 3.0^{a}	51.8 ± 3.2^{ab}	31.9 ± 1.1^{a}
Wet	No warm	29.2 ± 3.3^{A}	61.8 ± 6.6^{A}	23.0 ± 2.4^{B}	27.7 ± 0.4^{AB}
Wet	Warm	37.8 ± 1.6^{a}	43.6 ± 1.4^{a}	37.1 ± 3.0^{b}	25.0 ± 0.1^{a}

Upper- and lower-case letters indicate significant ($P < 0.05$, Tukey's HSD) differences between precipitation treatments within the No Warm and Warm temperatures, respectively. Asterisk (*) indicates difference between No Warm and Warm within a specific precipitation treatment.

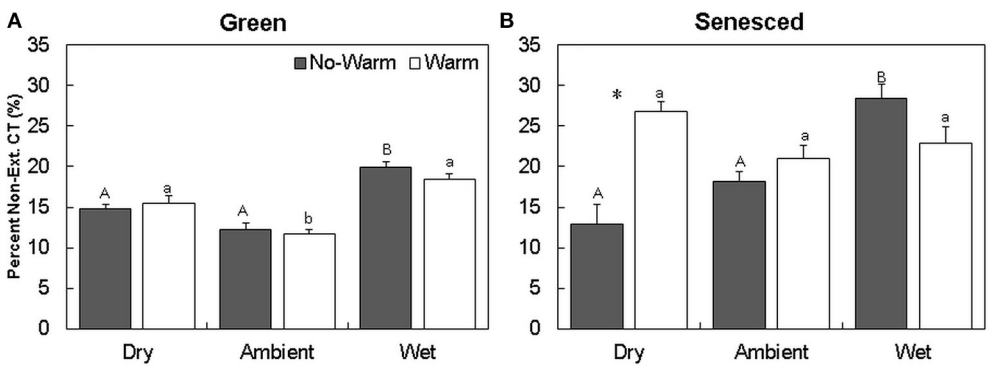

FIGURE 3 | Average (±SE) percent of non-extractable condensed tannins for green (A) and senesced **(B)** leaf tissue. Asterisks (*) above a set of columns indicate significant ($P < 0.05$) differences between the *No Warm* and *Warm* treatments within each precipitation treatment. Different uppercase letters indicate significant differences between precipitation treatments within the *No Warm* temperature regime. Different lowercase letters indicate significant differences between precipitation treatments within the *Warm* temperature regime.

having the lowest percentages of gallotannins (**Figure 4**). In the *Ambient* treatment, warming increased gallotannins in the extractable fraction in both the green and senesced leaf tissue (**Figures 4B,D**). In senesced tissue, warming in the *Ambient* treatment also increased the gallotannin percentage of the non-extractable fraction (this pattern did not occur in the green leaf tissue; $P = 0.09$, **Figures 4A,C**). In the *Wet* treatment, senesced leaves had more gallotannins (11.8%, *No Warm*; 7.7% *Warm*) in the non-extractable fraction than green leaf tissue.

DISCUSSION

Climatic stresses that disrupt cellular functioning in plants initiate readjustments in metabolic pathways, which in turn facilitate the acclimation of plants to their new environment.

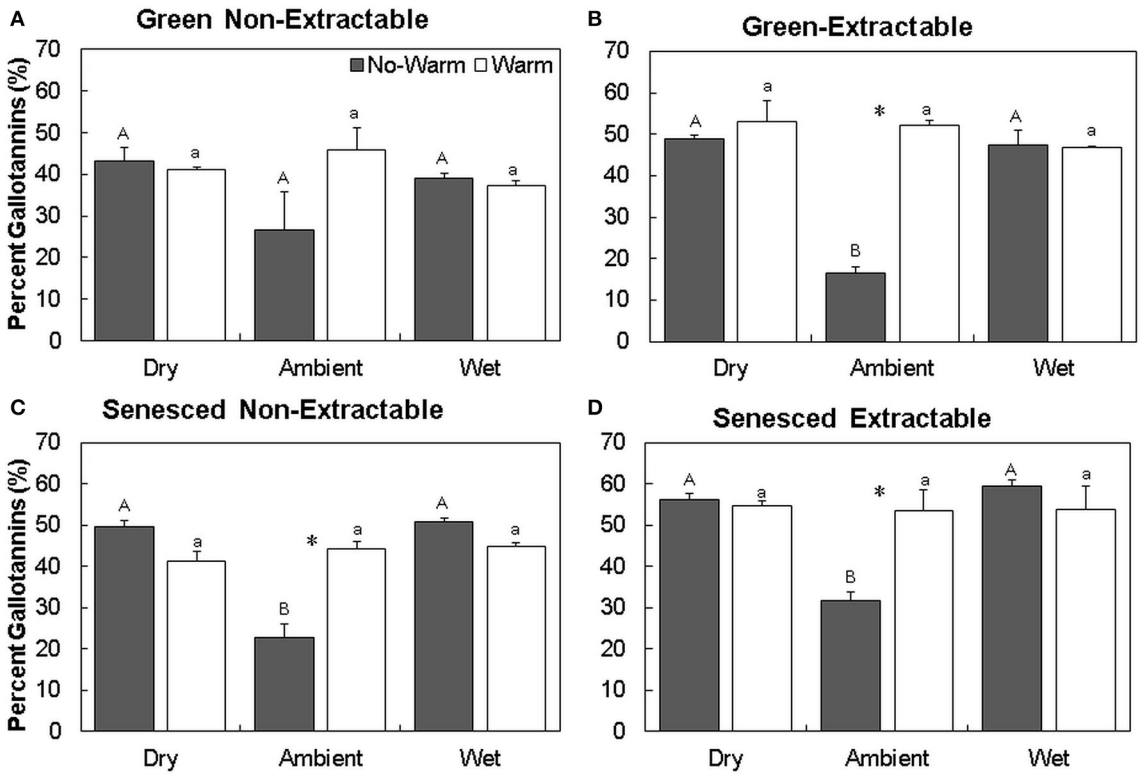

FIGURE 4 | Average (±SE) percent gallotannins in non-extractable and extractable hydrolysable tannins for both green (A,B) and senesced **(C,D)** leaf tissue. Asterisks (*) above a set of columns indicate significant ($P < 0.05$) differences between *No Warm* and *Warm* treatments within each precipitation treatment. Uppercase letters indicate significant differences between precipitation treatments within the *No Warm* temperature regime. Lowercase letters indicate significant differences between precipitation treatments within the *Warm* temperature regime.

The resulting reprogramming of important metabolic pathways, including the phenylpropanoid pathway, citric acid cycle, glycolysis, and urea cycle, results in the upregulation of several metabolite classes such as amino acids, phenolic acids, sugars, organic acids, sugar alcohols, polyamines, and polyols (Bohnert et al., 1995; Peñuelas et al., 2013; Suseela et al., 2015). Drought and warmer temperatures have been shown to influence many different morphological (yield, growth) and physiological (rate of photosynthesis, stomatal conductance, leaf pigmentation, water potential, protein concentrations, etc.) responses in plants (Benjamin and Nielsen, 2006; Rennenberg et al., 2006; Praba et al., 2009; Anjum et al., 2011). However, the influence of environmental stressors on the content and composition of polymeric defense compounds across green and senesced plant tissues is less well-known.

In this study, climate-induced changes in leaf tannin composition influenced not only the quantity of tannins, but also the proportion of HTs to CTs, monomer composition and mean degree of polymerization of CTs, composition of HTs, the association of tannins within the cells (extractable and non-extractable), and the composition of the tannins remaining in the senescent leaves. In general, plants growing in favorable conditions (*Ambient***No Warm*, *Wet*) produced less tannins per unit leaf mass than those growing in more stressful conditions (*Dry*, *Ambient***Warm*). Increase in tissue content of tannins with increasing environmental stress has been frequently reported (Bussotti et al., 1998; Cohen and Kennedy, 2010). Reallocation of nutrients in plants based on the favorability of growth and nutrient conditions is expected under two major hypotheses: the carbon/nutrient balance and the growth/differentiation balance (Herms and Mattson, 1992). The increase in tannin production under *Dry***Warm* conditions could be a defense strategy in plants to protect the resources that they have already acquired (Herms and Mattson, 1992; Wright et al., 2010; Massad et al., 2014). At the same time, in the *Wet* treatments, because growth conditions were presumably closer to optimal, the decrease in tannin production could be a result of the plant preferentially allocating C for growth instead of defense (Bryant et al., 1983; Tuomi, 1992). Also, a greater concentration of tannins binding with cellulose and lignin matrices (Zucker, 1983; Bussotti et al., 1998) could potentially create a physical impedance to cell expansion. A similar allocation pattern of photosynthates for growth, rather than for defense compounds, has been previously reported in other tree species (Donaldson et al., 2006), where the plant growth was negatively correlated with phenolics and CTs. In a previous study conducted at BACE, the tannin content in senesced leaves of *Acer rubrum* was greater in *Dry* than in *Ambient* treatments, while the senesced leaves from *Wet* and the *Ambient* treatments had similar tannin contents (Tharayil et al., 2011). This contrasting pattern could be attributed to a difference

in the physiological responses of the two species to stress, and also to differences in precipitation patterns between the two growing seasons. In the 2009 growing season (April to October; Tharayil et al., 2011), 720 mm of rain fell, as opposed to 606 mm during the 2013 growing season. Further, only half as much rain fell in the last 4 months of the 2013 growing season (July to October) as fell during that period in 2009 (210 mm and 428 mm, respectively). The larger amount of precipitation received by *A. rubrum* during the 2009 growing season would have led to a similar physiological water status between *Ambient* and *Wet* treatments, which could have resulted in similar tannin content between these two precipitation treatments (Tharayil et al., 2011). In the present study, the scarcer and less evenly distributed precipitation of 2013 likely exposed *Q. rubra* in *Ambient* to a greater physiological water stress, which could explain the observed similarity in tannin content between *Ambient* and *Dry* treatments.

Along with the total quantity of tannins, climate influenced both the monomer composition and mean degree of polymerization of tannins, the two parameters that are seldom investigated despite their regulatory influence on the potential biological reactivity of tannins (Zucker, 1983; Kraus et al., 2003b; Tharayil et al., 2011; Triebwasser et al., 2012). Reactivity of CT is a function of the hydroxylation pattern of the B-ring, and tannins with trihydroxy B-rings (PD) are more reactive than those with dihydroxy B-ring (PC). Both *Wet* and *Dry* treatments, despite their contrasting influences on total content of CTs, reduced the relative proportion of PD units. Compared to PCs, the PD units are formed only during later parts of cell development (Stafford et al., 1989). Thus, the lower accumulation of the PDs in wet climates could indicate a metabolic modification in CT biosynthesis. Under the metabolic stress in *Dry* conditions the triphenolic vicinal hydroxyls predispose PDs to oxidation and polymerization reactions (Close and Mcarthur, 2002; Aron and Kennedy, 2008) resulting in a lower proportion of PDs. This could partly explain the lower proportion of PDs in *Dry* treatments despite a higher production of CTs. Compared to *Ambient* and *Dry* treatments, the proportion of PD units and the mean degree of polymerization were higher under *Wet* treatments. The biological reactivity of tannins is a function of their degree of polymerization, where tannins with greater chain length have a higher capacity to complex with and precipitate proteins. Thus, though the leaves that were formed under ambient growing conditions and *Wet* treatments had a lower tannin quantity, these tannins would have a greater capacity to defend herbivory due to their higher biological reactivity contributed by a greater polymerization and higher proportion of PD units. Environmental conditions have been shown to influence the degree of polymerization of tannins (Cohen and Kennedy, 2010). A lower polymerization of tannins exposed to non-optimal growing conditions has been reported before (Tharayil et al., 2011) in senesced leaves of *Acer rubrum* from the same site. In *Vitis vinifera*, environmental stressors, especially moisture deficit, have been reported to increase the mean degree of polymerization of tannins (Kennedy et al., 2002; Cohen and Kennedy, 2010). In *Onobrychis viciifolia* the influence of drought on the mean degree of polymerization of CTs was dependent on otogeny of the plant (Malisch et al., 2016). These results indicate that the influence of environment on molecular composition of tannins could be regulated by the physiological stress perceived by the plant (as opposed to the magnitude of the treatment applied), the identity of the species, as well as their growth stage.

Distributions of tannins within leaves are highly species-specific (Kraus et al., 2003a), and within a species the cellular localization of tannins, herein operationally defined as solvent extractable and non-extractable tannins, can change based on environmental conditions (Gagné et al., 2006). In green leaves, CTs are usually stored in the cell vacuoles (Stafford, 1988; Bussotti et al., 1998; Marles et al., 2003), and thus are sequestered away from potential interactions with the structural matrix and plant metabolic compounds. During leaf maturation the tannins penetrate the cell walls (Bussotti et al., 1998), which may result in an increase in the fiber-bound proportion of tannins. These complexes can protect the cell wall or organelle against microbial attack, and can also delay the decomposition after senescence of the tissue (Zucker, 1983). Irrespective of the warming treatment, the leaves from the *Wet* treatment had a greater proportion of total CT that was non-extractable during the sequential solvent extraction. While we cannot be certain of the exact complexation of the tannins, some of them may be in association with cellulose and pectin (Le Bourvellec et al., 2009; Padayachee et al., 2012a,b; Jakobek, 2015) in the cell walls and middle lamellae. A high affinity of PCs binding to pectin has been observed (Le Bourvellec et al., 2009; Watrelot et al., 2013; Jakobek, 2015), meaning that tannins in the *Wet* treatment with the highest percentage of PCs and greater chain-length could have a greater complexation capacity with cell wall components. Alternatively, the greater chain-length and higher proportion of PC units would have resulted in lower extractability of tannins from the leaf tissues, resulting in the observed lower content of tannins in *Wet* treatments. Also, the *Wet* treatments, despite a lower content of CTs, have a higher percentage of HTs, which have a higher enzyme inhibition capacity compared to CTs (Triebwasser et al., 2012). Thus, overall, the leaves formed under *Wet* treatments would have a similar herbivore deterrence contributed by tannins, despite a lower content of CTs in the tissue.

Climate influenced the proportion of HTs and CTs. Across most treatments, the dominant type of tannin in *Q. rubra* was CT, with lower content of HTs. A similar, lower proportion of HT in red oak species has been reported before (Barbehenn et al., 2005). Compared to the *Ambient*NoWarm* control, all climate treatments had higher percentages of HTs, which may reflect the ease of maintenance, mobility, and greater responsiveness of HTs relative to CTs (Zucker, 1983; Shure et al., 1998). Because HTs are less associated with the structural matrix of the plants than CTs, they offer less resistance to normal cell growth and expansion, making them a preferable type of defense compound over CTs in environments that favor active plant growth (Zucker, 1983), as evidenced by the higher percentages of HTs in the *Wet* treatments. HTs are also thought to be metabolically cheaper; the metabolic cost for the formation of proanthocyanidin (monomers of CT) is 0.395 ATP equivalent g^{-1}, where as that of HTs is 0.27 equivalent g^{-1} (Lewis and Yamamoto, 1989).

Both gallotannins and ellagitannins also exhibit antioxidant properties (Barbehenn et al., 2006), and thus may play a role in the inhibition of higher radical formation in leaves, especially under climatic stress. Ellagitannins can also be highly variable in structure (Zucker, 1983). This could have specific relevance inhibiting digestive enzymes in herbivore digestive tracts (Zucker, 1983), which would be an effective defense mechanism against herbivory for the plant. In most climates in this study, the green leaves exhibited higher content of ellagitannins, however, the *Ambient*No Warm* control had the highest percentage of ellagitannins compared to all other treatments, suggesting that the alteration of climate in any way can significantly alter the proportion of certain types of tannins present, either by production rates or utilization of certain hydrolysable tannin structural components, such as the glucose molecule (Zucker, 1983).

During tissue senescence, following the disruption of membrane-bound vesicles, CTs can form insoluble complexes with proteins and cell wall carbohydrates (Kraus et al., 2003a; Marles et al., 2003), which could contribute to an increase in the percent of non-extractable CTs in the senesced tissue. Differences in the content of tannins in green leaves compared to senesced leaves observed in this study may be the result of different overall resorption strategies of the plants. Considering their protein complexation capacity, polymeric nature, and the C-C linkages, the CT would less amenable to enzyme mediated catalysis and subsequent resorption. Due to their lower complexity and the presence of more labile ester bonds, the gallotannins would be more amenable to resorption during tissue senescence than CTs. Compared to the green tissues, the greater mobilization of HTs during senescence is reflected in the lower proportion of HTs in the senesced tissues across the treatments. The CT content of the *Wet* treatments were similar in both green and senesced tissues, which could be primarily attributed to the greater degree of polymerization and a greater PC content of tannins produced in this treatment, which would have resulted in a greater association of these tannins with the cell wall.

Tannins can complex with carbohydrates and proteins and this complexation can persist after the leaf senesces, potentially delaying decomposition. With the increase in CTs and non-extractable CTs for some of the treatments, this might mean that those leaves would break down more slowly, lose less material to leaching, and possibly provide more sustained nutrient input into the soil. The higher tannin content of foliage observed under less favorable growing conditions could be partly countered by the changes in allocation patterns at the tree level, since trees exposed to climatic stress often produce less leaf biomass. However, the reduced carbon input through litterfall in such ecosystems, coupled with higher content of phenolic compounds in these tissues, might impose a greater constraint on nutrient cycling in these soils. Extractable phenolics and tannins are lost rapidly from the leaves, suggesting that leaching is a primary route for loss of tannins from leaves (Benner et al., 1988; Schofield et al., 1998; Hernes et al., 2001), and lower molecular weight tannins are lost more rapidly than higher molecular weight tannins (Schofield et al., 1998). Thus, the *Wet* treatments with their higher length CTs could better retain some of the tannin-related defense capabilities which could interfere with decomposition processes. However, the overall herbivory and decomposition processes are influenced not only by the content of anti-nutrients in the tissues, but also by their overall nutrient content (Suseela et al., 2013; Almuzini et al., 2017), which in turn is also regulated by environmental stressors.

Overall, our study elucidates multiple factors that regulate the production and seasonal change of tannins in *Q. rubra*. In agreement with the nutrient-balance hypothesis, the tannin concentrations in tissues formed under favorable climates were lower, but the tannins produced were more complex and potentially more protective due to higher chain lengths of CTs and greater proportions of HTs. When exposed to climatic stress, *Q. rubra* responded by producing a greater quantity of tannins that were of shorter chain length. The differential influence of the environment on the production dynamics of plant metabolites is important in the context of global changes and carbon and nitrogen cycle feedbacks. Although over longer time scales climate modifies ecosystem processes through shifts in species composition, over shorter time scales the physiological adaptations of plants to changing climate could influence soil C and nutrient cycling through changes in chemical composition of biomass. These changes in tannin content and composition may alter forest dynamics, not only by influencing decomposition and nutrient cycling dynamics, but also by regulating herbivore dynamics.

AUTHOR CONTRIBUTIONS

ST and NT contributed to the conceptualization of the project and design of the experiment, JD provided the experimental framework, ST collected and analyzed the data, CP and NT guided the analyses, ST drafted the article, all authors contributed to the critical revision of the article.

ACKNOWLEDGMENTS

We thank Vidya Suseela two reviewers for their critiques and thoughtful remarks on earlier drafts of this manuscript. We also thank Carol Goranson for maintaining the BACE field site, and Vidya Suseela for reoptimizing the tannin extraction protocols for this experiment. This research was supported by the National Science Foundation Grant (DEB-1145993) to NT and JD. The BACE infrastructure was supported by grants to JSD from NSF (grant DEB-0546670) and the U.S. Department of Energy's Office of Science (BER), through the Northeastern Regional Center of the National Institute for Climatic Change Research and the Terrestrial Ecosystem Sciences program. This is Technical Contribution No. 6549 of the Clemson University Experiment Station and is Purdue Climate Change Research Center Publication number 1709. This material is based upon work supported by NIFA/USDA, under project number SC-1700538.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00423/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 08 June 2017
doi: 10.3389/fpls.2017.00959

Infestation of Broad Bean (*Vicia faba*) by the Green Stink Bug (*Nezara viridula*) Decreases Shoot Abscisic Acid Contents under Well-Watered and Drought Conditions

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OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Jürgen Kreuzwieser, Albert Ludwig University of Freiburg, Germany
Christine Helen Foyer, University of Leeds, United Kingdom

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *31 January 2017*
Accepted: *22 May 2017*
Published: *08 June 2017*

Citation:
Ederli L, Brunetti C, Centritto M, Colazza S, Frati F, Loreto F, Marino G, Salerno G and Pasqualini S (2017) Infestation of Broad Bean (Vicia faba) by the Green Stink Bug (Nezara viridula) Decreases Shoot Abscisic Acid Contents under Well-Watered and Drought Conditions. Front. Plant Sci. 8:959. doi: 10.3389/fpls.2017.00959

The response of broad bean (*Vicia faba*) plants to water stress alone and in combination with green stink bug (*Nezara viridula*) infestation was investigated through measurement of: (1) leaf gas exchange; (2) plant hormone titres of abscisic acid (ABA) and its metabolites, and of salicylic acid (SA); and (3) hydrogen peroxide (H_2O_2) content. Furthermore, we evaluated the effects of experimentally water-stressed broad-bean plants on *N. viridula* performance in terms of adult host–plant preference, and nymph growth and survival. Water stress significantly reduced both photosynthesis (A) and stomatal conductance (g_s), while infestation by the green stink bug had no effects on photosynthesis but significantly altered partitioning of ABA between roots and shoots. Leaf ABA was decreased and root ABA increased as a result of herbivore attack, under both well-watered and water-deprived conditions. Water stress significantly impacted on SA content in leaves, but not on H_2O_2. However, infestation of *N. viridula* greatly increased both SA and H_2O_2 contents in leaves and roots, which suggests that endogenous SA and H_2O_2 have roles in plant responses to herbivore infestation. No significant differences were seen for green stink bug choice between well-watered and water-stressed plants. However, for green stink bug nymphs, plant water stress promoted significantly lower weight increases and significantly higher mortality, which indicates that highly water-stressed host plants are less suitable for *N. viridula* infestation. In conclusion two important findings emerged: (i) association of water stress with herbivore infestation largely changes plant response in terms of phytohormone contents; but (ii) water stress does not affect the preference of the infesting insects, although their performance was impaired.

Keywords: ABA, hydrogen peroxide, *Nezara viridula*, photosynthesis, salicylic acid, *Vicia faba*, water deficit

INTRODUCTION

Climate change is increasing aridity in the Mediterranean basin (Dai, 2011; Hewitson et al., 2014), where many regions have experienced the most severe droughts on record in more recent years. Recurrent exposure to heavy and prolonged drought is dramatically increasing the vulnerability of natural ecosystems and causing massive failures in the whole agricultural sector. The imposition of water stress in plants during drought leads to inhibition of photosynthesis, which is caused by both diffusional and metabolic limitations (Centritto et al., 2003; Flexas et al., 2004). Photosynthesis is the major source of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and singlet oxygen, even in the absence of stress. Under conditions of drought, ROS production is increased largely because of increased photorespiration, which leads to oxidative stress (Noctor et al., 2014). ROS are important secondary messengers in local and systemic signaling pathways in plants that trigger plant acclimation responses to abiotic and biotic stresses through interactions with phytohormones (Cruz de Carvalho, 2008).

Abscisic acid (ABA) is a phytohormone that is extensively involved in plant responses to abiotic stress, and especially water stress. Water stress induces accumulation of ABA in leaves and triggers downstream responses that can confer water stress tolerance to plants; e.g., stomatal closure, with the consequent feedback on photosynthesis and the ROS accumulation mentioned above. ABA-induced ROS accumulation also triggers up-regulation of the antioxidant defense systems (Jiang and Zhang, 2002). Many abiotic stresses in plants can also alter the endogenous concentrations of salicylic acid (SA), which again triggers activation of ROS-scavenging antioxidants (He et al., 2002; Erasalan et al., 2007; War et al., 2011; Sadeghi et al., 2013). For example, it has been reported that endogenous SA increases in *Phillyrea angustifolia* plants challenged with water stress, and that SA contents are positively correlated with those of α-tocopherol, which acts as a ROS and lipid-peroxyl-radical scavenger (Munné-Bosch and Peñuelas, 2003).

As plant responses to water stress might involve changes in the levels of constituents that determine the plant 'quality' (e.g., amino acids, organic acids, sugars, other compounds that limit negative osmotic effects), the performance and host preference of infesting herbivores might also be affected. To explain the complexity of the interactions of insect herbivores (hereafter simply called herbivores) with plants, two main hypotheses have been proposed. The plant stress hypothesis predicts that drought stress increases the hydroxylation of proteins, which subsequently increases the levels of free amino acids; this will, in turn, enhances insect growth and reproduction (White, 1984; Mattson and Haack, 1987). Alternatively, the plant vigor hypothesis suggests that vigorous (unstressed) plants will be more nutritious for herbivores (Price, 1991). Moreover, the impact of water stress on herbivores might depend on insect feeding mode (e.g., phloem feeding, chewing), or on stress occurrence over time (i.e., pulsed, continuous) (Huberty and Denno, 2004; Pineda et al., 2016). In nature, plants frequently experience simultaneous exposure to water stress and biotic stress.

Plants can respond to herbivore attacks by activating direct and indirect defenses. Direct defenses include limiting the food supply, reducing the nutrient value and insect preference, disrupting physical structures, and inhibiting chemical pathways of the insect. Indirect defenses include emission of a bouquet of volatile compounds, known as host-induced synomones, which can attract parasitoids (Kessler and Baldwin, 2001; Schoonhoven et al., 2005; Dicke, 2009; Ode, 2013). Plant synomones can be induced by insect feeding and egg deposition (Colazza et al., 2010; Hilker and Meiners, 2010; Fatouros et al., 2016). In the tritrophic system that is constituted by broad bean (*Vicia faba*)–green stink bug (*Nezara viridula* L. *Heteroptera*: *Pentatomidae*)–egg parasitoid [*Trissolcus basalis* (Wollaston) *Hymenoptera*: *Platygastridae*], the release of plant synomones is indeed induced by the combination of *N. virudula* feeding and oviposition (Colazza et al., 2004a,b). *N. viridula* is an extremely polyphagous piercing-sucking herbivore that feeds on the sap of xylem, phloem and cells, and lays egg masses mainly on the abaxial leaf surface.

Recent data from our laboratory have demonstrated that the egg parasitoid *T. basalis* shows selection toward water-stressed plants over well-watered pants (Salerno et al., 2017). However, whether water stress also influences green stink bug infestation has not been investigated to date. Moreover, the induction of direct plant defenses by green stink bug infestation remains unknown. SA, ABA, and ROS are also known to be signaling molecules in plant defenses against herbivores (Reymond, 2013), and it has been reported that SA is up-regulated and jasmonic acid (JA) is down-regulated after stink bugs have fed on soybean seeds (Giacometti et al., 2016). However, no reports are available on the involvement of such plant molecules in signaling pathways induced by green stink bug feeding and oviposition. For this purpose, H_2O_2 (the predominant ROS) accumulation was assessed here as an element of direct plant defenses, and SA and ABA were measured as putative components of plant signal transduction (Reymond, 2013). In addition, water stress strongly impacts on primary and secondary plant metabolism, to affect growth, health and feeding behavior of interacting herbivores (Mattson and Haack, 1987; Gutbrodt et al., 2012; Copolovici et al., 2014). Thus, we investigated whether *N. viridula* performance and preference are also affected by plant water stress. The present study was designed to test the hypothesis that water stress alters plant responses to herbivore infestation through stress-related hormones and modulation of photosynthetic parameters.

MATERIALS AND METHODS

Plant Material

Seeds of broad-bean plants (*V. faba* cv. 'Superaguadulce') were immersed for 24 h in a slurry of water and non-cultivated soil (1:4) to favor root nodulation. Seeds were planted into plastic pots (9 cm × 9 cm × 13 cm) filled with a mixture of agriperlite (Superlite, Gyproc Saint-Gobain, PPC Italia, Italy), vermiculite (Silver, Gyproc Saint-Gobain, PPC Italia, Italy) and sand (1:1:1), and grown in a climate-controlled chamber with a 12-h photoperiod, photosynthetic photon flux density

(*PPFD*) of 400 μmol m^{-2} s^{-1}, day/night air temperatures of 24°C/20°C ± 2°C, and relative humidity from 60 to 75%. Plants were watered daily to the water capacity of the pot, and fertilized once a week with an aqueous solution of low N fertilizer with the 5:15:45 ratio of N-P-K (1.4 g L^{-1}; Plantafol, Valagro, Italy).

Insect Rearing

A colony of *N. viridula* collected in fields near Perugia (Italy) was established under controlled conditions (temperature, 24 ± 2°C; relative humidity, 70 ± 5%; light/ dark, 16 h/8 h) in plastic cages (50 cm × 30 cm × 35 cm), with mesh-covered holes for ventilation (diameter, 5 cm). The insects were fed regularly with a diet of sunflower seeds and seasonal fresh vegetables.

Water Stress and Herbivore Treatment

The water stress treatment was applied to 15-day-old plants with approximately four fully expanded leaves, and two independent experiments were performed. On the day prior to water stress onset, the plants were fully irrigated and the excess water was allowed to drain off overnight. After draining, the pots were weighed to 1.0 g precision on a digital balance (model QS32A; Sartorius Instrumentation, Ltd, Germany), to determine the pot weight (PW) at the pot maximum water capacity ($Initial_{PW}$). The pots were wrapped in plastic bags to prevent evaporation from the soil, and weighed daily during the experiment to determine the daily pot weight ($Daily_{PW}$). Progressive soil water deprivation was expressed as the fraction of transpirable soil water (FTSW) (Sinclair and Ludlow, 1986; Brilli et al., 2013). The water stress cycle ended when transpiration of the stressed plants decreased to ∼10% of the mean transpiration of the control plants, which was achieved 12 days after withholding water. The pots were then weighed to determine the final weight ($Final_{PW}$) of the water-stressed plants. The FTSW was then calculated as in Equation (1):

$$\text{FTSW} = (Daily_{PW} - Final_{PW})/(Initial_{PW} - Final_{PW}). \quad (1)$$

During the 12 days of water deprivation, the plants were grown to the seven fully expanded leaf. Well-watered plants were used as controls: four plants at the beginning of the treatment (i.e., control), and four plants at the end of the 12 days of water deprivation (i.e., developmental control). Biochemical analyses and gas exchange measurements were performed on both the control and developmental control plants. The developmental control measurements taken at the end of the experiments showed no significant variations with respect to control plants (data not shown).

Each of four plants for treatment as control ($FTSW_{100}$) and water-stressed ($FTSW_{80}$, $FTSW_{50}$, $FTSW_{10}$) were exposed to three gravid *N. viridula* females for 24 h. During this period, the insects walked freely, fed and oviposited on the whole plants. Twenty-four hours after insect removal, the leaves with egg masses were used for biochemical analyses and gas exchange measurements.

Gas Exchange and Chlorophyll Fluorescence Measurements

Gas exchange and chlorophyll fluorescence measurements were performed on four plants per water treatment, using a portable infrared gas analyser equipped with an integrated fluorometer (LI-6400; Li-Cor, Inc., Lincolin, NE, United States), with a 2 cm^2 leaf chamber. Measurements were performed on the central portion of the first four fully expanded leaves of the plants, at $FTSW_{100}$, $FTSW_{80}$, $FTSW_{50}$, and $FTSW_{10}$. The measurements were made at the CO_2 concentration of 390 μmol mol^{-1}, relative humidity of 35–45%, and leaf temperature of 25°C. An outer gasket was added to the LI-6400 leaf clamp, to create a buffer zone in which the H_2O and CO_2 gradients between the in-chamber air and the pre-chamber air were minimized by the supply of the infrared gas analyser exhaust air (Rodeghiero et al., 2007). Photosynthesis (A), stomatal conductance (g_s) and chlorophyll fluorescence were measured simultaneously at PPFD = 800 μmol m^{-2} s^{-1}. After determining steady-state fluorescence (F_s), the maximum fluorescence (F_m') was measured by applying a saturating light pulse (10,000 μmol m^{-2} s^{-1}; 0.8 s duration), and the minimal level of fluorescence (F_0') was measured by switching off the actinic light after the saturating pulse. The photosystem-II operating photochemical efficiency (Φ_{PSII}) was calculated according to Equation (2) (Genty et al., 1989):

$$\Phi_{PSII} = (F_m' - F_s)/F_m'. \quad (2)$$

The photochemical quenching (qP), related to the proportion of open PSII reaction centeres was determined according to Equation (3) (Schreiber, 1986):

$$qP = (F_m' - F_s)/(F_m' - F_0'). \quad (3)$$

Finally, the electron transport rate (ETR) was calculated according to Equation (4):

$$ETR = \Phi_{PSII} \times PPFD \times 0.5 \times 0.87, \quad (4)$$

where the coefficient 0.5 assumes equal distribution of the absorbed photons between PSI and PSII, and 0.87 is the leaf absorbance coefficient (Krall and Edwards, 1992). The measurements were conducted on a total of eight replicates.

Hormones and H_2O_2 Quantification

Biochemical analyses were performed on leaf and root samples collected from four different non-infested and infested plants subjected to the FTSW treatment. In non-infested plants, the four leaves (from the first to the fourth completely expanded leaves) and root apical portions of each single plant were sampled. For the biochemical analyses of the plants infested with green stink bug, leaves with eggs (with the exception of cotyledons and leaves that were not completely expanded) and roots were collected. The leaf and root samples were rapidly frozen by immersion in liquid nitrogen.

Total SA was extracted and quantified as reported by Di Baccio et al. (2012). For quantification of ABA and its metabolites,

fresh leaf tissue (300–350 mg) was added to 50 ng deuterated ABA (d^6-ABA), 50 ng d^5-ABA-glucose ester (d^5-ABA-GE), 50 ng d^3-phaseic acid (d^3-PA), and 50 ng d^3-dihydrophaseic acid (d^3-DPA). The sample was extracted with 3 mL CH_3OH/H_2O (1:1 [v/v], pH 2.5 with HCOOH) at 4°C for 30 min. The supernatant was partitioned with 3 mL × 3 mL *n*-hexane, and the aqueous- methanolic phase was loaded onto C18 cartridges (Sep-Pak; Waters, Milford, MA, United States), and washed with 2 mL water, pH 2.5. Free-ABA and ABA-GE were then eluted with 1.2 mL ethylacetate, and the eluate was dried under nitrogen, and rinsed with 500 μL CH_3OH/H_2O (1:1 [v/v], pH 2.5 with HCOOH). Identification and quantification of free-ABA and ABA metabolites were performed using liquid chromatography–electrospray ionization-tandem mass spectrometry. The samples (3 μL) were injected into the chromatograph (LC1200; Agilent Technologies, Santa Clara, CA, United States), which was coupled to a triple quadrupole mass spectrometer detector equipped with an electrospray ionization source (6410; Agilent Technologies) operating in negative ion mode. The metabolites were separated in a C_{18} column (Poroshell; 3.0 mm × 100 mm, 2.7 μm i.d.; Agilent Technologies) at a flow-rate of 0.3 mL min^{-1} and using a linear gradient solvent system from 95% solvent A (0.1% HCOOH in H_2O) to 100% solvent B (CH_3CN/MeOH, 1:1 [v/v], with 0.1% HCOOH), over 30 min. Quantification was conducted in multiple reaction mode, as reported by López-Carbonell et al. (2009).

For determination of H_2O_2, fresh plant tissue (500 mg) was ground to a powder in liquid nitrogen and homogenized in 2 mL 0.2 M $HClO_4$ in a pre-cooled pestle and mortar. The extract was centrifuged at 10,000 × *g* for 10 min at 4°C. The acidic supernatant was neutralized to pH 6.5–7.0 with 0.2 M NaOH, and centrifuged at 3,000 × *g* for 2 min, to sediment insoluble material. To remove pigments, antioxidants, polyphenolics and other interfering substances, activated charcoal was added, and the extract was centrifuged at 11,000 × *g* for 5 min at 4°C. The supernatant was filtered through PTFE membranes (0.45 μm; Millipore), and the H_2O_2 concentrations were immediately determined using the xylenol orange assay, as reported previously (Pasqualini et al., 2003), with the calculations using a standard curve prepared with known concentrations of H_2O_2. Biochemical data were expressed on a dry weight basis by weighing parallel samples of fresh plant tissues (FW) and drying them at 70°C for at least 72 h to determine the dry weight (DW). The specific leaf water content (SWC), as g H_2O g^{-1} DW, was calculated according to the following formula:

$$SWC = (FW - DW)/DW. \quad (5)$$

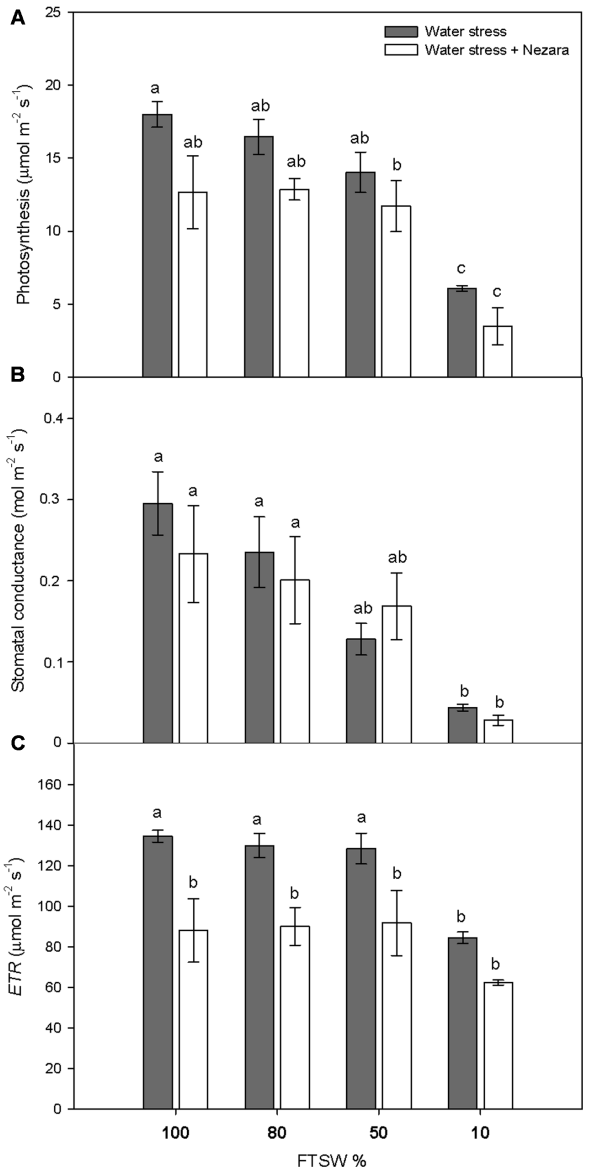

FIGURE 1 | Gas exchange measurements in well-watered and water-stressed broad bean plants with or without *Nezara viridula* infestation. Leaf photosynthesis **(A)**, stomatal conductance **(B)**, and electron transport rate (*ETR*) **(C)**, of *Vicia faba* plants under developing water stress (gray bars) and with an associated presence of *N. viridula* infestation (white bars). Measurements were made at 100% (well-watered plants), and 80, 50, and 10% of fraction of transpirable soil water (FTSW). Data are means ± SE ($n = 8$). Different letters indicate significant differences ($P < 0.05$).

Nezara viridula Host Preference and Performance

Nezara viridula females had the choice of where to lay their eggs, in terms of $FTSW_{100}$ and water-stressed ($FTSW_{80}$, $FTSW_{50}$, or $FTSW_{10}$) broad-bean plants. One control and one water-stressed plant were placed together inside a glass box (36 cm × 34 cm × 50 cm) with the above-ground parts isolated from the pots using a plastic panel. Fifteen gravid *N. viridula* females were released into the box and allowed to move freely under controlled conditions (temperature, 26 ± 1°C; relative humidity, 30 ± 5%; light/dark, 12 h/12 h). After 24 h, the number of females on each plant and the number of egg masses deposited per plant were counted. In total, 10 pairwise comparisons between $FTSW_{100}$ and each of $FTSW_{80}$, $FTSW_{50}$, and $FTSW_{10}$ were carried out. The percentages of adult choice and egg masses deposited were recorded. To determine whether these three levels

of water stress affected the herbivore growth, *N. viridula* nymphs were placed on $FTSW_{100}$ and $FTSW_{80}$, $FTSW_{50}$, and $FTSW_{10}$ plants. After placing the above-ground organs inside a tissue bag, each plant was inoculated with 10 nymphs (3rd instar) that had been weighed previously on a microbalance (P Series; Exacta, Germany). The nymphs were allowed to feed on the individual plants for 1 week under controlled conditions (temperature, 26 ± 1°C; relative humidity, 30 ± 5%; light/dark 15 h/9 h). To maintain a constant FTSW during the entire bioassay period, the transpired water was returned to the pots every 2 days. At the end of the experiment, the following were recorded: nymph weight increases (i.e., % difference between final and initial weights in relation to the initial weight), and nymph mortality (i.e., % dead nymphs in relation to total nymphs placed on each plant at the beginning of the experiment). Moreover, the live nymphs were weighed and the mean weight for each plant was calculated to record the nymph weight increase (i.e., % difference between final and initial weights in relation to initial weight). This bioassay was performed with 10–13 individual plants per water stress level.

Statistical Analysis

The data for gas exchange and biochemical analyses underwent factorial analysis of variance (ANOVA; two-way maximum interactions) to determine the effects of the interactions between water stress and insect infestation on all of the dependent variables. *Post hoc* multiple comparisons were carried out using Tukey honest significant difference tests, to analyze the differences among the treatment means for the physiological and biochemical data. *N. viridula* choice and oviposition were compared statistically by parametric paired *t*-tests for dependent samples. Nymph weight increase (%) and nymph mortality (%) were analyzed using one-way factorial ANOVA. For *post hoc* comparisons, Dunnett tests were used to compare water-stressed and control plants (Statsoft Inc., 2001). Before analysis, Box–Cox transformations were used to reduce the data heteroscedasticity (Sokal and Rohlf, 1998).

RESULTS

Gas Exchange and Chlorophyll Fluorescence Measurements

Under well-watered conditions ($FTSW_{100}$), the SWC was 13.51 ± 1.44 g H_2O g^{-1} DW. During the progression of water stress, SWC did not change significantly for mild ($FTSW_{80}$) and moderate ($FTSW_{50}$) water stress. Under severe water stress ($FTSW_{10}$), SWC dropped significantly to 7.85 ± 0.13 g H_2O g^{-1} DW ($P \leq 0.001$). Photosynthesis (*A*) (**Figure 1A**) and stomatal conductance (g_s) (**Figure 1B**) of *V. faba* plants significantly decreased with increasing water stress, both in the absence and presence of *N. viridula*. The FTSW thresholds at which *A* and g_s began to decline rapidly where different, as g_s declined at a higher rate with respect to *A*, and the difference with respect to well-watered plants was significantly different already at $FTSW_{50}$ (**Figure 1B**). Under severe water stress conditions ($FTSW_{10}$), the *A* and g_s reductions compared to well-watered leaves were around 65 and 85%, respectively. The response of the ETR to soil drying (**Figure 1C**, *ETR*) mirrored that of *A*, with a decrease of about 37% at $FTSW_{10}$, from the pre-stress values. *N. viridula* infestation had small, and non-significant, effects on the responses of *A* and g_s to water stress. In contrast, *ETR* significantly decreased in plants infested by *N. viridula*, by 35% (at $FTSW_{100}$) and 26% (at $FTSW_{10}$), with respect to non-infested plants at comparable FTSW.

ABA Metabolism

The leaf and root ABA and ABA-GE contents (**Figure 2**) and the content of the ABA catabolites phaseic acid (PA) and dihydrophaseic acid (DPA) (**Figure 3**) were analyzed in plants under water stress and for the interaction between water stress and *N. viridula* infestation.

Along the water stress treatments, free ABA content in the leaves increased significantly at $FTSW_{80}$ and $FTSW_{50}$, and decreased significantly at $FTSW_{10}$, compared to well-watered plants (**Figure 2A**). In roots, a significant decrease in free ABA content was observed at each stage of water stress (**Figure 2B**). The infestation with *N. viridula* caused contrasting responses on leaf and root contents of free ABA in plants before and during water stress. In leaves of infested plants, the free ABA content was significantly lower than in non-infested plants at $FTSW_{100}$, $FTSW_{80}$ and $FTSW_{50}$, whereas there was no difference between treatments at $FTSW_{10}$ (**Figure 2A**). In roots, the content of free ABA at $FTSW_{100}$ was more than double for the infested compared to non-infested plants. Under mild water stress ($FTSW_{80}$) this difference was even higher. However, as the water stress became more severe ($FTSW_{50}$, $FTSW_{10}$), the root free ABA content dropped in plants infested by *N. viridula* (**Figure 2B**). Leaf ABA-GE contents (**Figure 2C**) showed generally opposite trends to those of root free ABA in response to water stress, with significant decreases at $FTSW_{80}$ and $FTSW_{50}$ (by ∼40%), while returning to pre-stress levels at $FTSW_{10}$. In the roots, there was no clear pattern of ABA-GE in response to water stress, as ABA-GE only significantly increased (by ∼40%) at $FTSW_{80}$. *N. viridula* infestation induced a significant further reduction in leaf ABA-GE, especially in well-watered leaves, but also at different water stress levels. However, when comparing only plants infested by *N. viridula*, the ABA-GE content was significantly higher at $FTSW_{10}$ than at any other FTSW (**Figure 2C**). In the roots of plants infested by *N. viridula*, the ABA-GE contents increased significantly compared to non-infested plants at each water stress level, and was similarly high across all FTSW (**Figure 2D**).

Water stress significantly reduced the leaf content of PA, by 73% at $FTSW_{80}$ and $FTSW_{50}$, but by only 29% at $FTSW_{10}$ (**Figure 3A**). The PA content also decreased in the roots in response to the water stress, although it was significantly lower than in well-watered plants (by ∼35%) only at $FTSW_{80}$ (**Figure 3B**). *N. viridula* infestation significantly decreased the leaf PA content of well-watered plants (by ∼60%) (**Figure 3A**), but did not affect its content in roots (**Figure 3B**). Furthermore, *N. viridula* infestation also modified the response to water stress. The leaf PA content showed a declining trend as water stress increased in severity (significant only at $FTSW_{10}$). An opposite, significantly increasing trend occurred in water-stressed roots,

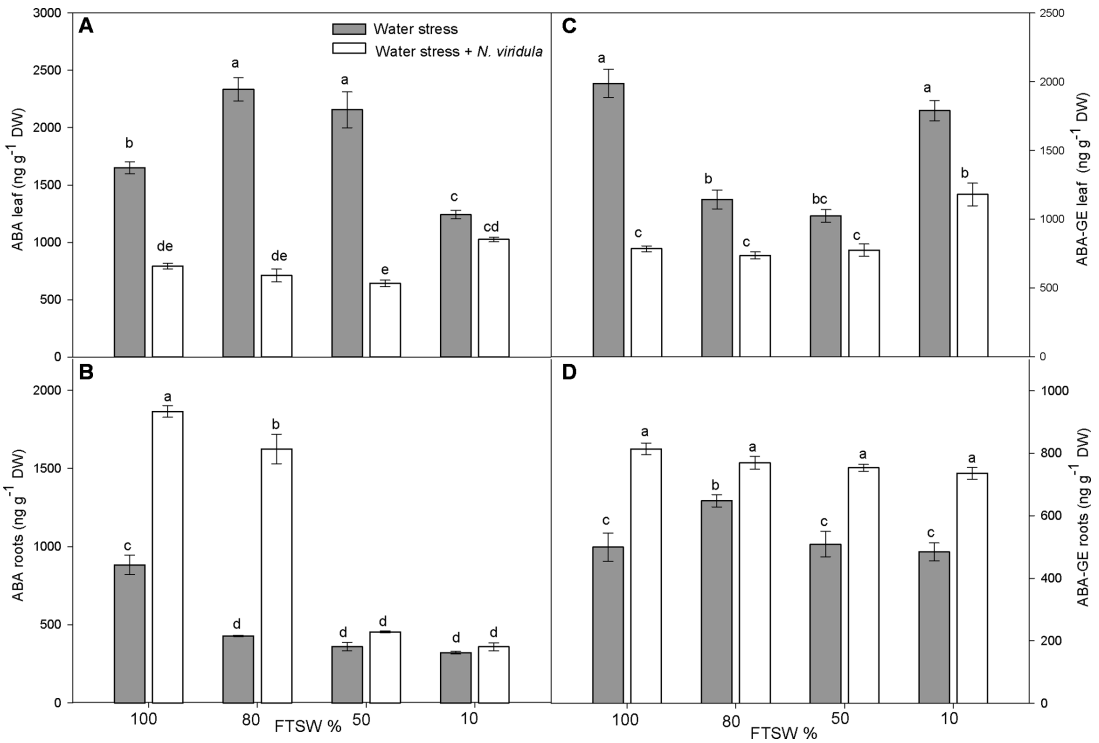

FIGURE 2 | Quantitative measurements (ng g^{-1} DW) of ABA and its metabolite ABA-GE in well-watered and water-stressed broad bean plants with or without *N. viridula* infestation. Abscisic acid (ABA; **A,B**) and abscisic acid glucose ester (ABA-GE; **C,D**) contents in leaves **(A,C)** and roots **(B,D)** of *V. faba* plants under developing water stress (gray bars) and with an associated presence of *N. viridula* infestation (white bars). Measurements were made at 100, 80, 50, and 10% of FTSW. Data are means ± SE (n = 8). Different letters indicate significant differences ($P < 0.05$).

which resulted in about a threefold increase in the PA content at $FTSW_{10}$. The DPA content was also affected by water stress, although significant increases were only seen at $FTSW_{10}$ for both leaves (by 388%) (**Figure 3C**) and roots (by 110%) (**Figure 3D**). No effects of *N. viridula* infestation on DPA contents were evident at $FTSW_{100}$ for both leaves and roots. However, similar to PA, as the water stress increased in severity, the DPA contents showed a declining trend in leaves and an opposite significant increasing trend in roots. At $FTSW_{10}$, the root DPA content increased by more than fourfold after *N. viridula* infestation (**Figure 3D**).

H_2O_2 and SA

Water stress did not significantly affect the H_2O_2 contents of non-infested plants for either leaves (**Figure 4A**) or roots (**Figure 4B**). However, *N. viridula* infestation significantly stimulated the production of H_2O_2 in well-watered and water-stressed leaves, until the stress reached $FTSW_{10}$ (**Figure 4A**). A similar trend was observed in infested plant roots (**Figure 4B**). SA showed similar trends in response to water stress in non-infested leaves (**Figure 5A**) and roots (**Figure 5B**), peaking under mild water stress ($FTSW_{80}$), and then dropping again when the water stress further increased. The infestation of *N. viridula* caused a very large increase in SA content in well-watered leaves and in roots at $FTSW_{80}$. The amount of SA was also higher in infested than in non-infested leaves and roots at $FTSW_{50}$.

Nezara viridula Host Preference and Performance

The water stress did not affect the choice of host plant by the female *N. viridula* ($FTSW_{80}$, 50.1 ± 4.9%; $FTSW_{50}$, 51.5 ± 5.4%; $FTSW_{10}$, 49.9 ± 5.0%), and similarly, no differences were seen in egg-mass distribution between well-watered and water-stressed plants ($FTSW_{80}$, 52.9 ± 10.9%; $FTSW_{50}$, 52.1 ± 9.7%; $FTSW_{10}$, 49.6 ± 7.8%). The nymph weight increase was not affected in plants under mild water stress ($FTSW_{80}$), but was significantly reduced for $FTSW_{50}$ and $FTSW_{10}$ (**Figure 6A**). A significant increase in the mortality of *N. viridula* nymphs was only seen in response to severe water stress ($FTSW_{10}$) (**Figure 6B**).

DISCUSSION

As climate change continues, droughts and insect populations are predicted to increase in many parts of the world (Oliver and Morecroft, 2014). It is therefore important to understand how such changes can affect the agroecosystems. The present study suggests that in a typical legume crop in temperate areas: (i) association of water stress with herbivore infestation greatly changes the plant responses in terms of phytohormone contents; although (ii) water stress does not change the plant preference of the infested insects, although it does greatly impair the nymph vitality after feeding on leaves.

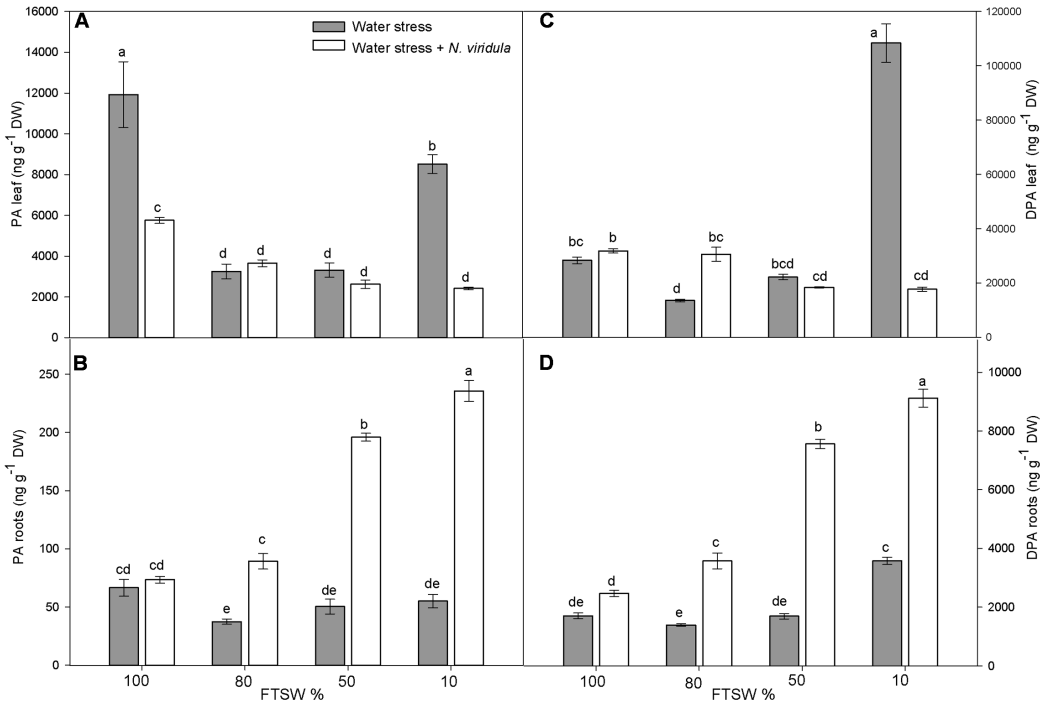

FIGURE 3 | Quantitative measurements (ng g^{-1} DW) of ABA catabolites PA and DPA in well-watered and water-stressed broad bean plants with or without *N. viridula* infestation. Phaseic acid (PA; **A,B**) and dihydrophaseic acid (DPA; **C,D**) in leaves **(A,C)** and roots **(B,D)** of *V. faba* plants under developing water stress (gray bars) and with an associated presence of *N. viridula* infestation (white bars). Measurements were made at 100, 80, 50, and 10% of FTSW. Data are means ± SE ($n = 8$). Different letters indicate significant differences ($P < 0.05$).

As expected, water stress induced a significant decrease in the photosynthetic parameters. In several studies where water stress was expressed as a function of FTSW, A and g_s were reduced in parallel; e.g., for poplar (Brilli et al., 2007; Centritto et al., 2011) and eucalyptus (Brilli et al., 2013). In the present study, with respect to A (Brilli et al., 2007), g_s showed slightly higher curve inflection, from where it began to decline rapidly (i.e., the threshold at which the second stage of the plant responses to FTSW started; Sinclair and Ludlow, 1986). This indicates that A is primarily influenced by CO_2 diffusive limitations when water stress is not severe (i.e., $FTSW_{80}$ and $FTSW_{50}$ in the present study) (Cornic, 2000; Lawlor and Cornic, 2002; Centritto et al., 2003; Brilli et al., 2007; Flexas et al., 2013). Generally, the combined effects of biochemical and diffusional factors appear to limit A only under severe water stress ($FTSW_{10}$) (Lawlor and Cornic, 2002), whereas *N. viridula* infestation had a limited negative effect on A and g_s of well-watered and water-stressed leaves. However, the infestation significantly reduced *ETR* across all FTSW levels, which implies damage to the photochemistry of photosynthesis during insect feeding, which will probably be related to damage to photosynthetic pigments. Herbivory effects on photosynthetic parameters are controversial and might be different according to the insect feeding behaviors. Chewing insects can cause extensive damage to plant tissues, whereas piercing-sucking insects (such as *N. viridula*) generally induce minimal physical damage (War et al., 2013). However, previous studies on plant injury caused by insects with piercing–sucking mouthparts have also shown that A and g_s can decrease or increase as a result of herbivore feeding (Meyer and Whitlow, 1992; Kerchev et al., 2012). Studies that have shown increases or maintenance of the photosynthetic activity indicate a compensatory response of the plants; i.e., increased A of non-infested leaf parts (Crawley, 1989; Welter, 1989). Maintenance of A might also indicate an increased allocation of energy and carbon-based resources to the plant defensive systems (Schwachtje and Baldwin, 2008). Indeed, tolerant lines of barley and wheat can be discriminated from susceptible lines on the basis of their maintenance of A under herbivore infestation (Franzen et al., 2007; Gutsche et al., 2009).

Water stress induced changes in the contents of ABA and its catabolites in both leaves and roots of *V. faba*. The increased free ABA contents in leaves at mild to moderate water stress ($FTSW_{80}$, $FTSW_{50}$) were correlated to low levels of its oxidized catabolites, and to increased hydrolysis of its conjugate form, ABA-GE. Increased free ABA content might be the cause for the g_s reduction as the water stress increases (Davies and Zhang, 1991). It should be noted, however, that free ABA content increased even in leaves under mild water stress ($FTSW_{80}$) with no significant reduction of g_s seen. This early increase in leaf free ABA might be associated to the concurrent decrease in root free ABA, because ABA is transported to the aerial part as a mechanism for the transmission of a chemical signal to indicate the declining soil water status (Sauter et al., 2001; Zhang et al., 2006). Moreover, the

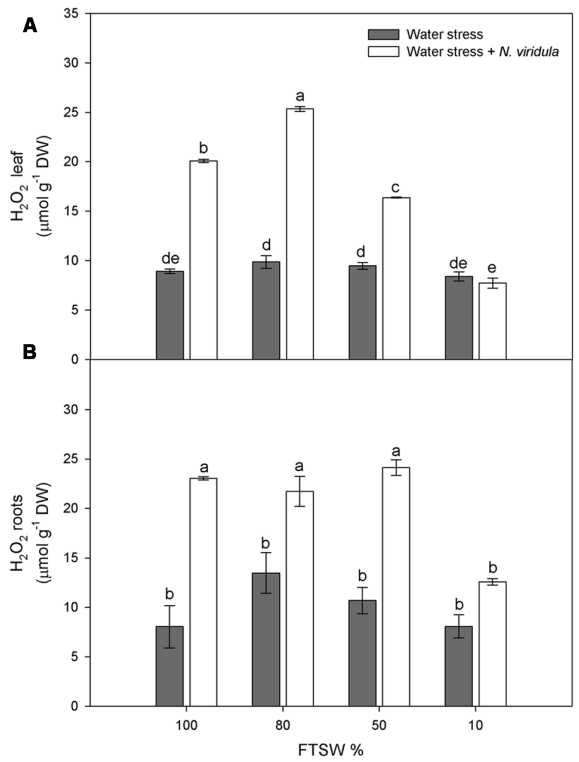

FIGURE 4 | Quantitative measurements (µmol g^{-1} DW) of H_2O_2 in well-watered and water-stressed broad bean plants with or without *N. viridula* infestation. Quantitative measurements of H_2O_2 in leaves **(A)** and roots **(B)** of *V. faba* plants under developing water stress (gray bars) and with an associated presence of *N. viridula* infestation (white bars). Measurements were made at 100, 80, 50, and 10% of FTSW. Data are means ± SE (n = 8). Different letters indicate significant differences ($P < 0.01$).

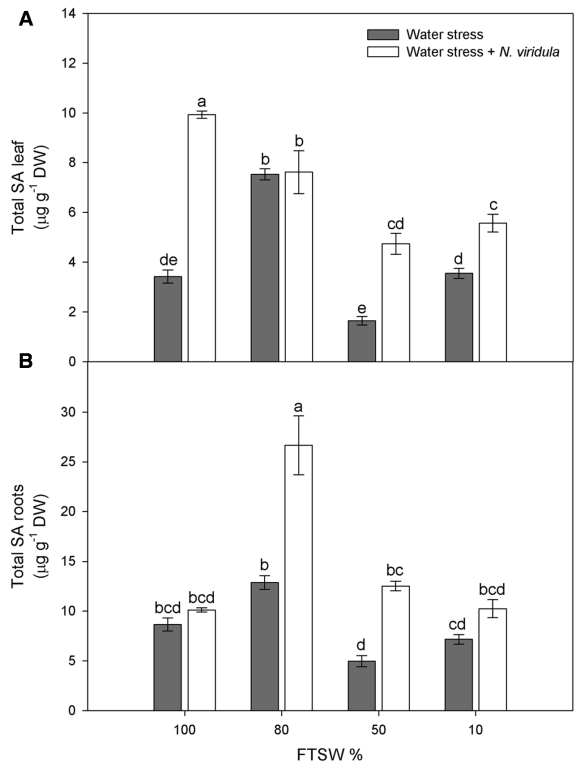

FIGURE 5 | Quantitative measurements (µg g^{-1} DW) of total SA in well-watered and water-stressed broad bean plants with or without *N. viridula* infestation. Total salicylic acid (SA) contents in leaves **(A)** and roots **(B)** of *V. faba* plants under developing water stress (gray bars) and with an associated presence of *N. viridula* infestation (white bars). Measurements were made at 100, 80, 50, and 10% of FTSW. Data are means ± SE (n = 8). Different letters indicate significant differences ($P < 0.01$).

relationship between free ABA and g_s was lost under severe water stress ($FTSW_{10}$), when the catabolism of ABA increased and the physiological functions of the plants were compromised, possibly because of reduced xylem flow (Atkinson et al., 2015) and a further drop in leaf water potential (Brodribb and McAdam, 2013). Low xylem flow might also explain the stable root ABA content even under severe water stress.

The trends seen for the leaf ABA-GE contents were specular of those of free ABA at all stages of stress, which suggests a 'buffer' function for this physiologically inactive form of free ABA (Jiang and Hartung, 2008). ABA-GE can be cleaved by a dehydration-inducible β-glucosidase that contributes to the regulation of free ABA content in leaves (Sauter et al., 2002; Lee et al., 2006; Xu et al., 2012). In the roots, the ABA-GE content remained constant during the water stress, and did not appear to be involved in fine control of the free ABA content. Conversely, in the plants infested by *N. viridula*, the free ABA content showed very large changes. The main trends were a reduction of leaf free ABA at all water contents, and an increase in root free ABA in the controls ($FTSW_{100}$) and under mild water stress ($FTSW_{80}$) conditions. These trends were confirmed by the levels of the ABA catabolites, PA and DPA, which remained low in leaves during stress progression, whereas they increased significantly in roots. It is known that ABA influences JA biosynthesis and JA-dependent gene expression, thereby activating resistance to herbivores (Adie et al., 2007; Bodenhausen and Reymond, 2007; Vos et al., 2013). The down-regulation of ABA synthesis and signaling in leaves can be interpreted as part of the *N. viridula* attack strategy to inhibit JA synthesis and JA-dependent defense gene expression, and therefore keep the defenses of the host plant low.

The importance of oxidative stress in plant water stress responses is widely acknowledged (Smirnoff, 1993; Cruz de Carvalho, 2008; Miller et al., 2010). This can range from oxidative damage to the role of ROS in local and systemic signaling. Increased ROS production under water stress has been associated with inhibition of photosynthesis. Stomatal closure induced by water stress restricts CO_2 uptake, which in turn favors photorespiratory production of H_2O_2 in the peroxisomes, and the production of superoxide, H_2O_2 and singlet oxygen by the photosynthetic electron transport chain (for review, see Noctor et al., 2014). Furthermore, H_2O_2 enhancement under stress might induce acclimation/defense responses triggered by ABA-mediated stomatal closure (Pei et al., 2000).

However, many studies have shown no changes in H_2O_2 contents in response to water stress (Moran et al., 1994; Porcel and Ruiz-Lozano, 2004). This is also the case for the present

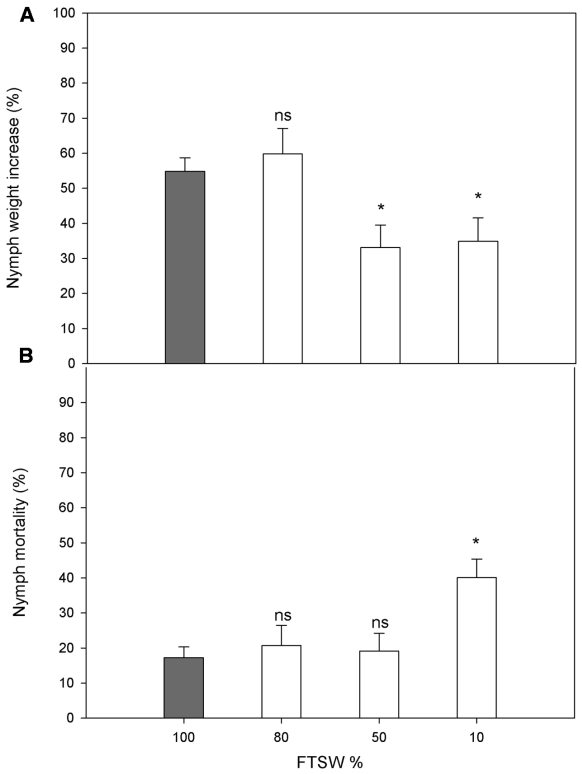

FIGURE 6 | Performance of *N. viridula* nymphs in well-watered and water-stressed plants. Weight increase **(A)** and mortality **(B)** of *N. viridula* nymphs feeding on *V. faba* plants under developing water stress. Measurements were made at 100% (n = 11), 80% (n = 10), 50% (n = 13), and 10% (n = 11) of FTSW. Data are means ± SE. White bars are compared with gray bar (well-watered), $^*P < 0.05$; ns, no significant difference.

study, as the H_2O_2 contents of both leaves and roots were not affected by the water stress, despite stomatal closure and the consequent inhibition of photosynthesis. Activation of the ROS scavenging enzymes is under the control of SA, which promotes plant tolerance to abiotic stress (He et al., 2002; Erasalan et al., 2007; War et al., 2011; Sadeghi et al., 2013). The significant increase in leaf SA content under mild water stress ($FTSW_{80}$) might be the signal that promotes the induction of antioxidant enzymes that can counteract H_2O_2 accumulation under water stress, and under combined water stress and *N. viridula* infestation. Interestingly, the highest stimulation of leaf SA after infestation with *N. viridula* was observed here in the well-watered plants. Thus, while both abiotic and biotic stresses can induce SA biosynthesis, there were no additive effects under the co-occurring stress. Many studies have shown that in tobacco and cucumber leaves inoculated with necrotising pathogens, there is export of SA into the phloem (Métraux et al., 1990; Rasmussen et al., 1991; Yalpani et al., 1991; Shulaev et al., 1995). Similarly, the absence of an additive increase in SA in these infested and water-stressed *V. faba* leaves might be due to partial reallocation of the SA toward the roots, where SA indeed showed a large increase. The observation that both H_2O_2 and SA accumulate in leaves where a green stink bug has laid at least one batch of eggs suggests that oviposition causes a localized response with a strong similarity to a hypersensitive response (Little et al., 2007; Bruessow et al., 2010). It remains to be clarified whether this response to egg deposition constitutes a direct defense or a mechanism to anticipate the threat posed by the future feeding of the larvae.

The final focus of this study was to evaluate how water stress of *V. faba* influences pest infestation and nymph growth performance. The highly debated relationships between plant stress, plant quality, and herbivore performance has generated several hypotheses (White, 1984; Price, 1991; Huberty and Denno, 2004). Under the experimental conditions also used in the present study, water stress induction of plant volatiles attracted more individuals of the egg parasitoid *T. basalis* (Salerno et al., 2017). However, here *N. viridula* females showed no preference in terms of where they laid their eggs according to the well-watered and water-stressed *V. faba* plants. The neutral behavioral choice of *N. viridula* toward the water-stressed plants did not correspond to the performance of the herbivore larvae, which was significantly lowered by the water stress. Thus, the water-stressed hosts were less suitable for the *N. viridula*. Reductions in lepidopteran larvae performance and aphid population densities due to water stress in host plants have been reported (Huberty and Denno, 2004; Badenes-Perez et al., 2005; Nachappa et al., 2016). This might be due to reduced biosynthesis of the primary metabolites (e.g., carbohydrates, proteins) caused by increased limitation of photosynthesis or early plant senescence under water stress, or by insufficient water availability in the drying plant tissues (Showler, 2013). Indeed, these effects were observed in the present study when the water stress was already relatively severe, as the photosynthetic activity and SWC dropped significantly. The negative effects of water stress on insect growth and survival might also be due to accumulation of 'anti-nutrients.' A negative effect on egg production of the SA analog benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester was reported for the herbivore mite *Tetranychus urticae* (Choh et al., 2004). Also, as SA is a metabolite that can accumulate in leaves in response to water stress, it might have been involved in reductions in the nutritional properties of the leaves or the feeding capacity of the insects. However, this hypothesis must be tested in further experiments.

Overall, our data show that water stress and *N. viridula* infestation individually trigger the SA pathway. However, the water stress does not affect the H_2O_2 contents, which were only increased by the *N. viridula* infestation. In contrast, ABA signaling has a role under water stress, and is down-regulated by *N. viridula* infestation. Furthermore, under our experimental conditions, the decrease in *N. viridula* performance suggests that this herbivore is adversely affected by severe water stress. These findings provide a better understanding of how plants respond to combined abiotic and biotic stresses.

AUTHOR CONTRIBUTIONS

All of the authors participated in the conception and design of the experiments; LE, GS, FF, GM, and CB performed the

experiments and analyzed the data; SP, LE, MC, and FL wrote the manuscript; and all authors interpreted the results and revised the manuscript.

FUNDING

This work was supported by the national project MIUR – PRIN 2010–2011 [grant number 20105XLAXM_004]; the Fondazione Cassa di Risparmio Perugia [grant number 2015.0337.021 Ricerca Scientifica e Tecnologica]; and the "Fondo di Ateneo per la Ricerca di Base 2015" financed by University of Perugia.

ACKNOWLEDGMENT

The authors thank Dr. Chris Berrie for critical appraisal and editing of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

REVIEW
published: 02 March 2017
doi: 10.3389/fpls.2017.00278

Interactive Effects of UV-B Light with Abiotic Factors on Plant Growth and Chemistry, and Their Consequences for Defense against Arthropod Herbivores

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Ultraviolet-B (UV-B) light plays a crucial role in plant–herbivorous arthropods interactions by inducing changes in constitutive and inducible plant defenses. In particular, constitutive defenses can be modulated by UV-B-induced photomorphogenic responses and changes in the plant metabolome. In accordance, the prospective use of UV-B light as a tool to increase plant protection in agricultural practice has gained increasing interest. Changes in the environmental conditions might, however, modulate the UV-B -induced plant responses. While in some cases plant responses to UV-B can increase adaptation to changes in certain abiotic factors, UV-B-induced responses might be also antagonized by the changing environment. The outcome of these interactions might have a great influence on how plants interact with their enemies, e.g., herbivorous arthropods. Here, we provide a review on the interactive effects of UV-B and light quantity and quality, increased temperature and drought stress on plant biochemistry, and we discuss the implications of the outcome of these interactions for plant resistance to arthropod pests.

Keywords: blue light, drought, far-red light, herbivores, photosynthetically active radiation, plant defenses, temperature, ultraviolet-B light

OPEN ACCESS

Edited by:
Massimiliano Tattini, Consiglio Nazionale Delle Ricerche (CNR), Italy

Reviewed by:
Lucia Guidi, University of Pisa, Italy
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *05 December 2016*
Accepted: *15 February 2017*
Published: *02 March 2017*

Citation:
Escobar-Bravo R, Klinkhamer PGL and Leiss KA (2017) Interactive Effects of UV-B Light with Abiotic Factors on Plant Growth and Chemistry, and Their Consequences for Defense against Arthropod Herbivores. Front. Plant Sci. 8:278. doi: 10.3389/fpls.2017.00278

INTRODUCTION

As sessile organisms, plants can respond to simultaneous or sequential changes in abiotic conditions by modulating their physiology and, consequently, chemistry. Plant adaptive responses to external variations in growing conditions can have a profound effect on their responses to biotic stresses (Gouinguené and Turlings, 2002; Goel et al., 2008; Gutbrodt et al., 2011; Nguyen et al., 2016). In particular, light exerts a great impact on how plants are protected against herbivores or pathogens (reviewed by Ballaré, 2014). Light can be used as a powerful tool to increase plant resistance against herbivorous arthropods and, eventually, plant yield. Accordingly, for many crop species, manipulation of light conditions in greenhouses have become a common technique used by growers to increase plant performance, or to control photomorphogenic processes such as flowering (see for a review, Vänninen et al., 2010). In this regard, the prospective use of the ultraviolet-B (UV-B) light component of the solar radiation to enhance crop protection against

pests and pathogens, as well as crop production, has gained increasing interest (Wargent and Jordan, 2013).

Ultraviolet-B (UV-B) light (280–315 nm) constitutes only a small fraction of solar radiation reaching the Earth's surface. It represents, however, a crucial light signal to which plants can respond and develop specific photomorphogenic responses (Jenkins, 2009; Robson et al., 2015). Among these responses, changes in the morphology, physiology, and production of secondary metabolites are commonly described. The UV-B specific photoreceptor UV RESISTANT LOCUS (UVR8) regulates these photomorphogenic responses by controlling the expression of genes involved in the inhibition of hypocotyl elongation, DNA repair, antioxidative defense, and production of phenolic compounds that can act as UV-screening molecules (Rizzini et al., 2011). In order to reduce the oxidative damage and the penetration of UV light to photosynthetic cell layers, plants can accumulate flavonoids and phenylpropanoids in the leaf epidermis, and in both the palisade and spongy mesophyll tissues (Mazza et al., 2000; Agati et al., 2013).

Adaptive responses to changing UV-B conditions play an important role in plant–herbivores interactions as well. UV-B-mediated changes in plant architecture, physiology, and/or chemistry can alter herbivorous arthropod's performance and preference. In most cases, these UV-B-mediated induced physiological changes lead to the reinforcement of plant defenses. For example, increased production of UV-B-protective secondary metabolites and/or the reinforcement of plant cell walls induced by UV-B were proposed to affect plant colonization by herbivorous arthropods (Mazza et al., 1999, 2013; Rousseaux et al., 2004; Caputo et al., 2006; Foggo et al., 2007; Kuhlmann and Müller, 2010; Mewis et al., 2012; Zavala et al., 2015). However, in spite of the increasing literature of UV-B effects on plant–insect interactions, our current understanding is still hampered by the lack of an integrated approach that allows us to predict plant responses to diverse and changing environmental conditions. This aspect is of great importance when aiming for more environmental friendly agronomic practices and optimization of culture conditions. Modifications of plant chemistry and/or physiology by UV-B light can determine the responses of plants to other environmental variables, and vice versa (**Figure 1**). In natural conditions plants have to cope with constant variations in light intensity and quality, as well as with variations in abiotic factors such as increased temperature and reduced water availability. In some cases, responses to UV-B and to variations in these abiotic conditions converge to increase plant adaptation and, in addition, increase resistance to biotic stresses. However, antagonistic interactions between these responses may also occur and they may decrease plant defenses. Studies addressing these interactive effects on plant biochemistry and, eventually, the degree of resistance to arthropod herbivores are, however, lacking. In this review, we provide an overview on the existing knowledge on the single and interactive effects of UV-B and light quantity and quality, increased temperature and drought stress on plant growth and chemistry. We particularly focus on the possible implications for plant performance and protection against herbivorous arthropods.

EFFECTS OF UV-B-MEDIATED INDUCED SECONDARY METABOLITES ON PLANT DEFENSES AGAINST HERBIVORES

Ultraviolet-mediated induction of phenolic compounds is one of the most common described plant responses that can directly alter the feeding of herbivorous insects. For instance, solar UV-B-mediated induction of the isoflavonoid glycosides daidzin and genistin in soybean (*Glycine max*) pods was reported to be negatively correlated with the percentage of damaged seeds by the stink bugs *Nezara viridula* and *Piezodorus guildinii* (Zavala et al., 2015). This was explained by the fact that isoflavonoids, a type of compounds restricted to plants of the Fabaceae family, are one of the main chemical defenses against herbivorous arthropods in soybeans. Also, chlorogenic acid, a phenolic acid induced by solar UV-B in *Nicotiana attenuata* (Đinh et al., 2013) is reported to participate in plant defenses against insects. Oxidation of chlorogenic acid by plant polyphenol oxidases (PPOs) and peroxidases occurs after disruption of plant tissues caused by herbivory. This results in the production of highly reactive quinones that can covalently bind to leaf proteins and inhibit their digestion by the herbivore (War et al., 2012). Also, Đinh et al. (2013) described that not only phenolic acids, but also the activity of defensive proteinase inhibitor proteins and levels of diterpene glucosides in *N. attenuata* plants were induced by solar UV-B. Interestingly, those authors demonstrated that the UV-B-mediated induction of a specific diterpene glycoside played a major role in *N. attenuata* defenses against the mirid *Tupiocoris notatus*. Hence, UV-B can modulate the production of different plant chemicals varying in their effects on plant resistance. Likewise, Mewis et al. (2012) described the UV-B-mediated induction of two different plant defense-related metabolites, flavonoids, and glucosinolates, in broccoli (*Brassica oleracea*) sprouts. This induction positively correlated with higher levels of resistance against the caterpillar *Pieris brassicae* and the aphid *Myzus persicae*. Glucosinolates produced by plants belonging to the order of Brassicales are nitrogen- and sulfur-containing glucosides that are hydrolyzed by myrosinases upon tissue disruption. The resulting hydrolyzed compounds, i.e., mainly isothiocyanates and nitriles, possess high toxicity against some herbivorous arthropods (Jeschke et al., 2015). However, whether UV-B-mediated induction of glucosinolates, alone or in combination with flavonoids, is responsible for the enhanced resistance against those herbivores has not been fully addressed. These examples highlight the complexity of the interactions between the UV-B-induced chemical defenses and herbivorous arthropods. Nevertheless, we can speculate that the overlapping plant responses to UV-B and herbivore's attack might have a similar impact on plant defenses. For instance, this would be the case of common UV-B and herbivory-mediated induction of chlorogenic acid in *N. attenuata* plants (Izaguirre et al., 2007). In the same study, however, the flavonoid rutin was

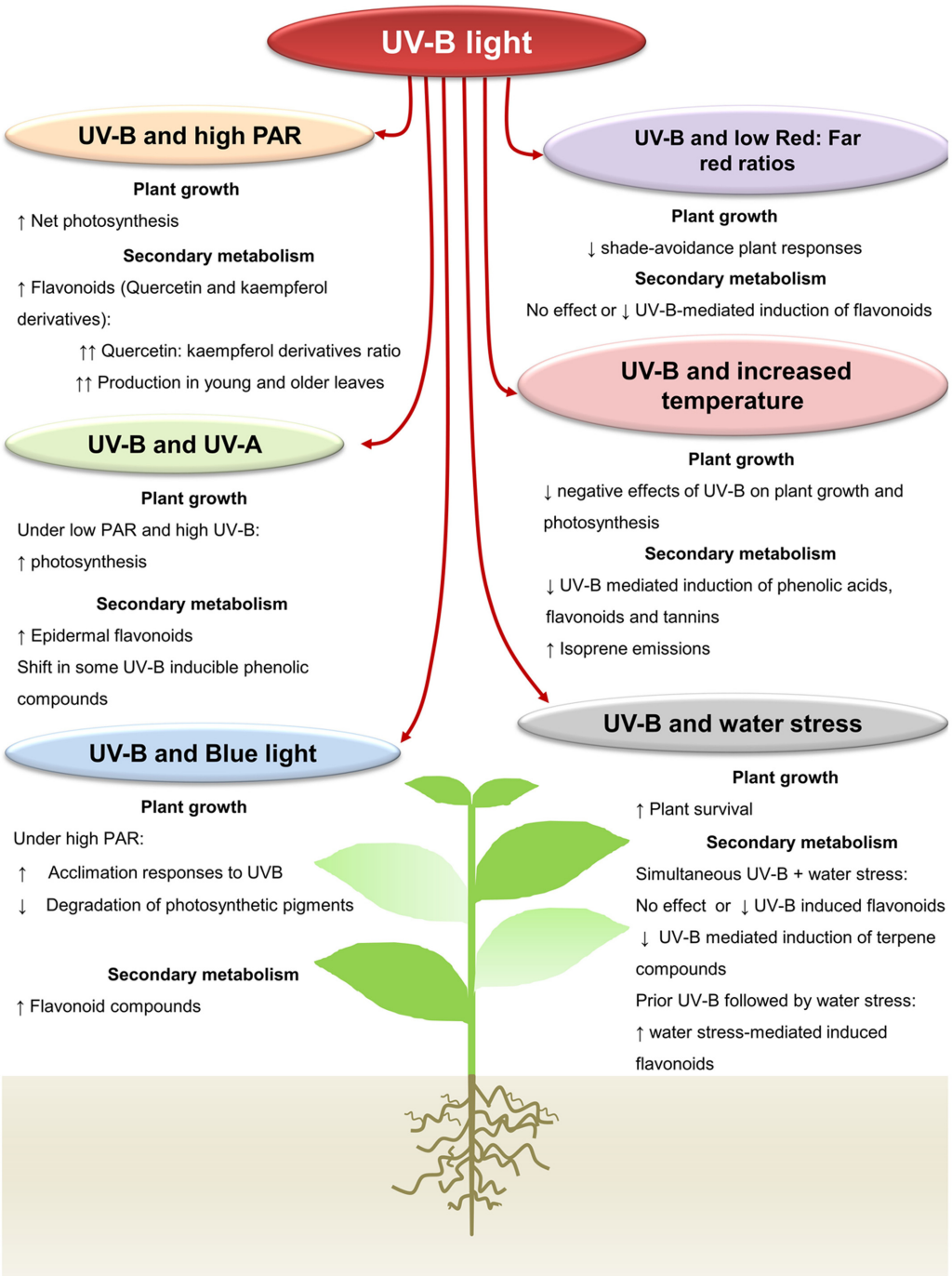

FIGURE 1 | Interactive effects of UV-B light with other abiotic factors on plant growth and production of plant secondary metabolites. Under high Photosynthetic active radiation, UV-B light increases the net plant photosynthesis in several plant species. Higher production of flavonoids can be induced under both UV-B and high PAR in young and old plant leaves. UV-A radiation has a positive effect on the photosynthesis when plants are exposed to UV-B. Higher epidermal flavonoids are detected in plants under both UV-A and B radiations in some plant species. Exposition of plants to blue light prior or subsequent to UV-B also increases the acclimation responses to UV-B by reducing the degradation of photosynthetic pigments. Antagonistic responses between UV-B radiation and low-Red:far-red ratios have been reported. UV-B can inhibit the shade avoidance associated responses under low-Red:far-red ratios. Likewise, a low-Red:far red ratio can reduce the UV-B-mediated induction of plant flavonoids. Increased temperature increases acclimation of plants to UV-B, though it can reduce the UV-B-mediated induction of plant phenolics. Under combined UV-B radiation and increased temperature, however, higher emission of the plant volatile isoprene can be detected in some plant species. Similarly, under UV-B and water stress conditions, a positive effect on plant survival is reported. Production of UV-B-induced flavonoids can be modulated by the application of UV-B prior or subsequent to water stress.

induced by UV-B, but not by herbivory. Increases in the levels of rutin, and also kaempferol derivatives, is a common response to UV-B in many plant species. Though these compounds have been reported to confer anti-herbivore properties, their role in plant defenses have been only addressed in a few studies, and these effects seem to depend on their concentration *in planta*. For instance, low rutin (quercetin-3-*O*-β-rutinoside) concentrations acted as phagostimulants to some polyphagous insects (e.g., *Schistocerca americana*, *Schistocerca albolineata,* and *Melanoplus differentialis*), but high concentrations deterred their feeding (reviewed by Simmonds, 2001). Yet, the degree of resistance given by an increase in these UV-B-induced compounds might also depend on the herbivore species. While increased susceptibility of *Arabidopsis* plants to the specialist caterpillar *P. brassicae* was associated with a significant reduction of kaempferol-3,7-dirhamnoside, no effect was observed for the specialist aphid *Brevicoryne brassicae* (Onkokesung et al., 2014).

In addition to the effect of UV-B on constitutive defenses (i.e., prior herbivore attack), UV-B has been demonstrated to alter the magnitude of the inducible plant defenses upon herbivory. When challenged by the feeding of arthropod herbivores, plants can perceive and display specific defense responses that are mainly regulated by the phytohormones jasmonic acid (JA), salicylic acid (SA), ethylene (ET), and abscisic acid (ABA) (Pieterse et al., 2012). Fine tuning plant defense responses is ultimately achieved by the cross-talk between JA, SA, ET, ABA, and other phytohormones. Activation of these signaling pathways is herbivore-species specific, and it leads to the production of defensive compounds such as secondary metabolites (e.g., alkaloids, glucosinolates, terpenes) and defensive proteins (e.g., proteinase inhibitors and PPOs) that deter herbivore's feeding or alter its performance. In particular, activation of JA-associated defenses has been associated with increased resistance against leaf-chewing, piercing-sucking and some phloem feeding arthropods. In line with this, Đinh et al. (2013) demonstrated that UV-B exposure of *N. attenuata* plants enhanced the JA burst and altered the accumulation of toxic 17-hydroxygeranyllinalool diterpene glycosides after infestation with the mirid *T. notatus*. Constitutive levels of JA, JA-isoleucine (JA-Ile) and ABA were not altered by the presence of solar UV-B, but herbivore-mediated induced JA defenses were augmented (i.e., primed) and, ultimately, plant resistance. Similarly, Demkura et al. (2010) demonstrated that UV-B-mediated induction of plant resistance to thrips (*Thrips tabaci* and *Frankliniella* spp.) in wild tobacco (*N. attenuata*) depended on the increased plant sensitivity to JA. Notably, though UV-B irradiated *N. attenuata* plants impaired in the JA pathway increased rutin and chlorogenic acid production, they did not display augmented resistance against thrips. This was explained by the necessary induction of the anti-herbivore PPOs, controlled by JA signaling, and whose preferred enzymatic substrate is chlorogenic acid. Therefore, the extent to which the plant's chemical changes induced by UV-B confer antiherbivore properties can be highly related to the UV-B-mediated modulation of induced plant defenses.

INTERACTIVE EFFECTS OF UV-B AND ABIOTIC FACTORS ON PLANT GROWTH, CHEMISTRY AND DEFENSES AGAINST HERBIVORES

UV-B and Photosynthetically Active Radiation

Several studies have addressed the role of photosynthetically active radiation (PAR) (400–700 nm) in the modulation of plant sensitivity and photomorphogenic responses to UV-B radiation and vice versa. Direct, e.g., increased photorepair, photoreactivation and levels of photoprotective compounds, as well as indirect mechanisms, e.g., leaf anatomical changes, have been postulated to explain the UV protective effects of high PAR light conditions (Cen and Bornman, 1990; Deckmyn and Impens, 1997; Bolink et al., 2001; Krizek, 2004; Hoffmann et al., 2015). However, recent experimental evidence suggests that high PAR and UV-B might have a synergistic and positive effect on plant photoprotection. Prior exposure to UV-B has been shown to increase the net photosynthesis after subsequent exposition to high-light intensity conditions in lettuce (*Lactuca sativa*) (Wargent et al., 2011, 2015). Likewise, UV-B stimulated photosynthesis rates in Swedish ivy (*Plectranthus coleoides*) by increasing CO_2 assimilation rate, stomatal conductance and internal CO_2 concentration under high, but also low, PAR conditions (Vidović et al., 2015). Notably, under natural sunlight conditions, photo-inhibition (i.e., light-induced inactivation of photosystem II) in pumpkin (*Cucurbita pepo*) has been suggested to be caused by the UV-A, but not the UV-B component of solar radiation (Hakala-Yatkin et al., 2010). Moreover, this photoinhibitory effect is suggested to be attenuated by UV-B-inducible screens, i.e., accumulation of phenolic compounds in the plant epidermis.

Light intensity or distinct PAR levels have been demonstrated to influence the inducibility of plant responses triggered by contact with herbivore's cues, but also to affect constitutive plant defenses. Gouinguené and Turlings (2002) showed that increasing light intensities positively correlated with increased volatile production in herbivore- induced corn (*Zea mays*) plants. This might be correlated with an increase in the hormone-signaling involved in these defense responses. For instance, an enhanced generation of JA precursors has been described under high light conditions (Frenkel et al., 2009). In tomato, constitutive levels of defensive leaf trichome densities and their associated allelochemicals were induced by increased light intensity, which correlated with augmented resistance against the caterpillar *Manduca sexta* (Kennedy et al., 1981). Furthermore, high PAR has been reported to induce other leaf secondary metabolites, such as flavonoids and phenolic acids, which might affect plant–insect interactions. A synergistic effect in the production of these compounds is often reported when both high PAR and UV-B irradiance is applied to plants (Götz et al., 2010; Guidi et al., 2011; Barnes et al., 2013; Müller et al., 2013; Vidović et al., 2015). This suggests a common acclimation response of plants to both light signals (Wargent et al., 2015) and, therefore, also their

possibly positive effect on plant defenses against biotic stresses. Interestingly, when both high PAR and UV-B irradiances are applied, a greater increase in the concentration of flavonoids is detected in old plant leaves. For instance, while high PAR induced the accumulation of flavonoids in young leaves of barley (*Hordeum vulgare*) plants, a combined treatment with high UV-B increased the production of these compounds in older leaves as well (Klem et al., 2012). Similarly, higher flavonoid production in young, but also in older leaves of silver birch (*Betula pendula*) plants grown under ambient PAR and UV-B have been reported (Morales et al., 2013). The fact that older leaves experience an increase in the content of phenolic compounds might have repercussions for plant protection against herbivores. Some herbivore arthropods show a high feeding and oviposition preference for older parts of the plants over the young ones. Some examples are the whitefly *Bemisia tabaci* (Zhang and Wan, 2012) and the thrips *Frankliniella fusca* on tomato (*Solanum lycopersicum*) (Joost and Riley, 2008), *F. occidentalis* on *Senecio* hybrids (*Senecio jacobaea* × *Senecio aquaticus*) (Leiss et al., 2009) and tomato (Mirnezhad et al., 2010), and the larvae of *Spodoptera litura* on radish (*Raphanus sativus*) (Yadav et al., 2010). We can, therefore, speculate that an encounter of the herbivore with better protected old leaves might negatively impact their performance and/or survival.

Besides the enhancement of constitutive defenses in older parts of the plant by combined high PAR and UV-B conditions, it remains unknown whether this positive effect also extends to an increase in the capacity of older plant parts to respond to herbivore's attack. Old plant leaves are reported to be less responsive to herbivore-mediated induced defenses, which might affect direct and indirect (i.e., attraction of natural enemies of the herbivore) defense responses. For example, the predator *Phytoseiulus persimilis* was reported to be attracted to the emitted-volatiles of spider mites-infested young leaves of cucumber plants, but less to infested old leaves (Takabayashi et al., 1994). These indirect induced defenses are controlled by JA and SA signaling pathways (Ament et al., 2004). A higher induction of these defenses in young leaves with respect to older ones might explain these differences. As UV-B can prime JA-mediated induced defenses against insects, we might hypothesize that combined high PAR and UV-B conditions do not antagonize each other, but they rather might have a positive and/or synergistic effect on these inducible plant defenses. This is an aspect that needs further research.

UV-B and UV-A

Ultraviolet-A (315–400 nm) constitutes the major component of the solar UV spectrum. Plants perceive and respond to UV-A by inducing photomorphogenic responses that, in some cases, resemble those triggered by UV-B. For example, stem elongation and leaf enlargement were decreased under ambient UV-A in cucumber (*Cucumis sativus*) (Krizek et al., 1997) and lettuce (Krizek et al., 1998). Interestingly, UV-A can interact with UV-B to modulate plant responses. For instance, UV-A can mitigate the deleterious effects of UV-B on the photosynthetic apparatus under low PAR conditions (Adamse et al., 1994), as demonstrated in barley (Štroch et al., 2015, cluster bean (*Cyamopsis tetragonoloba*) (Joshi et al., 2007, 2013) and the woody shrub *Pimelea ligustrina* (Turnbull et al., 2013).

In contrast to the well-known effects of UV-B on plant–insect interactions mediated by changes in plant quality, the role of UV-A has not been well explored so far. The effects of UV-A on constitutive chemical defenses, however, can differ from those induced only by UV-B. For instance, higher accumulation of epidermal flavonoids was not stimulated by UV-A, but by a combined UV-A and UV-B treatment in silver birch (Morales et al., 2010, 2011) and *Arabidopsis* (Morales et al., 2013). These results suggested a major role of UV-B on the induction of flavonoids. However, UV-A has been described to modulate the UV-B associated responses in plants. For instance, in turnip hypocotyls, while both UV-A and B induced anthocyanin biosynthesis, the pattern of anthocyanin accumulation along the hypocotyl greatly differed depending on the wavelengths of UV applied (Zhou et al., 2007; Wang et al., 2012). Also, Morales et al. (2010) described different changes in the abundance of specific flavonoids when UV-A or UV-B were depleted. Under exclusion of UV-B, young silver birch leaves accumulated less of six epidermal flavonoids (i.e., myricetin-3-galactoside, quercetin-3-galactoside, quercetin-3-rhamnoside, and kaempferol-3-rhamnoside), while UV-A exclusion decreased the accumulation of only quercetin-3-galactoside and quercetin-3-arabinopyranoside. Likewise, Wilson et al. (2001) reported that UV-A reduced the production of UV-B-inducible flavonoids in rape by shifting the abundance of particular quercetin compounds. A common regulatory component of plant responses to both types of UV was therefore proposed. In particular, Morales et al. (2013) suggested that the UV-photoreceptor UVR8 may be involved in the UV-A regulation of individual metabolites in *Arabidopsis*. This was supported by the necessary activation of UVR8 for UV-A induction of kynurenic and chlorogenic acids, tryptophan, phenylalanine, kaempferol and kaempferol-3-rhamnoside in *Arabidopsis* (Morales et al., 2013). How these UV-A and UV-B interactions might affect plant responses to herbivory is still unknown. However, we might hypothesize that changes in the abundance of plant specific phenolics may alter the feeding behavior of herbivorous arthropods. This might be illustrated by the experiments performed by Hamamura et al. (1962). These authors demonstrated that while the quercetin-3-*O*-glucoside acted as a feeding stimulant for the silkworm (*Bombyx mori*) in white mulberry (*Morus alba*) leaves, another quercetin glucoside, quercetin-3-*O*-rhamnoside, deterred larval feeding, and 3-*O*-rutinoside did not have any effect at all.

UV-B and Blue Light

Blue light (400–500 nm) regulates diverse plant processes such as phototropism, photomorphogenesis, stomatal opening, and leaf photosynthetic functioning (Whitelam and Halliday, 2008). During plant growth it constitutes an essential part of the development of higher plants. For instance, increasing levels of supplemental blue light are positively correlated with leaf photosynthesis, even under low light irradiances in cucumber (Hogewoning et al., 2010). Under red light conditions, it has been reported that supplemental blue light can enhance dry

matter production in radish, lettuce, and spinach (*Spinacia oleracea*) (Yorio et al., 2001; Johkan et al., 2010), as well as leaf photosynthesis in pepper (Brown et al., 1995) and rice (*Oryza sativa*) (Matsuda et al., 2004). Yet, the intensity of combined red and blue light conditions was suggested to determine the energy efficiency and the net photosynthesis rate in tomato (Fan et al., 2013).

Supplemental blue light in plants prior to, simultaneously with, or subsequent to UV-B exposure, can prevent the damaging effects of high UV-B radiation, therefore showing certain similarities with the effects described for high PAR. For instance, blue light (i.e., 62% of PAR) increased the acclimation of pepper and cucumber plants to UV radiation under high light intensity conditions (Adamse et al., 1994; Hoffmann et al., 2015). This has been explained by a lower degradation of photosynthetic related pigments (chlorophyll a and b, and carotenoids) by UV (Hoffmann et al., 2015), as well as the increase in epidermal flavonols when plants were grown under enriched blue light radiation (Adamse et al., 1994; Ebisawa et al., 2008; Son and Oh, 2013; Hoffmann et al., 2015; Ouzounis et al., 2015; Siipola et al., 2015). These observations have led to some authors to propose blue light as the major constituent of the sunlight responsible for the upregulation of the epidermal content of flavonoids (Ouzounis et al., 2014; Siipola et al., 2015). If so, its importance in the reinforcement of plant defenses against herbivorous arthropods might be highly overlooked. In line with this, diminished blue light was reported to reduce the accumulation of quercetin derivatives in apical, cauline and basal leaves of pea (*Pisum sativum*) (Siipola et al., 2015) which, as discussed previously, might influence plant protection against herbivores in older and, therefore, more susceptible plant leaves. Also, the distribution of flavonoid compounds in the plant under different solar/blue radiation might not only differ in young and old leaves, but also within the leaf cell layers. For example, flavonoid accumulation in shade leaves of the green olive tree (*Phillyrea latifolia*) has been reported to occur mainly in the adaxial epidermal layer. However, in sun leaves of this tree flavonoids also accumulated in sub-epidermal cells leading to a steeper gradient in flavonoid concentration from the adaxial epidermis to the inner spongy layers (Tattini et al., 2000; Agati et al., 2002). A deeper distribution of these compounds within the plant leaf might alter the performance of herbivores that feed preferentially on the mesophyll cell layers while avoiding the epidermis, such as the larvae of leaf miners (Sinclair and Hughes, 2010) or cell-content feeders as thrips (Chisholm and Lewis, 1984) and spider mites (Helle and Sabelis, 1985).

Though plant perception and responses to blue and UV-B light have been addressed in several studies (see review by Huché-Thélier et al., 2016), their interactive effects on feeding and/or survival of herbivorous arthropods have not been investigated so far. Yet, the similarities in the plant responses triggered by both light signals suggests that the positive UV-B effect on plant defenses against herbivores might not be counteracted by blue light, but the contrary. Supporting this hypothesis, a synergistic effect between blue and UV-B on the production of UV-B-induced flavonoids has been reported to occur under both light irradiances. This is the case of the production of anthocyanins, which was significantly enhanced under combined blue light and UV-B radiation conditions in the hypocotyls of turnip seedlings (Wang et al., 2012). This might be explained by the reported synergy in the induction of key genes involved in flavonoid biosynthesis such as *chalcone synthase* (*CHS*) in *Arabidopsis* (Fuglevand et al., 1996; Wade et al., 2001) and turnip (*Brassica rapa*) (Wang et al., 2012), as well as *flavonol synthase* in lettuce (Ebisawa et al., 2008), under combined blue and UV-B light conditions. However, whether these transcriptomic responses also extend to augmented plant responses against herbivorous arthropod is still unknown.

UV-B and Far-Red Light

Far-red (FR) light (700–780 nm) modulates a wide range of physiological responses in plants. Higher FR radiation or low-Red (R):FR ratios, resulting from shade conditions, constitute a signal of competition for light in dense plant canopies (Ballaré, 1999). In shade-avoidance species, such as *Arabidopsis*, typical plant responses to low R:FR ratios are principally regulated by the phytochrome B and include hyponasty (i.e., more vertical orientation of the leaves) and enhanced stem and petiole elongation (reviewed by Pierik and de Wit, 2013).

In general, plant morphological and biochemical features under low R:FR conditions have been associated with weaker defense responses against herbivores (Izaguirre et al., 2006; Kegge et al., 2013). For instance, in FR-supplemented tobacco (*N. longiflora*) plants, the caterpillar *M. sexta* grew faster than on ambient light-treated plants (Izaguirre et al., 2006). Likewise, growth of *S. frugiperda* caterpillar in *Arabidopsis* was increased when plants were grown under enriched FR conditions (Moreno et al., 2009). This was explained by the R:FR- mediated suppression of inducible plant defenses controlled by the JA and SA defense-related hormone signaling pathways (Wit et al., 2013; Radhika et al., 2010). However, constitutive defenses can be also affected. For instance, Cortés et al. (2016) recently demonstrated that density of defensive trichomes was reduced in the stems of tomato mutants defective in the perception of red light by the phytochrome B. Moreover, these authors also reported lower concentration of leaf flavonoids in the tomato mutants. In line with this, inactivation of phytochrome B by low R:FR ratios has been shown to negatively regulate induction of *CHS* in *Arabidopsis* (Wade et al., 2001).

Recently, it has been demonstrated that UV-B perception by plants blocks the signal transduction triggered by low R:FR conditions (Hayes et al., 2014; Mazza and Ballaré, 2015). These results agree with a previous study by Tegelberg et al. (2004) showing that under combined supplemental UV-B and FR light conditions, concentrations of quercetins, kaempferols, and chlorogenic acid were increased by UV-B irrespective of the FR treatment in silver birch seedlings. However, UV-B-mediated induction of plant flavonoids can depend on the FR doses plants are exposed. Gerhardt et al. (2008) observed a suppression of UV-B-mediated induction of flavonoids in FR light pre-irradiated *B. napus* plants when increasing the amount of FR in the spectrum. These authors also described that under supplemental UV-B and moderate levels of FR, higher contents of kaempferol glycosides were detected, while levels of quercetin glucosides were

reduced. Overall, these results suggest that the magnitude of the UV-B or FR light signal can determine the outcome of plant chemical responses and, therefore, constitutive plant defenses against herbivores. In addition, as UV-B has been shown to increase plant responsiveness to JA-defenses, a negative trade-off between plant responses to low R:FR ratios and UV-B light might be expected. However, whether the negative effects of low R:FR ratio on plant defenses can be neutralized by the positive impact on constitutive and/or inducible plant defenses of UV-B is an aspect that needs further research.

UV-B and Increased Temperature

Temperature is a key parameter regulating many processes of plant physiology. Increases in temperature are reported to lead to increased plant growth, as an effect of enhanced photosynthetic rates (Tollenaar, 1989; Saxe et al., 2001; Nybakken et al., 2012). Though there are many studies describing the effect of temperature and UV-B on plants separately, there is limited research on their combined effects on plant physiology and/or chemistry. Most of the these studies, however, report a compensatory effect of enhanced temperature on UV-B-mediated inhibition on plant growth. In studies with sunflower (*Helianthus annuus*) and maize (*Zea mays*), for instance, Mark and Tevini (1996) showed that an increase in temperature (28–32°C) resulted in higher values of absolute growth parameters regardless of UV-B treatment. Moreover, these authors described that higher temperature compensated the negative effect of UV-B on plant growth. Similar findings were reported by Han et al. (2009) for dragon spruce (*Picea asperata*) seedlings. Enhanced UV-B reduced growth, chlorophyll content and net photosynthesis rate, but these effects were alleviated by higher temperature. On the other hand, pre-exposition to low and ambient doses of UV-B promoted heat tolerance in cucumber (Teklemariam and Blake, 2003) and conifer seedlings (L'Hirondelle and Binder, 2005).

Temperature is a very important factor affecting herbivore performance directly (Bale et al., 2002), or indirectly by altering the host plant quality (Grinnan et al., 2013). For instance, temperature-dependent changes in primary and secondary plant metabolites were suggested to explain the negative effects of increased temperature (17–25°C) on *Pieris napi* larvae development on *Sinapis alba* (Bauerfeind and Fischer, 2013). Enhanced temperature conditions, however, have shown to decrease the content of phenolic compounds in dark-leaved willow (*Salix myrsinifolia*) (Veteli et al., 2002; Paajanen et al., 2011; Nybakken et al., 2012) and Norway spruce (*Picea abies*) (Virjamo et al., 2014). This negative regulation of plant phenolics is most probably caused by the temperature-dependent regulation of genes involved in their biosynthesis. Transcript levels of essential regulators of flavonoids in *Arabidopsis* are reported to strongly increase under decreasing temperatures (Olsen et al., 2009; Dao et al., 2011). Furthermore, a slower degradation of quercetin and kaempferol glycosides has been described under lower temperatures (Olsen et al., 2009). Under both UV-B and enhanced temperature the negative effect on plant phenolics persists in dark-leaved willow plants, where combined increased temperature (2°C above ambient) and UV-B decreased the content of phenolic compounds (i.e., chlorogenic and cinnamic acids) (Nybakken et al., 2012). In addition to phenolic acids, tannins have been reported to be negatively affected by the interaction between temperature and UV-B. For instance, in European aspen (*Populus tremula*) seedlings, Randriamanana et al. (2015) observed a genotype-specific reduction of soluble condensed tannins when both UV-B and temperature (13.7–24°C) were augmented. In the same study, flavonoid production was induced by enhanced UV-B, but diminished by increased temperature. Nevertheless, there are some studies in the literature that did not describe negative interactions between enhanced temperature and UV-B on plant phenolics (Lavola et al., 2013; Neugart et al., 2014). Lavola et al. (2013) described that UV-B increased the accumulation of condensed tannins, quercetin derivatives, rhamnosylated kaempferol and phenolic acids in silver birch seedlings, but these compounds were not affected by elevated temperature (21.9–24.4°C) (Lavola et al., 2013). This might be explained by a lower responsiveness of flavonoid biosynthesis-related genes when variations in temperature occur in that specific range (Olsen et al., 2009). Still, how UV-B and temperature interact at the transcriptional level regulating the production of phenolic compounds is an aspect that has not been investigated yet.

As previously described, flavonoids might determine plant resistance against herbivorous arthropods, and a reduced UV-B-mediated induction of these compounds might influence these interactions. However, condensed tannins, in turn, are not clearly associated with negative effects on insect herbivore performance (reviewed by Barbehenn and Constabel, 2011). Only a few studies have reported negative correlations between the presence of condensed tannins and insect feeding and/or performance. Moreover, higher production of condensed tannins was reported to increase caterpillar feeding in transgenic hybrid aspen (*Populus tremula* × *tremuloides*) (Boeckler et al., 2014) and thrips damage in poplar (Mellway and Constabel, 2009). Tannins can precipitate proteins only at low pH. While the gut of vertebrate animals has a low pH, the gut of many arthropods is highly alkaline. Tannins might also be oxidized in the guts of insects, generating quinones that might link to proteins and make them non-digestible for the insect. However, recent reports showed that condensed tannins are the least oxidatively active, and that some condensed tannins even inhibit the pro-oxidant activity of ellagitannins (hydrolysable tannins) (reviewed by Salminen and Karonen, 2011). We can, therefore, consider that a reduction in condensed tannins might not have direct effects on plant–insect interactions. However, Madritch and Lindroth (2015) recently showed that variation in condensed tannin concentration is correlated with plant nitrogen recovery following a severe defoliation event, such as that caused by herbivory. From this point of view, reduced condensed tannins might influence plant tolerance to insect herbivores, i.e., by reducing re-growth capacity, rather than defense.

Though it seems that increased warming might cause a reduction in plant protection against herbivorous arthropods by diminishing the UV-B-mediated accumulation of phenolics, other defense-related compounds are reported to increase under incremental temperatures. Emission of volatile organic

compounds (VOCs) can be positively influenced by temperature, as reported for monoterpenes in sunflower and beech trees (*Fagus sylvatica*) (Schuh et al., 1997), and for the sesquiterpene β-caryophyllene in orange trees (*Citrus sinensis*) (Hansen and Seufert, 2003). Besides protecting plants from abiotic stresses, plant volatiles are crucial in plant–arthropod interactions. For instance, the sesquiterpene β-caryophyllene has been described as a key component in the attraction of plant enemies to insect infested-maize plants (Köllner et al., 2008). Interestingly, a positive interaction between UV-B and enhanced temperature on VOCs production has been recently described by Maja et al. (2016). They reported higher isoprene emissions in European aspen under enhanced UV-B radiation (31% above ambient) but only in combination with increased temperature (ambient +2°C) conditions. Isoprene is the dominant VOC released to the atmosphere by vegetation, though not emitted by all plant species (Kesselmeier and Staudt, 1999). Apart from its antioxidant capacity, isoprene can mediate plant–herbivore interactions. For instance, Laothawornkitkul et al. (2008) demonstrated that the emission of isoprene in tobacco (*N. tabacum*) deterred feeding of *M. sexta* caterpillars. The ecological importance of these interactions for plant–insect interactions, however, remains to be determined.

UV-B and Drought Stress

The interrelation between drought stress and UV-B, and their combined action on plant physiology has been amply studied (see Bandurska et al., 2013, for review). When moderate UV-B and drought conditions occur simultaneously or sequentially, both can interact synergistically to increase plant tolerance and, consequently, plant survival. These responses were associated to enhanced production of antioxidant proteins, UV-B absorbing compounds and higher leaf cuticle thickness, among others. However, less is known about the outcome of drought stress and UV-B interactions on the production of plant chemical defenses and their effects on plant–herbivore interactions.

Drought stress has been shown to greatly influence plant resistance and defense responses against herbivores (see review by Foyer et al., 2016). However, these drought-induced plant responses have shown no clear pattern in their effects on insects. Though some phytophagous insects benefit from water-stressed hosts (Mewis et al., 2012; Tariq et al., 2013), this abiotic stress can also interact negatively with the performance of herbivorous arthropods (Nguyen et al., 2016; Pineda et al., 2016). Positive effects might be explained by the increased content of amino acids and soluble sugars (Mewis et al., 2012). In turn, negative effects have been proposed to be explained by the reduction in turgor pressure, water content, plant growth, and higher concentration of allelochemicals. For instance, flavonoids and anthocyanins have been reported to accumulate in wheat (*Triticum aestivum*) (Ma et al., 2014), and soluble phenols in pea (Alexieva et al., 2001), under drought stress conditions. Interestingly, constitutive levels of glucosinolates have been reported to be affected by drought stress as well. For instance, Mewis et al. (2012) described increased levels of flavonoids and glucosinolates in drought-stressed *Arabidopsis* plants. In the same study, however, the generalist aphid *M. persicae* performed better in plants subjected to drought, which suggested that induction of glucosinolates did not have a great impact on these interactions. Conversely, Pineda et al. (2016) described that drought conditions decreased the population growth of *M. persicae* in *Arabidopsis*. These authors also showed that this negative effect was maintained in *Arabidopsis* mutants defective in the production of glucosinolates, suggesting the existence of other mechanisms involved in these interactions. However, whether other drought-induced secondary metabolites had a role in *Arabidopsis* resistance to aphids was not further investigated. In this regard, not only phenolic compounds, but higher terpene and benzenoid emissions have been described for some plant species subjected to drought stress or combined drought and herbivory (Copolovici et al., 2014; Weldegergis et al., 2015). Mono- and sesquiterpenes can protect plant membranes against peroxidation and water stress-induced reactive oxygen species by acting as potent antioxidants (Tattini et al., 2015), but they also are important mediators in the interactions of plants with herbivores and the herbivore's natural enemies. Hence, the blends of volatiles released by herbivore-infested plants provide natural enemies cues to locate their prey. In line with this, though the effect of drought-mediated induction of plant volatiles on direct plant responses against herbivores has not been clarified yet, their possible impact on indirect plant defenses was recently explored. Weldegergis et al. (2015) showed that drought stress augmented the emission of volatile compounds in *Mamestra brassicae*-infested *B. oleracea* plants. They described that the parasitic wasp *Microplitis mediator* showed equal preference for plant volatiles emitted from *M. brassicae*-damaged plants and plants exposed to combined drought and herbivory. In another study, however, Tariq et al. (2013) reported that plants exposed to both root herbivory and drought negatively affected the preference of a aphid parasitoid for aphid-infested plants. This was explained by the modification of the aphid-mediated induced volatile blend in simultaneously drought stressed- and root feeder-infested plants.

The interactive effects of drought and UV-B on the production of secondary metabolites do not show a clear pattern. Prior treatment with high ratio of UV-B to PAR treatment enhanced the production of flavonoids in pea plants that were subsequently subjected to drought conditions (Nogués et al., 1998). Conversely, simultaneous enhanced UV-B treatment and drought stress dramatically reduced the UV-B-mediated induction of anthocyanins and flavonols in barley (Bandurska et al., 2012) and pea (Alexieva et al., 2001). These contrasting effects might be explained by different experimental conditions, i.e., different levels of drought stress, but also by plant-species specific responses. Regarding VOCs, only a few studies have addressed the effect of combined UV-B and water stress on VOCs emission. Some authors have described that UV-B can alter emissions or increase endogenous leaf accumulation of VOCs (Tiiva et al., 2007; Llusia et al., 2012; Alonso et al., 2015). When combined with drought, however, Alonso et al. (2015) reported lower UV-B-mediated induction of terpene compounds in grapevine (*Vitis vinifera*). Conversely, Llusia et al. (2012) showed that terpene emissions were altered by increased UV-B and water stress in a species-specific manner in Mediterranean species of xerophytes (*Daphne gnidium*, and *Pistacia lentiscus*)

and mesophytes (*Ilex aquifolium* and *Laurus nobilis*). While in one of these species UV-A+B increased terpene emissions, water stress only had a positive effect in another species, and combined water stress and UV-A+B conditions elicited a stronger response. In summary, though drought and UV-B can strongly modulate plant constitutive defenses through changes in secondary metabolites, more effort is needed to elucidate the impact of these chemical changes in plant resistance against herbivores.

In addition, whether the single effects of drought or UV-B on plant induced defenses against herbivores can differ from the plant responses triggered by simultaneous drought and UV-B is an aspect that needs to be elucidated. Remarkably, drought can increase JA accumulation and JA-induced defenses in plants (Nguyen et al., 2016), suggesting that combined drought and UV-B effects on plant responses to insect herbivores might not neutralize each other.

Future Perspectives

Implementation of systems that can modulate UV-B irradiances in greenhouses to increase crop protection against pests is promising. However, modern agriculture is highly dependent on other environmental variations (Mittler and Blumwald, 2010). First, environment conditions have a high influence on plant growth and yield and, second, they influence the outbreaks of crop pests and how plants respond to these attackers. Thus, agricultural systems located in different areas of our planet have to face different climate challenges. Adaptation to these diverse environments requires knowledge to predict the outcome of crop production when UV-B is applied and, accordingly, to implement measures that can benefit plant's performance. For instance, the agricultural challenges in higher altitude and temperate regions differ greatly from those in tropical and subtropical zones. While in the first case limited solar radiation and low temperatures determine agronomy's practices, in the second high PAR, high temperature, high UV-B radiation, reduced water availability and increasing pest's outbreaks are the principal challenges for the sustainability of crops. In middle and higher latitude regions, the cold conditions reduce the survival of arthropod pests, but in order to increase the productivity under limited light and low temperatures the maintenance costs of the greenhouse are higher. Importantly, knowledge on light perception and responses by plants to the interactive effects of UV-B light with other abiotic conditions can help to optimize culture conditions. For instance, understanding the effects of UV-B:PAR ratio on plant chemistry has been shown to be fundamental to avoid plant stress, and to promote desirable photomorphogenic responses (reviewed by Wargent and Jordan, 2013). This is of special importance in greenhouses installed in higher latitudes of temperate areas, where low PAR levels during winter require the use of supplemental light systems (see review by Vänninen et al., 2010).

In warmer areas, pest's outbreaks have a predominant influence on crops productivity. In order to increase protection against pests, traditional greenhouses are generally built with plastic materials that also block the transmission of UV-A and -B light. Lack of UV-B, but specially UV-A, blocks the orientation of some insects inside the greenhouse, such as thrips and whiteflies, which can result in reduced plant damage and transmission of virus diseases (Mazza et al., 1999; see also review by Johansen et al., 2011). These responses, however, are species-specific, and UV-B has been demonstrated to induce avoidance responses in other arthropod pests. Such is the case of the spider mite *Tetranychus urticae* (Barcelo, 1981). Hence, we can speculate that the use of UV-B-transmitting films might be beneficial in areas where this pest is predicted to experience strong upsurges. Though discussion of direct effects of UV-B and other biotic factors on behavior of herbivorous arthropods is not the main goal of this review, their interactions cannot be overlooked when aiming for integrated pest management practices. Increasing evidence presented here, however, suggests that the beneficial effects of UV-B on plant physiology and resistance to pests might also confer as much benefits as the exclusion of this UV light signal from the greenhouse environment.

Ultraviolet-B can interact positively with high PAR, blue light, temperature and water stress to increase plant performance and constitutive chemical defenses. Under mild water stress, for instance, the use of UV-B-transmitting films might alleviate the stress responses of crop plants (Bandurska et al., 2013). Water stress responses are, in some cases, associated with plant susceptibility to herbivorous arthropods. Whether UV-B can ameliorate the negative effects of water restrictions on plant resistance would require investigating different aspects of plant–insects interactions. First, how changes in constitutive defenses alter the insect's preference and performance in the host plant. Second, how inducible plant defenses controlled by plant's hormone signaling are modulated by drought and UV-B. The use of mutant plants deficient in constitutive production of secondary metabolites and/or in herbivore-mediated induced defenses can shed light on the mechanisms operating in UV-B and abiotic factors interactions. Also, to unravel these complex interactions the use of different experimental settings is required. The use of growth cabinets for UV-B supplemental or exclusion experiments exposes plants to unrealistic conditions when compared to greenhouse environments. However, their controlled conditions provide some advantages, as they facilitate the specific assessment of interactions between abiotic factors, and to determine the mechanisms behind. Then, assessment of these effects under greenhouse conditions would be a further step to verify the implementation of new agronomic practices. Remarkably, current literature on the effect of UV-B on plant–insect interactions has mainly focused on a few model plants and crop species, such as *A. thaliana, N. attenuata,* broccoli (*B. oleracea*) and soybean (*G. max*). No studies on the effect of UV-B on plant resistance against herbivores have been described for economically important crop species such as tomato, and less is known about the interactive effects of UV-B and abiotic factors on these interactions. Additionally, how different varieties of the same crop differ in their responses to changing UV-B and abiotic conditions such as drought, heat and light intensity/quality, and how these affect their capacity to protect against major pests is a another question that needs to be investigated.

One of the major limitations in agricultural systems is the area crops plants have available to grow. This leads to reduction in

the inter-plant distances, and results into crowded plant canopies. As a consequence, this environment is enriched in far-red and deficient in red light, which promotes shade-avoidance responses in plants (see review by Ballaré, 2009). Herbivorous arthropods take advantage of this situation, as they find better shelter to escape from natural enemies, protection from direct UV-B damaging effects, and weaker plant defenses. As described here plant responses to UV-B and low-Red:far-red ratios are reported to counteract each other. We hypothesize that implementation of supplemental UV-B within plant canopies might constitute a promising alternative to increase crop protection against pests. Moreover, UV-B-mediated reinforcement of plant defenses against herbivores can increase plant yield in the absence of pesticides, as demonstrated by Mazza et al. (2013) in soybean (*G. max*). Though negative trade-offs between plant growth and defenses are generally the rule, UV-B-mediated reinforcement of constitutive and inducible plant defenses against herbivores might optimize the usage of plant resources. In other words, UV-B-induced production of secondary metabolites can inhibit colonization of insects, thus reducing the energy investment to replace the plant tissues consumed by herbivores (Karban, 2011). Similarly, UV-B-mediated priming of inducible plant defenses, which results in stronger and faster defense responses could stop herbivore infestations at earlier stages, reducing the negative effects on plant growth and yield (Frost et al., 2008). In this regard, it would be interesting to investigate whether a blue light-enriched environment might optimize these UV-B-mediated plant responses. As blue light can increase the photosynthetic capacity of plants, the availability of substrates for production of secondary metabolites might be augmented. Nevertheless, this is an aspect that needs further research.

In summary, modulation of UV-B light in agriculture systems constitutes a promising tool to increase crop production and protection against pests. However, the inherent complexity of the UV-B-mediated effects on plant–herbivore interactions when the crosslink effects of different abiotic conditions are considered demands for a better understanding of plant responses to these changing environment conditions.

AUTHOR CONTRIBUTIONS

RE-B wrote the first concept of the manuscript and made **Figure 1**. PK and KL provided ideas and discussions points and contributed to the final manuscript.

FUNDING

This project is funded by STW Perspective grant program "Green Defense against Pests" (GAP) (Ref. 13553).

ACKNOWLEDGMENTS

We thank the companies involved in the GAP project: Rijk Zwaan, Duemmen Orange, Dekker Chrysanten, Deliflor and Incotec for financial support.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REVIEW
published: 09 February 2017
doi: 10.3389/fpls.2017.00172

Abiotic Stress Responses and Microbe-Mediated Mitigation in Plants: The Omics Strategies

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *07 September 2016*
Accepted: *27 January 2017*
Published: *09 February 2017*

Citation:
Meena KK, Sorty AM, Bitla UM, Choudhary K, Gupta P, Pareek A, Singh DP, Prabha R, Sahu PK, Gupta VK, Singh HB, Krishanani KK and Minhas PS (2017) Abiotic Stress Responses and Microbe-Mediated Mitigation in Plants: The Omics Strategies. Front. Plant Sci. 8:172. doi: 10.3389/fpls.2017.00172

Abiotic stresses are the foremost limiting factors for agricultural productivity. Crop plants need to cope up adverse external pressure created by environmental and edaphic conditions with their intrinsic biological mechanisms, failing which their growth, development, and productivity suffer. Microorganisms, the most natural inhabitants of diverse environments exhibit enormous metabolic capabilities to mitigate abiotic stresses. Since microbial interactions with plants are an integral part of the living ecosystem, they are believed to be the natural partners that modulate local and systemic mechanisms in plants to offer defense under adverse external conditions. Plant–microbe interactions comprise complex mechanisms within the plant cellular system. Biochemical, molecular and physiological studies are paving the way in understanding the complex but integrated cellular processes. Under the continuous pressure of increasing climatic alterations, it now becomes more imperative to define and interpret plant–microbe relationships in terms of protection against abiotic stresses. At the same time, it also becomes essential to generate deeper insights into the stress-mitigating mechanisms in crop plants for their translation in higher productivity. Multi-omics approaches comprising genomics, transcriptomics, proteomics, metabolomics and phenomics integrate studies on the interaction of plants with microbes and their external environment and generate multi-layered information that can answer what is happening in real-time within the cells. Integration, analysis and decipherization of the big-data can lead to a massive outcome that has significant chance for implementation in the fields. This review summarizes abiotic stresses responses in plants in-terms of biochemical and molecular mechanisms followed by the microbe-mediated stress mitigation phenomenon. We describe the role of multi-omics approaches in generating multi-pronged information to provide a better understanding of

plant–microbe interactions that modulate cellular mechanisms in plants under extreme external conditions and help to optimize abiotic stresses. Vigilant amalgamation of these high-throughput approaches supports a higher level of knowledge generation about root-level mechanisms involved in the alleviation of abiotic stresses in organisms.

Keywords: abiotic stress, genomics, metabolomics, microbes, multi-omics, plant–microbe interactions

INTRODUCTION

Adverse climatic conditions creating abiotic stresses are among the principal limiting factors for decline in agricultural productivity (Padgham, 2009; Grayson, 2013). As per the FAO report (2007), only 3.5% of the global land area has left unaffected by any environmental constraint[1]. Dominant abiotic stresses comprise drought, low/high temperature, salinity and acidic conditions, light intensity, submergence, anaerobiosis and nutrient starvation (Wang et al., 2003; Chaves and Oliveira, 2004; Agarwal and Grover, 2006; Nakashima and Yamaguchi-Shinozaki, 2006; Hirel et al., 2007; Bailey-Serres and Voesenek, 2008). Water deficit (drought) has affected 64% of the global land area, flood (anoxia) 13% of the land area, salinity 6%, mineral deficiency 9%, acidic soils 15% and, cold 57% (Mittler, 2006; Cramer et al., 2011). Out of the world's 5.2 billion ha of dryland agriculture, 3.6 billion ha is affected by the problems of erosion, soil degradation and salinity (Riadh et al., 2010). Ruan et al. (2010) estimated salt affected soils to impact upon 50% of total irrigated land in the world costing US$12 billion in terms of loss (Flowers et al., 2010). Similarly, global annual cost of land degradation by salinity in irrigated lands could be US$ 27.3 billion due to loss in crop production (Qadir et al., 2014). The detrimental effect of salinity on plant growth is well established. The area under ever-increasing salinization has almost reached 34 million irrigated hectares (FAO, 2012)[2]. Although any accurate estimation of agricultural loss (reduction of crop production and soil health) in terms of agro-ecological disturbances due to abiotic stresses could not be made, it is evident that such stresses affect large land areas and significantly impact qualitative and quantitative loss in crop production (Cramer et al., 2011).

Plants frequently cope up with the rapid fluctuations and adversity of environmental conditions because of their intrinsic metabolic capabilities (Simontacchi et al., 2015). Variations in the outside environment could put the plant metabolism out of homeostasis (Foyer and Noctor, 2005), and create necessity for the plant to harbor some advanced genetic and metabolic mechanisms within its cellular system (Apel and Hirt, 2004; Gill and Tuteja, 2010). Plants possess an array of protective mechanisms acquired during the course of evolution to combat adverse environmental situations (Yolcu et al., 2016). Such mechanisms cause metabolic re-programming in the cells (Heil and Bostock, 2002; Swarbrick et al., 2006; Shao et al., 2008; Bolton, 2009; Massad et al., 2012) to facilitate routine bio-physico-chemical processes irrespective of the external situations (Mickelbart et al., 2015). Many times plants get facilitated in reducing the burden of environmental stresses with the support of the microbiome they inhabit (Turner et al., 2013a; Ngumbi and Kloepper, 2014).

Microbial life is the most fundamental and live system on the earth. Being important living component of the soils, they naturally become integral part of the crop production system as soon as a seed comes into the soil to start its life cycle. Microorganisms are important inhabitants of seeds also, and proliferate as the seeds grow in the soils to form symbiotic associations at the surface or endophytic interactions inside the roots, stems or leaves. Plant microbiome provides fundamental support to the plants in acquiring nutrients, resisting against diseases and tolerating abiotic stresses (Turner et al., 2013a). Microbial intrinsic metabolic and genetic capabilities make them suitable organisms to combat extreme conditions of the environment (Sessitsch et al., 2012; Singh et al., 2014). Their interactions with the plants evoke various kinds of local and systemic responses that improve metabolic capability of the plants to fight against abiotic stresses (Nguyen et al., 2016). A testament to the important attributes of the microbial interactions with plants is significant number of accumulating pieces of evidence that suggest in-depth mechanisms based on plant–microbe interactions that offer modulation of cellular, biochemical and molecular mechanisms connected with stress tolerance (Bakker et al., 2012; Onaga and Wydra, 2016). Growing interest in uncultured microbes, especially from the rhizosphere of the crop plants, depleted and degraded soils, soils with disturbed fertility status and endophytic communities that potentially represent 'obligate endophytes' inhabiting plant tissues deciphered multi-phasic functions associated with the stress tolerance in microbial communities. The advent of next-generation sequencing (NGS) facilities supported gradually increasing metagenomic work and consequently led to the accumulation of greater amount of data for functional characterization of microbial communities in the soils (Bulgarelli et al., 2012).

Work on plant–microbe interactions at biochemical, physiological and molecular levels established that microbial associations largely direct plant responses toward stresses (Farrar et al., 2014). For dissecting deeper interaction mechanisms and connecting the changes at molecular levels with the tolerance responses against stresses, biological data based on the multi-omics approaches were generated (Kissoudis et al., 2014). The data generation and analysis was supported by the advancements in the high-end instrumentation and computational integration which helped to decipher individual signal molecules, proteins, genes and gene cascades to connect

[1]http://www.fao.org/docrep/010/a1075e/a1075e00.htm

[2]http://www.fao.org/docrep/meeting/024/md324e.pdf

them with the gene networks/pathways for their function description. Technological developments also facilitated understanding of gene editing systems, RNAi-mediated gene silencing, mutant technology, proteomic analysis and metabolite profiling to reveal voluminous molecular information that helped in improving our understanding of microbe-mediated mitigation strategies of abiotic stresses in plants (Yin et al., 2014; Luan et al., 2015). Multi-omics approaches have emerged as a holistic and integrated analytical strategies for the dissection of one of the most complex and dynamic living system of microbial interactions with plants and modulating the consequences developed in the plants to help them overcome stresses. In this review, we aim at summarizing the implications of abiotic stresses and plant responses generated thereafter in terms of biochemical and molecular mechanisms followed by the microbe-mediated stress mitigation processes. We further describe the role of multi-omics approaches in establishing understanding of plant–microbe interactions that help plants optimize abiotic stresses.

HOW DO ABIOTIC STRESSES AFFECT PLANTS?

Plants need light, water, carbon and mineral nutrients for their optimal growth, development and reproduction. Extreme conditions (below or above the optimal levels) limit plant growth and development. An unfavorable environment comprising extreme high or low of temperature, salinity and drought pose a complex set of stress conditions. Plants can sense and react to stresses in many ways that favor their sustenance (Crane et al., 2011; Ahmad et al., 2015; Jiang et al., 2016). They remember past exposure to abiotic stresses and even mechanisms to overcome them in such a way that responses to repeated stresses can be modified accordingly (Hilker et al., 2015). However, the underlying molecular mechanisms are primarily unknown. The most obvious effect of unfavorable conditions initially appear at the cellular levels after that, physiological symptoms are observable. Water stress adversely affects physiological status of plants including the photosynthetic capability (Xu and Zhou, 2006). Prolonged water stress decreases leaf water potential and stomatal opening, reduces leaf size, suppresses root growth, reduces seed number, size, and viability, delays flowering and fruiting and limits plant growth and productivity (Osakabe et al., 2014; Xu et al., 2016) (**Figure 1**). Therefore, plants have smartly evolved different mechanisms to minimize consumption of optimal water resources and manage their growth till they face adverse conditions (Osakabe et al., 2013). Exposure to low or high light intensities diminishes physiological process and adversely influences growth and development of plants. Excess light induces photooxidation that increases the production of highly reactive oxygen intermediates to manipulate biomolecules and enzymes (**Figure 1**). Under severe conditions, loss in plant productivity is observed (Li et al., 2009). Both freezing (cold) injury and/or an increase in temperature are major cause of crop loss (Koini et al., 2009; Pareek et al., 2010). Various edaphic factors like acidity, salinity, and alkalinity of soils (Bromham et al., 2013; Bui, 2013), pollutant contamination and anthropogenic perturbations (Emamverdian et al., 2015) severely affect plant development and adversely influence crop production (**Figure 1**). Different levels of acidic conditions badly influence soil nutrients and limit their ease of availability due to which plants become nutrient deficient and lose their normal physiological pattern of growth and development (Rorison, 1986). Early exposure to salinity leads to ion toxicity within the cell followed by disruption of osmotic balance when stress prolonged for longer duration. Combined effect of these ionic as well as osmotic shocks result into altered plant growth and development (Munns and Tester, 2008). Tolerance to salinity stress needs to maintain or quickly adjust both osmotic and ionic homeostasis within the cells. For combating salinity, plants usually try to avoid high saline environments by keeping sensitive plant tissues away from the zone of high salinity or by exuding ions from roots or compartmentalize ions away from the cytoplasm of physiologically active cells (Silva et al., 2010). Plants under extreme cold conditions survive either through avoiding super cooling of tissue water or through freezing tolerance. Certain species of plants have developed an ability to tolerate super-cooling or freezing temperatures by increasing their anti-freezing response within a short photoperiod, a process called cold acclimation (Thomashow, 2010).

After sensing the stress stimuli, plants exhibit an immediate and effective response to initiate a complex stress-specific signaling cascade (Chinnusamy et al., 2004; Andreasson and Ellis, 2010). Synthesis of phytohormone like abcisic acid, jasmonic acid, salicylic acid and ethylene (Spoel and Dong, 2008; Qin et al., 2011; Todaka et al., 2012), accumulation of phenolic acids and flavonoids (Singh et al., 2011; Tiwari et al., 2011), elaboration of various antioxidants and osmolytes and activation of transcription factors (TFs), are initiated along with the expression of stress-specific genes to mount appropriate defense system (Koussevitzky et al., 2008; Atkinson et al., 2013; Prasch and Sonnewald, 2013). Though many of the mechanisms related to stress tolerance in plants are known, our knowledge regarding '*on-field response*' of the plants to simultaneous exposure to multiple stresses is still in quite an infancy.

The most crucial aspect in mitigating stress in plants is to understand fine level molecular machinery and its networks operative under stress conditions. This includes elaborative elucidation of abundance of metabolic pathways and their regulatory genes in the plant varieties. Identification of multigenic traits involved in stress responses, exploration of linked markers for such genes, and investigation of the probabilities to pool out important genes through breeding programs is the current focus of stress mitigation strategies. Other strategies that have put forward for the alleviation of abiotic stresses in plants include the use of various biomolecules of plant and microbial origin. These approaches are opening new gateways for scientists to dig out novel methods to alleviate the abiotic stresses in field grown plants.

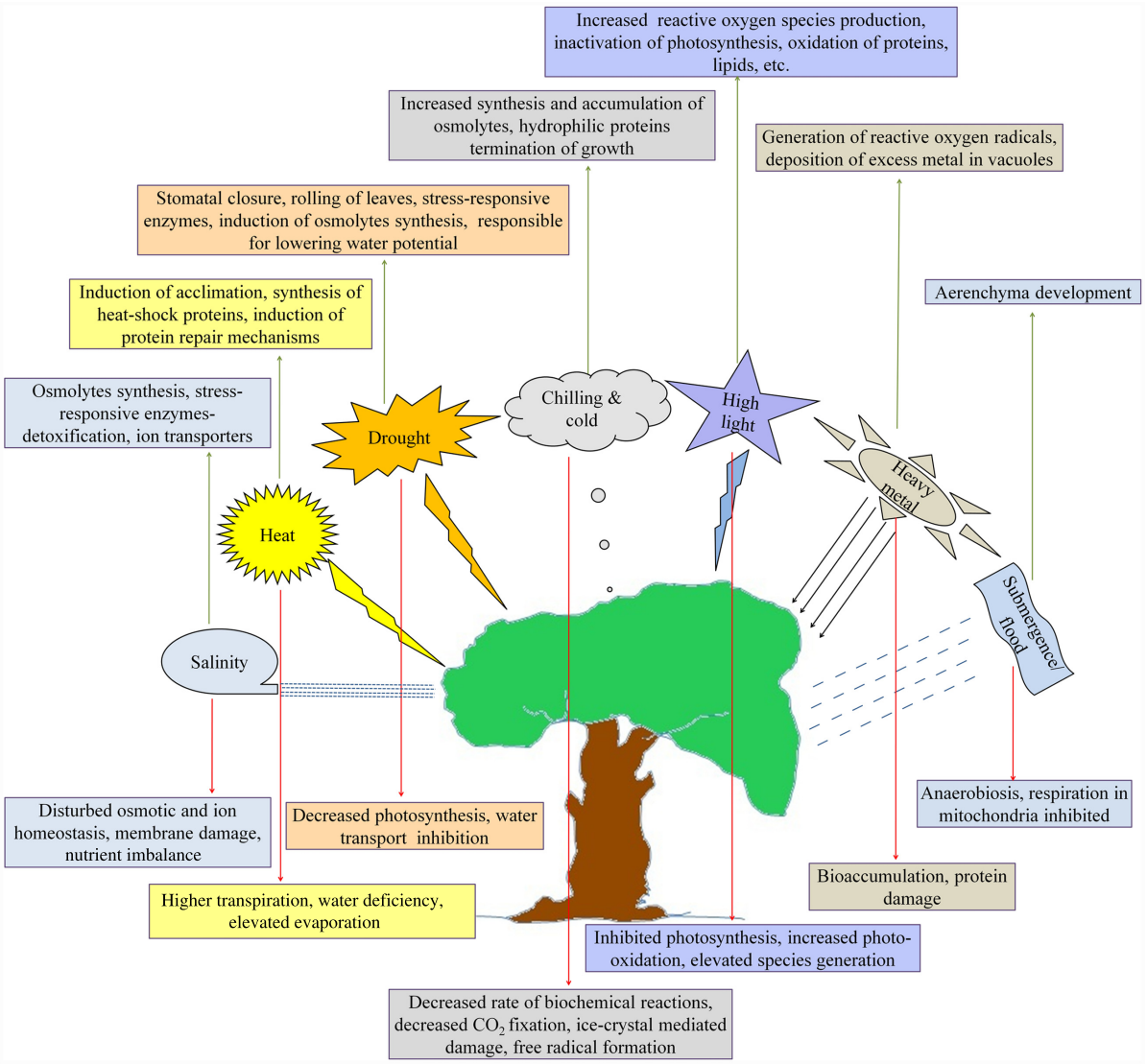

FIGURE 1 | Diverse abiotic stresses and the strategic defense mechanisms adopted by the plants. Though the consequences of heat, drought, salinity and chilling are different, the biochemical responses seem more or less similar. High light intensity and heavy metal toxicity also generate similar impact but submergence/flood situation leads to degenerative responses in plants where aerenchyma are developed to cope with anaerobiosis. It is therefore, clear that adaptive strategies of plants against variety of abiotic stresses are analogous in nature. It may provide an important key for mounting strategic tolerance to combined abiotic stresses in crop plants.

PHYSIOLOGICAL AND MOLECULAR RESPONSES OF PLANTS AGAINST STRESSES

Plants smartly sense, manage, maintain or escape changing environmental conditions (**Figure 1**). Their perception to environmental stimuli and responses to abiotic stresses involve an interactive metabolic crosstalk within diverse biosynthetic networks and pathways. Root architecture is thought to be more sensitive in sensing abiotic stimuli and reacting accordingly in the soils (Khan M.A. et al., 2016). It is a complex phenomenon that involves dynamic and real-time changes at genetic, transcriptomic, cellular, metabolic and physiological levels (Atkinson and Urwin, 2012). The foremost and direct impact of drought stress, frost, salinity and heat is creation of water deficient conditions within cells followed by a parallel development of biochemical, molecular and phenotypic responses against stresses (Cushman et al., 1990; Almoguera et al., 1995; Xu and Zhou, 2006). In the environment, the stresses experienced by the plants may be many, so as the complexity of their responses to multiple stresses in comparison to individual stress. The complexity lies in activating specific gene expression followed by metabolic programming in cells in response to individual stresses encountered. Tolerance, defense

or susceptibility to stresses is a dynamic event involving multiple stages of plant's development. Rather than imposing an additive effect on plants, abiotic stress responses may reduce or enhance susceptibility of plants toward biotic stresses caused due to pests or pathogens (Rizhsky et al., 2004). This becomes more important when we take into account agricultural crops because, in many agricultural systems, most crops grow in suboptimal environmental conditions that are limiting to the genetic potential of the plants for growth and development (Bray et al., 2000). Defense, repair, acclimation and adaptation are the major components of resistance responses toward stresses.

Plants are vulnerable to water stress. Environmental changes like rewatering or cycled water conditions are created most frequently in the globally changing climatic conditions (Xu et al., 2010). Under severe water deficit conditions, peroxidation may be induced leading to negative impact on antioxidant metabolism (Bian and Jiang, 2009; Xu et al., 2014). Rewatering further decreases the level of peroxidation and restores growth and development of new plant parts and stomatal opening. In roots, both drought and rewatering lead to high accumulation of H_2O_2 (Bian and Jiang, 2009). Drought responses vary from plant to plant in terms of the activity of superoxide dismutase (SOD) enzyme that plays a central role in antioxidant metabolism (Xu Z. et al., 2015). In bluegrass, SOD activity remains unaffected by drought conditions and gene expression of FeSOD and Cu/ZnSOD is down-regulated. In Alfalfa nodules, FeSOD and CU/ZnSOD are up-regulated by moderate drought, implicating that responses differ from species and tissues (Foyer and Noctor, 2005; Naya et al., 2007). An elevated level of salts present in the soil is detrimental to the plant cells, and different cells in a tissue respond differently to the stresses caused due to salinity (Voesenek and Pierik, 2008). Stressed cells irrespective of their location, whether at the root surface or within internal tissues, influence their neighbors and cause a change in their gene expression pattern over the stress duration (Dinneny et al., 2008). A drastic decrease in the osmotic potential of the soil occurs due to the elevated salt levels, the ultimate result of which is ion toxicity coupled with water stress in the plants. This situation can affect the vitality of the plants by suppressing seed germination and growth of the seedlings, hamper senescence of the plants and finally cause death (McCue and Hanson, 1990). The role of Salt Overly Sensitive (SOS) stress signaling pathway consisting of three majorly involved proteins SOS1, SOS2, and SOS3 is well demonstrated (Hasegawa et al., 2000). Salinity conditions cause decrease in the levels of aromatic amino acids like cysteine, arginine and methionine. Proline accumulation within the cells is a well-known alleviation strategy from salinity stress (Matysik et al., 2002). Similarly, generation of nitric oxide (NO), activation of antioxidant enzymes and compounds, modulation of hormones, accumulation of glycine betaine and polyols are some other changes within plants due to salinity stress (Gupta and Huang, 2014). This principally happens due to unavailability of water and mutilation in the nutrient availability caused due to high salt concentrations that create much damage to plant tissues and ultimately affect productivity.

Due to continued rise in global temperature, heat stress is becoming an important agricultural problem as it badly affects crop production. Rising temperature has an adverse impact on morpho-anatomical, physiological, biochemical and genetic changes in plants. A thorough understanding of physiological responses of plants to heat and mechanisms of tolerance could lead to strategic development of better approaches for crop production management (Wahid et al., 2007). Heat affects plants at different developmental levels, and high temperature causes reduced seed germination, loss in photosynthesis and respiration and decrease in membrane permeability (Xu et al., 2014). Alterations in the level of phytohormones, primary and secondary metabolites, enhancement in the expression of heat shock and related proteins and production of reactive oxygen species (ROS) are some prominent responses of plants against heat stress (Iba, 2002) (**Figure 1**). Mitigation strategies in plants against heat stress involve activation of mechanisms that support maintenance of membrane stability and induction of mitogen-activated protein kinase (MAPK) and calcium-dependent protein kinase (CDPK) cascades (Wang and Li, 2006). Besides, scavenging of ROS, accumulation of antioxidant metabolites and compatible solutes, chaperone signaling and transcriptional modulation are certain parallel activities that help cells to sustain heat stress (Wahid et al., 2007).

Multiple stress conditions impose more beneficial impacts on plants compared to that posed in presence of individual stress alone. Combination of stresses ultimately reduce the detrimental effect of each other thereby, increasing the probability of better survival of plants. Iyer et al. (2013) demonstrated that the cumulative impact of drought and accumulation of ozone (O_3) in plants resulted in better tolerance. The combined affect was attributed to decreased values of stomatal conductance. Elevated concentration of reduced glutathione and ascorbic acid effectively scavenge ROS, thereby causing a considerable drop in the total ROS content. However, it is a difficult task to infer response pattern of a plant against any single stress, particularly when it is growing in the field from the cumulative impact of environmental stresses. Multiple stresses occur simultaneously in field conditions and so, multifaceted mechanisms exist in the plants to cope-up with rapidly fluctuating adverse situations. Although much efforts have been made to assess plant responses toward single stress conditions (Rizhsky et al., 2002, 2004; Mittler, 2006; Mittler and Blumwald, 2010; Alameda et al., 2012; Atkinson and Urwin, 2012; Kasurinen et al., 2012; Srivastava et al., 2012; Perez-Lopez et al., 2013; Rivero et al., 2013), attempts to assess the impact of combined stress conditions on crop plants under simulated laboratory trials are limited. This particularly limits our knowledge and understanding of plant responses to combined stresses and prediction of cumulative stress tolerance mechanisms in laboratory or field conditions.

Phytohormones are crucial for the plant growth and development but they critically play role in the abiotic stress tolerance (Wani et al., 2016). Gene expression profiling revealed that prioritization of signals done by protein switches like kinases, TFs and G-proteins are mostly regulated by hormones

(Depuyd and Hardtke, 2011; Yao et al., 2011). Plants typically channel their physiological resources toward adapting to abiotic stress which makes them more susceptible to biotic stresses like herbivory and disease attack (Zabala et al., 2009; Hey et al., 2010). ABA-dependent abiotic stress response pathways are predominant. Other defense pathways rooted through salicylic acid, jasmonic acid or ethylene also trigger plants for abiotic stress response. For example, triggering ROS production to minimize loss during abiotic stress may prevent plants from biotrophic pathogen attack, but it makes plants more prone for necrotrophic pathogens. The other hormone, JA is effective for defense responses to necrotrophic pathogens and associated to ISR by beneficial microbes (Matilla et al., 2010). Study of omics may help in understanding these complex plant–microbe interactions and harvesting associated and linked understanding.

MICROBE-MEDIATED MITIGATION OF ABIOTIC STRESSES

Microbial interactions with crop plants are key to the adaptation and survival of both the partners in any abiotic environment. Induced Systemic Tolerance (IST) is the term being used for microbe-mediated induction of abiotic stress responses. The role of microorganisms to alleviate abiotic stresses in plants has been the area of great concern in past few decades (de Zelicourt et al., 2013; Nadeem et al., 2014; Souza et al., 2015). Microbes with their potential intrinsic metabolic and genetic capabilities, contribute to alleviate abiotic stresses in the plants (Gopalakrishnan et al., 2015). The role of several rhizospheric occupants belonging to the genera *Pseudomonas* (Grichko and Glick, 2001; Ali et al., 2009; Sorty et al., 2016), *Azotobacter* (Sahoo et al., 2014a,b), *Azospirillum* (Creus et al., 2004; Omar et al., 2009), *Rhizobium* (Alami et al., 2000; Remans et al., 2008; Sorty et al., 2016), *Pantoea* (Amellal et al., 1998; Egamberdiyeva and Höflich, 2003; Sorty et al., 2016), *Bacillus* (Ashraf et al., 2004; Marulanda et al., 2007; Tiwari et al., 2011; Vardharajula et al., 2011; Sorty et al., 2016), *Enterobacter* (Grichko and Glick, 2001; Nadeem et al., 2007; Sorty et al., 2016), *Bradyrhizobium* (Fugyeuredi et al., 1999; Swaine et al., 2007; Panlada et al., 2013), *Methylobacterium* (Madhaiyan et al., 2007; Meena et al., 2012), *Burkholderia* (Barka et al., 2006; Oliveira et al., 2009), *Trichoderma* (Ahmad et al., 2015) and cyanobacteria (Singh et al., 2011) in plant growth promotion and mitigation of multiple kinds of abiotic stresses has been documented. Recently, Pandey et al. (2016) have demonstrated the role of *Trichoderma harzianum* on stress mitigation in rice genotypes due to upregulation of aquaporin, dehydrin and malonialdehyde genes along with various other physiological parameters. Rhizobacteria-induced drought endurance and resilience (RIDER) that includes changes in the levels of phytohormones, defense-related proteins and enzymes, antioxidants and epoxypolysaccharide have been observed for microbe-mediated plant responses. Such strategies make plants tougher toward abiotic stresses (Kaushal and Wani, 2016). The selection, screening and application of stress-tolerant microorganisms, therefore, could be viable options to help overcome productivity limitations of crop plants in stress-prone areas. Enhanced oil content in NaCl affected Indian mustard (*Brassica juncea*) was reported by *Trichoderma harzianum* application which improved the uptake of essential nutrients, enhanced accumulation of antioxidants and osmolytes and decreased Na^+ uptake (Ahmad et al., 2015). Parallel to such reports, up-regulation of monodehydroascorbate reductase in *Trichoderma* treated plants was demonstrated. It was also confirmed by mutant studies that *Trichoderma* ameliorates salinity stress by producing ACC-deaminase (Brotman et al., 2013). In barley and oats, *Pseudomonas* sp. and *Acinetobacter* sp. were reported to enhance production of IAA and ACC-deaminase in salt affected soil (Chang et al., 2014). Palaniyandi et al. (2014) reported alleviation of salt stress and growth promotion by *Streptomyces* sp. strain PGPA39 in 'Micro-Tom' tomato plants. *Burkholderia phytofirmans* strain PsJN mitigates drought stress in maize (Naveed et al., 2014b), wheat (Naveed et al., 2014a) and salt stress in *Arabidopsis* (Pinedo et al., 2015).

The rhizosphere comprises the fraction of soil in vicinity of the plant roots. It constitutes a soil microenvironment in the proximity of root region where the average count of microorganisms is very high than rest of the bulk soil. It is, therefore, obvious that plant roots with a diversity of their nutrient, mineral and metabolite composition, could be a major factor responsible for attracting microorganisms to accumulate and associate alongside. The secretion of root exudates by plants is a vital factor for microbial colonization within the rhizosphere. Chemotactic movement of microorganisms toward the root exudates plays the role of dragging force for the microbial communities to colonize on the roots. While utilizing the rhizosphere-microenvironment around plant roots, the PGPRs may act as biofertilizers, phytostimulators or biocontrol agents depending upon their inherent capabilities, mode of interaction and competitive survival conditions. Growth promoting bacteria stimulate plant growth by employing several broadly categorized direct and indirect mechanisms (Braud et al., 2009; Hayat et al., 2010). Direct mechanisms include synthesis of bacterial compounds which facilitate uptake of essential nutrients and micronutrients from the soil along with the production of plant growth regulators, e.g., iron and zinc sequestration, siderophore production, phosphorus and potassium solubilisation, plant hormone production, and atmospheric nitrogen fixation. On the other hand, indirect mechanisms involve antagonistic activity toward plant pathogenic organisms, production of HCN and antifungal compounds and tolerance against abiotic stresses. Besides this, the bacteria can induce systemic resistance in plants by their metabolites acting as extracellular signals, which subsequently trigger a series of internal processes. Eventually, the translocated signal is perceived by the distant plant cells triggering the activation of the defense mechanism. Besides bacteria, fungi particularly the mycorrhiza are also important plant growth promoters. These are principally divided into mycorrhizal fungi and vesicular-arbuscular mycorrhizal (VAM) fungi. These fungi remain associated with the host plant externally (ectomycorrhizae) or they may form endosymbiotic associations (VAM). These fungi form extensive networking of very fine hyphae, thus increasing overall nutrient uptake by the roots. The root fungal endophyte *Piriformospora indica*

induces salt tolerance in barley (Baltruschat et al., 2008) and drought tolerance in Chinese cabbage (Sun et al., 2010) by increasing the levels of antioxidants and improving many other aspects (Franken, 2012). The potential of microbial interactions with the plants have, therefore, multipronged role. At one end, microbes induce local or systemic stress alleviation response mechanisms in plants to sustain under abiotic stress conditions while at the other end, they help plants to maintain their growth and development through fixation, mobilization and/or production of nutrients, hormones and organic phytostimulant compounds. Such multifaceted action of microorganisms or their communities makes them strong, viable and vital options for abiotic stress mitigation strategies in crop plants.

Several mechanisms highlighting the role of microbes in abiotic stress alleviation have been proposed. Soil-inhabiting microbes belonging to genera *Achromobacter, Azospirillum, Variovorax, Bacillus, Enterobacter, Azotobacter, Aeromonas, Klebsiella* and *Pseudomonas* have been shown to enhance plant growth even under unfavorable environmental conditions (Pishchik et al., 2002; Hamdia et al., 2004; Mayak et al., 2004; Arkhipova et al., 2007; Barriuso et al., 2008a,b; Dardanelli et al., 2008; Belimov et al., 2009; Ortiz et al., 2015; Kaushal and Wani, 2016; Sorty et al., 2016). Literature relating to the involvement of microbes for the alleviation of abiotic stressors signifies the importance of microbes in this area (**Table 1**). All such soil bacteria that are capable of inducing plant growth under variety of physicochemical and environmental conditions are classified cumulatively as plant growth promoters (PGP). There exists different mechanisms by which microbes induce plant growth. The plant-growth regulating molecules predominantly, indole acetic acid (IAA) are synthesized in shoot and accumulated in the actively growing regions of roots. The IAA and other auxins have growth-stimulating effect in terms of cell elongation resulting in root growth initiation. Moreover, these molecules also promote the development of lateral roots. Higher concentrations of auxins, on the other hand, are known to have a negative impact on root growth (Jackson, 1991; Sorty et al., 2016). A similar situation can also happen due to increased synthesis of ethylene (Jackson, 1991). The rhizosphere colonizing bacteria were reported to perform in a similar manner, and produce phytohormones to enhance plant growth (Bowen and Rovira, 1991; Timmusk and Wagner, 1999; German et al., 2000; Belimov et al., 2007). Evidences from recent agricultural practices witness that the PGPRs not just help in mitigation of environmental stresses, but also improve yield of diverse crop plants including rice, maize, barley and soybean (Tapias et al., 2012; Sharma et al., 2013; Sen and Chandrasekhar, 2014; Suarez et al., 2015). A mechanism of salt tolerance imposed by *Pseudomonas* sp. PMDzncd2003 on rice germination under salinity stress is demonstrated. Better root colonizing capability of *Pseudomonas* sp. along with its ability to produce exopolysaccharides (EPS) leads to enhanced tolerance toward salinity (Sen and Chandrasekhar, 2014). Similarly, Khan A. et al. (2016) have shown that inoculation of *Bacillus pumilus* improved rice growth in response to salinity and high boron stresses. A possible mechanism was suggested, that higher expression of antioxidant enzyme machinery in the presence of bacterial inoculant may lead to cell protection in stress conditions. More efforts are now needed to dissect molecular mechanisms involved in the communication between plant and bacterial colonizers.

MULTI-OMICS APPROACHES TO ADDRESS ALLEVIATION OF ABIOTIC STRESS

The ecology of plant–microbe interaction is very complicated and interwoven system. It is important to understand the fine-tuning and integration of diverse signals generated by microbial interactions in the plants for advantage in crop improvement. A plant has to combat multiple biotic and abiotic stresses in the environment. Multiple stress factors produce complex defense signals in plants and therefore, the result of plant–microbe interaction can be decided by prioritization of physiological pathways in plants (Schenk et al., 2012). Interaction of microbes with plant roots evoke multipronged responses in local and/or in distal plant parts at physiological, biochemical and molecular level. Such responses at all levels have their interconnections with the stress; many are parallel to stress responses while others are adverse. For dissecting the mechanisms, multi-omics approaches can be applied to address the challenging task of deciphering changes in plants at genetic, proteomic or metabolomic level (**Figure 2**). Entwined with the advances in bioinformatics, the data-driven science of multi-omics has improved our knowledge in understanding the microbial community composition and their functional behavior in complex environments like rhizosphere, where inter-connections among microbial communities direct plant responses toward stresses. Recently, meta-omics approaches including metagenomics, metatranscriptomics and metaproteomics have emerged as promising tools to address microbial communities and functions within a given environment at a deeper level (de Castro et al., 2013).

GENOMICS

Abiotic stress alleviation by altering crop genetics is of paramount importance and is a challenging issue that requires extensive breeding programs (Grainger and Rajcan, 2013). Low heritability and environmental variations make such breeding programs even more challenging (Manavalan et al., 2009). Strategic marker-assisted breeding is efficient in accelerating tolerance in cultivars. Understanding about genomic loci governing traits responsible for tolerance and availability of molecular markers tightly linked with it is a prerequisite for marker assisted selection (Xu et al., 2012). A large amount of genomic data in the form of sequenced genomes and expression profiles are thus, impetuous for breeding for stress alleviation (Sonah et al., 2011; Tomar et al., 2014). Use of genomics-based technologies has made a great impact in crop improvement programs. Use of molecular markers in crop improvement for the accumulation of silicon (Si) in rice

TABLE 1 | Microbe-mediated abiotic stress tolerance in plants.

Abiotic stress	Microbe inoculation	Plant	Tolerance strategy	Reference
Salt	*Bacillus subtilis* GB03	*Arabidopsis thaliana*	Tissue-specific regulation of sodium transporter *HKT1*	Zhang et al., 2008
Salt	*Pseudomonas simiae*	*Glycine max*	4-nitroguaiacol and quinoline promote soybean seed germination	Vaishnav et al., 2016
Salt	*Pseudomonas syringae* DC3000, *Bacillus* sp. strain L81, *Arthrobacter oxidans*	*Arabidopsis thaliana*	SA-dependent pathway	Barriuso et al., 2008b
Salt	Root-associated plant growth-promoting rhizobacteria (PGPR)	*Oryza sativa*	Expression of salt stress-related *RAB18* plant gene	Jha et al., 2014
Salt	Cyanobacteria and cyanobacterial extracts	*Oryza sativa*, *Triticum aestivum*, *Zea mays, Gossypium hirsutum*	Phytohormones as elicitor molecule	Singh, 2014
Salt	*Pseudomonas koreensis* strain AK-1	*Glycine max* L. Merrill	Reduction in Na^+ level and increase in K^+ level	Kasotia et al., 2015
Osmotic stress	*Bacillus megaterium*	*Zea mays*	High hydraulic conductance, increased root expression of two ZmPIP isoforms	Marulanda et al., 2010
Osmotic stress	*Glomus intraradices* BEG 123	*Phaseolus vulgaris*	High osmotic root hydraulic conductance due to increased active solute transport through roots	Aroca et al., 2007
Salt	*Glomus etunicatum*	*Glycine max*	Increased root but decreased shoot proline concentrations	Sharifi et al., 2007
Salt	*Burkholderia, Arthrobacter* and *Bacillus*	*Vitis vinifera*, *Capsicum annuum*	Increased accumulation of proline	Barka et al., 2006
Drought	*Rhizobium tropici* and *Paenibacillus polymyxa* (Co-inoculation)	*Phaseolus vulgaris*	Upregulation of genes involved in stress tolerance	Figueiredo et al., 2008
Salt	*Glomus fasciculatum*	*Phragmites australis*	Accumulation of carbohydrates	Al-Garni, 2006
Salt	*Glomus intraradices*	*Glycine max*	Accumulation of carbohydrates	Porcel and Ruiz-Lozano, 2004
Salinity	*Azospirillum brasilense* and *Pantoea dispersa* (Co-inoculation)	*Capsicum annuum*	High stomatal conductance and photosynthesis	del Amor and Cuadra-Crespo (2012)
Salinity	*Glomus intraradices* BAFC 3108	*Lotus glaber*	Decreased root and shoot Na^+ accumulation and enhanced root K^+ concentrations	Sannazzaro et al., 2006
Salinity	*Glomus clarum* *Glomus etunicatum*	*Vigna radiata*, *Capsicum annuum*, *Triticum aestivum*	Decreased Na^+ in root and shoot and incesaed concentration of K^+ in root	Rabie, 2005; Daei et al., 2009; Kaya et al., 2009
Salinity	*Bacillus subtilis*	*Arabidopsis*	Decreased root transcriptional expression of a high-affinity K^+ transporter (*AtHKT1*) decreasing root Na^+ import	Zhang et al., 2008
Salinity	*Glomus intraradices* BEG121	*Lactuca sativa*	Reduced concentration of ABA	Aroca et al. (2008)
Salinity	*Pseudomonas putida* Rs-198	*Gossypium hirsutum*	Prevented salinity-induced ABA accumulation in seedlings	Yao et al., 2010
Salinity	*Azospirillum brasilense* strain Cd	*Phaseolus vulgaris*	Stimulation of persistent exudation of flavonoids	Dardanelli et al., 2008
Salinity	*Bacillus subtilis*	*Lactuca sativa*	Root-to-shoot cytokinin signalling and stimulation of shoot biomass	Arkhipova et al., 2007
Drought	*Burkholderia phytofirmans* *Enterobacter* sp. FD17	*Zea mays*	Increased photosynthesis, root and shoot biomass under drought conditions	Naveed et al., 2014b
Drought	*Bacillus thuringiensis* AZP2	*Triticum aestivum*	Production of volatile organic compounds	Timmusk et al., 2014
Drought	*Pseudomonas chlororaphis* O6	*Arabidopsis thaliana*	Production of 2R,3R butanediol- a volatile compound	Cho et al., 2008
Drought	*Pseudomonas putida* strain GAP-P45	*Helianthus annuus*	Epoxypolysaccharide production	Sandhya et al., 2009
Drought	*Bacillus licheformis* strain K11	*Capsicum annum*	Stress related genes and proteins	Lim and Kim, 2013

(Continued)

TABLE 1 | Continued

Abiotic stress	Microbe inoculation	Plant	Tolerance strategy	Reference
Drought	*Bacillus cereus* AR156, *B. subtilis* SM21 and Serratia sp. XY21	*Cucumis sativa*	Production of monodehydro ascorbate, proline, and antioxidant enzyme, expression of genes	Wang et al., 2012
Heat	*Bacillus amyloliquefaciens, Azospirillum brasilence*	*Triticum aestivum*	Reduced regeneration of reactive oxygen species, preactivation of heat shock transcription factors, changes in metabolome	El-Daim et al., 2014
Heat and drought	*Curvularia proturberata* isolate Cp4666D	*Dichanthelium lanuginosum, Solanum lycopersicum*	Colonization of roots	de Zelicourt et al., 2013
Arsenic toxicity	*Staphylococcus arlettae*	*Brassica juncea*	Increased soil dehydrogenase, phosphatase and available phosphorus	Srivastava et al., 2013
Pb/Zn toxicity	*Phyllobacterium myrsinacearum*	*Sedum plumbizincicola*	Resistance to 350mg/L Cd, 1000 mg/L Zn, 1200 mg/L Pb	Ma et al., 2013
Zn toxicity	*Pseudomonas aeruginosa*	*Triticum aestivum*	Improved biomass, N and P uptake and total soluble protein	Islam et al., 2014
Zn toxicity	*Enterobacter intermedius* MH8b	*Sinapis alba*	ACC deaminase, IAA, hydrocyanic acid, P solubilization	Plociniczak et al., 2013
Cd, AS, Cu, Pb and Zn toxicity	*Pseudomonas koreensis* AGB-1	*Miscanthus sinensis*	ACC deaminase, IAA production	Babu et al., 2015
Zn toxicity	*Pseudomonas brassicacearum, Rhizobium leguminosarum*	*Brassica juncea*	Metal-chellating molecules	Adediran et al., 2016
Hg toxicity	*Photobacterium* spp.	*Phragmites australis*	IAA, mercury reductase activity	Mathew et al., 2015

to enhance the tolerance of plant for abiotic stress is in vogue. Ma et al. (2004) used PCR-based markers for microsatellite (RM5303) and expressed sequence tag (EST, E60168) in mapping Si transporter gene during a bulk segregant experiments.

Besides crop breeding programs, a significant level of abiotic stress alleviation in plants can be achieved through the manifestations of plant-microbe interaction also. Omics approaches help to have deep insight into the mechanisms of established plant–microbe interactions (**Figure 2**). In a study of *Trichoderma*-plant interaction (*T. atroviride* and *T. harzianum* with tomato), Tucci et al. (2011) reported the impact of genotypic characteristics of plants for modulation of microbe–plant interaction leading to its effect on plant growth and stress alleviation. Growth promoting and stress alleviating activity of *T. atroviride* on tomato is demonstrated through degradation of IAA in the rhizosphere and ACC deaminase activity (Gravel et al., 2007). A putative sequence of ACC-deaminase found in *Trichoderma* genome was confirmed by gene silencing through RNAi (Viterbo et al., 2010; Kubicek et al., 2011). Expression of dicarboxylate transporter LjALMT4 responsible for carbohydrate translocation in plants was reported in *Lotus japonicas* genome by Takanashi et al. (2016). The gene silencing strategy of Hb1 gene to enhance NO production and up-regulation of CBF regulon could be a way to engineer crops in improving cold tolerance (Sehrawat et al., 2013). Kumari et al. (2015) reported IST to salinity in soybean by *Pseudomonas* sp. AK-1 and *Bacillus* sp. SJ-5 inoculation. Results indicated that superior tolerance to salt stress may be observed due to proline accumulation and lipoxygenase activity.

Koussevitzky et al. (2008) reported that Apx1, a gene coding for cytosolic ascorbate peroxidase 1 is specifically required for tolerance to drought and heat stress in *Arabidopsis*. Ectoine is a compatible osmolyte responsible for salt tolarance in *Halomonas elongata* OUT30018. Three genes for ectoine biosynthesis were cloned and transferred to tobacco plant (*Nicotiana tabacum L.*) cv Bright Yellow 2 (BY2) which caused increase in tolerance to hyperosmotic shock by accumulation of ectoine and exhibited normal growth under such conditions (Nakayama et al., 2000). Identifying the genes and their regulation helps breeders in generating better varieties for stress tolerance. Use of multi-omics strategies yield highly efficient and reliable outcome that are useful to facilitate methodical experiments (**Figure 2**). Chilling tolerance in *Miscanthus* grass is a desirable trait that often varies in different cultivars. Molecular expression of relevant genes for the accumulation of carbohydrates creates differentiation among varieties for chilling tolerance. The impairment of tolerance among varieties can be predicted by molecular marker of sensitive genes like TF MsCBF3 expressed in sensitive genotypes (Purdy et al., 2013).

Stress due to submergence affects more than 15 million hectares of rainfed lowland rice in different parts of Asia (Neeraja et al., 2007). Thirteen percent of the total land area of the world is affected by problems of flooding or anoxia (Cramer et al., 2011). In rice, submergence tolerance is governed by a single major quantitative trait locus (QTL) found on chromosome 9 (Toojinda et al., 2003). Neeraja et al. (2007) used molecular markers for *Sub1* gene in backcross breeding program with recurrent parant *Swarna*. This *Sub1* provides tolerance in sensitive mega varieties. *Sub1A* is now confirmed of being the primary contributor to submergence tolerance (Septiningsih et al., 2009). This QTL has provided a great opportunity for marker assisted backcrossing (MAB) for developing submergence tolerance in mega varieties.

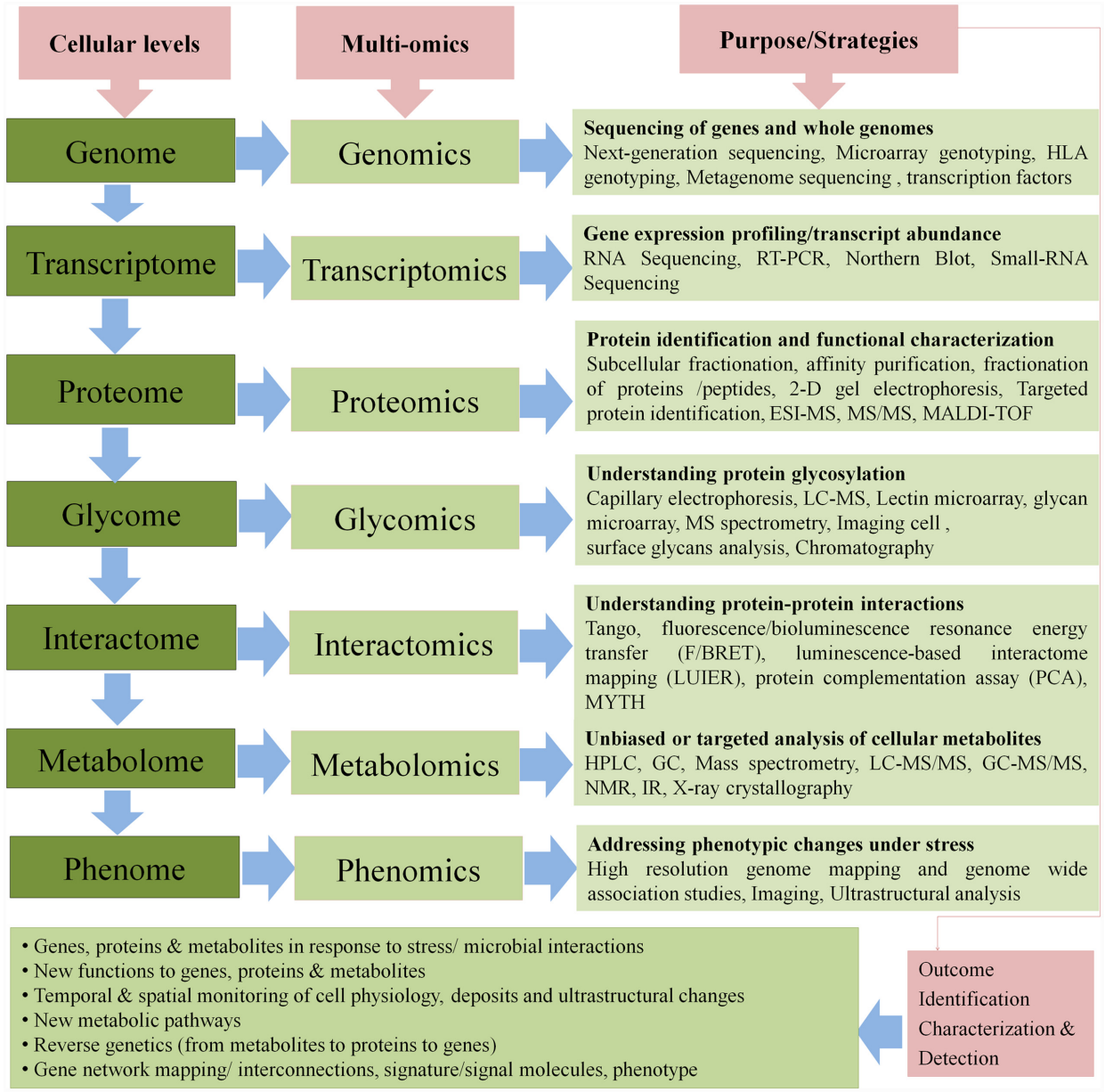

FIGURE 2 | Cellular level components, multi-omics approaches to address different levels and the strategies that help identify the outcome of the impact of abiotic stresses or impact of microbial-interactions.

Genomic analysis of both the host and associated microbial communities especially phyllosphere-associated microbial communities permits to access the system involved for the smooth functioning of associative interactions (**Figure 2**). Several studies have outlined the role of different genes from associated bacteria. Plants donate indispensible moleculr counterparts to facilitate and maintain the biological system involved at the associative interface. The genotypic diversity of plants has significant influence on the interactive process. The response of the roots of *SUNN1 Medicago truncatula* toward elevated levels of nitrate gets markedly affected with the advent of associative rhizobia because *SUNN1* exhibited no impact showing the response of *SUNN1* under limiting nitrogen environment in presence of associative rhizobia (Jin et al., 2012). There are evidences on the influence of *Nod* factors from associative microbes on the pattern of root development and smooth functioning of symbiotic association (Olah et al., 2005; Oldroyd, 2013). Unlike nodulating plants, widely cultivated cereals lack a system to acquire nitrogen with the help of nodulation. Some diazotrophic microbes manage to enter and colonize root tissues via mechanical injuries caused during root growth (Gaiero et al., 2013). However, knowledge of such interactions is scarce.

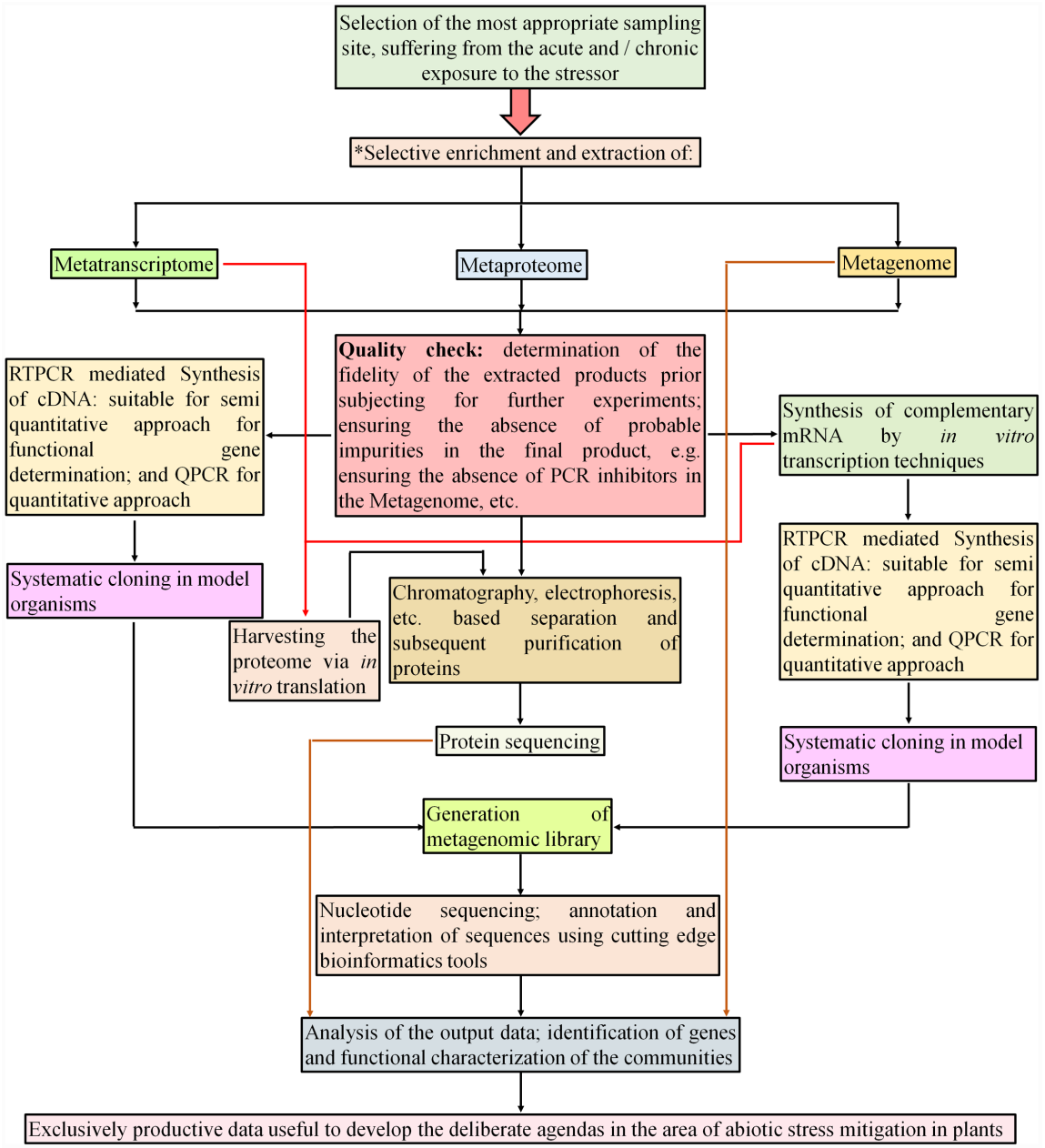

FIGURE 3 | Meta-omics approaches to exploit yet-unexplored environmental population of microbial communities that have major impact on plant roots and support plants against stresses. Metatranscriptomics and metaproteomics are relatively new approaches to characterize functional attributes of microbial communities that have not yet been cultured. The approach could generate a deeper snapshot of major metabolic pathways and interactions and dominance of functional microbial communities in the rhizosphere of crop plants facing multiple environmental stresses. (*Enrichment techniques in metagenomics. In order to trace out less abundant genes from the environment, these techniques are usually employed. In induced enrichment approach, the physico-chemical factors such as nutrients, temperature, acidity/alkalinity, xenobiotic compounds, etc. (Eyers et al., 2004; Bertrand et al., 2005) are used to enrich the respective populations *in situ*. These factors are either directly implemented in the microbial habitat itself or used in simulated *in situ* laboratory conditions. The natural sample enrichment is mainly dependent on executing fine criteria while proceeding for sampling of an environment. The naturally predominating bio-geo-physico-chemical situations need to be considered, as they are the key factors for selective natural enrichment of genes, e.g., sites contaminated with xenobiotic compounds and habitats with extreme environments can be expected to yield the genes participating in the metabolism of xenobiotic compounds and the genes participating in environmental stress tolerance respectively. The enrichment of nucleic acids from natural environment is principally carried out for the samples containing insufficient quantities of nucleic acids. It involves techniques such as affinity capture, differential expression analysis, stable isotope probing, e.g., addition of ^{13}C labeled carbon source in the habitat. For the samples with low density of biomass, whole genome amplification technique is recommended to yield relatively larger quantity of nucleic acids (Abulencia et al., 2006; Binga et al., 2008). These approaches may work better with the samples collected from highly saline/sodic/drought affected, barren soils, where it is virtually difficult to cultivate the crop. The stress-genes of the little microbial community thriving in such harsh environments may provide novel guidelines for stress alleviation strategies in the crop).

METAGENOMICS

The culture-independent approach for the analysis of microbial communities has been a powerful tool for resolution of yet-uncultured, unseen microbial diversity that plays various role in the plant rhizosphere (Chen and Pachter, 2005) (**Figure 3**). The approach referred to as metagenomics enables the user to acquire data related to the habitat-specific distribution of microbial communities with plant growth promoting (PGP), biocontrol, antibiotic producing and xenobiotic degrading traits. The approach helps to elevate likelihood of successfully directed attempts made to explore novel culturable flora from particular niches (Handelsman et al., 2007). High-throughput metagenomic sequencing is proving to be an extremely useful tool for improved understanding of PGP rhizobacterial communities (**Figure 3**). In a study on potato endophytes, two types of ACC-deaminase genes (*acdS*) homologous to that of *Pseudomonas fluorescens* for stress alleviation were found from PCR analysis. Analysis of clones present in metagenomic libraries helped in identifying entire *acdS* operon from uncultivated endophyte and revealed a transcriptional regulator gene *acdR* at upstream of *acdS*. This operon was found prominently in the genus *Burkholderia* (Nikolic et al., 2011). *Escherichia coli* clones from a pond water metagenomic library were studied to identify salt tolerance genes in uncultivable bacteria by growing at inhibitory NaCl concentrations of 750 mM. Genes from two clones encoding for proteins similar to a putative general stress protein (*GspM*) having GsiB domain with a putative enoyl-CoA hydratase (*EchM*) identified to have a role in salt tolerance. After purification, *EchM* was found to have crotonyl-CoA hydratase activity (Kapardar et al., 2010). These genes are of great utility in developing salt tolerant recombinant bacteria and also transgenic plants. Metagenomic study of an acid mine drainage 250 m belowground revealed the presence of mechanisms of adaptation to cold. Genes related with the survival at low temperature like anti-freeze protein, cold-shock proteins, compatible solutes production pathways and pH homeostasis were found in the metagenome of acid mine drainage (Liljeqvist et al., 2015). Such metagenomics data help to finding out new genes and mechanisms for cold stress alleviation. The role of bacterial endophytes that reside inside roots is largely unexplored because endophytic microbes which are cultured successfully represent only a fraction of the whole bacterial community that inhabit root interiors. Sessitsch et al. (2012) described endophytic bacterial residents of rice roots with the help of metagenomics approach. Metagenomic sequences obtained from endophytic cell extracts revealed that metabolic processes pertaining to the endophytic life style and functional features like quorum sensing and detoxification of ROS have their role in improving plant stress resistance (Sessitsch et al., 2012). Microbial communities have a fundamental impact on plant health and productivity. As a community, microbes interact with each other and with the host. This is a key phenomenon that increases resistance to diseases and stresses. To know the microbiome composition and describe its diversity and function, global approaches like metagenomics, metatranscriptomics and metaproteomics are being applied (**Figure 3**). Metagenomics also reveals functional potential of microbial communities in terms of the abundance of the genes involved in particular metabolic processes linked with stresses or stress alleviation mechanism. Similarly, metatranscriptomics can reveal kingdom-level changes in rhizosphere microbiome structure (Turner et al., 2013b) and metaproteomics can reflect community-wide gene expression, protein abundance and putative proteins that can be linked with functions after bioinformatics analysis (Turner et al., 2013b). Diversity profiling and colonization studies using metagenomics can also reveal quantitative colonization of a given host under the influence of stressor. This can yield valuable knowledge regarding stressor-induced alterations in the taxonomic and functional diversity of colonizing population if coupled with metatranscriptomic analysis (Turner et al., 2013b) (**Figure 3**). The coupled analysis thus achieved significantly help in understanding mitigation of stressor-influence over colonization process.

TRANSCRIPTOMICS

Comparison of transcriptome profiles is helpful in identifying different sets of transcripts responsible for differences between two biologically different expressions in various conditions (Bräutigam and Gowik, 2010). Use of mRNA sequencing analysis and microarray technique to generate transcriptome level information is one of the important methodologies employed for studying plant-microbe interactions (Akpinar et al., 2015; Budak and Akpinar, 2015; Wang et al., 2016). Next-generation RNA sequencing study on *Sinorhizobium meliloti* revealed induction of genes for stress response in IAA overproducing strains (Defez et al., 2016). This study compared transcription profile of two *S. meliloti* strains, wild-type strain-1021 and an IAA overproducing derivative RD64. The genes coding for sigma factor RpoH1 and other stress responses were found to induce IAA overproducing strain of *S. meliloti*. Alavi et al. (2013) identified spermidine as a novel plant growth regulator during abiotic stress by transcriptome analysis of rapeseed and its symbiont *Stenotrophomonas rhizophila*.

Different miRNAs in rice, *Medicago, Phaseolus, Arabidopsis* and other plants have a regulatory role under abiotic stresses like drought, salinity and cold (Trindade et al., 2010). miRNAs are non-coding RNAs of 19–23 nucleotide length having regulatory role in several biological processes (Budak et al., 2015). Regulatory role of miR393 was found for salinity tolerance in *Arabidopsis* overexpressing osaMIR393 exhibit tolerant to salt excess (Gao et al., 2011). Zhao et al. (2009) reported miR169 alleviating salinity and drought stress in rice by modulating expression of a nuclear transcription factor YA (*NF-YA*). In tomato, plants overexpressing miR169c which controls expression of gene(s) involved in stomatal activity confer drought tolerance (Zhang et al., 2011). *Bvu-miR13* regulates WD-repeat proteins which plays crucial role in stress tolerance in cucumber (Li et al., 2014). Apart from regulating TFs, miRNAs also target stress signaling pathways which are responsible for root development, leaf morphogenesis and stress response (Curaba et al., 2014). Thirteen mature miRNAs were identified using *in silico* approach in *B. vulgaris* plants (Li et al., 2015).

The activity of superoxide dismutases SOD1 and SOD2 mRNAs are targeted by miR398 that has a role in reducing ROS and secondary effects of abiotic stress (Kantar et al., 2011). Diverse classes of miRNAs alleviate stress by regulating differential cellular responses and metabolic processes like transcriptional regulation, auxin homeostasis, ion transport and apoptosis (Li et al., 2010). miRNA is also found to regulate aluminum stress response in plants (Lima et al., 2011). Comparison of miRNA expression in two different rice subspecies, *japonica* and *indica* differing in aluminum tolerance was done. RT-qPCR approach revealed 16 different kinds of responses of miRNA indicating a complex response under aluminum stress.

UV-B radiation and flooding (hypoxia) affects plants by inducing irreversible damage by generation of ROS (Blokhina and Fagerstedt, 2010). The up-regulation of SOD proteins and miR398 down-regulation is crucial under oxidative stress in *Arabidopsis* (Sunkar et al., 2006). Induction of miR398 and down-regulation of miR395 was observed in alleviating UV-B stress in *Populus tremula* (Jia et al., 2009). Low temperature severely affects sugar beet seedlings and sugar recovery from final harvest. Transcriptome profile of cold stressed plants was done by high throughput RNA sequencing from leaves and roots (Moliterni et al., 2015). Up-regulation of *CBF3* was reported from root tissues faster than the leaf tissues. Genes from *AP2/ERF* family that were known to participate in jasmonic acid mediated responses were also upregulated during cold stress (Licausi et al., 2013).

PROTEOMICS

Proteins play a crucial role in expressing plant stress responses since they directly reflect shaping of a phenotypic trait. Proteomic studies therefore, have become powerful tools for the exploration of physiological metabolism and protein–protein interactions in microbes and plants (**Figures 2** and **3**). The implications of proteomics is important for intra- and inter-microbial species and host–microbe interactions, where host-mediated signaling and tactic responses of related microorganisms are involved (Kosova et al., 2015). Such studies lead to generate a deeper understanding of the regulation of biological system by identifying several proteins as signal of changes in physiological status due to stress or factors responsible for stress alleviation (Silva-Sanchez et al., 2015). Therefore, a comparative analysis in stressed, non-stressed and microbe-associated plants can help to identify protein targets and networks. Proteomic studies for stress responses in crops have been studied extensively in plants including *Arabidopsis,* wheat (*Triticum aestivum*), durum wheat (*Triticum durum*), barley (*Hordeum vulgare),* maize (*Zea maize*), rice (*Oryza sativa*), soybean *(Soybean max),* common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), oilseed rape *(Brassica napus*), potato (*Solanum tuberosum*) and tomato (*Lycopersicon esculentum*) (Liu et al., 2015; Kosova et al., 2015; Xu J. et al., 2015; Wang et al., 2016). Such studies reflected dynamic alternations in protein functional groups, proteins of signaling and regulatory pathways, TFs, protein metabolism, protein–protein interactions at interface, proteins and enzymes conferring several stress-related compounds, functions of structural proteins associated with the cell wall and cytoskeleton and identification of putative proteins using bioinformatics tools (Kosova et al., 2015).

Chen et al. (2015) assessed mechanisms of cold acclimation in alfalfa by proteomic analysis in cold tolerant (ZD7) and cold sensitive lines (W5). Cassava, a tropical crop sensitive to low temperature can modify its metabolism and growth to adapt to the cold stress. Proteomic study was carried out to understand the mechanism behind cold-tolerant process. Twenty differential proteins were found to have similar patterns in apical expanded leaves of cultivars SC8 and Col1046. Expression of proteome profile was found to link closely with changes in photosynthetic activity and peroxiredoxin expression levels. Principle component analysis reflected that electrolyte leakage (EL), chlorophyll content, and malondialdehyde (MDA) accumulation were the physiological indexes in determining cold tolerance in cassava (An et al., 2016).

A leucine-rich repeat receptor kinase (*Srlk*) was reported to function as an upstream regulator of salinity responsive genes in *Medicago truncatula.* It was found to be involved in sensing salinity stress and its response (de Lorenzo et al., 2009). This study revealed an interesting mechanism of sensing salinity stress. Based on proteome profile of barley at different water stress conditions, Ghabooli et al. (2013) proposed that *P. indica* mitigate drought stress by photosynthesis stimulation releasing energy and higher antioxidant production. Wang et al. (2016) screened a novel gene *Ds-26-16* from the cDNA library of 4M salt-stressed *Dunaliella salina*. This gene was found to confer salt tolerance in *E. coli*, *Haematococcus pluvialis* and tobacco. Proteomics data by iTRAQ studies reflected that *Ds-26-16* up-regulates TFs for stress responses like ROS alleviation, osmotic balance, and energy metabolism in *E. coli.*

The diversity of metabolic pathways existing amongst the microbes makes them more responsive toward stress conditions. It is important in the protocols implemented for the elucidation of plant-elaborated responses against stress. Unlike routine proteomics approaches which are more focused toward a single organism, the role of metaproteomics that deals with multiple metabolic interactions occurring simultaneously in an ecosystem is the need of the time (**Figure 3**). This could help to resolve better significance of interdependence between various microbial communities in an agro-ecosystem alongwith their interactions with the host plant. The protocols for protein extraction from the environmental samples are most important success-milestone in the area of metaproteomics (**Figure 3**). Recent advancements in protein sequencing are the key step for the identification of proteins from diverse species (Cordwell et al., 1995, 1997; Shevchenko et al., 2001). The complexity of metaproteome makes resolution and analysis quite difficult. However, recent approaches in extraction and analysis of successful environmental metaproteome could yield decisive output and establish a better correlation among the omics data and response mechanisms among organisms toward stresses (Schulze, 2005; Schweder et al., 2008).

Most of the environmental proteomic experiments are limited to model organisms cultured. They particularly highlight the

exceptional ability of the organisms, e.g., tolerance to salinity, sodicity, temperature, low water availability, toxic metals and radiation etc. The proteomic studies of the organisms lead toward better understanding of fine mechanisms being executed by them. Moreover, the same also stands helpful for the confirmation of the probability of their exploitation for expected induction of the said metabolism in diverse environments. The laboratory experiments allow a better grasp of the protein profile in a controlled environment, however, it contrasts the fact that the expression profile varies with changes in environmental conditions.

The Haloarchea and Halobacteria are gaining strong attention in present era due to their ability to thrive in high salt environments. PGP ability of organisms can be implemented conveniently in saline and sodic soils for the alleviation of respective stresses encountered by the crops. This will prove beneficial for yield improvement in stress-prone areas. Harvesting and implementing metabolites that can confer halotolerance from microorganisms growing in the area of high salt stress with other combined stresses may find important applications in the crop improvement programs. Culturing of these organisms under *in situ* stress conditions in laboratory is the simplest approach to induce the production of effective metabolites that when applied on plants, could impart tolerance against stresses. Similarly, to cope with the most agonizing problem of xenobiotic compounds, the genus *Pseudomonas* is the best considered one, particularly because of its unique ability to degrade enormous amount of carbon sources, especially of xenobiotic nature. The hydrocarbon degradation by *Pseudomonas* is well known. The proteomic experiments for *Pseudomonas* have been designed principally to focus on the recalcitrant, xenobiotic compounds in addition to the toxic organic pollutants (Lupi et al., 1995; Reardon and Kim, 2002; Kim et al., 2007). Species of *Pseudomonas* have been well characterized for their PGP traits such as siderophore production (Ferret et al., 2014; Cunrath et al., 2015), secretion of plant growth stimulating substances (Pereira and Castro, 2014; Balcazar et al., 2015; Sorty et al., 2016), and biocontrol against phytopathogenic organisms (Chet and Inbar, 1994; Natsch et al., 1994; Acebo-Guerrero et al., 2015; Wang et al., 2015). Characteristic versatile metabolic scope and unique biofilm forming ability of the members of this genus (Kertesz et al., 1993; Sauer et al., 2002, 2004; Arevalo-Ferro et al., 2005; VerBerkmoes et al., 2006) permits these species to thrive well under diverse environmental conditions, thus making them most effective inoculants for field application.

The proteomic exploration of methylotrophic bacteria is also an active area of interest today. Methylotrophs constitute a major portion of phylosphere community, typically leaf surface, where one-carbon substrate, methanol is easily available via transpiration activity. Pink-pigmented facultative methylotrophic (PPFM) bacteria are predominant and explored largely for their ability to release plant-growth regulation molecules (Meena et al., 2012; Araujo et al., 2015; Dourado et al., 2015). Many studies have successfully demonstrated PGP potential of these organisms under various conditions (Tani et al., 2012; Yim et al., 2013). Detailed investigations about the proteomic insights of these characteristic phyllosphere-community members helped to get novel ideas regarding involvements of proteins in survival mechanism of organisms under relatively harsh environments, generally encountered on leaf surfaces, where in addition to intense radiation, there exists frequent scarcity of nutrients. Additionally, their potential to secrete plant-growth regulators may come to a large-scale execution. It is, therefore, needful to elucidate deep molecular insights of PGP microbial communities, chiefly involved in stress alleviation to acquire the data regarding mechanisms involved in such processes. The identification of proteins involved in these processes is sufficient to create a boom in stress alleviation strategies at the molecular level where direct implementation of active molecules were thought upon instead of employing the whole organism.

In plants, the study of protein expression of different lines is helpful in selecting cold-tolerant lines for crop improvement. It is evident from earlier studies that cold-tolerant lines showed 14 differential proteome expressions in cold acclimation of sunflower (Balbuena et al., 2011). Proteome analysis also reveals possible mechanisms for chilling mitigation in plants and cross tolerance mechanisms (Yuan et al., 2015; Meng et al., 2016). Once the database of responsive and blocked genotypes is made for particular abiotic stress, it can be used as a marker in differentiating stress responsive genotypes. Santos et al. (2016) has made GeLC–MS/MS based proteomic profiling for large-scale identification of proteins from *Araucaria angustifolia* embryogenic cell lines. In total, 106 proteins were differentially expressed between the responsive and blocked type lines for abiotic stress. Two proteins at early stage were identified to be related with blocked cell lines only. These proteins can be an indicative to blocked cell lines at early stage of plant development.

METABOLOMICS

The scope of metabolomics involves characterization of all the metabolites elaborated by an organism under the influence of given environmental conditions. The metabolome of an organism directly correlates with diverse pathways being operated inside the cell which in turn reflects the availability of corresponding genetic information. The metabolome varies largely with alterations in surrounding environment that induce direct physiological alterations in an organism (Bundy et al., 2005). Similar situations of physiological state are expected in those organisms which are supposed to thrive well under the stress conditions. It is, therefore, important to acquire detailed knowledge of metabolome of an organism both in normal and under-stress physiological status, the subtraction of which will yield the presence/absence of typical signature metabolites of interest. This will be helpful in identifying alterations induced within the pathways and induction of typical stress-inducible genes (**Figure 2**). Metabolomics is increasingly being used for generating deep insights into abiotic stress responses (Jorge et al., 2015; Jia et al., 2016). Recent high throughput developments in the area of molecular detection techniques have given boost to metabolomics studies (Hollywood et al., 2006; Morrow, 2010). Studies highlight the presence of different bioactive chemicals (Burns et al., 2003; Ketchum et al., 2003) in plant metabolome.

This observation correlates with the reports pertaining to the identification of various signal molecules secreted by plants to attract and induce important biochemical pathways in colonizing microbial population (Zhang and Cheng, 2006; Micallef et al., 2009).

Trichoderma spp. produce auxins which stimulate plant growth by alleviating stress (Contreras-Cornejo et al., 2009). Two secondary metabolites, harzianolide and 6-pentyl-a-pyrone of *Trichoderma* was reported to exhibit auxin-like effects in etiolated pea stem (Vinale et al., 2008) and enhance plant growth. Variations induced by changing environmental situations in plant metabolism also affect secretion pattern and nature of secreted molecules (Martínez-Cortés et al., 2014) thereby affecting the level of root colonization. Microbial molecular signaling mechanisms in the rhizosphere are also affected in a similar manner but this is yet to be explored.

Plants accumulate different metabolites like trehalose, glycine betain, IAA etc. in response to abiotic stresses. Allen et al. (2009) reported that mere accumulation of a specific compound does not indicate stress tolerance, but it is the adjustment of flux to different pathways of defense and growth which decides tolerance. Modulation of stoichiometry and metabolism is reported as mechanisms to maintain optimum fitness in plants (Rivas-Ubach et al., 2012). Time-series experiments with *Arabidopsis thaliana* indicated that metabolic activities respond more quickly than that of transcriptional activities to abiotic alterations (Caldana et al., 2011).

The conditions, available within surrounding environment influence pathways operating in the microbial cell, thereby affecting the metabolome. It is evident that the same must affect their overall performance in surrounding microenvironment and within the ecosystem to a greater extent (Tringe et al., 2005; Raes and Bork, 2008; Jiao et al., 2010) in terms of the interactions evident within and between the inhabitants therein. Microbial metabolic products have been involved in both direct as well as indirect plant growth promotion. It is well known that many of the rhizosphere bacteria show the ability to produce plant growth stimulating biomolecules like cytokinins, gibberelins, etc. (Williams and de Mallorca, 1982; Robin et al., 2006). Variety of microbial metabolites including IAA, gibberelins, siderophores serve the purpose. Recently the IAA produced by *Pseudomonas* sp., *Rhizobium* sp., *Enterobacter* sp., *Pantoea* sp., *Marinobacterium* sp., *Acinetobacter* sp., and *Sinorhizobium* sp., has been shown to influence the germination and seedling growth in wheat under saline conditions (Sorty et al., 2016). Similarly, the strains of *Bacillus* sp. having phosphate solubilizing potential successfully improved the yield and quality of fennel in semiarid saline soil (Mishra et al., 2016). The solubilization of phosphate is mainly attributed to the low molecular weight organic acids produced by the microbes. Microbial siderophores also play an important role toward the biological availability of iron to plant roots, for instance, the siderophores produced by *Pseudomonas fluorescens* C7 successfully supplemented the iron to *Arabidopsis thaliana* (Vansuyt et al., 2007). Although the siderophore production by the microbes seems influenced by biogeochemical factors, they also help in the alleviation of the stress imposed by heavy metals (Diels et al., 2002). Many microbes show high degree of environmental dependency for optimal siderophore production. Sorty and Shaikh (2015) reported reduced iron uptake by both sediment as well as soil magnetotactic bacteria under acidic conditions and the probable cause was attributed to the conversion of Fe^{+++} to Fe^{++} under acidic conditions that could have interfered with the siderophore mediated iron uptake system. This signifies the need of keen evaluation of *in situ* mechanisms influencing microbial metabolism. Moreover, the majority of these metabolites are yet to be identified. The cutting edge metabolomics technology could serve as a powerful tool for the evaluation of these metabolites and environmental interventions in the microbial metabolism *in situ*. Many microbes from the ecosystem show interdependence with respect to the substrate utilization and metabolite exchange forming the basis of succession. Moreover, the same is applicable in the area of biodegradation of recalcitrant as well as xenobiotic compounds too, where co-metabolism is shown to play the principle role. This involves simultaneous oxidation of non-substrate compounds with that of true substrates during vigorous growth of bacteria. The members belonging to the genera *Pseudomonas, Flavobacterium, Bacillus, Azotobacter, Microbacterium, Hydrogenomonas, Achromobacter* and *Xanthomonas* are predominant co-metabolisers in the ecosystem (Beam and Perry, 1973). This property of co-metabolism has significant implications in the studies depicting biochemical pathways, particularly involved in the metabolism of polycyclic and polyaromatic compounds (Horvath, 1972; Chauhan et al., 2008). Metabolomics studies of these processes provide the information on the enzymes involved in the conversion and pathways they participate in, thereby raising the probability of their large-scale exploitation to the sites where the native ecosystems have encountered the stress conditions due to the accumulation and/or contamination of xenobiotic and recalcitrant compounds. Many hydrocarbons such as *p*-isopropyltoulene, *n*-butylcyclohexane, *n*-butylbenzene, ortho and para xylene etc. are actively co-metabolized by the members of genus Nocardia (Davis and Raymond, 1961; Raymond et al., 1967). The inimitable metabolic power of such organisms highlight strong implementable ability of PGP members of such genera to remediate stresses imposed by contaminated soils on crop and thus pave the way for bioremediation.

The quantitative metabolomics studies also permit measurements of cellular processes with high accuracy and precision (Noack and Wiechert, 2014). The high-throughput mass spectrometric profiling of cellular metabolites of plant-associated microbes under the influence of stressors could reveal the level of interference by the stressor in the overall cellular homeostasis (**Figure 2**). The communication between plants and soil microbial community represents a bilateral process involving root exudates and microbial-elaborated signal response molecules (Oldroyd and Downie, 2008; Inceoglu et al., 2011; Peiffer et al., 2013). The augmentation of rhizosphere with exogenous microbial metabolites also needs prior insights into the microbial metabolism. This includes the ratio of cellular abundance, biomolecules elaborated under normal and optimal circumstances, quantitative leakage, participation of plant signals in the cascade and resulting counter

response of microbes. It could be thoughtful to enrich such biomolecules in the rhizosphere that are down-regulated due to the influence of the stressor. Similar is applicable to the probable management of stressor-responsive biomolecules influencing overall communication process between the host and microbe. The altered plant root exudation under the influence of stressor fails to induce cascades described above in microbial systems that transpire otherwise.

The accessibility of nucleotide data has been one of the value additions for metabolomics studies (NCBI). This ensures smoothening of future experiments targeting systems biological perspectives. The recruitment of microbial-originated biomolecules and/or *in vitro* synthesized metabolites under simulated conditions in the phyllosphere have been demonstrated recently (Sorty et al., 2016). This mainly deals with the fact that live microbes, under stressed environment fail to express vital genes for PGP activity. However, the impact of enrichment of rhizosphere with the appropriate quantity of such metabolites needs thorough evaluation. The insights to host metabolomics are also beneficial to acquire knowledge regarding the influence of host on post-colonization metabolism of microbes. This could open the gateway for simulation of highly complex endophytic environment.

Rhizosphere community also represents multifaceted system involving biogeochemical cycling and exchange of nutrients, leaving an excellent platform for gaining deep insights in to the systems microbiology. Enormous pathways are simultaneously operated by diverse members of microbial community. Environmental factors have maximum influence on the smooth operation of such pathways. Arrival of stressor/altered environmental situation ultimately diverts overall functionality of microbial system, thereby inducing variation in the community structure. The understanding of biochemical links within and between the members of an ecosystem is necessary to acquire the phenotype-level knowledge in a given biogeochemical state of event (Breitling et al., 2008).

CONCLUSION

Plant responses toward various abiotic stresses and microbe-mediated stress mitigation strategies in plants have been studied on sound grounds of molecular, biochemical, physiological and ultrastructural parameters. Such studies have been carried out encompassing different omics approaches (genomics, metagenomics, metatranscriptomics, proteomics, metaproteomics and metabolomics) that strengthened our understanding behind the mechanisms of microbial interactions, gene cascades and metabolic pathways, accumulation and enhancement of various metabolites, proteins, enzymes and up- and down regulation of different genes. Such studies could yield dynamic data related to combined responses of plants to multiple stresses, and the same is also pertinent with the naturally associated or artificially inoculated microorganisms. These studies provide new directions for improvising the existing protocols in the field of plant–microbe interactions under stress, and use of microorganisms and microbial metabolite molecules for alleviation of diverse stresses encountered by plants. The expected outcomes are facilitating germination, superior sustenance, enhanced ability to combat adverse conditions of environment and superior yield in plants because of the use of microbe-elaborated molecules. Due to limitations regarding the sustenance of microbes in diversity of stress environments and variable responses at phenotypic level, it is always suitable to implement microbe-derived natural products that are capable of performing expected job of stress mitigation irrespective of the environmental situations. To conclude, we strongly advocate that there is a need to put greater attention on in-depth studies pertaining to identification, trait characterization, compatibility assessment, delivery methods and impact of application of microbes isolated from diverse environmental conditions for the mitigation of abiotic stresses in crop plants. We need to find out new roles for microbial metabolites that are being produced under stressed environmental conditions. Established evidences exist to support the role of microbe-mediated plant interactions in stress mitigation under diverse climatic and edaphic conditions. However, more focused omics-based research data generation following integrated approaches encompassing genomics, metagenomics, proteomics and metabolomics studies on specific plant–microbe-abiotic stress system will be needed to resolve many facts behind precise mechanisms of stress tolerance/mitigation in the crop plants.

AUTHOR CONTRIBUTIONS

KM proposed concept, KM and AS collected data and wrote the manuscript, KC and UB collected data, AP and PG added abiotic stress in plants, DS, RP, PS, HS, KK, VG, and PM contributed for omics data and edited the manuscript.

ACKNOWLEDGMENTS

Financial assistance from Indian Council of Agricultural Research (ICAR), India under Application of Microorganisms in Agriculture and Allied Sectors (AMAAS-NBAIM/AMAAS/2014-15/1a(6)/223) and DST-SERB Young Scientist scheme (SB/YS/LS-218/2013) is gratefully acknowledged.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 14 March 2017
doi: 10.3389/fpls.2017.00339

Linking Changes to Intraspecific Trait Diversity to Community Functional Diversity and Biomass in Response to Snow and Nitrogen Addition Within an Inner Mongolian Grassland

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OPEN ACCESS

Edited by:
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Reviewed by:
Zhenzhu Xu, Institute of Botany (CAS), China
Lina Fusaro, Sapienza University of Rome, Italy

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *10 January 2017*
Accepted: *27 February 2017*
Published: *14 March 2017*

Citation:
Mao W, Felton AJ and Zhang T (2017) Linking Changes to Intraspecific Trait Diversity to Community Functional Diversity and Biomass in Response to Snow and Nitrogen Addition Within an Inner Mongolian Grassland. Front. Plant Sci. 8:339. doi: 10.3389/fpls.2017.00339

In recent years, both the intraspecific and interspecific functional diversity (FD) of plant communities have been studied with new approaches to improve an understanding about the mechanisms underlying plant species coexistence. Yet, little is known about how global change drivers will impact intraspecific FD and trait overlap among species, and in particular how this may scale to impacts on community level FD and ecosystem functioning. To address this uncertainty, we assessed the direct and indirect responses of specific leaf area (SLA) among both dominant annual and subordinate perennial species to the independent and interactive effects of nitrogen and snow addition within the Inner Mongnolian steppe. More specifically, we investigated the consequences for these responses on plant community FD, trait overlap and biomass. Nitrogen addition increased the biomass of the dominant annual species and as a result increased total community biomass. This occurred despite concurrent decreases in the biomass of subordinate perennial species. Nitrogen addition also increased intraspecific FD and trait overlap of both annual species and perennial species, and consequently increased the degree of trait overlap in SLA at the community level. However, snow addition did not significantly impact intraspecific FD and trait overlap of SLA for perennial species, but increased intraspecific FD and trait overlap of annual species, of which scaled to changes in community level FD. We found that the responses of the dominant annual species to nitrogen and snow additions were generally more sensitive than the subordinate perennial species within the inner Mongolian grassland communities of our study. As a consequence of this sensitivity, the responses of the dominant species largely drove impacts to community FD, trait overlap and community biomass. In total, our study demonstrates that the responses of dominant species in a community to environmental change may drive the initial trajectories of change to community FD and functioning.

Keywords: life history, community assembly, functional diversity, intraspecific variance, trait overlap

INTRODUCTION

Functional diversity (FD) can be a strong predictor of ecosystem processes (Albert et al., 2010; Jung et al., 2014; Lamanna et al., 2014), and has allowed ecologists to scale up the functional traits of individuals to the community level (de Bello et al., 2013; Kazakou et al., 2014). Previous findings suggest that communities with large interspecific FD are more likely to have species with traits adapted to novel environmental conditions, of which may facilitate rapid changes in the composition of community level traits through species turnover (Kraft et al., 2007; Weiher et al., 2011). As for intraspecific trait variability (ITV), previous research has typically assumed that ITV is small compared to interspecific variation (Kraft et al., 2008). However, recent evidence suggests that ITV may account for as much as 25% of the total trait variation on average within communities at the global scale (Siefert and Ritchie, 2016). Moreover, recent studies have highlighted the notion that ITV is likely to be important for contributing insight toward rules governing plant community assembly and ecosystem functioning (Violle et al., 2012; Auger and Shipley, 2013; Hulshof et al., 2013; Siefert et al., 2015).

For quantitative assessments of ITV, Violle et al. (2012) provided a framework for how to calculate internal filtering with using the $T_{IP/IC}$ metric. $T_{IP/IC}$ is the ratio of variation of trait values among individuals within populations (IP) to variation of trait values among individuals (IC) in the community, and thus is a strong indicator for assessing trait overlap between species for a given level of interspecific FD (Violle et al., 2012). Communities with high $T_{IP/IC}$ indicate that species within the community have more similar traits, and vice versa. This assessment is also consistent with niche-based model approaches. As in the niche-based model, the dissimilarity or similarity within and between coexisting species can provide insight as to how the available resources are partitioned among species within the community (Weiher and Keddy, 1995; Cornwell and Ackerly, 2009; Mason et al., 2011).

For plant communities with highly uneven species abundances, the ITV of dominant species within the community may have a significant effect on community FD due to such species occupying larger niche space (Johnson et al., 2015). According to the 'mass ratio hypothesis' (Grime, 1998), the extent to which a trait of a plant species impacts ecosystem functioning is posited to be related to the contribution of a species to community composition and/or productivity. The underlying assumption of this hypothesis is that weighting traits by species abundance will improve the scaling of individual responses to community and ecosystem processes (Grime, 1998; Lavorel and Garnier, 2002). As a consequence, the function of an ecosystem likely depends to a great extent on the functional traits of the dominant species or functional groups within communities (Breza et al., 2012). Therefore, the intraspecific variation of dominant species may largely determine community trait space and the ability to obtain resources (Johnson et al., 2015), and may also impact other properties such as community biomass (Li et al., 2016). For example, dominant species with higher specific leaf area (SLA), photosynthetic efficiency, and thus resource acquisition have been shown to produce higher leaf biomass that directly scales to the community level, leading to a strong association between the dominant species traits and SLA at community scale, of which may further scale to community productivity (Li et al., 2016). As such, environmentally driven changes to key traits such as SLA may very well scale up to changes to the community and ecosystem level.

Interannual fluctuations of snow have increased due to increases in climate variability associated with global climate change, while anthropogenic nitrogen deposition has increased dramatically since the industrial revolution (Galloway et al., 2004; Bobbink et al., 2010; Liu et al., 2013). Anthropogenic nitrogen deposition includes the input of reactive nitrogen from the atmosphere to the biosphere from such sources as gasses, dry deposition and in precipitation as wet deposition. Increased nitrogen deposition will impact biodiversity at the global scale (Sala et al., 2000), and thus may also impact both ITV and FD within communities, of which can be represented by the variation in $T_{IP/IC}$.

Intraspecific trait variability has been demonstrated to mediate the effects of climatic changes, such as extreme drought, on subalpine grassland communities (Jung et al., 2014), yet the dynamics of ITV may differ among species with different life histories. More specifically, annual species and perennial species often exhibit different patterns of responses to environmental change, which has been shown to impact species turnover and community productivity (Mao et al., 2012). For annual species, nitrogen addition has been observed to reduce the proportion of belowground biomass and increase aboveground biomass (Cai et al., 2005; Qi et al., 2011; Yan et al., 2013), while only significantly increasing the belowground biomass of perennial grasses (Song et al., 2012). Further, in low nutrient habitats annual species have been reported to have higher reproductive outputs than perennial species (Ploschuk et al., 2005).

Snow is a potential source of water via soil moisture and thus is critical for plant ecophysiological processes, such as biomass production (Wipf, 2010). Previous studies have shown that increased snow may be more important for perennials, and thus increased snowfall in winter could lead to changes in species composition due to greater benefits to perennial species (Chen et al., 2008; Aerts et al., 2009; Liu et al., 2011). For example, in the alpine meadow of the Qinghai–Tibet Plateau, increased snowfall increased the aboveground biomass of perennial grasses, yet had little effect on annual species (Shen et al., 2002). Although there is some evidence to suggest that snow and nitrogen deposition have interactive ecological effects (Zatko et al., 2016), few studies have analyzed the interaction between long-term, continuous nitrogen addition and interannual snow addition. Moreover, although the effects of nitrogen on intraspecific FD and interspecific FD have been previously documented (Koerner et al., 2016; Siefert and Ritchie, 2016), few studies have examined the effects on both intraspecific and interspecific FD, and in particular the role of dominant versus subordinate species responses in driving impacts at the community level. To address these uncertainties, we exposed a grassland ecosystem to multiple years of increased nitrogen and snow additions.

The research site of this study is located in northern Inner Mongolia, which has come to be dominated by annual species due to land degradation (Mao et al., 2012). Therefore, we posited that changes to the ITV and/or FD of annual species may largely determine impacts at the community level. In this area, severe land desertification and subsequent wind erosion leads to loss of clay and silt particles, resulting in soil infertility and coarsening, and thus decreased soil water holding capacity and soil inorganic nitrogen availability (Zhou et al., 2008). Such dynamics make these already drought-prone and nutrient-poor plant communities increasingly subject to water and nutrient limitation.

Nitrogen addition may favor species that allocate more biomass to aboveground carbon assimilation organs (Bai et al., 2010; Koerner et al., 2016). However, the responses of plant species to nitrogen deposition is highly variable (Diekmann and Falkengren-Grerup, 2002). However, the effect of nitrogen addition on annual plant has been observed to be stronger than perennial species, with snow more strongly impacting perennial species (Wipf, 2010; Lü et al., 2014). Thus, if a community is exposed to both of these drivers, there will likely be trade-offs between snow and nitrogen effects on species performance within the community, particularly if the community is comprised of both perennial and annual species. In accordance with prior findings, we hypothesized that snow addition would significantly affect the intraspecific variation and traits of overlap of perennial species. However, we posited that nitrogen addition would increase the intraspecific and traits overlap of annual species in the community. This hypothesis lead to three key predictions:

(1) Nitrogen addition will more strongly affects annual plants via increased ITV, leading to higher $T_{IP/IC}$.
(2) Snow addition will more strongly affected perennial plants via increased ITV and thus $T_{IP/IC}$, yet the effect will disappear after cessation of snow addition.
(3) Changes in the ITV of the dominant annual species will more strongly scale to effects on community ITV and biomass than changes to the ITV of subordinate annual plants.

MATERIALS AND METHODS

Experimental Design

The nitrogen and snow addition experiment was established in the Horqin Sandy Grassland of eastern Inner Mongolia, China (42°55′N, 120°42′E) on 5 October 2009 and ran through 10 October 2014. The total soil nitrogen content in the top 0–30 cm ranged from 0.057 to 0.199 (%), and the soil bulk density ranged from approximately 1.29–1.59 g cm^{-3} (Mao et al., 2012). Soil nitrogen and snow were treated as limiting factors that reflect both stressed and unstressed habitats depending on their availability. For the soil nitrogen, stressed conditions represented ambient conditions due to the characteristically low nutrient levels (represented by N_0). For the unstressed condition, we added extra 536 g (equal to 100 kg ha y^{-1}) of $(NH_2)_2CO$ to each plot to represent high nutrient levels (represented by N_1). For snow, control plots received no additional snow (represented by W_0). For the high snow level, we added snow equivalent to 100 mm precipitation (weight conversion) applied during the winter (represented by W_1).

Nitrogen was added each May from 2009 to 2014, and snow was added in the winter from December 2009 to April 2010, from December 2010 to April 2011, and from December 2011 to April 2012. Snow was collected outside of the experimental plots during snowfall in winter and added to the each W_1 plots evenly. The snow added was equal to 30% of the average annual precipitation during the growing season from the last 20 years (Mao et al., 2012).

We used a randomized complete block design, consisting of 24 10 m × 10 m plots. We employed two nitrogen and two snow treatments, as well as their interactions. The four experimental treatments are as follows: (1) control (N_0W_0), (2) high nitrogen and low snow (N_1W_0), (3) high nitrogen and high snow (N_1W_1), and (4) low nitrogen and high snow (N_0W_1). Each treatment was replicated six times.

Leaf Traits and Biomass Measurements

Vegetation surveys were carried out during peak biomass accumulation in August from 2010 to 2014. In order to prevent edge effects, the quadrats were set at least 1 m from the edge, with the size of each sampling plot 1 m × 1 m. Species richness, abundance and the e were measured in each plot. Aboveground biomass was clipped, dried at 65°C and weighed to the nearest 0.01 g. Root-type and cluster perennial species, like *Pennisetum purpureum* and *Cleistogenes squarrosa*, were used to calculate the number of ramets. All communities were located in a grazing-free enclosure.

Leaf samples were collected in August 2010–2014 from the same plots used to assess SLA (kg m^{-2}). We randomly chose three individuals of the selected plants from the six plots of each treatment for leaf functional traits analysis. A single fully expanded mature leaf was selected from three individuals of each species and used to determine leaf area and dry mass (drying for 48 h at 65°C) (Cornelissen et al., 2003; Perez-Harguindeguy et al., 2013). Selected species were determined to be dominant based on their abundance by weight within each given community. The percent abundance of dominant annual species was higher than 80% of the community among all plots.

We selected SLA at the species level to describe the total FD at the community level. We chose this trait because it is directly related to plant competitive ability with respect to different levels of nitrogen availability, and reflects trade-offs between tolerances of environmental stressors (e.g., nutrient or climatic) (Lepš et al., 2011). High SLA also indicates a fast rate of resource uptake (Wright et al., 2004; Laughlin, 2014), and thus increases in SLA will likely enhance plant-atmosphere gas exchange (Lepš et al., 2011; Koerner et al., 2016).

Functional Diversity Indices

The total community FD, within species FD, and between-species FD were quantified for each treatment (utilizing R code by de Bello et al., 2011). This method can be used with multi

and single trait approaches (Albert et al., 2010; de Bello et al., 2011). The contribution of intraspecific variability to community variability was assessed via $T_{IP/IC,}$ which is the ratio of within-species (IP) variance to total community variance (IC) (Violle et al., 2012). All FD was calculated on the basis of three groups of species data; the first set of data was based on all species in each community. The second data set was based only on the annual plants that were determined as dominant in the community. The third data set was based only on all perennial species that were determined as subordinate in the community (Mao et al., 2012). A null modeling method was used to analyze the trait overlap for each community within the four different treatments.

To make sure that the observed pattern of $T_{IP/IC}$ responses to different environmental variables was non-random (Mason et al., 2011; Kumordzi et al., 2015a), trait overlap (i.e., $T_{IP/ICexpected}$) was calculated by randomizing SLA values between all individuals in the given community. We utilized the equation $T_{IP/IC}SES = [T_{IP/ICobserved} - \text{mean}\ (T_{IP/ICexpected})]/\text{sd}\ (T_{IP/ICexpected})$ (for further details see Kumordzi et al., 2015a). We then developed a large matrix that takes into account both species relative abundance and richness within the community.

Statistical Analyses

We analyzed the effect of nitrogen and snow on biomass of annual and biomass of perennial species using analysis of variance (ANOVA). We also analyzed the intraspecific FD, interspecific FD and total FD at the community scale (de Bello et al., 2011). A null model was used to analyze the significance ($p < 0.05$) of intraspecific FD, interspecific FD and total FD at the community scale, annual species scale and perennial species scale, and was calculated as the difference between the observed and expected values (Kumordzi et al., 2015a). We used both R statistical software (R Development Core Team, 2014) and SPSS (version 19.0; SPSS Inc., Chicago, IL, USA) to analyze the data.

RESULTS

Nitrogen addition increased community biomass, the biomass of annual species, yet reduced the biomass of perennial species (**Figures 1**, **2**). Compared with the control treatment, community biomass increased by 11.5, 49.8, 32.4, 49.4, and 17.7% after adding nitrogen in 2010, 2011, 2012, 2013, and 2014, respectively (**Figure 1**). In 2011, nitrogen addition increased annual species' biomass by 128%. Surprisingly, the main effect of snow addition had no significant effect on biomass at the community, annual species or perennial species level (**Figure 2**). The interaction of nitrogen addition and snow addition significantly increased community biomass and the biomass of annual species. For example, in 2010, the interaction of nitrogen addition and snow addition increased 43.3% biomass of annual species, compared to control treatment (**Figure 2**).

One year after cessation of snow addition, there was still an interaction between nitrogen and snow additions. In 2013 the biomass of annual species in N_1W_1 treatment was 45.6% higher than snow additions alone. In 2014, nitrogen addition and the interaction of nitrogen and snow addition increased the biomass of annual species by 61.3 and 64.9%, respectively, compared to the control treatment (**Figure 2**). In 2010, 2011, and 2012, the biomass of perennial species at the nitrogen addition treatment decreased by 89.1, 235.9, and 20.6%, respectively, as compared with the control treatment. However, this trend began to weaken in the fourth year of nitrogen addition, and consequently nitrogen addition did not significantly reduce the biomass of perennial species in 2013.

We chose the first year of snow addition in 2010, and the last year of snow addition in 2012, and the second year following cessation of snow addition in 2014 to analyze the SLA of all coexisting species in the community. The results show that the effects of nitrogen addition, snow addition, and their interaction on intraspecific FD are significant, while the effects on the interspecific FD and total FD were comparatively weak (**Figure 3**). However, these effects differed among annual and perennial species. For example, snow addition in 2010 and 2012 increased intraspecific variation in annual species yet had no significant effect on perennials (**Figure 4**). Conversely, the interaction of nitrogen and snow addition dramatically affected the intraspecific variation of perennial species, yet not annual species in 2010 and 2012 (**Figure 4**). Despite these results, the nitrogen addition, snow addition and the interaction of nitrogen and snow ultimately had little effect on interspecific variation. However, in 2014 N addition did significantly impact the interspecific variation of annual species, while the interaction of nitrogen addition and snow addition affected the perennial interspecific variation significantly.

The trends of $T_{IP/IC}$ after nitrogen addition and snow addition treatment were similar to that of intraspecific FD and total FD. The effects of nitrogen addition on $T_{IP/IC}$ of the community, $T_{IP/IC}$ of annual species and $T_{IP/IC}$ of perennial species were significant (**Table 1**). After snow addition, the $T_{IP/IC}$ of annual species increased markedly despite no impacts to biomass. However, $T_{IP/IC}$ of annual species returned to near control levels after cessation of snow additions. Snow addition had no significant effect on the $T_{IP/IC}$ of perennial species. Although the trend of biomass and community level ITV had no significant correlations, changes to the ITV and biomass of dominant plants were consistent with each other.

DISCUSSION

The Effect of Nitrogen Addition on Intraspecific FD and Interspecific FD

We observed shifts in the trait overlap between annual species and perennial species in response to key global change drivers of the study region, of which further underscores the potential role of niche packing as a central mechanism for the coexistence of plant species (Kraft et al., 2008; Mason et al., 2011; Le Bagousse-Pinguet et al., 2015; Fajardo and Siefert, 2016). Due to land desertification and subsequent wind erosion, the available soil nitrogen in this grassland is very low (Zhou et al., 2008). As a

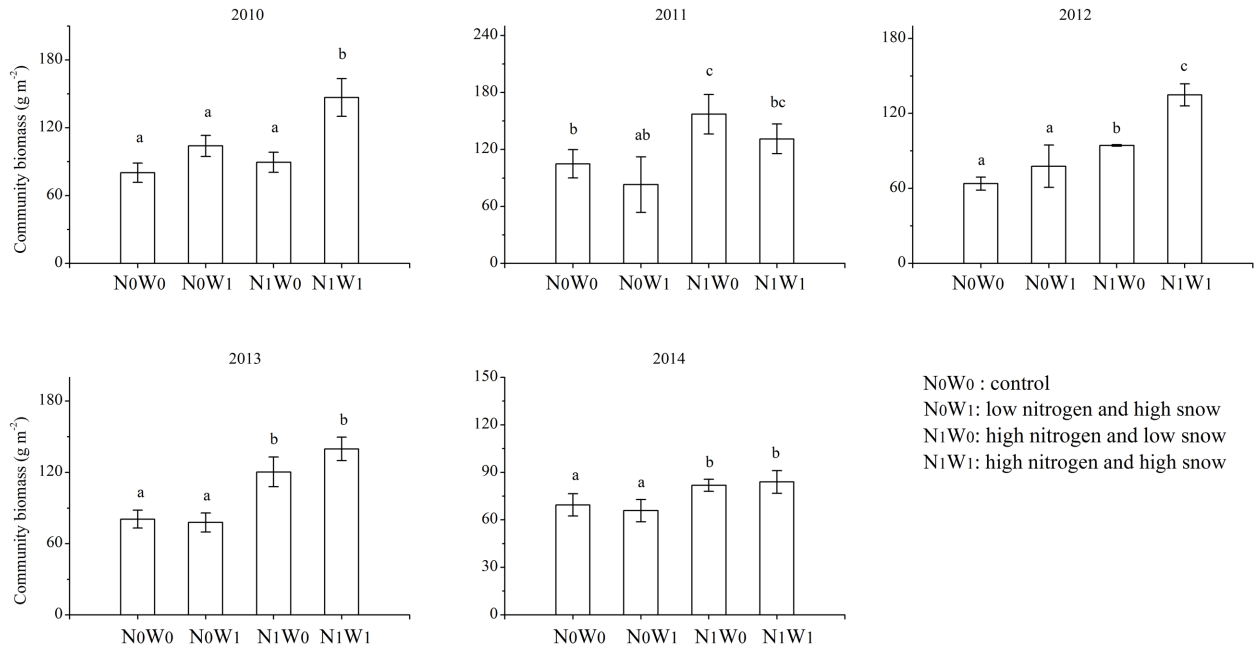

FIGURE 1 | The productivity of 2010–2014 at community scale. Significance are indicated by different letters ($P < 0.05$).

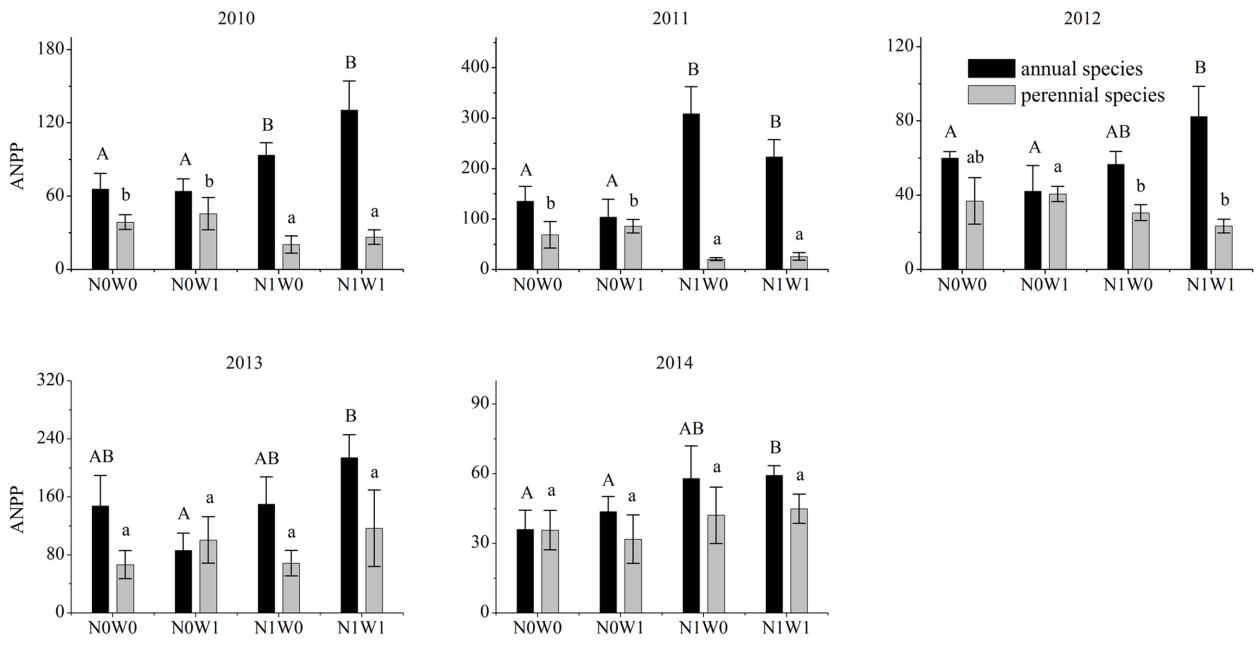

FIGURE 2 | ANPP of annual species and perennial species of 2010–2014. Significance are indicated by different same type letters ($P < 0.05$).

result of this limitation, the majority of the dominant species in this grassland are annual species, which are also sensitive to changes in nitrogen availability (Bai et al., 2010; Mao et al., 2012).

Indeed, in the plant communities of our study, the dominant annual species were highly sensitive to nitrogen addition (Mao et al., 2012). We observed that increased species trait overlap within the community due to nitrogen addition was mainly caused by increases to the ITV of the dominant annual species. In this nutrition-poor habitat, nitrogen addition alleviates nutrient limitation and thus alters the role of environmental filtering as a mechanism of community assembly (Chapin et al., 1986; den Haan et al., 2016), as nitrophilic

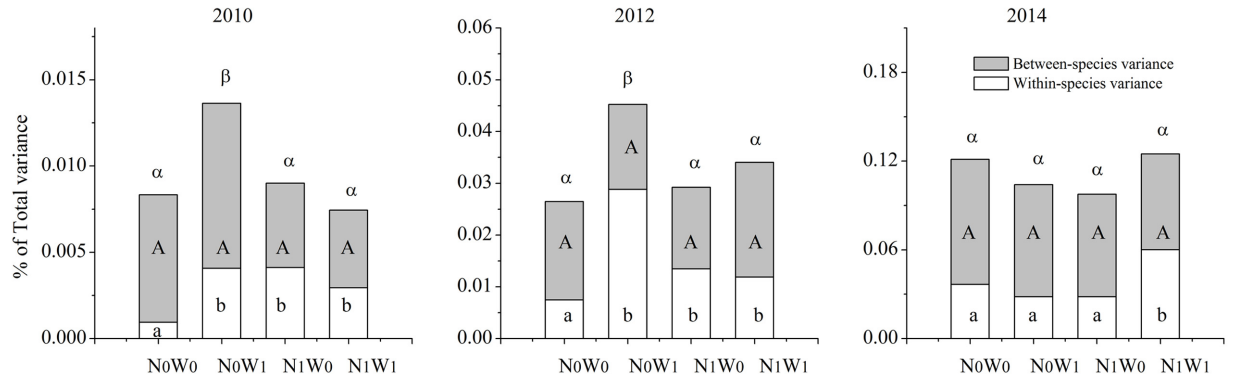

FIGURE 3 | Partitioning of the total functional diversity at community in to intraspecific functional diversity (transparent) and interspecific functional diversity (light gray) for SLA. Significance are indicated by different same type letters ($P < 0.05$).

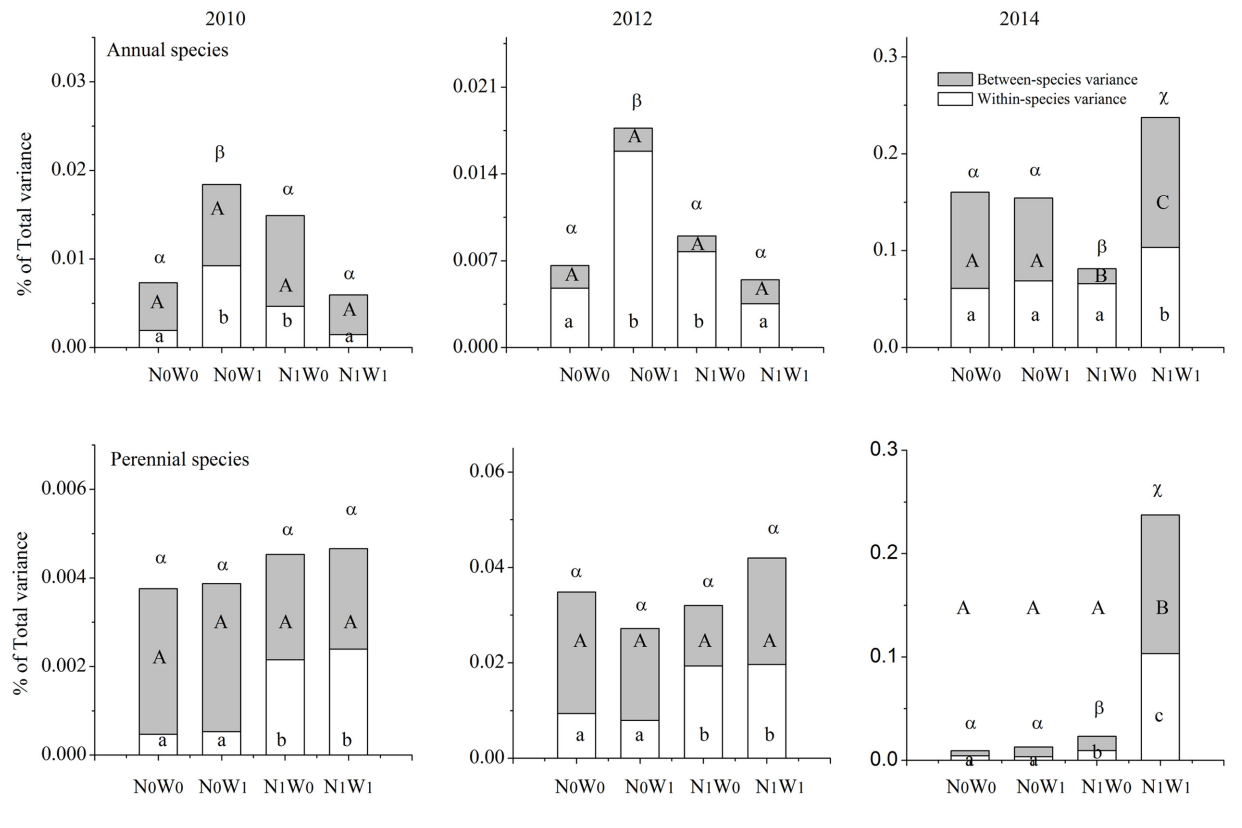

FIGURE 4 | Partitioning of the total functional diversity of annual species and perennial species in to intraspecific functional diversity (transparent) and interspecific functional diversity (light gray) for SLA. Significance are indicated by different same type letters ($P < 0.05$).

plants may show more advantages as nitrogen levels increase (Menge et al., 2015). Moreover, coexisting species are likely to exhibit more nitrophilic traits in the community after nitrogen addition, and as a result trait overlap will likely increase.

The responses of the subordinate perennial species to nitrogen addition was different than annual species (**Supplementary Figure S1**). It is likely that light competition increased after nitrogen addition (Hautier et al., 2009; Okubo et al., 2012). This may then increase the upper limit of the SLA range, and further increase ITV via greater trait overlap among individuals. However, biomass of the dominant perennial species increased quickly after nitrogen addition and thus may have ultimately heightened competitive exclusion of perennial species in the community. This dynamic may operate to reduce trait overlap. The trait overlap of perennial species

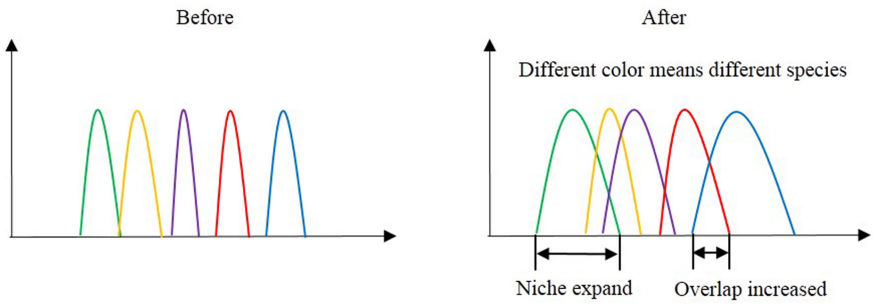

FIGURE 5 | Niche overlap before and after nitrogen addition and snow addition.

TABLE 1 | The trait overlap of observed community, annual species and perennial species.

	Treatments\$T_{IP/IC}$	2010	2012	2014
Community	N_0W_0	0.240	0.342	0.268
	N_0W_1	**0.445***	0.468	0.382
	N_1W_0	**0.427***	**0.680***	**0.451***
	N_1W_1	**0.442***	0.384	**0.442***
Annual species	N_0W_0	0.272	0.467	0.420
	N_0W_1	**0.521***	**0.652***	0.309
	N_1W_0	**0.405***	**0.675***	**0.534***
	N_1W_1	0.321	**0.564***	0.406
Perennial species	N_0W_0	0.340	0.366	0.462
	N_0W_1	0.251	0.276	0.309
	N_1W_0	**0.686***	**0.465***	**0.534***
	N_1W_1	**0.632***	**0.451***	0.406

*All the data is log-transformed; Significance are indicated by star symbol (*P < 0.05).*

is likely the result of the trade-off between the overlap increase caused by nitrogen addition and the overlap decrease caused by the increase in the intensity of competition. The trait overlap of perennial species increased after nitrogen addition (Le Bagousse-Pinguet et al., 2015), which indicates that environmental filtering played a more obvious role in driving increased trait overlap as compared to interspecific competition (Kumordzi et al., 2015a). These results are consistent with hypothesis i.

We did not find a detectable decrease in interspecific FD variation after nitrogen addition. This result contradicts with the prediction that nitrogen addition will impact species composition, and thus potentially increase the functional trait diversity at community scale (Weiher et al., 2011; Siefert and Ritchie, 2016) by extending the niche space of species in the community at the interspecific level (Kumordzi et al., 2015a). There may be two reasons for this result. First, the dominant species in our sandy grassland communities are annual forb species. The intraspecific variability of these species increased sharply after nitrogen addition, which may have obscured the impacts caused by interspecific FD variation. For example, in Qinghai Tibet the dominant species maximize resource use after nitrogen addition through intraspecific variability in SLA to occupy greater niche space, thus allowing more nitrogenous plants to coexist (Li et al., 2016) and leading to higher $T_{IP/IC}$ in the community. Second, the species richness is very low in our study area. In species poor communities, the intraspecific variation is typically much higher than species-rich communities (Mao et al., 2012), while in comparison the interspecific variation is relatively small.

The Effect of Snow Addition on Intraspecific FD and Interspecific FD

We found that snow did not change the intraspecific FD variation and trait overlap of the subordinate perennial species, which is not consistent with hypothesis ii. However the dominant annual species in the community increased their intraspecific FD variation and trait overlap after snow addition. The reason for the weak effects of snow on perennial species may be that, firstly, snow addition in this study did not directly affect the growth of perennials. The increase of snow in winter increases the thickness of snow, soil temperature and soil water supply in early spring, and thus affects plant growth and biomass allocation (Chen et al., 2008; Wipf, 2010). However, in the study area of Inner Mongolia, strong continuous winds blow accumulated snow rapidly, while the sandy soil also has poor water holding capacity (Yao et al., 2013). As a consequence, water produced after snowmelt may migrate to deep soil layers very quickly, where plant roots may not be able to fully access this resource.

Second, although snow addition slightly increased available soil available nutrients, this did not impact perennial species. This may be due to the fact that the perennial species in our study were mostly clonal plants, and that redistribution of available nutrients among individual clones could reduce the importance of small-scale spatial heterogeneity for FD and community assembly (Gundale et al., 2011). Patterns of snowfall are likely to change within the Inner Mongolian region, with these changes are less certain as compared to temperature change. One key prediction is that the amount of snowfall will increase each time, yet that the interval between events will also increase (IPCC, 2013). In this snowfall change scenarios, different functional groups of plants show different response strategies. Thus it may be the case that the response of annuals to snow changes is more pronounced

in arid and semiarid regions, while perennial responses may comparatively insensitive to snow changes (Mao et al., 2012).

The change of trait overlap of annual species was more obvious after snow addition, and the variation in this pattern was similar to the changes at the community scale. This result is consistent with the 'mass ratio hypothesis,' which posits that the function of the ecosystem is largely determined by the traits of the dominant species or functional groups (Grime, 1998; Johnson et al., 2015). Therefore, the ITV of SLA for the dominant annual species is likely to play a more important role in determining patterns of community level FD than that of subordinate species (Lavorel and Garnier, 2002; Mao et al., 2012; Volf et al., 2016). The different variance pattern of annual species and perennial species suggests that changes to the niche space occupied by the dominant species or functional group will likely affect niche packing at the community level (Breza et al., 2012; Li et al., 2016). These contrasting responses of intraspecific FD and interspecific FD between dominant and subordinate species also suggests that species niche packaging plays an important role in maintaining the FD of plant communities (Johnson et al., 2015).

Although the effects of snow addition alone on intraspecific FD and interspecific FD of sandy grassland ecosystems were weak, there was an interaction between snow addition and nitrogen addition. This interaction remained 1 or 2 years after cessation of snow addition. Even if the interaction between snow addition and nitrogen addition are mostly driven by the effect of nitrogen alone, increasing snowfall amount may lend insight to potential variability in the effects of nitrogen on the community (**Figure 4**). For instance, the interaction of snow addition and nitrogen addition on intraspecific variability are significant, while the effect on interspecific FD was weak. There were temporally different responses between annual species and perennial species in N_1W_1 treatment. For instance, the interaction of nitrogen and snow had no significant effect on intraspecific FD and interspecific FD of annual species in 2010 and 2012 when both nitrogen and snow were added, yet affected the intraspecific FD and interspecific FD of perennial species. This is clearly different from the addition of nitrogen alone.

The Variation of $T_{IP/IC}$ and Biomass

The response of intraspecific variation, $T_{IP/IC}$ and biomass of the dominant annual species to nitrogen addition were related to trends at the community level, and the response of the annual species differed from that of the subordinate perennial species (**Figure 5** and **Supplementary Figure S2**). This result supports our third hypothesis. Previous studies have shown that communities with higher intraspecific FD, potentially indicative of high phenotypic plasticity or genetic variation, may also have increased capacity to respond to environmental change through changes in traits within the same species (Grime, 2006). In this study, the biomass allocation of dominant species changed after nitrogen addition, and more nutrients were likely distributed to the leaves, which expanded the range of leaf traits and increased leaf, species and community biomass. Conversely, the effect of nitrogen addition on perennial plants was relatively weak and even negative (Mao et al., 2012). We also used standardized major axis tests to analyze the variation of annual species and perennial species after nitrogen addition. The results indicate that the resource utilization axis of the annual species shifted to the direction of nitrogen use after nitrogen addition, and that the community showed more nitrophilic functional traits (e.g., high SLA and leaf nitrogen content) as the ratio of leaf weight to total weight increased significantly (Mao et al., 2012). This effect in turn increased the biomass of the community, without significant changes in biomass allocation of perennial species after nitrogen addition.

The biomass of the community and biomass of annual species increased at nitrogen addition treatment and N_1W_1 treatment, yet interestingly the biomass of perennial species decreased significantly. This contrasting result suggests that different community components, such as dominant versus subordinate species, will likely have differential responses to environmental change drivers (Fargione and Tilman, 2006; Kumordzi et al., 2015b). Moreover, depending on a species role in the community, the response of a species to environmental change may or may not translate to detectable impacts at the community level. For example, community biomass was mainly affected by the biomass of dominant annual species, which showed the same trend with the $T_{IP/IC}$ of annual plants, despite a negative trend with the changes of subordinate perennial plant biomass. This further suggests that changes in the functional traits and productivity of dominant and subordinate species may help to more accurately understand which component of the community has a greater impact on community assembly and potentially ecosystem processes.

CONCLUSION

We analyzed the effects of nitrogen addition and snow addition on the intraspecific and interspecific variation of the community, annual species, and perennial species. We studied how changes to these environmental factors may influence coexistence, community FD and biomass. First, nitrogen addition increased the biomass, intraspecific variation and total FD of the annual plants, and eventually increased it's $T_{IP/IC}$. Nitrogen addition reduced the biomass of and intraspecific variation, and trait overlap of perennials, yet did not change total FD. Second, snow addition did not change the biomass of annual species, but increased intraspecific variation at the community scale by increasing the intraspecific variation of annuals. Snow addition also had no effect on the biomass, ITV and trait overlap of perennial plants. In total, the ITV of SLA of the dominant annual plant species, a trait related to plant resource acquisition capacity, partially informed changes to total community FD and biomass. Thus, global change-driven trait shifts among dominant species within plant communities, particularly if they are species-poor, are likely to underlie the initial changes to community FD and function.

AUTHOR CONTRIBUTIONS

WM carried out this research, collected the field data and draft the manuscript, carried out all data analysis. TZ conceived of the study, designed the study and AF helped draft the manuscript; All author gave final approval for publication.

ACKNOWLEDGMENTS

This work was supported by the National Science and Technology Basic Resource Survey (SQ2016FY491000700), National Natural Science Foundation of China [41671208, 31270501], West Light Foundation of Chinese Academy of Science [Y529891001], and Foundation of China Postdoctoral Science [2014T70947, 2013M542403]. We also gratefully acknowledge two reviewers for helpful comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00339/full#supplementary-material

FIGURE S1 | The variance of richness, abundance and coverage between annual species and perennial species.

FIGURE S2 | The variance extent of intraspecific functional diversity and productivity.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 15 June 2017
doi: 10.3389/fpls.2017.01020

Losing the Warning Signal: Drought Compromises the Cross-Talk of Signaling Molecules in *Quercus ilex* Exposed to Ozone

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: 21 February 2017
Accepted: 29 May 2017
Published: 15 June 2017

Citation:
Cotrozzi L, Pellegrini E, Guidi L, Landi M, Lorenzini G, Massai R, Remorini D, Tonelli M, Trivellini A, Vernieri P and Nali C (2017) Losing the Warning Signal: Drought Compromises the Cross-Talk of Signaling Molecules in Quercus ilex Exposed to Ozone. Front. Plant Sci. 8:1020. doi: 10.3389/fpls.2017.01020

Understanding the interactions between drought and acute ozone (O_3) stress in terms of signaling molecules and cell death would improve the predictions of plant responses to climate change. The aim was to investigate whether drought stress influences the responses of plants to acute episodes of O_3 exposure. In this study, the behavior of 84 Mediterranean evergreen *Quercus ilex* plants was evaluated in terms of cross-talk responses among signaling molecules. Half of the sample was subjected to drought (20% of the effective daily evapotranspiration, for 15 days) and was later exposed to an acute O_3 exposure (200 nL L^{-1} for 5 h). First, our results indicate that in well-water conditions, O_3 induced a signaling pathway specific to O_3-sensitive behavior. Second, different trends and consequently different roles of phytohormones and signaling molecules (ethylene, ET; abscisic acid, ABA; salycilic acid, SA and jasmonic acid, JA) were observed in relation to water stress and O_3. A spatial and functional correlation between these signaling molecules was observed in modulating O_3-induced responses in well-watered plants. In contrast, in drought-stressed plants, these compounds were not involved either in O_3-induced signaling mechanisms or in leaf senescence (a response observed in water-stressed plants before the O_3-exposure). Third, these differences were ascribable to the fact that in drought conditions, most defense processes induced by O_3 were compromised and/or altered. Our results highlight how *Q. ilex* plants suffering from water deprivation respond differently to an acute O_3 episode compared to well-watered plants, and suggest new effect to be considered in plant responses to environmental changes. This poses the serious question as to whether or not multiple high-magnitude O_3 events (as predicted) can change these cross-talk responses, thus opening it up possible further investigations.

Keywords: climate change, holm oak, mediterranean plant species, phytohormones, hypersensitive response, stress combination

INTRODUCTION

Mediterranean plants are threatened by several abiotic stress factors [e.g., warming, drought, tropospheric ozone (O_3), UV radiation, salinity] due to environmental changes characterized by new types of stress conditions and stress combinations, which are expected to be more extreme in the Mediterranean than in other areas worldwide (Matesanz and Valladares, 2014; Gray and Brady, 2016; Guidi et al., 2017). Today, drought is the major factor limiting plant performance, and revealing the mechanisms that enable plants to survive or acclimatize to such conditions is crucial (Claeys and Inzé, 2013). On the other hand, O_3 is by far the most phytotoxic air pollutant with deleterious effects on growth and productivity (Vainonen and Kangasjärvi, 2015; Cotrozzi et al., 2016a; Yang et al., 2016). Both drought and O_3 are co-occurring, increasing stressors in future climate change scenarios (Bates et al., 2008). Given that high-level O_3 episodes and drought often occur together in Mediterranean areas, especially during the summer, their interaction needs to be understood (Iyer et al., 2013; Cotrozzi et al., 2016b). O_3 enters the leaf through open stomata, then drought-triggered stomatal closure limits O_3 uptake, thereby limiting foliar damage (Panek et al., 2002). However, other studies have highlighted that these factors have a synergistic effect, with increased O_3 sensitivity observed in droughted plants (Alonso et al., 2014; Pollastrini et al., 2014). The response of plants to a combination of stresses is species-specific and depends on the intensity and duration of each stress factor (Ramegowda and Senthil-Kumar, 2015).

Plant exposure to acute O_3 (high O_3 concentration within a short period) commonly occurs during hot, dry Mediterranean summers (Matesanz and Valladares, 2014), which often results in a programmed cell death (PCD) response. This is a physiological process that selectively targets and eliminates unwanted cells in response to a variety of biotic and abiotic stimuli (Apel and Hirt, 2004). PCD resembles the hypersensitive response (HR) observed in several plant-pathogen interactions, which often precedes the acquisition of a systemic resistance by plants (Kangasjärvi et al., 1994; Rao et al., 2000; Pellegrini et al., 2013; Vainonen and Kangasjärvi, 2015; Pellegrini et al., 2016). O_3 entering the leaves first induces a biphasic oxidative burst with a massive, rapid and transient increase in apoplastic reactive oxygen species (ROS), which is the main event leading to PCD activation (Langebartels et al., 2002) Similarly, an oxidative burst was usually observed in plants under drought (Smirnoff, 1993; Miller et al., 2010; Noctor et al., 2014) and the drought-triggered ROS production can elicit acclimatory events (Smirnoff, 1993). However, HR-like response has never been observed following drought stress. Therefore, the role of ROS in cell signaling and in regulating gene expression is a key aspect (Baxter et al., 2014), in particular in plant, subjected to abiotic stresses, including O_3 and drought (Wilkinson and Davies, 2010).

The signaling pathways activated by O_3 are integrated into a complex regulatory system involving ROS, plant hormones [e.g., ethylene (ET) and abscisic acid (ABA)], signaling molecules [e.g., salicylic acid (SA) and jasmonic acid (JA)], and secondary messengers (e.g., Ca^{2+}). Signaling and cell death in O_3-exposed plants have been reviewed by several authors (e.g., Rao et al., 2000; Rao and Davis, 2001; Kangasjärvi et al., 2005; Tamaoki, 2008; Vainonen and Kangasjärvi, 2015; Carmody et al., 2016; Pellegrini et al., 2016). Different plants use many hydraulic and chemical signals to tune their sensing of water deficit (Wilkinson and Davies, 2010). Thus, the interactions between drought and acute O_3 stress in terms of signaling molecules and cell death need to be studied in depth in order to improve predictions of plant acclimation/adaptation strategies to climate change (Carmody et al., 2016). Signaling in acute O_3 exposure has mainly been studied in the test plant *Arabidopsis thaliana*, however, few works have evaluated these mechanisms in tree species [e.g., on hybrid poplar, by Kock et al. (2000)].

To the best of our knowledge, no studies have assessed signaling molecules and cell death in Mediterranean tree species exposed to O_3. O_3 can also be used as a non-invasive tool to mimic signaling pathways triggered by active apoplastic ROS formation induced by pathogens (Vainonen and Kangasjärvi, 2015), also enabling conclusions to be drawn on drought-biotic stress interactions. The responses of Mediterranean species to the interaction of drought and O_3 have yet to be extensively investigated as shown by the scarce information available in the literature (Kurz et al., 1998; Vitale et al., 2008; Calderòn Guerrero et al., 2013; Alonso et al., 2014; Cotrozzi et al., 2016b), especially in relation to acute exposure to the pollutant.

Holm oak (*Quercus ilex* L.) is probably the most widely studied Mediterranean evergreen tree species which has been defined as 'drought-avoidant' and 'water saver' with regard to its ecophysiological behavior (Bussotti et al., 2002), although adverse impacts of drought have also been reported in this species (e.g., Gimeno et al., 2008; Cotrozzi et al., 2016b). This species has also been referred to as the most tolerant to O_3 stress among several other *Quercus* species (Calatayud et al., 2011). In a previous study carried out by this research group (Cotrozzi et al., 2016b), *Q. ilex* subjected to drought (30% of the effective daily evapotranspiration) and/or chronic O_3 (80 nL L^{-1}, 5 h d^{-1}, for 77 consecutive days) showed that the major determinant was the water deficit; however, oxidative stress (revealed by a significant build-up of MDA by-products) occurred only when drought was applied with O_3 (Cotrozzi et al., 2016b).

In the present study, we evaluated the behavior of *Q. ilex* saplings, subjected or not to drought, and later exposed to acute O_3 exposure by characterizing different components of O_3 stress signaling. Our aim was to answer the following questions: (i) can acute O_3 exposure initiate an HR? (ii) What role do phytohormones and signaling molecules play in the perception and transduction of drought and/or O_3 stress? (iii) Do drought conditions compromise/alter the signaling responses to acute O_3 exposure?

MATERIALS AND METHODS

Plant Material and Experimental Design

Three-year old *Q. ilex* saplings grown under field conditions were potted in 6.5-L pots with growing medium containing a mixture of standard soil Einhetserde Topfsubstrat ED 63 (Sinntal-Altengronau, Germany) and sand (3.5:1, in volume),

according to Cotrozzi et al. (2016b). Two weeks before the beginning of the O_3 treatment, 42 plants (WS) received 20% of the effective daily evapotranspiration (calculated by the average 24-h weight loss of five well-watered plants), whereas another 42 plants (WW) were kept at field water capacity. The two groups of plants were then subdivided into four sets (WW-O_3, WS-O_3, WW+O_3, WS+O_3; 21 plants per set) and transferred into four controlled fumigation facilities (temperature 23 ± 1°C, relative humidity 85 ± 5% and photon flux density of 530 μmol photons m^{-2} s^{-1} at plant height provided by incandescent lamps with L/D 14:10 photoperiod; lights were switched on from 7:00 to 21:00 to simulate environmental light conditions).

WW-O_3 and WS-O_3 plants were randomly distributed into two chambers, whereas WW+O_3 and WS+O_3 plants were randomly distributed in the other two chambers. After one week of acclimation, WW+O_3 and WS+O_3 plants were exposed to an acute O_3 stress (200 nL L^{-1}, 5 h day^{-1}, in the form of a square wave between the 2nd and the 7th h of the light period). On the other hand, WW-O_3, WS-O_3 plants were maintained under charcoal-filtered air, in which the O_3 concentration was less than 5 nL L^{-1}. During the O_3-exposure, environmental factors were maintained as reported above.

The O_3 exposure was performed according to Lorenzini et al. (1994) with minor modifications to avoid pseudo-replications. At the end of the drought exposure, plant water status was evaluated. Photosynthetic parameters were measured at 0, 5, 24 and 48 h from the beginning of the O_3 exposure (FBE, From the Beginning of Exposure). Five fully expanded mature leaves per plant per treatment were taken at 0, 1, 2, 5, 8, and 24 h FBE, stored at –20°C and subsequently used for chemical analyses, with the exception of ET determination, which was performed immediately. At the same measuring times, staining, and microscopic assays were also performed on fresh material.

Water Status of Plants

Pre-dawn leaf water potential ($PD\Psi_W$) was measured on three plants per treatment (one fully expanded mature leaf per plant) with a pressure chamber (PMS model 600, PMS Instrument Company, Albany, OR, USA). On the very same plants, relative water content (RWC) was calculated (one fully expanded mature leaf per plant) as: RWC (%) = (FW-DW)/(TW-DW) × 100, where FW is the fresh weight, TW is the turgid weight after rehydrating samples for 24 h, and DW is the dry weight after oven-drying samples at 85°C for 24 h.

Gas Exchange and Chlorophyll *a* Fluorescence Measurements

Gas exchange and chlorophyll *a* fluorescence measurements were determined between 10:00 and 13:00 (solar time) on one fully expanded mature leaf per plant, on three plants per treatment. CO_2 assimilation rate (A), stomatal conductance to water vapor (g_s) and intercellular CO_2 concentration (C_i) in light-saturated conditions and ambient CO_2 concentration were measured using an Infrared Gas Analyzer (LI-COR Inc., Lincoln, NE, United States) as reported by Cotrozzi et al. (2016b). Modulated chlorophyll *a* fluorescence of photosystem II (PSII) was measured with a PAM-2000 fluorometer (Walz, Effeltrich, Germany) on the same leaves used for the gas exchange after 40 min of dark adaptation using a dark leaf clip provided by the producer. The maximal PSII photochemical efficiency [$F_v/F_m = (F_m – F_0)/F_m$] and the photochemical efficiency in light conditions [$\Phi_{PSII} = (F_m' – F_s)/F_m'$)] were calculated (Genty et al., 1989). Maximal fluorescence, F_m, when all PSII reaction centers were closed, was determined by applying a saturating light pulse (0.8 s) at 8,000 μmol m^{-2} s^{-1} in dark-adapted leaves. Fluorescence was induced with actinic light (about 480 μmol m^{-2} s^{-1}), superimposed with 800 ms saturating pulses (10,000 μmol m^{-2} s^{-1}) at 20 s intervals to determine maximal fluorescence in the light-adapted state (F'_m). Minimal fluorescence in the light-adapted state (F'_0) was determined immediately after turning off the actinic source in the presence of a far-red (>710 nm) background for 10 s to ensure maximal oxidation of PSII electron acceptors. The saturation pulse method was used to analyze the quenching components, as described by Schreiber et al. (1986).

Staining and Microscopic Assays

For the detection of dead cells, Evan's blue staining was used according to Tonelli et al. (2015). Leaf material was boiled for 1 min in a mixture of phenol, lactic acid, glycerol and distilled water (1:1:1:1, in vol.) containing 20 mg mL^{-1} Evan's blue and, after clarification with an aqueous chloral hydrate solution, examined under a light microscope (DM 4000 B, Leica, Wetzlar, Germany). To detect H_2O_2 accumulation, fresh leaf samples were stained with 3,30-diaminobenzidine (DAB) following Tonelli et al. (2015). Leaf parts were incubated for at least 8 h in a DAB solution (1 mg mL^{-1}) in HCl adjusted to pH 5.6. The samples were then soaked in 70% (v/v) boiling ethanol and clarified overnight in a solution of 2.5 g L^{-1} aqueous chloral hydrate solution. The cellular H_2O_2 accumulation was visualized under a light microscope as a reddish-brown precipitation.

ROS determination

H_2O_2 production was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA, United States), according to Pellegrini et al. (2013). Spectrofluorimetric determinations were performed with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter Perkin Elmer, Waltham, MA, United States) at 530 and 590 nm (excitation and emission resorufin fluorescence, respectively). O_2^- concentration was measured according to Tonelli et al. (2015), after extraction with a Tris-HCl buffer (50 mM, pH 7.5), with a spectrophotometer (6505 UV-Vis, Jenway, United Kingdom) at 470 nm, and using a buffer solution as a blank.

Phytohormone and Signaling Molecule Bioassays

Two minutes after excision, ET production was measured by enclosing six intact leaves (cut a few millimeters below the petiole by a scalpel) in air-tight glass containers (80 mL). Gas samples (2 mL) were taken from the headspace of containers after 1 h incubation at room temperature. Separations were

performed with a gas chromatograph (HP5890, Hewlett-Packard, Ramsey, MN, United States) equipped with a stainless steel column (150 × 0.4 cm i.d. packed with Hysep T) and a flame ionization detector. Analytical conditions were as follows: injector and transfer line temperature at 70 and 350°C, respectively, and carrier gas nitrogen at 30 mL min^{-1} (Pellegrini et al., 2013). SA was determined according to Vitti et al. (2016) with some minor modifications. High performance liquid chromatography (HPLC) separations were performed with a liquid chromatograph (Dionex, Sunnyvale, CA, United States) equipped with a reverse-phase Dionex column (Acclaim 120, C18 5 μm particle size, 4.6 mm i.d. × 150 mm length) and RF 2000 Fluorescence Detector. Analytical conditions were as follows: excitation and emission at 305 and 407 nm, respectively, mobile phase containing 0.2 M sodium acetate buffer, pH 5.5 (90%) and methanol (10%), and the flow-rate at 0.8 mL min^{-1}. JA was determined according to Pellegrini et al. (2013). HPLC separations were performed with the Dionex column described above and a UVD 170U UV/VIS detector. Analytical conditions were as follows: absorbance at 210 nm, mobile phase containing 0.2% (v/v) acidified water, and the flow-rate at 1 mL min^{-1}. ABA was measured after extraction in distilled water (water:tissue ratio, 10:1) overnight at 4°C. The indirect ELISA determinations, based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA, as described by Trivellini et al. (2011), were performed at 415 nm with a microplate reader (MDL 680, Perkin-Elmer, Waltham, MA, United States).

Proline Content

Proline content was measured as reported in Cotrozzi et al. (2016b), after extraction with sulfosalicylic acid (3%, v/v). Spectrophotometric determinations were performed at 520 nm, using toluene as a blank.

Statistical Analysis

Three repeated experiments were set up following a randomized design and the experimental plot consisted of one plant per container. Ecophysiological and biochemical measurements were carried out on three replicates for each treatment. The normality of data was preliminary tested by the Shapiro–Wilk *W* test. The effects of drought exposure vs. well-watering were analyzed by the Student's *t*-test. The effects of O_3 on ecophysiological parameters were tested using one-way repeated measures ANOVA with treatment ($WW+O_3$, $WS+O_3$) as the variability factor. The effects of O_3 on biochemical parameters were evaluated by two-way ANOVA with treatment and time as variability factors. For both ecophysiological and biochemical analyses, Fisher's LSD was used as the post-test, with a significance level of $P \leq 0.05$. Since data obtained by control plants maintained in filtered air ($WW\text{-}O_3$ and $WS\text{-}O_3$) did not show significant differences during the time course (*data not shown*), a comparison among means was carried out using as $WW+O_3$ and $WS+O_3$ plants controls before beginning the fumigation. Analyses were performed by NCSS 2000 Statistical Analysis System Software (Kaysville, UT, United States).

RESULTS

Effects of Drought Stress

After 15 days of drought, plants did not show visible foliar injury. Physiological responses are reported in **Table 1**. In WS plants, $PD\Psi_W$ decreased significantly to -4.0 MPa at the end of water deprivation compared to WW controls (–0.5 MPa). However, no changes in RWC were recorded in WS leaves. The net carbon gain was reduced by about 73% in response to drought, which was a larger effect compared with the reduction of g_s (–50%). Values of C_i increased in WS leaves (+7%). Chlorophyll fluorescence measurements revealed a reduction in Φ_{PSII} (–39%) and qP (–18%) in WS compared to WW leaves, but no changes in the F_v/F_m ratio. An increase of qNP (+30%) was found after drought stress.

The biochemical responses at the end of drought exposure are summarized in **Table 2**. In comparison to the controls, H_2O_2 levels did not change in WS leaves, while accumulation of O_2^- was 1.6-fold higher under drought. A strong increase in

TABLE 1 | Water status and ecophysiological parameters in *Quercus ilex* plants well-watered (WW) or water stressed (20% of the effective evapotranspiration daily for 15 days, WS).

		WW	WS	*P*
$PD\Psi_W$	(–MPa)	0.5 ± 0.06	4.0 ± 0.70	**
RWC	(%)	86 ± 7.4	82 ± 1.7	ns
A	(μmol CO_2 m^{-2} s^{-1})	7.4 ± 0.23	2.0 ± 0.13	***
g_s	(mol H_2O m^{-2} s^{-1})	0.16 ± 0.001	0.08 ± 0.008	***
C_i	(μmol CO_2 mol^{-1})	284 ± 2.1	304 ± 8.1	*
F_v/F_m		0.83 ± 0.003	0.84 ± 0.004	ns
Φ_{PSII}		0.36 ± 0.008	0.22 ± 0.045	**
qP		0.60 ± 0.008	0.49 ± 0.034	**
qNP		0.64 ± 0.027	0.83 ± 0.043	**

*Data are shown as mean ± standard deviation. Asterisks show the significance of Student's t-test: ***P ≤ 0.001, **P ≤ 0.01, *P ≤ 0.05, ns P > 0.05. Φ_{PSII}, photochemical efficiency in light conditions; A, CO_2 assimilation rate; Ci, intercellular CO_2 concentration; F_v/F_m, potential PSII photochemical efficiency; g_s, stomatal conductance to water vapor; $PD\Psi_W$, pre-dawn leaf water potential; qP, photochemical quenching coefficient; qNP, non-photochemical quenching coefficient; RWC, relative water content.*

TABLE 2 | Biochemical parameters in *Quercus ilex* plants WW or water stressed (20% of the effective evapotranspiration daily for 15 days, WS).

		WW	WS	*P*
H_2O_2	(μmol g^{-1} DW)	0.18 ± 0.011	0.17 ± 0.004	ns
O_2^-	(nmol min^{-1} g^{-1} DW)	24.0 ± 0.20	38.7 ± 1.47	***
ET	(pl g^{-1} FW h^{-1})	136 ± 15.0	226 ± 10.8	**
SA	(nmol g^{-1} DW)	7.1 ± 1.04	4.3 ± 0.08	**
JA	(μmol g^{-1} DW)	3.5 ± 0.08	24.0 ± 0.35	***
ABA	(nmol g^{-1} DW)	4.2 ± 0.03	2.8 ± 0.28	***
Pro	(mmol g^{-1} DW)	0.23 ± 0.001	0.32 ± 0.010	***

*Data are shown as mean ± standard deviation. Asterisks show the significance of Student t-test: ***P ≤ 0.001, **P ≤ 0.01, ns P > 0.05. ABA, abscisic acid; DW, dry weight; ET, ethylene; FW, fresh weight; H_2O_2, hydrogen peroxide; JA, jasmonic acid; O_2^-, superoxide anion; Pro, proline; SA, salicylic acid.*

Pro content (+39%) was observed in WS leaves. The endogenous concentration of ABA and SA measured in WS leaves decreased significantly at the end of the experimental period (–33% and –39%, respectively). However, the JA and ET amounts accumulated by WS leaves increased significantly (about 7-fold and +66%, respectively).

Influence of Drought Stress on the Response to Acute O_3 Exposure

Macroscopic and Microscopic Ozone-Induced Symptoms

At the end of the O_3 treatment (alone and in combination with drought), leaves appeared macroscopically symptomless. However, O_3-injuries were already detectable at the microscopic level after 5 h FBE, as confirmed by the appearance of dead cells observed in WW+O_3 and WS+O_3 (**Figures 1A–D**). Histological staining showed local accumulation of H_2O_2 evidenced by reddish-brown areas in O_3-treated material (**Figures 1G,H**) (regardless of drought stress; **Figures 1E,F**).

Ozone-Induced Physiological Responses

The photosynthetic rate in light saturation conditions decreased strongly following O_3 exposure in both WW+O_3 and WS+O_3 plants, and especially under drought (–23 and –41% in WW+O_3 and WS+O_3 plants, respectively) (**Figure 2A**). However, A values continued to decrease only in WW+O_3 leaves, also after the end of fumigation, reaching values of about 4 μmol CO_2 $m^{-2}s^{-1}$ at 48 h FBE with a reduction of about 50% compared to the values determined before O_3 exposure (**Figure 2A**). WS+O_3 plants showed low levels of A already before the beginning of O_3 exposure (–73% in comparison with WW plants) and these values did not decrease further after the fumigation (**Figure 2A**). Ozone also induced a strong decrease in g_s in WW+O_3 and even more in WS+O_3 leaves at the end of the exposure (-13 and -38% in WW+O_3 and WS+O_3 leaves, respectively) and the effect of O_3 on g_s remained at 24 and 48 h FBE (**Figure 2B**). However, in WS+O_3 plants, g_s values were 50% lower than those recorded in WW+O_3 plants. Finally, C_i values increased significantly following O_3 exposure in both WW+O_3 and WS+O_3 leaves (+8%) although the values recorded in WS+O_3 leaves were significantly higher compared to those found in WW+O_3. In both WW+O_3 and WS+O_3 leaves, the C_i values reached at the end of the exposure were maintained up to the end of the experimental period, although a slight decrease was observed for WW+O_3 leaves at 24 h FBE (**Figure 2C**).

Actual Φ_{PSII} decreased at the end of exposure in both WW+O_3 and WS+O_3 leaves (-19 and -62%, respectively). However, Φ_{PSII} recovered completely 48 h FBE in both WW+O_3 and WS+O_3 leaves (**Figure 2D**). In WW+O_3 plants, qP values were higher than those observed before the beginning of exposure, both at 24 and 48 h FBE (+19 and +14%, respectively) (**Figure 2E**). Conversely, in WS+O_3 plants, qP values decreased at the end of exposure (-47%), but recovered completely from 24 h onward (**Figure 2E**). Values of qNP increased in WW+O_3 leaves at the end of the fumigation (+24%), and similar values were maintained until 48 h FBE (**Figure 2F**). Conversely, in WS+O_3 plants, qNP decreased at 24 and 48 h FBE (–14 and –8%, respectively, in comparison with the pre-treatment values) (**Figure 2F**). These mechanisms were sufficient to protect PSII from photoinhibition given that the decrease in F_v/F_m observed at the end of exposure in both WW+O_3 and WS+O_3 leaves was completely recovered 48 h FBE (data not shown).

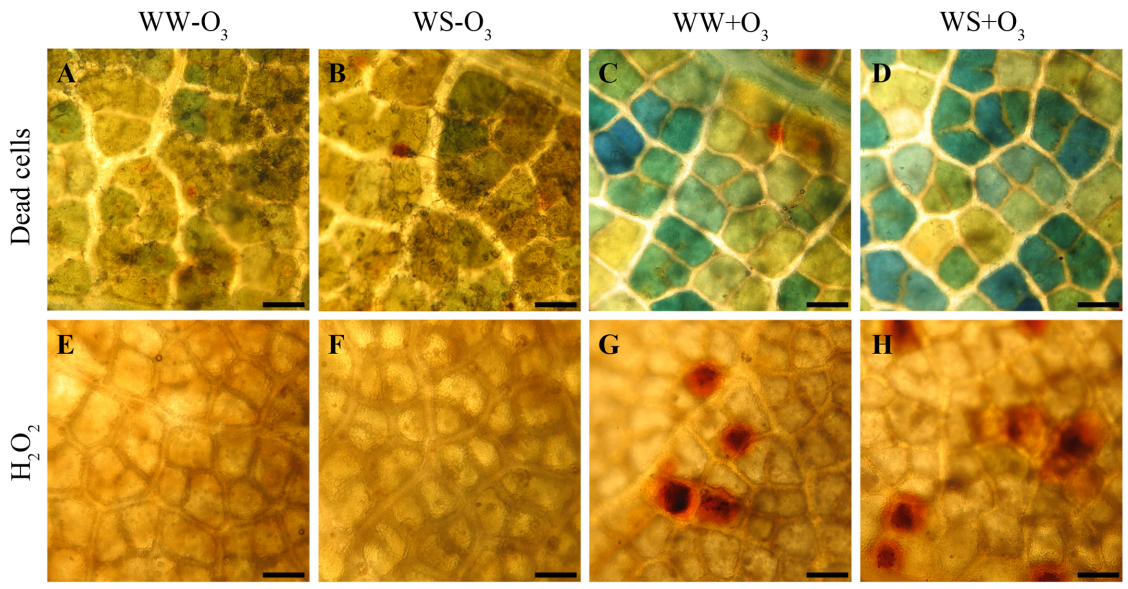

FIGURE 1 | Localization of dead cells visualized with Evans blue staining **(A–D)** and of hydrogen peroxide (H_2O_2) visualized the 3,3′-diaminobenzidine (DAB) uptake method **(E-H)** in *Quercus ilex* leaves (i) well-watered (WW) and exposed to charcoal filtered air (WW-O_3); (ii) water stressed (20% of effective evapotranspiration daily for 15 days) and exposed to charcoal filtered air (WS-O_3); (iii) well-watered and exposed to acute ozone (200 nL L^{-1} for 5 h) (WW+O_3); (iv) water stressed and O_3 fumigated (WS+O_3). The assays were performed 96 h FBE. Bars 50 μm.

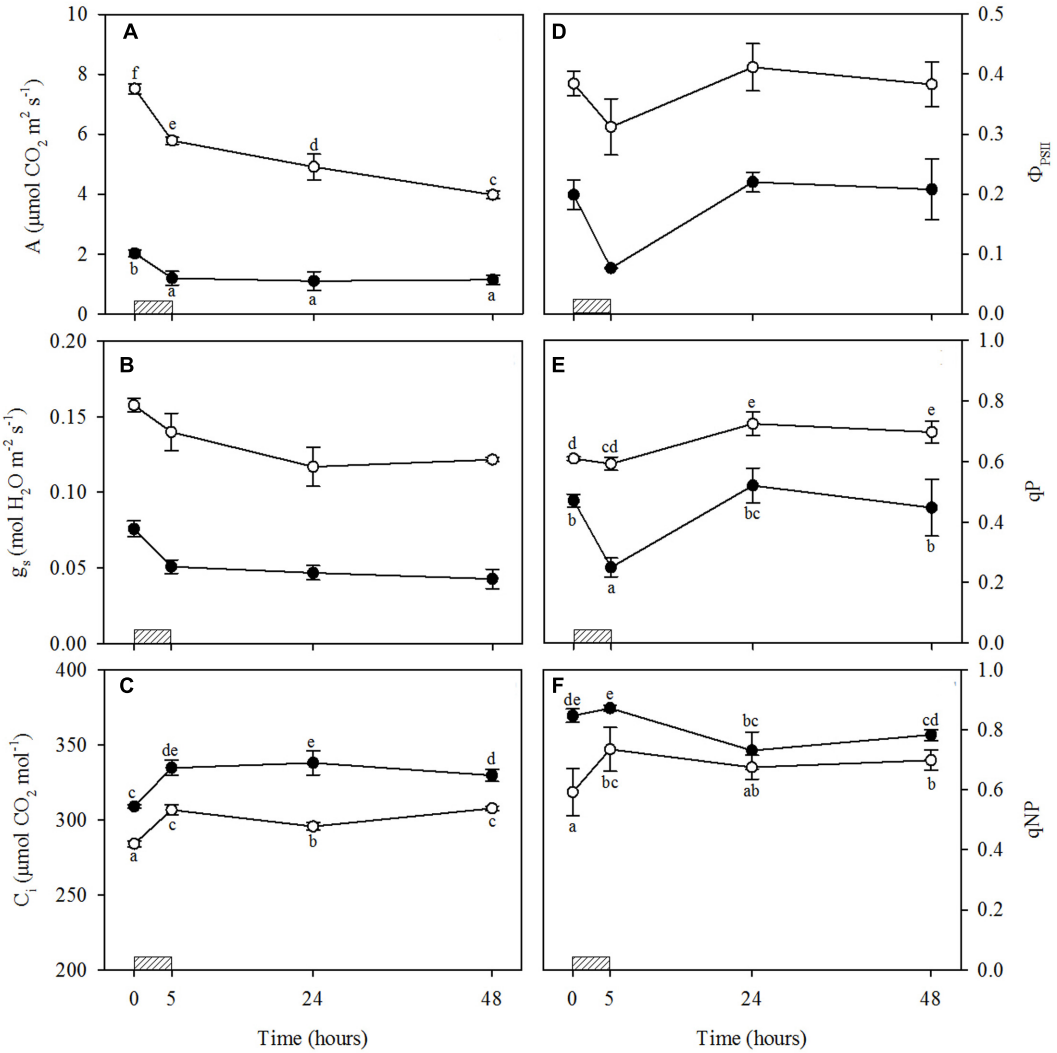

FIGURE 2 | Time course of leaf gas exchange and chlorophyll fluorescence parameters in *Quercus ilex* plants well-watered (open circle) or water stressed (20% of the effective evapotranspiration daily for 15 days, closed circle) and exposed to acute ozone (200 nL L^{-1} for 5 h). Data are shown as mean ± standard deviation. The measurements were carried out 0, 5, 24 and 48 h from the beginning of exposure. According to the one-way repeated measures ANOVA with treatment as variability factor, different letters indicate significant differences ($P = 0.05$). The absence of letters in B and D indicates not significant interaction between variability factors (see Supplementary Table S1). **(A)** CO_2 assimilation rate (A); **(B)** stomatal conductance to water vapor (g_s); **(C)** intercellular CO_2 concentration (C_i); **(D)** photochemical efficiency in light conditions (Φ_{PSII}); **(E)** photochemical quenching coefficient (qP); **(F)** non-photochemical quenching coefficient (qNP). The thick bottom line indicates the time (5 h) of ozone exposure.

Ozone-Induced ROS Accumulation

A biphasic time course of H_2O_2 production in response to O_3 was observed irrespective of drought stress (**Figures 3A** and Supplementary Table S2). In both WW+O_3 and WS+O_3 plants, H_2O_2 content increased at 2 h FBE (+33 and +43% as compared to time 0, respectively), showed a significant decline at 5 h FBE, and increased again at 8 h FBE. This second peak was higher in WW+O_3 than in WS+O_3 plants (+62% vs. +38%, compared to their respective time 0). In addition, only in WW+O_3 leaves was the second peak higher than the first, and H_2O_2 levels at 24 h FBE remained higher than those at time 0 (+24%).

The time patterns of O_2^- induced by O_3 were notably different in relation to water stress (**Figures 3B** and Supplementary Table S2). In WW+O_3 conditions, O_2^- content also exhibited a clear biphasic time course. It peaked already at 1 h FBE (+47%, compared to the beginning of O_3-exposure), and again at 8 h FBE (+75%), although it remained higher than time 0 at 2 and 5 h FBE (+20 and +38%, respectively). At 24 h FBE, the O_2^- content decreased at the same levels as time 0. Conversely, in WS+O_3 plants (where O_2^- levels were already higher in WS+O_3 than WW+O_3 plants), O_2^- content decreased during the first two hours of O_3 treatment (−20 and −14%, after 1 and 2 h FBE, respectively). It then peaked at 5 h FBE (+22%), decreased again

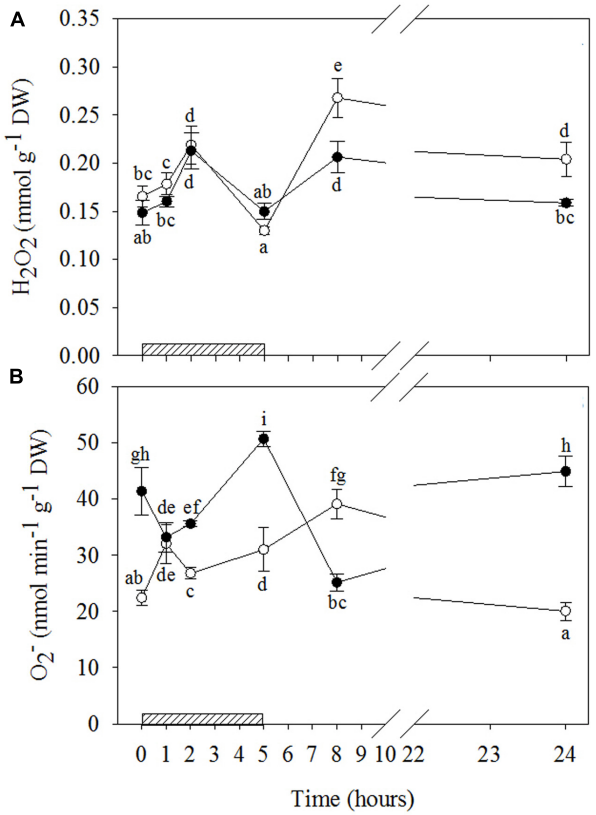

FIGURE 3 | Time course of reactive oxygen species (ROS) in *Quercus ilex* plants well-watered (open circle) or water stressed (20% of the effective evapotranspiration daily for 15 days, closed circle) and exposed to acute ozone (200 nL L^{-1} for 5 h). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P = 0.05$). DW, dry weight; H_2O_2, hydrogen peroxide **(A)**; O_2^-, superoxide anion **(B)**. The thick bottom line indicates the time (5 h) of ozone exposure.

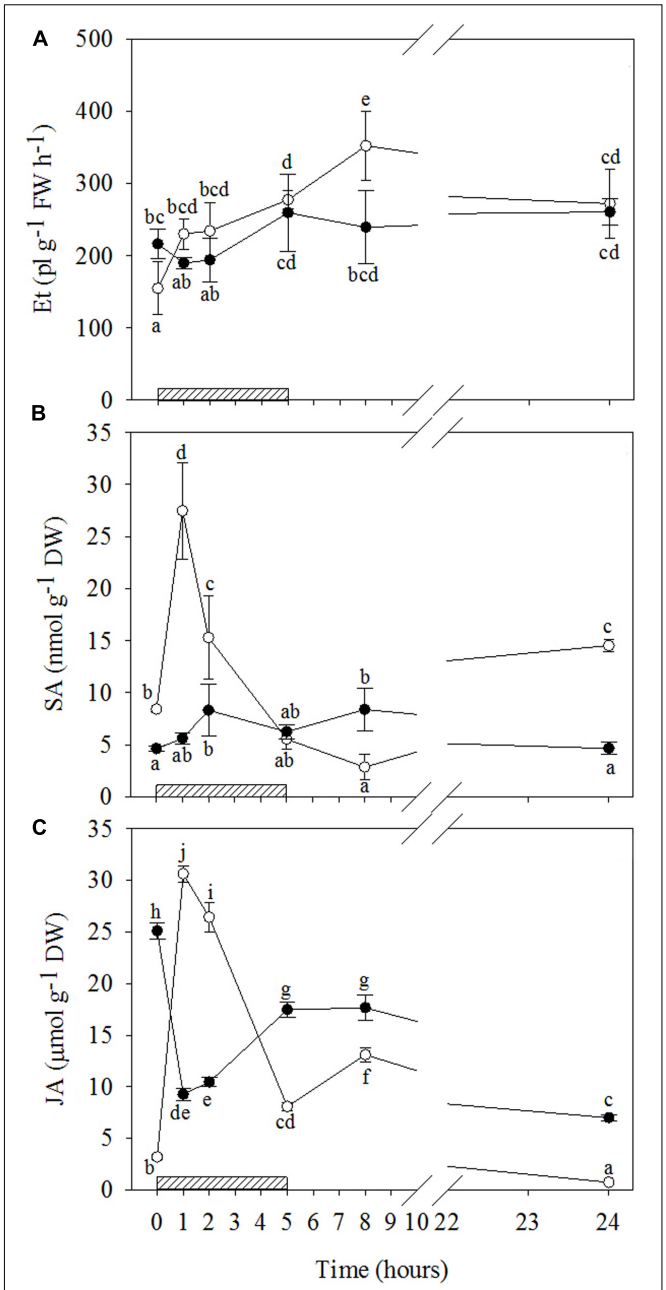

FIGURE 4 | Time course of phytohormones in *Quercus ilex* plants well-watered (open circle) or water stressed (20% of the effective evapotranspiration daily for 15 days, closed circle) and exposed to acute ozone (200 nL L^{-1} for 5 h). Data are shown as mean ± standard deviation. The measurements were carried out 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P = 0.05$). DW, dry weight; ET, ethylene **(A)**; FW, fresh weight; JA, jasmonic acid **(C)**; SA, salycilic acid **(B)**. The thick bottom line indicates the time (5 h) of ozone exposure.

at 8 h FBE (reaching the lowest values of the whole treatment), and finally increased again reaching the levels shown at time 0.

Ozone-Induced Signaling Molecule Stimulation

The results of signaling molecules indicate that O_3 only significantly stimulated ET emission in WW+O_3 leaves (**Figures 4A** and Supplementary Table S2). In these plants, starting from 1 h of treatment onwards, ET emission values remained higher than those shown at the beginning of O_3-exposure throughout the treatment period, and reached the maximum 8 h FBE (+49, +52, +79, +128, and +76% after 1, 2, 5, 8, and 24 h FBE, respectively). Conversely, a clear biphasic time course of SA production was observed in response to O_3, irrespective of drought stress. However, the average concentration throughout the treatment and the changes induced by O_3 were higher and more pronounced in WW+O_3 than in WS+O_3 plants, respectively (**Figures 4B** and Supplementary Table S2). In WW plants, total SA levels increased already at 1 h FBE, reaching their maximum values (three-fold higher than before the O_3-treatment). They then progressively decreased to constitutive levels at 5 h FBE, and to even lower values at 8 h FBE, but increased again at the end

of the experiment (+73% compared to time 0). In WS+O_3 conditions, SA concentrations peaked at 2 and 8 h FBE (+79 and +80%, respectively), whereas SA levels were similar before the beginning of O_3 treatment than at the other analysis times.

The time patterns of JA induced by O_3 were also notably different in relation to drought (**Figures 4C** and Supplementary Table S2). A biphasic time course of JA production was shown by WW+O_3 plants. Similarly to SA (and ABA, as reported below), a first marked peak in JA levels (tenfold higher than controls) was shown by WW+O_3 plants at 1 h FBE. Then, JA progressively decreased until 5 h FBE (remaining at higher levels than those recorded at time 0), peaked again 8 h FBE (four times higher than time 0), and, finally, reached lower values than before the beginning of O_3-treatment at 24 h FBE. Conversely, in WS+O_3 plants (where JA levels, similarly to O_2^-, were already higher in WS+O_3 than WW+O_3 plants) a marked decrease in JA concentrations was observed starting from 1 h onwards (–63%, in comparison to controls). Throughout the period of O_3-treatment, the values of this phytohormone remained lower than those shown before the exposure, although a recovery was shown at 5 and 8 h FBE.

Ozone-Induced ABA and Osmolyte Accumulation

O_3 significantly stimulated ABA production only in WW+O_3 leaves (**Figures 5A** and Supplementary Table S2), where a clear biphasic response to the pollutant was observed. In comparison to the levels shown before the O_3 treatment, ABA in WW+O_3 leaves already increased at 1 h FBE (overall three times), showed no differences at 2 and 5 h FBE, slightly increased again at 8 h FBE (+44%) and, finally, reached lower values at 24 h FBE. In WW+O_3 conditions, O_3 induced a slight increase in Pro only at the first two hours of exposure (+46 and +33%, respectively at 1 and 2 h FBE), whereas during the post-fumigation period, Pro values remained lower than those at time 0 (**Figures 5B** and Supplementary Table S2). Conversely, in WS+O_3 plants, Pro content peaked after 1 h FBE (+96%, in comparison to controls), then declined at 2 h FBE (at the same concentrations shown before the beginning of O_3-exposure), increased again at 5 h FBE (+69%) and reaching a maximum at 8 h FBE, with the maximum values (sixfold higher than at time 0). Finally, Pro concentration of WS+O_3 leaves decreased at 24 h FBE, although they remained higher than before the O_3-exposure (more than two fold).

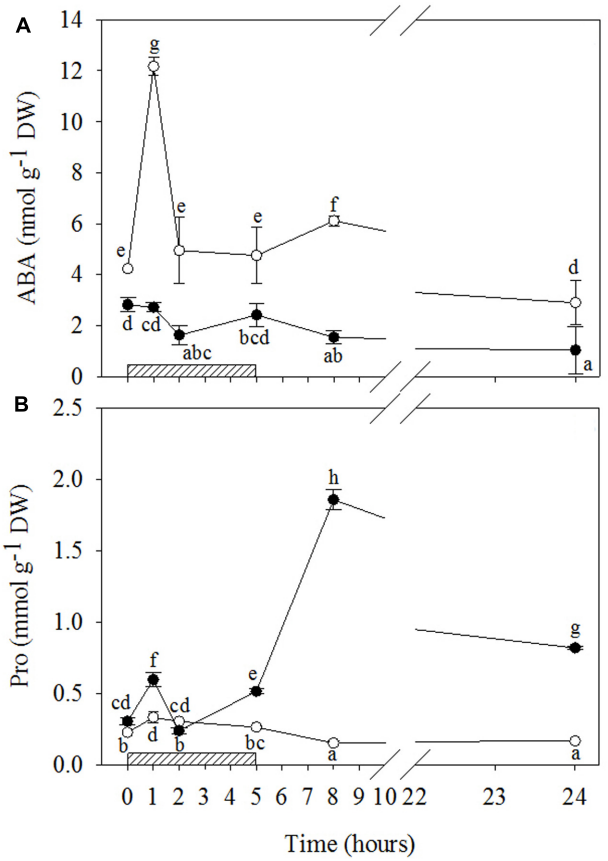

FIGURE 5 | Time course of abscisic acid (ABA; **A**) and proline (Pro; **B**) in *Quercus ilex* plants well-watered (open circle) or water stressed (20% of the effective evapotranspiration daily for 15 days, closed circle) and exposed to acute ozone (200 nL L^{-1} for 5 h). Data are shown as mean ± standard deviation. The measurements were carried out at 0, 1, 2, 5, 8, and 24 h from the beginning of exposure. According to the two-way ANOVA with treatment and time as variability factors, different letters indicate significant differences ($P = 0.05$). DW, dry weight. The thick bottom-line indicates the time (5 h) of ozone exposure.

DISCUSSION

In this study, the behavior of the Mediterranean evergreen *Q. ilex* subjected or not to drought, and later exposed to an acute O_3 exposure, was evaluated in terms of cross-talk responses among signaling molecules. The aim was to confirm or disentangle the hypothesis according to which drought stress influences the responses of plants to acute episodes of O_3 exposure.

Physiological and Biochemical Responses to Drought

Although drought induced a strong decrease in PDΨ_W (reaching lower values than those reported in a previous study; Cotrozzi et al. (2016b), attributable to different growing seasons between experiments), the RWC of WS leaves did not significantly change in comparison to the WW leaves. This indicates that a good level of leaf hydration was also maintained under drought conditions, RWC being a reliable indicator of leaf water content (Rosales-Serna et al., 2004). This result is in accordance with the accumulation of Pro, a metabolite that is considered an important compatible solute which (i) facilitates water absorption by increasing the cell osmotic potential (Ashraf and Foolad, 2007), and (ii) reduces cell damage (Filippou et al., 2014). The important role of Pro in response to water stress has already been reported in this species where Pro played a key role in the high plasticity of *Q. ilex* under a long period of moderate water stress (Cotrozzi et al., 2016b).

In WS plants, the strong decline in CO_2 photo-assimilation was attributable to coordinated and concomitant stomatal and mesophyll limitations, which is in line the results obtained by

several authors (e.g., Centritto et al., 2009; Flexas et al., 2013). The drop in A levels was higher than that in g_s, thus leading to lower values of intrinsic water use efficiency (data not shown), as already reported in this species when subjected to water stress (Cotrozzi et al., 2016b).

These outcomes indicate that CO_2 assimilation was strongly influenced not only by stomatal behavior but also by mesophyll limitations, as demonstrated by the increase in C_i. Drought compromised the PSII photochemical efficiency in light adapted leaves with decreases in Φ_{PSII} and qP levels, although this inhibition did not determine PSII photoinhibition, as confirmed by unchanged values in the F_v/F_m ratio. In WS conditions, the lower CO_2 assimilation rate induced, in turn, a reduced consumption of ATP and NADPH synthesized into the chloroplasts and, consequently, led to an excess of excitation energy in the thylakoid membrane, which was only partially dissipated, via non-photochemical mechanisms (increase in qNP). The remaining excess of reducing power in WS plants altered the ROS levels (although H_2O_2 did not change, a strong increase of O_2^- was observed). This led to a modification in phytohormones and other signaling molecule cross-talk in terms of (i) promoting oxidative stress and (ii) modulating leaf senescence (Munné-Bosch and Alegre, 2004; Miller et al., 2010; Baxter et al., 2014), which is a defense commonly activated in response to both a plethora of abiotic and biotic stresses (Wingler and Roitsch, 2008).

Among phytohormones, the important roles of ABA and ET in plant responses to drought is well known (Munné-Bosch and Alegre, 2004) as ABA represents the most important regulator of stomata functioning (Wilkinson and Davies, 2002), whereas ET is a shoot growth inhibitor and a promoter of ripening, senescence and abscission (Abeles et al., 1992). However, ET can inhibit ABA-induced stomatal closure (Tanaka et al., 2005). Wilkinson and Davies (2009, 2010) reported that under stress-oxidative conditions, an ET-antagonism of the stomatal response to ABA occurs. This interaction was confirmed by our data (ABA decreased, while ET increased), suggesting that drought-induced ET biosynthesis could be considered a marker of leaf senescence (Dangl et al., 2000; Dolferus, 2014).

In addition, SA and JA have been shown to play a central role in leaf senescence (Abreu and Munné-Bosch, 2008), although they are well known for triggering defense reactions against biotrophic and necrotrophic pathogens such as induced resistance (Barna et al., 2012; Shigenaga and Argueso, 2016). In particular, SA and JA interact at physiological levels in many growth and developmental processes, and they play a role in controlling gene expression during leaf senescence (Abreu and Munné-Bosch, 2008). However, as only the JA levels increased in WS leaves, it is reasonable that only JA participated in senescence-associated signaling and degradative processes of membranes. The significantly higher levels of JA shown by WS compared to WW plants indicate that lipid peroxidation producing substrates for octadecanoid pathways was exacerbated in limited water conditions. In particular, JA could be a promoter of leaf senescence in response to drought, thus leading to stomatal closure and accumulation of osmo-compatible solutes (in our case, only Pro), in line with Dar et al. (2015).

Influence of Drought Stress on the Physiological and Biochemical Responses to O_3 Exposure

The physiological responses observed in O_3-stressed plants were similar to those shown at the end of water deprivation, and in accordance with a previous study by our research group on *Q. ilex* exposed to O_3 (Cotrozzi et al., 2016b). At the end of the fumigation, the O_3-induced stomatal closure found in both WW+O_3 and WS+O_3 leaves led to significant reductions in CO_2 assimilation. The more pronounced decrease in A in WW+O_3 compared to WS+O_3 leaves, as well as the lack of a further decrease in A observed in WS+O_3 plants throughout the recovery phase, was probably attributable to the very low CO_2 assimilation rate shown by water stressed plants before the beginning of the fumigation. The increase in C_i level in plants exposed to O_3 indicates that the pollutant gas influenced not only stomatal conductance but, as with after water stress, also the mesophyll activity. In fact, an impairment of PSII activity was recorded. Although F_v/F_m and Φ_{PSII} values decreased significantly after O_3 exposure in both WW+O_3 and WS+O_3 plants, the reduction was more pronounced in WS+O_3 plants. This behavior was linked to different quenching responses to the leaf-water status of plants. In WW+O_3 plants, where qP did not decrease, a mechanism aimed at dissipating the excess excitation energy was activated (qNP increased). By contrast, in WS+O_3 leaves, the O_3-induced decrease in qP was ascribable to the fact that qNP values were already high (probably at their maximum in relation to the capability of plants to activate this mechanism) after drought, and the leaves were not able to enhance this type of dissipation mechanism. The complete recovery of PSII photochemical activity during the recovery time after drought and O_3 exposure indicates that the decrease in PSII activity was sufficient to prevent the photosynthetic apparatus from undergoing irreversible damage.

Unlike the ecophysiological measurements, microscopic analyses highlighted significant differences between plants exposed (WW+O_3, WS+O_3) or not (WW and WS) to the gaseous pollutant. Although visible symptoms were not shown by any of the plants irrespectively of the applied treatment, DAB staining and Evan's blue incorporation observed in WW+O_3 and WS+O_3 leaves 5 h FBE indicated that H_2O_2 deposition and cell death had already occurred at the end of exposure. This confirms that O_3 resembles the HR occurring in incompatible plant-pathogen interactions (Iriti and Faoro, 2008; Vainonen and Kangasjärvi, 2015). An integrated perspective has been proposed to explain how phytohormones and signaling molecules might be involved in molecular events (namely lesion initiation, propagation, and containment) leading to O_3-induced HR-mimicking foliar symptoms (Overmeyer et al., 2003, 2005; Kangasjärvi et al., 2005). ROS, phytohormones and other signaling molecules have a pivotal role in both HR-mimicking responses induced by acute O_3 and in promoting leaf senescence under drought (as shown by WS plants). The trends of these molecules were monitored in both well-watered and drought-stressed plants during and after O_3 exposure, in order to test the hypotheses of this work.

Given that O_3 induces an endogenous, active and self-propagating ROS generation in the apoplast and a subsequent cellular oxidative burst, some authors have proposed that short-term O_3 exposure mimics pathogen infection (Rao et al., 2000; Kangasjärvi et al., 2005; Carmody et al., 2016). The two O_3-induced H_2O_2 peaks observed in our saplings, irrespectively of the water conditions, are analogous to the biphasic response usually observed during the establishment of the HR of plants against pathogens. The first H_2O_2 peak usually reflects elicitation by pathogen-associated molecular patterns, and the second reflects the interaction between a pathogen-encoded virulence gene product with a plant resistance gene (Mur et al., 2009). In our study, the first peak observed during the fumigation was attributable to O_3-decomposition, whereas the second peak, in the recovery period, could be entirely ascribable to the plant metabolism, in line with Mahalingam et al. (2006), Di Baccio et al. (2012), and Pellegrini et al. (2013) in herbaceous species.

Although the similarity in H_2O_2 profiles over time between WW+O_3 and WS+O_3 conditions, the divergence in the magnitude of their relative peaks (the second peak of the WW+O_3 plants was much greater than their first peak and greater than the second peak of the WS+O_3 plants, where the two peaks were not significantly different from each other) suggests that drought partially inhibited the response to O_3-stress. As H_2O_2 is one of the most important products of oxidative stress (Gill and Tuteja, 2010), it is reasonable to speculate that the biphasic trend of H_2O_2 observed in *Q. ilex,* irrespectively of drought stress, might reflect the biphasic oxidative burst in response to O_3, in line with several authors (e.g., Wohlgemuth et al., 2002; Di Baccio et al., 2012).

However, this hypothesis is strengthened by O_2^- only in WW+O_3 plants, where a biphasic time course of O_2^- levels was shown concurrently with H_2O_2. Kangasjärvi et al. (2005) reported similar temporal changes in ROS for O_3-sensitive genotypes of several species (e.g., tobacco, tomato, birch), whereas only a modest increase in the first hours of exposure was observed in O_3-tolerant genotypes. Conversely, the different O_2^- patterns of the WS+O_3 plants suggest a possible dual function of this radical depending on water stress. In fact, the significant decrease in O_2^- observed in WS+O_3 plants during the first hours of exposure suggests that under drought+O_3 superoxide anion may act as a precursor of H_2O_2 and even more toxic radical derivatives. However, the accumulation of O_2^- had already been induced by drought before the beginning of O_3-exposure. On the other hand, the marked increase in O_2^- content at the end of the exposure demonstrates that this radical may also be directly involved in the O_3-oxidative burst.

Reactive oxygen species should not be considered as exclusively deleterious and harmful. They can (i) play a key role in intracellular communication which triggers the acclimation ability, and (ii) indirectly orchestrate PCD (Mittler et al., 2011; Xia et al., 2015; Carmody et al., 2016). The amplification of ROS signals and the complete induction of defense genes seem to require signal molecules (Overmeyer et al., 2003). The differences observed in the present study in O_3-induced ROS extent dynamics in relation to water stress suggest a rather complex network of events in signal transduction, involving other molecules (e.g., phytohormones) and processes. Metabolites such as ET, ABA, SA and JA may interact at the physiological level in many growth and developmental processes, with a key role in controlling gene expression during leaf senescence. Most of the genes regulated by these metabolites are defense-related (Fossdal et al., 2007), participating therefore in responses to O_3 (Xu et al., 2015).

Under both biotic and abiotic stresses, SA is required for the induction of PCD, controlling and potentiating the oxidative burst together with ET, whereas JA is involved in limiting the spread of lesions (van Loon et al., 2006; Shigenaga and Argueso, 2016). There are three phases that highlight the influence of ABA on stress conditions (Rejeb I.B. et al., 2014). First, ABA induces stomatal closure, which leads to a reduction in water loss (in this phase, SA, JA and ET may not yet be activated and ABA can antagonize their induction). In the second step, there is a post-infection reaction- an intact ABA signaling pathway is required to increase callose accumulation in affected plants, and the presence of ABA can induce or repress additional callose accumulation depending on the environmental conditions. The third phase begins when pathogen-associated molecular patterns stimulate the accumulation of specific SA, JA, and ET hormones in order to regulate the defense reaction.

In our study, the patterns of phytohormones during and after O_3 treatment were completely different in WW+O_3 and WS+O_3 plants, showing how drought stress has a pivotal role in O_3 responses, and how these signal molecules may be altered in relation to water stress. The ET and ABA accumulations observed throughout the entire period of O_3 exposure occurred only in well-watered conditions. On the other hand, when plants had been previously subjected to water stress, their unchanged values suggest that ET and ABA were not involved in either signaling-responses to O_3, or senescence strategies (as shown for WS plants).

It is worth noting that (i) the maximal ET emission in WW+O_3 plants coincided with the second peak of H_2O_2 and O_2^-; (ii) the first peak of ABA (during O_3 treatment) preceded that of H_2O_2, suggesting that ABA could act as a stress messenger by inducing H_2O_2 (Jiang and Zhang, 2002) and consequently stomatal closure (as confirmed by the decrease in g_s values observed at the end of exposure), and (iii) the weaker second ABA peak (in the recovery phase) was concomitant with the maximum H_2O_2 and O_2^- levels and the maximal ET emission. These outcomes confirm a spatial and functional correlation between ROS and the accumulation of these phytohormones.

The SA induction observed, irrespectively of whether the plants had been subjected to drought or not, suggests that this metabolite is also an important modulator of O_3-induced responses (Pasqualini et al., 2002; Horváth et al., 2007). However, the differences between WW+O_3 and WS+O_3 plants show that the functioning of SA is dependent on water stress. In WW+O_3 conditions, the strong increase in SA during the first hours of the treatment and at the end of the O_3 fumigation, confirms the central role of this metabolite in lesion initiation and progression in response to O_3 (Tamaoki, 2008). In addition, the greater SA concentration corresponded with the maximal ABA stimulation and the first increase in ET, thus demonstrating the

synergistic action of these hormones in the regulation of defense reactions (Roychoudhury et al., 2013; Wang et al., 2013). By contrast, the biphasic time course of SA (similar to that of H_2O_2) shown by WS+O_3 plants (although slight) recalls the biphasic induction that develops during biotrophic pathogen infection (Mur et al., 2009). Here, the similarity in magnitude of the two SA peaks suggests that the first accumulation of this metabolite (concomitant with the first peak of H_2O_2) did not actuate the second increase in H_2O_2 and hence did not affect the level of plant defense.

The highest concentration of JA observed in WW+O_3 plants during the first hour of fumigation coincided with the initial increase in ET and the maximum accumulation of SA and ABA, thus also demonstrating a spatial and functional correlation between these compounds (Thaler et al., 2012). The significant O_3-induced decrease in JA levels observed in WS+O_3 plants during and after the exposure suggests that JA did not promote leaf senescence in O_3-treated leaves in spite of the high concentrations of this metabolite observed in WS+O_3 plants, not excluding the involvement of JA in senescence-associated signaling (Abreu and Munné-Bosch, 2008). In fact, the JA level in WS+O_3 plants increased again after the end of fumigation, reaching higher values than those found in the WW+O_3 counterpart during the recovery. JA is known to rapidly inhibit the expression of genes involved in photosynthesis by inducing chlorophyll loss and cellular changes that cause less photochemical damage (Santino et al., 2013).

Proline plays several roles in plant responses to abiotic and biotic stresses, and under stress its metabolism is affected by multiple and complex regulatory pathways which can profoundly influence cell death and survival in plants (Zhang and Becker, 2015). The slight increase in Pro observed in WS plants compared to WW likely indicates its role as an osmoprotectant. By the same token the O_3-induced increase in Pro observed in WW+O_3 plants only during the first hours of treatment and coinciding with the maximal ABA, SA and JA stimulation and the first increase in ET, H_2O_2 and O_2^-, suggests a potential cross-talk among signaling molecules in regulating Pro metabolism, as previously reported by Rejeb K.B et al. (2014). The role of Pro as an ROS scavenger has also been reported (Szabados and Savouré, 2009; Banu et al., 2010; Zhang and Becker, 2015). In WW+O_3 plants the lack of accumulation in Pro at 8 h FBE was concurrent with a strong increase in H_2O_2, whereas in WS+O_3 the huge increase in Pro at 8 h FBE suppressed the increase in H_2O_2 (which remained at the same levels shown at 1 h FBE), thus suggesting an H_2O_2-scavenging role in these water conditions. This mechanism was also confirmed at 24 h FBE. Several studies have attributed an antioxidant feature to Pro, suggesting the capability of Pro in O_2^- and H_2O_2 quenching (e.g., Szabados and Savouré, 2009; Wang et al., 2009).

AUTHOR CONTRIBUTIONS

The work presented here was carried out in collaboration among all authors. GL and RM defined the research theme and obtained funding. LC, DR, EP, MT, AT, and ML designed methods, carried out laboratory experiments and analyzed the data. LG, CN, and PV co-designed experiments, discussed analyses, interpreted the results, and wrote the paper. All authors have contributed to, seen and approved the manuscript.

ACKNOWLEDGMENTS

This study was supported by MIUR, Rome, project PRIN 2010–2011 "Planning the green city in the global change era: urban tree functions and suitability for predicted future climates (TreeCity)". The authors are grateful to Mr Andrea Bianchi and Ms Romina Papini for technical support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01020/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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MINI REVIEW
published: 26 July 2017
doi: 10.3389/fpls.2017.01281

Isoprene Responses and Functions in Plants Challenged by Environmental Pressures Associated to Climate Change

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Edited by:
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Reviewed by:
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Miguel Portillo-Estrada, University of Antwerp, Belgium

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *13 April 2017*
Accepted: *06 July 2017*
Published: *26 July 2017*

Citation:
Fini A, Brunetti C, Loreto F, Centritto M, Ferrini F and Tattini M (2017) Isoprene Responses and Functions in Plants Challenged by Environmental Pressures Associated to Climate Change. Front. Plant Sci. 8:1281. doi: 10.3389/fpls.2017.01281

The functional reasons for isoprene emission are still a matter of hot debate. It was hypothesized that isoprene biosynthesis evolved as an ancestral mechanism in plants adapted to high water availability, to cope with transient and recurrent oxidative stresses during their water-to-land transition. There is a tight association between isoprene emission and species hygrophily, suggesting that isoprene emission may be a favorable trait to cope with occasional exposure to stresses in mesic environments. The suite of morpho-anatomical traits does not allow a conservative water use in hygrophilic mesophytes challenged by the environmental pressures imposed or exacerbated by drought and heat stress. There is evidence that in stressed plants the biosynthesis of isoprene is uncoupled from photosynthesis. Because the biosynthesis of isoprene is costly, the great investment of carbon and energy into isoprene must have relevant functional reasons. Isoprene is effective in preserving the integrity of thylakoid membranes, not only through direct interaction with their lipid acyl chains, but also by up-regulating proteins associated with photosynthetic complexes and enhancing the biosynthesis of relevant membrane components, such as mono- and di-galactosyl-diacyl glycerols and unsaturated fatty acids. Isoprene may additionally protect photosynthetic membranes by scavenging reactive oxygen species. Here we explore the mode of actions and the potential significance of isoprene in the response of hygrophilic plants when challenged by severe stress conditions associated to rapid climate change in temperate climates, with special emphasis to the concomitant effect of drought and heat. We suggest that isoprene emission may be not a good estimate for its biosynthesis and concentration in severely droughted leaves, being the internal concentration of isoprene the important trait for stress protection.

Keywords: climate change, drought and heat stress, fast-growing plants, isoprene biosynthesis vs. isoprene emission, membrane protection, stomatal conductance

WHY ISOPRENE EMISSION MAY BECOME MORE RELEVANT IN A DRIER AND WARMER CLIMATE?

Isoprene (2-methyl-1,3-butadiene), the major volatile organic compound (VOC) emitted by biogenic sources, has driven attention because of its impact on atmospheric chemistry and climate (Atkinson, 2000). Globally, 0.5–0.6 Pg C are emitted as isoprene annually, accounting for 50% of total biogenic volatile organic compounds (BVOCs), and for 30% of non-methane hydrocarbons emissions (Guenther et al., 2006, 2012). Isoprene is highly volatile and reactive, and its emission by terrestrial plants can substantially affect the concentration of tropospheric ozone (O_3), the lifespan of methane, and the nucleation, condensation or coagulation of secondary aerosol(s) (Pike and Young, 2009; Ying et al., 2015). In the present global change scenario, isoprene emission (Iso_e) is of major concern for several reasons.

First, urban population is expected to increase by approximately 70% by 2050 (United Nations, 2015), and growing megacities are hotspots of atmospheric gaseous and particulate pollutants, with economic, sanitary and social consequences (Baudic et al., 2016). For example, air pollution, particularly tropospheric O_3 and particulate matter, was responsible of 34,143, and 17,800 excess deaths, in Italy and France, respectively, during 2010 (Global Burden of Disease [GBD], 2013; Mori et al., 2015; Baudic et al., 2016). While at null nitrogen oxide (NO_x) concentration, isoprene can even lower tropospheric [O_3], when levels of NO_x are high, a single isoprene molecule leads to the formation of several O_3 molecules (Zeng et al., 2008). In urban areas, where NO_x concentration is high (the so-called NO_x-saturation regime), O_3 production is highly responsive to VOCs (Sillman, 1999; Deguillaume et al., 2008; Ling et al., 2014). Thus, to limit O_3 pollution in NO_x-saturated urban sites, policy actions aimed at reducing VOC emission may be more effective and easier to actuate than the policies aimed at decreasing NO_x concentration (Baudic et al., 2016; Khedive et al., 2017).

Second, conversion of isoprene-emitting forest to low-emitting cropland to match the increasing demand for food, globally decreased isoprene concentration by 15% during the last century (Lathiere et al., 2010). However, the ongoing shift to bioenergy crops (e.g., giant reed) and short rotation forests (e.g., poplar) will likely increase isoprene load, particularly at regional scale (Hardacre et al., 2013; Sharkey and Monson, 2014). For example, in South East Asia, the 27 Mha expansion of land cultivated with oil palm, which can emit three times more isoprene than the native crops (Fowler et al., 2011), increased surface O_3 by 11% (Ashworth et al., 2012). Similarly, the expansion of short rotation forests (mainly poplar) in the temperate northern hemisphere triggers the increase in isoprene burden predicted for boreal Eurasia, North America, and China, where O_2/O_3 mixing ratios are expected to increase up to 2.26 ppb (Ashworth et al., 2012; Hardacre et al., 2013; Zenone et al., 2016).

Third, species from all taxonomic groups have spread around the world, mostly because of human activities. These biological invasions may alter the emission profile of volatiles. For instance, Llusià et al. (2010) have found a lower emission of isoprenoids in native species growing in Hawaii, compared to co-occurring alien species. This was attributed to the lower emission potential of native species relative to aliens, within any given phylogenetic line, though further research is required to upscale this phenomenon. Similarly, tree genera characterized by extensive speciation and hybridization have been reported to emit isoprene more frequently than their phylogenetically nearest non-speciose genera (Dani et al., 2014). Isoprene, being highly volatile (Henry's law constant of 7,780 Pa m^3 mol^{-1}, Harley, 2013), is a 'quick' metabolite capable of improving photosynthetic performance under physiological (non-stressful) (Pollastri et al., 2014) and under transient, usually mild-to-moderate, stress conditions (Loreto and Fineschi, 2015; Maja et al., 2016). Furthermore, it provides protection against generalist pests (Llusià et al., 2010; Harrison et al., 2013); thus alien species, which lack specialist parasites, may greatly benefit from being emitters (Laothawornkitkul et al., 2008; Mithofer and Boland, 2012).

Finally, Iso_e is exponentially linked to temperature, thus global warming is expected to increase the load of volatile compounds (Fares et al., 2011; Lahr et al., 2015). Nonetheless, a conclusive picture of the effect of climate change on Iso_e and hence on the chemistry of the atmosphere is far from being drawn, as the wide range of co-occurring environmental factors (e.g., rising CO_2) may have synergic or antagonistic effects on isoprene biosynthesis (Dieleman et al., 2012).

We focus our discussion on the effects of concomitant stress factors on the biosynthesis and emission of isoprene, with the aim of further exploring isoprene functional roles in hygrophylic plants challenged by 'novel' environmental pressures associated to climate change in temperate climates (e.g., Cfa, Cfb in Koppen Geiger classification).

EXPLORING THE SIGNIFICANCE OF ISOPRENE IN PLANTS CHALLENGED BY STRESS

The functional reasons for Iso_e are still a matter of debate (Sharkey and Monson, 2017). It was hypothesized that isoprene biosynthesis (Iso_s) evolved as an ancestral mechanism in plants adapted to high water availability, to cope with transient and recurrent oxidative stresses during their water-to-land transition (Vickers et al., 2009; Loreto et al., 2014). Consistently the tight association between Iso_e and species hygrophily suggests that Iso_e may be a favorable trait to cope with occasional exposure to stresses in mesic environments (Harrison et al., 2013; Monson et al., 2013; Loreto et al., 2014). Instead, xeric evergreen species inhabiting harsher environments, which require constitutive emissions over a longer time-scale level, generally produce compounds less volatile than isoprene, such as monoterpenes and sesquiterpenes (Loreto and Fineschi, 2015). Fast-growing hygrophilous *Quercus* species, such as most North American and some European oaks (e.g., *Q. robur*) emit isoprene, whereas isoprene is replaced by monoterpenes in xeric oaks, such as *Q. ilex*

and *Q. suber* (Loreto et al., 1998, 2009; Sharkey et al., 2008). This conforms the notion that marked differences in gene sequences encoding isoprene synthase have been found not only between plant groups, but also within each individual group (Dani et al., 2014), and suggests that environmental conditions may have contributed shaping the evolution of isoprenoid synthesis (Monson et al., 2013).

Several fast-growing, isoprene-emitting plants have moved to areas with harsher climate conditions than those of habitats they evolved (Owen et al., 2013). In many instances, extended periods of rainfall scarcity, which usually occur in combination with high both solar irradiance and air temperature may pose serious challenges to plant survival, not only to the profitable production of biomass. Furthermore, the suite of morpho-anatomical traits (e.g., low tissue density, thin cuticle, large vessels, high vein density, see Reich, 2014) does not allow a conservative water use in hygrophilic mesophytes and their ability to withstand combined stress conditions may greatly depend on the so-called metabolic plasticity, which mostly involves secondary metabolites (Tattini et al., 2015). There is evidence that the biosynthesis of isoprenoids is stimulated via ROS-signaling (Fanciullino et al., 2014). This may help explain why the biosynthesis of secondary metabolites, particularly of isoprene is generally uncoupled from photosynthesis (A_N) in drought-stressed leaves (Affek and Yakir, 2003; Loreto and Schnitzler, 2010; Centritto et al., 2011).

The lack of correlation between A_N and isoprene biosynthesis/emission becomes clearer when plants concurrently face multiple stresses. Indeed, there is compelling evidence that carbon sources alternative to recently fixed CO_2 may have particular significance when photosynthesis is constrained by stress (Brilli et al., 2007). These alternative carbon sources may include: non-structural carbohydrates (Kreuzwieser et al., 2002; Funk et al., 2004; Schnitzler et al., 2004); phosphoenolpyruvate imported from the cytosol (Rosenstiel et al., 2003; Fortunati et al., 2008; Jardine et al., 2010); re-fixation of respired CO_2 (Loreto et al., 2004); isoprenoid precursors from the cytosolic mevalonate pathway (Flügge and Gao, 2005); photorespiratory carbon (Jones and Rasmussen, 1975). Carbon derived from photorespiration may have particularly value in sustaining Iso_s when plants experience intense drought and heat stresses (Jardine et al., 2014). Drought stress depresses photosynthesis to a greater extent than photorespiration (Atkin and Macherel, 2009), particularly at high temperatures (Centritto et al., 2011), while elevated temperatures enhance both the substrate (DMADP) availability and the activity of isoprene synthase (Rasulov et al., 2010). Air temperature mostly regulates Iso_s in plants growing at light intensities that saturate photosynthesis (Monson, 2002; Mayrhofer et al., 2005; Fares et al., 2011; Niinemets and Sun, 2015), since Iso_e does not saturate even at very high photosynthetic photon flux density (>2000 μmol m^{-2} s^{-1}, Geron et al., 2006; Loreto and Schnitzler, 2010).

In plants concurrently experiencing water and heat stress, stomatal closure reduces latent heat and exacerbates sensible heat load (Tattini et al., 2015). In particular, hygrophilic isoprene-emitters steeply close stomata, even at moderate drought, to avoid tissue dehydration (Brilli et al., 2007; Tattini et al., 2015; Velikova et al., 2016). These are the conditions under which isoprene biosynthesis is largely stimulated. Isoprene has been reported to enhance drought resistance of many fast-growing species, including tobacco and poplars. In all cases, isoprene-emitting lines showed reduced depression of photosynthesis, and less oxidative damage than non-emitting lines, when exposed to drought (Ryan et al., 2014; Tattini et al., 2014; Vanzo et al., 2017).

ISOPRENE MODE OF ACTION: FACTS AND SPECULATIONS OF AN OPEN DEBATE

Because isoprene is costly for leaves (20 ATP and 14 NADPH for each molecule of isoprene produced by CO_2 fixation through photosynthesis) (Sharkey and Yeh, 2001) the great investment of leaves for Iso_s under stressful conditions must have functional reasons (Sharkey and Monson, 2017). Isoprene may play multiple functions in countering the detrimental effects of supernumerary photons reaching the chloroplast, when the leaf ability to process radiant energy to carbon fixation is severely constrained by environmental stressors (Loreto and Schnitzler, 2010). Isoprene is effective in preserving the integrity of thylakoid membranes (Velikova et al., 2011, 2015). *Populus* × *canescens* lines where Iso_s is suppressed displayed reduced photosynthetic electron transport rate (ETR) during heat stress, and did not recover photosynthesis at the level of the corresponding isoprene-emitting lines after relief from stress (Behnke et al., 2007). The protective functions of isoprene on membrane-associated processes (also observed under 'physiological' conditions, Pollastri et al., 2014) may not depend simply on the hydrophobic interaction between isoprene and the lipid acyl chains of membranes (Siwko et al., 2007), as isoprene concentration inside membranes is too low to effectively modulate their bulk lipid phase (Harvey et al., 2015). Benefits for membrane stability associated to Iso_e may also result from both the up-regulation of proteins associated with photosynthetic complexes (Velikova et al., 2014) and the enhanced biosynthesis of relevant membrane components, such as mono- and di-galactosyl-diacyl glycerols and unsaturated fatty acids (Velikova et al., 2015). In simpler terms, isoprene-induced improvement in the use of radiant energy to carbon fixation may reduce the risk of photo-oxidative stress in isoprene-emitting leaves. Protection of photosynthetic membranes may be induced by isoprene indirectly, as isoprene is also known to scavenge reactive oxygen species (ROS) (Loreto and Velikova, 2001; Affek and Yakir, 2002; Velikova et al., 2004). The antioxidant effect of isoprene is especially clear in the case of singlet oxygen ($^1O_2$), the most dangerous ROS in chloroplasts. This effect was empirically demonstrated by Velikova et al. (2004), and has now been theoretically framed (Zeinali et al., 2016). ROS scavenging inside leaves explains the formation of isoprene oxidation products, mostly methyl-vinyl-ketone and methacrolein, in plants exposed to a wide range of stressors, especially heat, in both controlled (Jardine et al., 2012, 2013) and field conditions (Cappellin et al., 2017).

Despite the large body of evidence summarized above, there are open questions that still challenge the idea that isoprene

might have a definite role in plant protection. Why did only about 20% of the plants worldwide develop the capacity to emit isoprene? (Loreto and Fineschi, 2015). Why are these plants spread all over biomes and climatic areas (Loreto and Fineschi, 2015), and are not concentrated where stress protection becomes more relevant for securing plant survival, growth, and reproduction?

In many instances, Iso_e increases under mild to moderate drought, but declines steeply when plants face severe drought (Brilli et al., 2007, 2013; Centritto et al., 2011; Tattini et al., 2014, 2015). Therefore, it has been hypothesized that isoprene plays a beneficial role only in response to mild stress, whereas non-volatile, more stable metabolites, produced through the same metabolic pathway of isoprene (the MEP pathway, i.e., carotenoids and abscisic acid), serve functions of greater significance when plants are challenged by severe stress. This is a revisited formulation of the "opportunistic hypothesis", firstly postulated by Owen and Peñuelas (2005). For example, in *Xerophyta humilis*, Iso_e ceased at 5% RWC, but zeaxanthin replaced isoprene to enhance membrane stability, thus allowing prompt chloroplast re-assembly upon re-watering of this resurrection plant (Beckett et al., 2012). Recent evidence suggests that isoprene may serve antioxidant functions (*sensu lato*) of increasing significance in plants concurrently challenged by drought and heat. Indeed, the activities of primary antioxidants, such as antioxidant enzymes, and the concentration of zeaxanthin may decrease in high light-exposed plants during the hottest hours of the day, whereas biosynthesis and emission of isoprene are promoted in the same conditions (Brunetti et al., 2015; Tattini et al., 2015).

Isoprene emission might even have a regulatory role, differentially setting the flow of carbon in the MEP pathway along stress progression. The transient increase of isoprene biosynthesis/emission in drought-stressed leaves might serve to use of excess reducing power, limiting the accumulation of dimethylallyl diphosphate (DMADP) and its consequent feedback down-regulation of the whole MEP pathway (Banerjee et al., 2013; Ghirardo et al., 2014). Sustained isoprene formation under stress conditions may also indirectly contribute to increase the carbon flux into the MEP pathway leading to the *de novo* biosynthesis of foliar abscisic acid, the stress hormone controlling stomatal aperture in drying soil to prevent water loss (Zhang and Davies, 1987). This effect might be exacerbated when plants concurrently face high solar irradiance and temperatures, which are known to increase the availability of DMADP (Mayrhofer et al., 2005; Sharkey and Monson, 2014). Thus, internal isoprene concentration (not isoprene emission) might 'prime for drought stress response,' triggering a general protective function that also involves changes in non-volatile isoprenoids, soluble carbohydrates and phenylpropanoids (Tattini et al., 2014). This may sustain the need of large metabolic adjustments of hygrophylic plants suddenly facing the unpredictable pressures imposed by "anthropogenic" planting sites, where microclimates can be very different from those where these species have evolved.

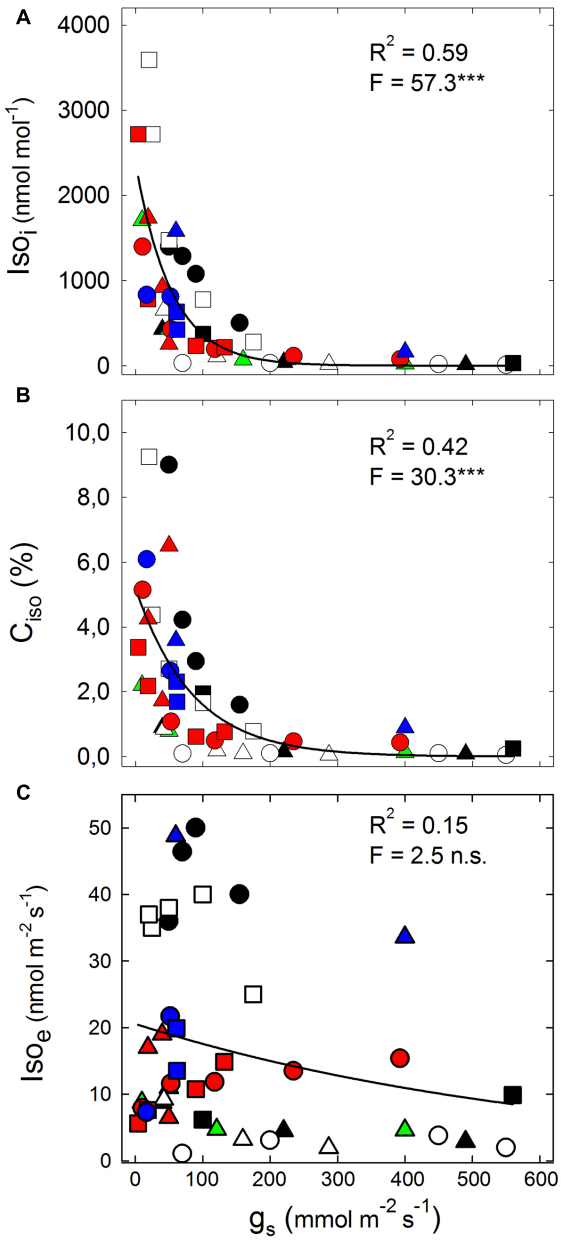

FIGURE 1 | The relationship between **(A)** internal isoprene concentration (Iso_i); **(B)** percent of fresh assimilated carbon lost as isoprene emission (C_{iso}); **(C)** isoprene emission (Iso_e) and stomatal conductance (g_s). Isoprene concentration was calculated using a simplified version of the equation proposed by Singsaas et al. (1997), as $Iso_i = 2.83 \times Iso_e/g_s$, where the factor 2.83 is the ratio of the diffusion coefficient of water vapor through air to that of isoprene through air; $C_{iso} = 5 \times (Iso_e, \mu mol\ m^{-2}\ s^{-1})/(A_N, \mu mol\ m^{-2}\ s^{-1}) \times 100$. Non-linear correlations have been drawn using the following exponential decay curve, $Iso_x = a^{-b \times x}$. Data points derive from the experimental data reported in: Brilli et al. (2007) (*Populus alba:* ●); Brilli et al. (2013) (*Eucalyptus citriodora*: ■); Funk et al. (2004) (*P. deltoides:* ▲); Tani et al. (2011) (*Quercus serrata:* ●); Tattini et al. (2014) (*Nicotiana tabacum:* ○); Tattini et al. (2015) (*Platanus* × *acerifolia:* ●); Velikova et al. (2016) (*Arundo donax:* ■); Pegoraro et al. (2004) (*Q. virginiana:* ▲); Dani et al. (2014) (▲, *E. occidentalis* and *E. camaldulensis:* ▲); Staudt et al. (2016) (*Q. pubescens:* ■); Marino et al. (2017) (*P. nigra:* □); Brunetti et al., personal communication, (*Moringa oleifera:* Δ).

IS ISOPRENE EMISSION A GOOD PROXY OF INTERNAL ISOPRENE AND OF PLANT STRESS RESPONSE?

Isoprene emission has been usually taken as a good estimate of Iso_s, but Iso_e might largely differ from Iso_s, e.g., as consequence of drought-induced declines in stomatal conductance (g_s). It has been hypothesized that stomata cannot control the emission of VOCs with high Henry's low constant, such as isoprene, even during rapid reductions in g_s (Niinemets and Reichstein, 2003). If Iso_s remains constant or even increases when stress induces stomatal closure, then the increased gradient between the internal and external concentration of isoprene should compensate for the increased resistance to isoprene outflow. However, under chronic or severe reductions of g_s, isoprene concentration inside the leaf (Iso_i) largely exceeds Iso_e (Velikova et al., 2016), and Iso_i might represent a more suitable estimate of Iso_s compared to Iso_e (Brunetti et al., 2015; Tattini et al., 2015).

It has been also speculated that lipid membranes are saturated with isoprene even at low emission rates (because isoprene is highly hydrophobic in its nature), and that any increase in Iso_s will increase isoprene diffusion through membranes rather than enhancing its membrane concentration (Vickers et al., 2009). However, recent results discussed above revisited this concept and showed that isoprene concentration in membranes is generally low (Harvey et al., 2015). Therefore, it cannot be excluded that steep reductions of g_s may induce large accumulation of isoprene inside leaves, on a short time-scale, thereby altering membrane composition (Velikova et al., 2015), while providing efficient antioxidant and priming functions.

The decline in g_s is a good proxy of drought stress severity in isohydric hygrophylic plants. The best-fit analysis reported in **Figure 1** shows a highly significant exponential decay of Iso_i with increasing g_s (**Figure 1A**), because drought stress strongly enhances Iso_i for $g_s < 200$ mmol m^{-2} s^{-1}, whereas Iso_i is unresponsive to higher g_s. The severity of drought also significantly correlates with the investment of freshly assimilated carbon (C_{iso}) to Iso_s (**Figure 1B**), indicating that a growing fraction of photosynthetic carbon sustains isoprene formation when the stress severely reduces g_s. C_{iso} has also been widely shown to positively correlate with the unbalance between the ETR and A_N (Morfopoulos et al., 2014). In fact, ETR/A_N often increases as drought become more severe, especially when A_N is constrained by diffusional limitations (at stomatal or mesophyll level) rather than by biochemical limitations, as observed in fast-growing mesophytes, which are usually strong isoprene emitters (Loreto et al., 2014; Haworth et al., 2016). In contrast, there is a poor correlation between Iso_e and g_s (**Figure 1C**). In our survey, Iso_e is almost unresponsive to mild and moderate drought-induced depressions in g_s, in both high (*Platanus* × *acerifolia*, *Populus nigra*, *P. deltoides*, on average Iso_e of 40.0 nmol m^{-2} s^{-1}) and low isoprene emitters (*Eucalyptus occidentalis*, *Nicotiana tabacum*, on average Iso_e of 4.0 nmol m^{-2} s^{-1}). In species with intermediate isoprene emission rates (on average Iso_e of 10.8 nmol m^{-2} s^{-1}), instead, Iso_e either declines (*P. alba*, *E. citriodora*) or increases (*Moringa oleifera*) following drought-induced depression of g_s. Data of our meta-analysis may help explain why isoprene emission fails representing the intensity of drought and heat stresses in current models (Harrison et al., 2013; Morfopoulos et al., 2013; Sharkey and Monson, 2014). We conclude that Iso_i and C_{iso}, representing isoprene accumulation inside leaves, may allow better estimation than isoprene emission of the functional responses of plants to stress. However, we are aware that accuracy of g_s measurements is inherently low for $g_s < 20$ mmol m^{-2} s^{-1} so that calculations of Iso_i (and to less extent of C_{iso} as well) have to be taken with some caution at very severe drought. The issue is of interest and merits further investigation.

AUTHOR CONTRIBUTIONS

AF wrote the first and second sections of the manuscript; CB wrote the second and third sections of the manuscript and conducted to the meta-analysis of data; FL contributed to manuscript writing and carefully reviewed the manuscript; MC and FF carefully reviewed the manuscript; MT drafted the manuscript and wrote section three and four of the manuscript.

FUNDING

This work was supported by the EU FP7-311929 project (Development of improved perennial non-food biomass and bioproduct crops for water stressed environments – WATBIO)

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 April 2017
doi: 10.3389/fpls.2017.00598

Contrasting Hydraulic Architectures of Scots Pine and Sessile Oak at Their Southernmost Distribution Limits

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Many temperate European tree species have their southernmost distribution limits in the Mediterranean Basin. The projected climatic conditions, particularly an increase in dryness, might induce an altitudinal and latitudinal retreat at their southernmost distribution limit. Therefore, characterizing the morphological and physiological variability of temperate tree species under dry conditions is essential to understand species' responses to expected climate change. In this study, we compared branch-level hydraulic traits of four Scots pine and four sessile oak natural stands located at the western and central Mediterranean Basin to assess their adjustment to water limiting conditions. Hydraulic traits such as xylem- and leaf-specific maximum hydraulic conductivity ($K_{S\text{-}MAX}$ and $K_{L\text{-}MAX}$), leaf-to-xylem area ratio (A_L:A_X) and functional xylem fraction (FX) were measured in July 2015 during a long and exceptionally dry summer. Additionally, xylem-specific native hydraulic conductivity ($K_{S\text{-}N}$) and native percentage of loss of hydraulic conductivity (PLC) were measured for Scots pine. Interspecific differences in these hydraulic traits as well as intraspecific variability between sites were assessed. The influence of annual, summer and growing season site climatic aridity (P/PET) on intraspecific variability was investigated. Sessile oak displayed higher values of $K_{S\text{-}MAX}$, $K_{L\text{-}MAX}$, A_L:A_X but a smaller percentage of FX than Scots pines. Scots pine did not vary in any of the measured hydraulic traits across the sites, and PLC values were low for all sites, even during one of the warmest summers in the region. In contrast, sessile oak showed significant differences in $K_{S\text{-}MAX}$, $K_{L\text{-}MAX}$, and FX across sites, which were significantly related to site aridity. The striking similarity in the hydraulic traits across Scots pine sites suggests that no adjustment in hydraulic architecture was needed, likely as a consequence of a drought-avoidance strategy. In contrast, sessile oak displayed adjustments in the hydraulic architecture along an aridity gradient, pointing to a drought-tolerance strategy.

Keywords: anisohydric, drought, functional xylem anatomy, isohydric, hydraulic conductivity, Mediterranean Basin, *Pinus sylvestris*, *Quercus petraea*

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Giai Petit, University of Padua, Italy
Dirk Vanderklein, Montclair State University, USA

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *09 December 2016*
Accepted: *03 April 2017*
Published: *20 April 2017*

Citation:
Martínez-Sancho E, Dorado-Liñán I, Hacke UG, Seidel H and Menzel A (2017) Contrasting Hydraulic Architectures of Scots Pine and Sessile Oak at Their Southernmost Distribution Limits. Front. Plant Sci. 8:598. doi: 10.3389/fpls.2017.00598

INTRODUCTION

Drought is a key factor of tree species' distribution in the Mediterranean Basin (Mitrakos, 1980; Cherubini et al., 2003), and climate projections for the next decades point to an increase in dryness in this region (Kirtman et al., 2013). For the most severe scenario of the last IPCC assessment report, an increase of ∼7°C in summer temperature and a decrease of ∼30% in April–September precipitation, as well as more intense summer droughts for the end of the 21st century are predicted (Collins et al., 2013). Climate change impacts may have global consequences in forest ecosystems, for instance, loss of ecosystem services (Anderegg et al., 2013); reduction of the terrestrial net productivity (Zhao and Running, 2010) and changes in sink–source carbon dynamics (Jones et al., 2009). In extreme cases, climate-change-induced negative effects can result in drought-induced tree mortality as reported worldwide by Allen et al. (2010).

Changes in climate may also extend the growing season due to higher spring temperatures (Menzel and Fabian, 1999; Menzel et al., 2006; Gordo and Sanz, 2010) and increase the water-use efficiency of the plants due to higher atmospheric CO_2 concentrations (De Kauwe et al., 2013). However, in drought-prone areas such as the Mediterranean Basin where water availability is already limited (Giorgi and Lionello, 2008) and land-use changes are increasing the competition for this scarce resource (Ruiz-Benito et al., 2013), the above mentioned potential benefits have to be balanced against projected water limitations and their consequences on tree growth (Andreu-Hayles et al., 2011; Peñuelas et al., 2011).

Water loss through the stomata is an unavoidable consequence of carbon assimilation in plants. This loss must be compensated by water pulled upward from the roots to the leaves under negative hydrostatic pressure (Pockman et al., 1995). Under dry conditions, cavitation occurs inside the xylem conduits reducing the total hydraulic conductivity of the plant. Long and more severe water limitations lead to the collapse of the plant hydraulic system and ultimately, to tree death (McDowell, 2011). However, plants can partly cope with water limitations through physiological regulations in different parts of the soil–plant–atmosphere continuum (Sperry et al., 2002; Martínez-Vilalta et al., 2014), such as changes in the rooting system, in the hydraulic architecture, and/or in the regulation of transpirational water loss [e.g., by decreasing leaf-to-sapwood area ratio and/or increasing sensitivity of stomata to vapor pressure deficit (VPD)] (Tyree and Ewees, 1991; Oren et al., 1999; Martínez-Vilalta et al., 2004). Few studies have focused on the intraspecific variability of some of these hydraulic traits (Martínez-Vilalta et al., 2009; Benito-Garzón et al., 2011; Violle et al., 2014; Anderegg and Hillerislambers, 2016).

Many temperate European tree species reach their southernmost distribution limits in the Mediterranean region where they face suboptimal environmental conditions compared to the center of the distribution area (Hampe and Petit, 2005). However, the environmental conditions that allowed temperate species to establish in this region are changing (Giorgi and Lionello, 2008; Mariotti, 2010). There is now ample evidence that current climate change is promoting a rearrangement of the geographic distributions of plant and animal species world-wide (Parmesan and Yohe, 2003). Furthermore, Mediterranean plant species that have developed mechanisms in response to dry summers may be favored under future climate change scenarios and consequently, temperate species would either have to acclimate or to migrate to higher altitudes/latitudes (Peñuelas and Boada, 2003; Galiano et al., 2010). Understanding how hydraulic traits may respond to different climatic conditions will improve our knowledge on physiological limits of the temperate and boreal species and the heterogeneity in the response along their distribution range.

Scots pine (*Pinus sylvestris* L.) and sessile oak (*Quercus petraea* (Matt.) Liebl.) constitute two of the main tree species in Europe with contrasting hydraulic architecture as well as different ecophysiological strategies to deal with water limitations. Scots pine tends to avoid water stress by a strict stomata control, which is considered as an isohydric behavior (Irvine et al., 1998; Leo et al., 2014; Salmon et al., 2015). Under drought conditions, Scots pine adjusts its leaf-to-sapwood area ratio, leaf-specific hydraulic conductivity and total leaf area (Sterck et al., 2008; Martínez-Vilalta et al., 2009). In contrast, sessile oak usually maintains high transpiration and stomatal conductance (anisohydric behavior) under moderate drought conditions (Bréda et al., 1993b; Epron and Dreyer, 1993; Aranda et al., 2000; Klein, 2014). The projected changes in climate may shift the Mediterranean area beyond the ecological niche of these two species (Hampe and Petit, 2005; Lenoir et al., 2008). In this context, the differences in hydraulic architecture and strategy to cope with drought may be decisive for the capacity of resistance and resilience and hence, in future species persistence in the region.

In this study, we compared branch-level hydraulic traits of four natural pine and oak stands each, located at the southernmost limit of their distribution areas in the western and central Mediterranean Basin. Our objectives were (1) to characterize the interspecific differences due to the diverging physiological strategies (isohydric vs. anisohydric); (2) to assess intraspecific variability across the limit of the species' distributions; and (3) to evaluate whether inter-site variability of hydraulic traits is linked to site aridity. Answering these questions would allow for a better understanding of the hydraulic strategies of the two species under natural conditions at the southernmost limits of the species distributions. The study was conducted during one of the warmest and driest summers in the region in the last six decades.

MATERIALS AND METHODS

Study Species and Sites

Scots pine is an evergreen conifer with a wide distribution range across Eurasia. Its distribution is the largest of all species of the genus *Pinus*, and even of the whole *Pinaceae* family (Boratynski, 1991). This broad ecological range suggests a high degree of structural and/or functional plasticity. Pine wood is made by non-specialized tracheids that perform conductive and structural functions. In contrast, sessile oak is a deciduous temperate tree

species predominantly distributed in central Europe. Oak wood is made up of vessels, tracheids, fibers, and parenchyma cells (Schweingruber, 1993). As a ring-porous species, the earlywood vessels support most of the water transport in the xylem, and in comparison to pine tracheids, they are by far more efficient in water transport (Tyree and Zimmermann, 2002).

Four natural stands of oak and four of pine were selected across the southernmost limits of both species distributions (**Figure 1**). The study sites cover a wide longitudinal area in the western and central part of the Mediterranean Basin (41.7° N – 44.9° N and 2.0° E – 11.9° E). They are located within the Mediterranean north and the Mediterranean mountains environmental zones (Metzger et al., 2005) characterized by a typical Mediterranean climate with a pronounced drop of precipitation during summer and one or two maxima of precipitation during the winter months.

For each site, annual sums of precipitation and mean annual temperature were extracted from the 0.25° gridded E-OBS dataset (Haylock et al., 2008) and are given as means in **Table 1** for the period 2011–2014, which also corresponds to the build-up time of the sampled branches. Mean annual temperatures ranged from 13.3°C to 15.5°C and 13.2°C to 16.1°C; and the annual sums of precipitation (P) ranged from 657.4 to 840.8 mm and 738.1 to 849.2 mm for the pine and oak sites, respectively. Potential evapotranspiration (PET) was calculated using the Hargreaves method (Hargreaves, 1994) included in the SPEI R package (Vicente-Serrano et al., 2010). Annual, summer and growing season P/PET and P-PET (climatic aridity indices) were calculated as proxies of mean potential drought stress for the studied period (Hogg, 1997; Knapp et al., 2008). However, results related to P-PET revealed identical patterns as P/PET and are not shown in this paper.

Collection of Samples and Measurements

The field campaign took place during the last 2 weeks of July 2015, which was regionally (Supplementary Figure S1), and globally (NOAA National Centers for Environmental Information, 2016), an exceptionally hot period. At each site, one low branch per tree was collected from six trees with a throw saw. For technical reasons, distal segments from the selected branches (>50 cm in length) instead of the whole branches were collected. The samples were labeled and carefully wrapped in large plastic bags with wet towels to minimize dehydration. Additionally, diameter at breast height and total height of the sampled trees were measured (Supplementary Table S1). After collection, the samples were sent by courier to our laboratory at the TU Munich. All hydraulic measurements were conducted within 3 days after collection of the branches in the field. Once in the laboratory, the branches were successively cut under water by trimming the ends of each segment with a fresh razor blade. Five-year old internodes (4 years old plus the current growing season), located within the 50-cm distal segments, were selected for the measurements to obtain comparable results among trees and sites. Diameter and length of the resulting segments were recorded for each sample (see Supplementary Table S2 for mean dimensions).

In order to measure native hydraulic conductivity, the stem segments were fitted to a tubing apparatus (Sperry et al., 1988) filled with a filtered (0.2 μm) and degassed solution of 20 mM KCl and 1 mM $CaCl_2$. The hydraulic conductivity (kg s^{-1}m MPa^{-1}) was calculated following the equation:

$$K_h = FL/\Delta P$$

where F is the flow rate (F, kg s^{-1}), L is the length of the segment (m) and ΔP the driving force (MPa). The gravity-induced water flow rate through the segments was recorded every 10 s with an electronic balance (Mettler-Toledo XS204DR, Mettler-Toledo AG, Greifensee, Switzerland) interfaced with a computer. Stem hydraulic conductivity (K_h/xylem area; kg m^{-1} s^{-1} MPa^{-1}) was then calculated as the flow rate for a given pressure gradient and normalized by dividing the hydraulic conductivity by the total xylem area. Since we cannot exclude the possibility that oak internodes contained long vessels that embolised after collection, native conductivities ($K_{S\text{-}N}$) are only reported for pine segments. In pine, the amount of native embolism (native percentage loss of hydraulic conductivity, PLC) was calculated as the percentage of $K_{S\text{-}N}$ relative to $K_{S\text{-}MAX}$. Native xylem embolism was reversed by applying two different methods for oak and pine, which were selected based in the results of previously performed tests. In the case of oak samples, branch segments were connected to a

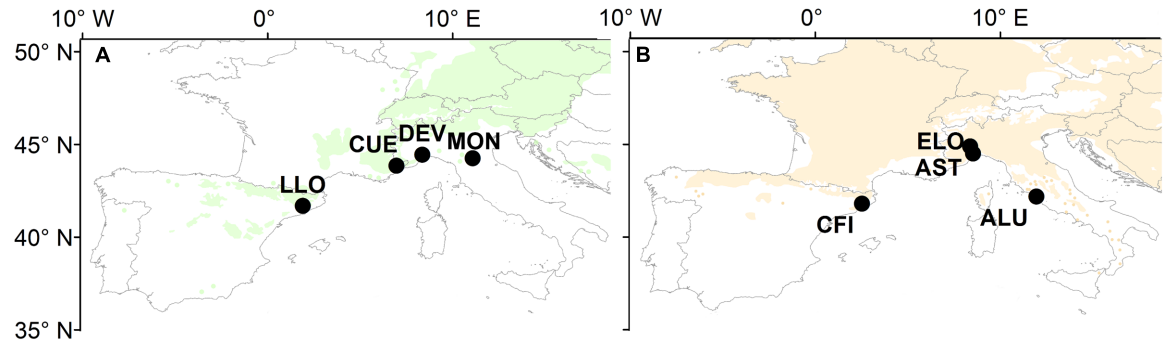

FIGURE 1 | Map of Europe showing the distribution of (A) Scots pine and **(B)** sessile oak and the location of the eight study sites (for details of the study sites, see **Table 1**). Maps are a modified version from those available at www.euforgen.org.

TABLE 1 | Geographical characteristics of the study sites and mean climatic variables derived from E-OBS dataset for the studied period (2011–2014) corresponding to the build-up time of the sampled branches.

Site	Site code	Country	Species	LAT (°N)	LON (°E)	Elevation (m a.s.l.)	T (°C)	P (mm)	PET (mm)	P/PET	P/PET JJA	P/PET season
Sant Llorenç	LLO	Spain	*P. sylvestris*	41.722	1.997	500	15.5	599.6	970.4	0.62	0.22	0.49
Cuébris	CUE	France	*P. sylvestris*	43.894	7.064	720	13.4	840.8	820.3	1.02	0.26	0.61
Devia	DEV	Italy	*P. sylvestris*	44.489	8.460	364	14.0	781.5	728.1	1.07	0.27	0.64
Monte Sole	MON	Italy	*P. sylvestris*	44.293	11.181	560	13.3	657.4	933.5	0.70	0.22	0.48
Can Figueroles	CFI	Spain	*Q. petraea*	41.767	2.477	800	13.2	738.1	940.7	0.78	0.26	0.60
Sasselo	ELO	Italy	*Q. petraea*	44.478	8.479	445	14.0	781.5	728.2	1.07	0.27	0.64
Asti	AST	Italy	*Q. petraea*	44.855	8.323	330	14.1	849.2	873.8	0.97	0.40	0.66
Allumiere	ALU	Italy	*Q. petraea*	42.155	11.909	620	16.1	834.7	994	0.84	0.21	0.48

LAT, latitude; LON, longitude; T, Mean annual temperature; P, sum annual precipitation; PET, potential evapotranspiration; P/PET, aridity index; P/PET JJA, summer aridity index, P/PET season, growing season aridity index.

tubing system and flushed with the measuring solution for 1 h at 70 kPa. The pine samples were submerged in measuring solution and vacuum infiltrated for 1 h. Vacuum infiltration gave better results in pines, probably because it prevents the aspiration of the pit membranes (Schulte et al., 2015). Afterward, the maximum hydraulic conductivity was measured.

Leaf-specific maximum hydraulic conductivity ($K_{L\text{-}MAX} = K_{H\text{-}MAX}$/leaf area; kg m^{-1} s^{-1} MPa^{-1}) was calculated as the ratio of maximum hydraulic conductivity and the cumulative leaf area supplied by the segment. All the leaves distal to the 5-year-old segment later on used for the hydraulic measurements were collected and scanned (Epson Expression 10000 XL, Seiko Epson Corporation, Suwa, Japan). The total leaf area was measured using the image processing software Image J (Schneider et al., 2012). $K_{L\text{-}MAX}$ is influenced by the leaf-to-xylem area ratio (A_L:A_X). Once the maximum hydraulic conductivity was determined, the stems were attached to a tubing system with a water reservoir and perfused with dye (0.1% crystal violet solution) to determine which parts of the xylem were functional after flushing or vacuum infiltration. Dye perfusions were conducted following the method described in Jacobsen et al. (2007). A pressure of ∼2 kPa was produced by lowering the water reservoir to 20 cm below the top of the dye solution, in order to stain the active xylem area. Cross sections were prepared from the center of each stem. The stained (functional) xylem area and the total xylem area (including non-stained xylem) were captured using a digital camera (Canon Rebel T2i, Canon, Krefeld, Germany) connected to a binocular microscope (Leica S6D, Leica camera AG, Wetzlar, Germany) and were measured with ImageJ (Schneider et al., 2012). Areas corresponding to the pith and the bark of the stems were excluded from the measurements when determining total xylem area and functional xylem area. The functional xylem fraction (FX) was expressed as the percentage of the ratio of the stained xylem area and the total xylem area. **Table 2** lists all branch-level traits with their respective units.

Data Analyses

Non-parametric approaches were chosen since the data were not normally distributed and could not be fit to a normal distribution by standard transformation techniques. Mann–Whitney (Wilcoxon) tests were performed to determine the significance of interspecific differences for all hydraulic traits. Pairwise Spearman's rank correlations were performed to evaluate the relation among hydraulic traits within species. Kruskal–Wallis tests with a posterior Bonferroni corrected Mann–Whitney analyses were used to test differences in the hydraulic properties among sites for each tree species. In addition, linear regression analyses were used to relate site-specific means of hydraulic traits and annual, summer and growing season site aridity. Summer site aridity was considered as the ratio between precipitation and PET from June to August, whereas growing season aridity included the same parameters from March to October. Although the data were not normally distributed, parametric linear regressions were chosen because homoscedasticity and normality of errors of the models met the normality assumptions. The statistical software R (R Core Team, 2015) was used to perform the analyses.

TABLE 2 | Branch-level traits measured.

Variable	Acronym	Units
Xylem-specific native hydraulic conductivity	$K_{S\text{-}N}$	kg m^{-1} s^{-1} MPa^{-1}
Native percentage of loss hydraulic conductivity	PLC	%
Xylem-specific maximum hydraulic conductivity	$K_{S\text{-}MAX}$	kg m^{-1} s^{-1} MPa^{-1}
Leaf-specific maximum hydraulic conductivity	$K_{L\text{-}MAX}$	10^{-4} kg m^{-1} s^{-1} MPa^{-1}
Leaf-to-xylem area ratio	A_L:A_X	10^3 m^2 m^{-2}
Functional xylem fraction	FX	%

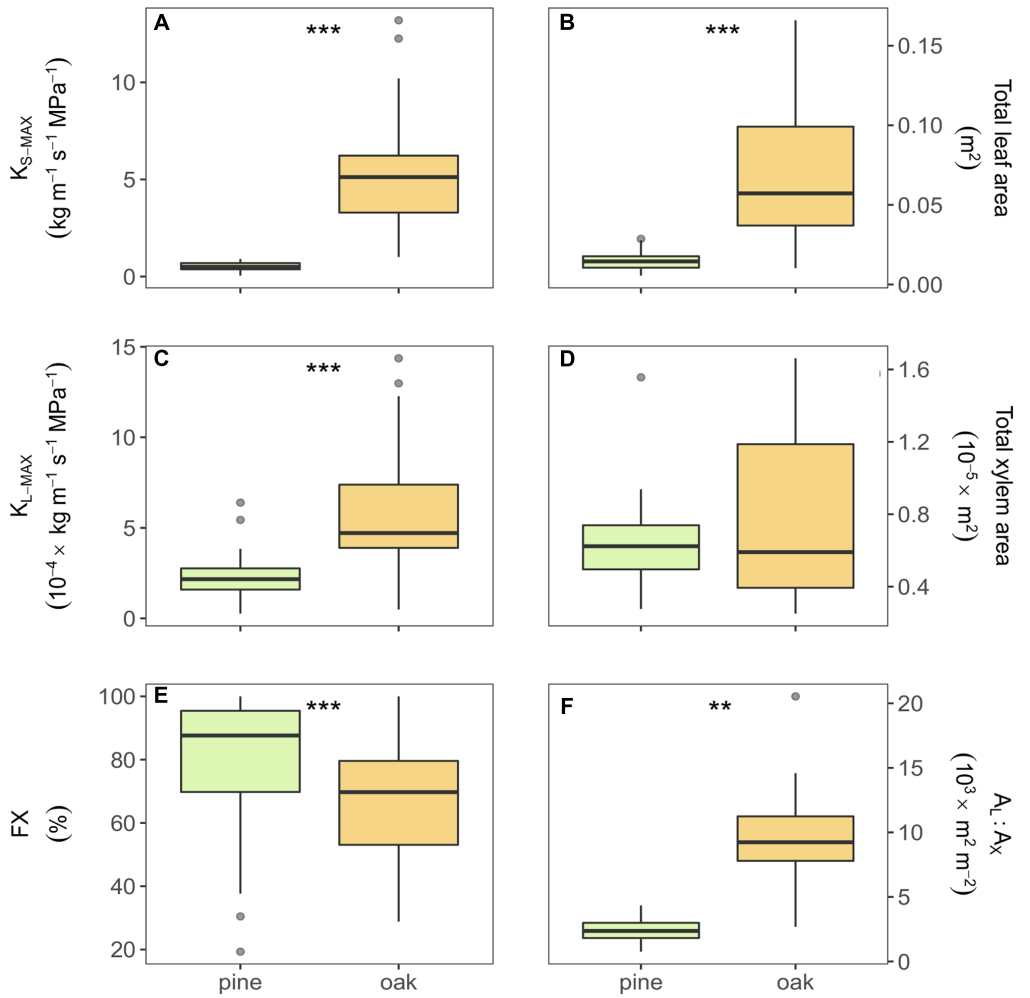

FIGURE 2 | Boxplots of the different hydraulic traits of Scots pine and sessile oak: (A) xylem-specific maximum hydraulic conductivity, **(B)** total leaf area, **(C)** leaf-specific maximum hydraulic conductivity, **(D)** total xylem area, **(E)** functional xylem fraction, and **(F)** leaf-to-xylem area ratio. Boxes represent 50% of the data (between the first and third quartiles). Median is shown as horizontal line. Bars extend from the box to the highest/lowest value within 1.5 * inter-quartile range (IQR). Points (outliers) are values > 1.5 * IQR. K_{S-MAX}, xylem-specific maximum hydraulic conductivity; K_{L-MAX}, leaf-specific maximum hydraulic conductivity; A_L:A_X, leaf-to-xylem area ratio; FX, functional xylem fraction. **Indicate p-value < 0.01 and ***indicate p-value < 0.001.

RESULTS

Interspecific Differences

All measured hydraulic variables differed significantly between oak and pine samples (**Figure 2**). Compared to pine, oak displayed eight and three times higher xylem area- and leaf area-specific maximum hydraulic conductivity (K_{S-MAX} and K_{L-MAX}), respectively. Oak branches showed six times higher values of total leaf area than pine branches, but similar values of total xylem area resulting in larger A_L:A_X in oak branches than in pine (**Figures 2B,D,F**). Additionally, pine branches had a higher FX than oak branches (**Figure 2E**). Almost all xylem of the pine samples was stained (functional in water transport after vacuum infiltration) whereas in oak, the inner rings were not stained meaning that they were no longer functional in water transport.

Inter-site Variability and Relation to Climate

There were no significant differences in the studied hydraulic traits among pine sites (**Figure 3** and Supplementary Table S3). Branches from the Italian site MON displayed the highest values of K_{S-N}, K_{S-MAX}, K_{L-MAX}, and FX while branches from LLO (the most arid site in Spain) showed the lowest values. All pine branches displayed low native PLC values. Branches from the French site CUE showed the lowest native mean PLC (3.59 %) while branches from MON showed the highest mean PLC (9.25 %). Consequently, no significant linear relation was found between the pine hydraulic traits and the annual, summer or growing season site aridity (**Table 3**).

In contrast to the results obtained for pine, most of the oak hydraulic traits differed across an aridity gradient (**Figure 4**). In particular, traits such as K_{S-MAX} and K_{L-MAX} significantly

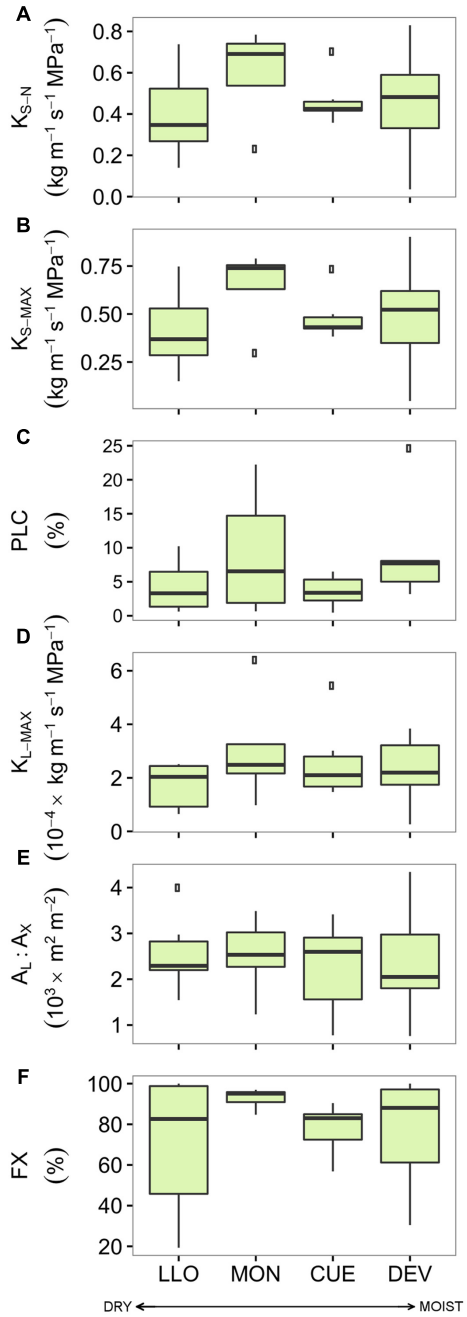

FIGURE 3 | Hydraulic traits of the four Scots pine populations sampled across an aridity gradient: (A) xylem-specific native hydraulic conductivity, **(B)** xylem-specific maximum hydraulic conductivity, **(C)** native percentage of loss hydraulic conductivity, **(D)** leaf-specific maximum hydraulic conductivity, **(E)** leaf-to-xylem area ratio, and **(F)** functional xylem fraction. Sites are ordered respect to P/PET annual (see **Table 1**). Boxes represent 50% of the data (between the first and third quartiles). Median is shown as horizontal line. Bars extend from the box to the highest/lowest value within 1.5 * inter-quartile range (IQR). Points (outliers) are values > 1.5 * IQR. Note: None of the differences among sites was significant (see Supplementary Table S3). K_{S-N}, xylem-specific native hydraulic conductivity; PLC, native percentage of loss hydraulic conductivity; K_{S-MAX}, xylem-specific maximum hydraulic conductivity; K_{L-MAX}, leaf-specific maximum hydraulic conductivity; A_L:A_X, leaf-to-xylem area ratio; FX, functional xylem fraction.

differed (Supplementary Table S4). Branches from ELO (the least arid site in Italy) exhibited the highest values of K_{S-MAX} and K_{L-MAX} while the ones from CFI (the most arid site in Spain) showed the lowest conductivity. Although there were significant differences in total xylem area among oak stands (Supplementary Figure S2), no site-specific differences in A_L:A_X were found. In addition, branches from the two less arid sites displayed higher fractions of functional xylem than branches from the two driest sites (**Figure 4D**).

These differences in hydraulic traits among oak sites were linked to the annual site aridity. Annual P/PET was positively related to K_{S-MAX} ($R^2 = 0.99$, $p < 0.01$), K_{L-MAX} ($R^2 = 0.92$, $p < 0.04$) and unrelated to A_L:A_X and FX ($p = 0.15$ and $p = 0.11$, respectively; **Table 3**). However, none of the hydraulic traits were related to site-specific summer or growing season aridity.

Relation among Hydraulic Traits

Branch hydraulic traits of pine samples were largely inter-correlated (Supplementary Figure S3). A highly significant positive correlation between K_{S-N} and K_{S-MAX} was found ($r = 0.99$, $p < 0.001$), as well as significant positive correlations of K_{S-N} and K_{S-MAX} with K_{L-MAX} and FX. Additionally, K_{L-MAX} showed a significant positive correlation with FX. However, K_{L-MAX} was the only trait showing a significant negative correlation with A_L:A_X ($r = -0.52$, $p < 0.05$).

Four out of the six possible correlations of oak hydraulic traits were significant. A highly significant correlation was found between K_{S-MAX} and K_{L-MAX} ($r = 0.84$, $p < 0.001$). FX was positively correlated to K_{S-MAX} ($r = 0.48$, $p < 0.05$), K_{L-MAX} and A_L:A_X ($r = 0.38$, $p < 0.1$ for both parameters).

DISCUSSION

Ecophysiological Differences between Species

The differences between the branch-level hydraulic traits of oak and pine are likely the result of the contrasting ecophysiological and hydraulic strategies of both species. According to the results of our study sites located at the southernmost limits of the species' distributions, oaks showed significantly higher hydraulic conductivities (K_{S-MAX} and K_{L-MAX}) than pines (**Figure 2**). Generally, earlywood vessels of oak are more efficient conduits due to their lower hydraulic resistance than pine tracheids (Tyree and Zimmermann, 2002; Sperry, 2003; Sperry et al., 2006). Thus, even with a smaller share of FX, oak branches may transport more water per xylem unit under optimum conditions. Our results showed that oaks had a larger total leaf area (**Figure 2B**) and, consequently, oak leaves may have a better water supply in terms of K_{L-MAX} than pine needles (**Figure 2C**). In line with the more efficient xylem, higher A_L:A_X ratios were found in oak (**Figure 2F**), suggesting that for the same xylem area, oak would be able to provide water to a larger leaf area than that of pines (e.g., Tyree and Zimmermann, 2002). However, these differences could be affected by the fact that the pine segments were longer than the maximum conduit length, and hence lumen and pit membrane hydraulic resistance was covered by our

TABLE 3 | Statistics of the linear models to explain different hydraulic traits by annual, summer and growing season aridity index (P/PET) for the studied period (2011–2014) across the study sites.

		P/PET annual		P/PET summer		P/PET growing season	
Species		R^2	*p*-value	R^2	*p*-value	R^2	*p*-value
Scots pine	$K_{S\text{-}N}$	0.01	0.89	0.06	0.75	0.09	0.70
	PLC	0.01	0.89	0.01	0.92	<0.01	0.98
	$K_{S\text{-}MAX}$	0.01	0.91	0.05	0.78	0.08	0.72
	$K_{L\text{-}MAX}$	0.03	0.82	<0.01	0.97	<0.01	0.97
	A_L:A_X	0.84	0.08	0.77	0.12	0.10	0.69
	FX	0.01	0.88	0.06	0.74	0.77	0.12
Sessile oak	$K_{S\text{-}MAX}$	0.99	<0.01	0.15	0.61	0.36	0.40
	$K_{L\text{-}MAX}$	0.92	0.04	0.06	0.76	0.34	0.42
	A_L:A_X	0.71	0.15	0.02	0.85	0.05	0.78
	FX	0.8	0.11	0.56	0.25	0.69	0.17

Summer aridity index considered values from June to August whereas the growing season aridity index considered values from March to October. $K_{S\text{-}N}$, Xylem-specific native hydraulic conductivity; PLC, Native percentage of loss hydraulic conductivity; $K_{S\text{-}MAX}$, Xylem-specific maximum hydraulic conductivity; $K_{L\text{-}MAX}$, Leaf-specific maximum hydraulic conductivity; A_L:A_X, leaf-to-xylem area ratio; FX, functional xylem fraction.

measurements; whereas some of the oak vessels are likely to be longer than the measured segments and thus basically only lumen resistance was measured. On the other hand and due to the longer mean internode's length in oak branches (around 8 vs. 5, see Supplementary Table S2), vessels may have widened axially for a longer distance from the apex than pines (Anfodillo et al., 2013).

Differences in FX may be interpreted by the species-specific sensitivity to embolism (Hacke and Sperry, 2001). Large earlywood vessels of ring-porous species are more prone to embolisms caused by winter frosts or freeze-thaw cycles (Sperry et al., 1994; Hacke and Sauter, 1996; Tyree and Cochard, 1996; Davis et al., 1999). Consequently, oaks lose the main part of their hydraulic conductivity during winter time and recover it through newly produced earlywood vessels in the next spring (Cochard and Tyree, 1990; Cochard, 2006). Latewood vessels of oaks remain functional for some years providing a minimum flow even when the earlywood vessels might fail (Cochard and Tyree, 1990). In contrast, pine showed larger percentages of functional areas (FX). Due to the conservative strategy of Scots pine, several rings are functional and involved in water transport. For instance, a study reported that the annual build-up of new xylem tissue just accounts for 15–20% of the total hydraulic conductivity (Urli et al., 2013). The correlations among hydraulic traits were comparable for both species in terms of direction and significance, except for the relation between A_L:A_X and $K_{L\text{-}MAX}$. This relation was negative for both species, as expected (Mencuccini and Grace, 1995; Martínez-Vilalta et al., 2009). However, we did not find any significant relation between A_L:A_X and $K_{S\text{-}MAX}$ as reported in other studies for pine (Martínez-Vilalta et al., 2004, 2009).

Trait Variation Related to Site Aridity

Pine did not show differences in any of the hydraulic traits across sites located at the southernmost edge of its distribution (**Figure 3**). Annual, summer and growing season site aridity were not linked to any hydraulic trait, although the range of mean P/PET values was wider for pine than for the oak sites. In contrast to our findings, Mencuccini and Grace (1995) suggested that A_L:A_X in pine was influenced by differences in water VPD in the air, and pines growing at warmer sites produced less leaf area per unit of sapwood. This adjustment would maintain a constant water potential gradient within the stem at sites with different VPD and avoid exceedingly low water potentials. Similar results were found by Martínez-Vilalta et al. (2009) assessing the variability of branch-level hydraulic traits of pine across Europe. However, the authors showed a low variability in pine hydraulic traits since A_L:A_X, $K_{L\text{-}MAX}$ and leaf carbon discrimination were the only traits out of eleven that were significantly associated with differences in moisture. Although our results did not reveal any significant differences in A_L:A_X among sites, the range is in agreement with that reported by Martínez-Vilalta et al. (2009) for southern pine populations. Even though aridity varies across sites at the southernmost limits of the species' distribution, the dominance of summer drought stress may force all trees to follow the same strategy. In fact, Stout and Sala (2003) found that intraspecific variability in A_L:A_X is only detectable across larger geographic gradients.

Our results revealed low PLC values across all pine sites (**Figure 3C**). The small loss of conductivity, even in one of the warmest summers in the region in the last 65 years, might be a consequence of the conservative stomatal behavior common in pines (e.g., Poyatos et al., 2007) and other conifers (e.g., Anderegg and Hillerislambers, 2016). This early drought-response mechanism of isohydric species may reduce hydraulic loss but forces trees to rely on carbon reserves (McDowell, 2011; Salmon et al., 2015). Other studies carried out at the southernmost limits of the species distributions reported that prolonged drought periods could lead to a near-zero gas exchange compromising the carbon uptake during the main part of the growing season (Poyatos et al., 2013). Although the mechanisms behind the regulation of the non-structural carbohydrates are still not well understood (Sala et al., 2010), such a non-favorable situation would foster carbon starvation, or at least

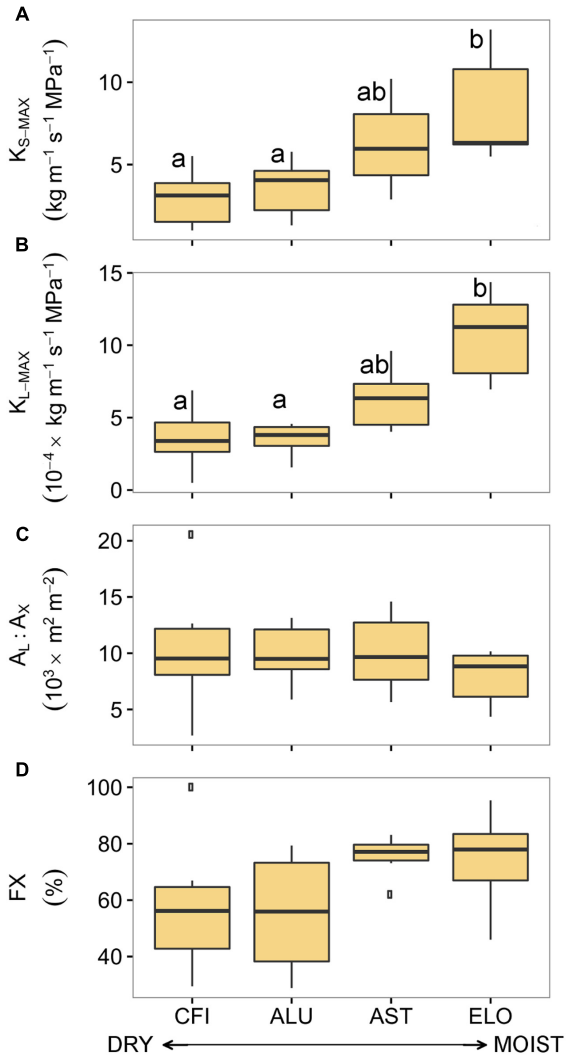

FIGURE 4 | Hydraulic traits of four sessile oak populations sampled across an aridity gradient: (A) xylem-specific maximum hydraulic conductivity, **(B)** leaf-specific maximum hydraulic conductivity, **(C)** leaf-to-xylem area ratio, and **(D)** functional xylem fraction. Sites are ordered respect to P/PET annual (see **Table 1**). Boxes represent 50% of the data (between the first and third quartiles). Median is shown as horizontal line. Bars extend from the box to the highest/lowest value within 1.5 * inter-quartile range (IQR). Points (outliers) are values > 1.5 * IQR. Letters denote significant differences between sites ($p < 0.05$) (see Supplementary Table S4). K_{S-MAX}, xylem-specific maximum hydraulic conductivity; K_{L-MAX}, leaf-specific maximum hydraulic conductivity; A_L:A_X, leaf-to-xylem area ratio; FX, functional xylem fraction.

make the trees more vulnerable to biotic attacks (McDowell, 2011).

Our results clearly point to a covariation between oak hydraulic traits and site aridity (**Table 3**). Relatively anisohydric species, such as oak, are able to track environmental fluctuations in water potential (Martínez-Vilalta et al., 2014). The lower K_{S-MAX} and K_{L-MAX} values observed at the drier sites were not associated with changes in A_L:A_X since the latter trait was similar across sites. Thus, part of the differences in conductivity among sites might be explained by changes in xylem traits such as smaller vessel diameters and/or more likely fewer functional vessels (**Figure 4D**). Earlywood vessel size is adjusted to drier conditions (Galle et al., 2010) and thus, might explain a larger portion of these differences in K_{S-MAX} and K_{L-MAX}. Adjustments in xylem architecture but not in leaf traits were also found for *Quercus pubescens* growing under water limited conditions (Sterck et al., 2008). Additionally, higher values of K_{L-MAX} have been related to a greater photosynthetic capacity, which could be a potential benefit at wetter sites (Brodribb et al., 2002).

The drought tolerance of oak supports low water potentials under dry conditions (Bréda et al., 1993a; Gieger and Thomas, 2002; Urli et al., 2013). Moreover, many aspects of the water use strategy and whole-plant physiology are linked to the structure and function of the root system. In this case, oak has a deep rooting system (Bréda et al., 1993b) and under dry conditions increases the proportion of fine-root biomass relative to leaf biomass (Bréda et al., 1995; Gieger and Thomas, 2002). This reinforces the relatively anisohydric strategy of oak during dry periods. Thus, carbon uptake may not be compromised under moderate drought conditions (Li et al., 2013). The accumulation of reserves during the growing season is essential for oak since the growth of new earlywood vessels will rely on the stored carbohydrates to restore the water pathway (Barbaroux and Bréda, 2002; Michelot et al., 2012). Moreover, and despite our limited understanding of xylem refilling (Sperry, 2013), part of the stored carbohydrates could be used to refill embolized xylem (Salleo et al., 1996), as found in a congeneric *Quercus* species (Taneda and Sperry, 2008). Our results suggests that oak is able to adjust the xylem architecture to site aridity.

However, recent studies highlighted the importance of the conduits axially increasing trend on hydraulic conductivity measurements (Petit et al., 2016). This axial conduit widening is a universal configuration of the xylem architecture (Anfodillo et al., 2013; Olson et al., 2014), and predicts that conduits should widen from stem/branch tip to the base, and, consequently also increases the theoretical hydraulic conductivity. In our case, the mean difference in the 5-year-old internode length among sites was 1 cm for pines and 2.7 cm for oaks (Supplementary Table S2), resulting in none significant differences in segment length among sites for both studied species ($p > 0.05$ for Krukal–Wallis test) (Supplementary Figure S4). Consequently, the effect of the path length on vessel size and therefore on the hydraulic conductivity, could be considered negligible in our specific case.

It should also be remarked that the characterization of site aridity in our study was just based on climatic variables. Although differences among sites are largely triggered by climatic conditions, other factors such as soil characteristics may also matter. On the other hand, water potentials could not be measured in the field during the sampling campaign and, consequently, we could not quantify the actual tension within the xylem during the extraordinary dry and hot spell of July 2015. The results shown here could be complemented with experiments under controlled conditions (i.e., dry-down experiments) to make more robust statements.

Future Perspectives

Most tree species operate with narrow hydraulic safety margins, which render them vulnerable to hydraulic failure (Choat et al., 2012). However, there are clear differences between angiosperms and gymnosperms (Johnson et al., 2012; Carnicer et al., 2013). Even taking into consideration the water potential inducing a 88% loss of stem conductivity (P88) to calculate these margins as recently proposed by Urli et al. (2013), conifers follow a safer strategy and show wider hydraulic safety margins than angiosperms.

The lack of variability in hydraulic traits across Scots pine populations found in this study suggests that this species performed a drought-avoidance survival strategy by closing stomata rather than investing in optimizing the hydraulic architecture to the harsh environmental conditions. This conservative strategy might compromise carbon gain during prolonged drought periods but, at the same time, preserves the integrity of the hydraulic system by avoiding possible damages during extreme events. On the other hand, the observed variability in the sessile oak hydraulic traits suggests that they are able to adjust to different levels of aridity. However, and despite the difficulty of measuring the loss of conductivity in oaks under natural conditions, such a drought-tolerance strategy might imply possible hydraulic failures under extreme climatic events.

A recent study on hydraulic traits at the dry-range limit in two of the most widely distributed species in North America, ponderosa pine (*Pinus ponderosa*) and trembling aspen (*Populus tremuloides*), revealed similar patterns (Anderegg and Hillerislambers, 2016). The authors suggested that the drought-avoidance strategy performed by ponderosa pine may lead to a range retreat as a response to a long-term drying trend. In contrast, trembling aspen performed a more drought-tolerant strategy by constructing a safer xylem, although such a hydraulic strategy may turn individuals more vulnerable to extreme events. Given the projected future drying of the Mediterranean Basin, impacts on pine and oak might be related to the intensity and the duration of drought events. Our results suggest that sessile oak holds the capacity to plastically adjust its hydraulic architectures to dryness, whereas Scots pine does not have such adaptable hydraulic structure, having both strategies different advantages but also disadvantages. Although recent publications have highlighted the ongoing replacement of pine by oak at the lower edge of the elevation range in some regions of the Mediterranean basin and inner alpine dry valleys (Galiano et al., 2010; Rigling et al., 2013; Vayreda et al., 2016), the intensity, duration and recurrence of forthcoming dry spells might be a crucial factor shaping the southernmost distribution limits of both tree species.

AUTHOR CONTRIBUTIONS

AM, ID-L, UH, HS, and EM-S conceived the ideas. ID-L and EM-S collected the samples. EM-S and HS carried out the analyses with help from UH. The writing of the article was led by EM-S and many contributions and comments were made by ID-L, UH, HS and AM.

FUNDING

This research was supported by IDDEC-Water03 (International Graduate School of Science and Engineering-TUM), MARGINS (Bavarian State Forestry Commission) and the European Research Council under the European Union's Seventh Framework Programme (FP7/2007-2013)/ERC grant agreement no. 282250. EM-S was supported by the Deutsche Forschungsgemeinschaft (DFG) through the TUM-IGSSE and FP//ERC 282250. ID-L acknowledges additional support from the project BOSSANOVA (S2013/MAE-2760) funded by Comunidad de Madrid.

ACKNOWLEDGMENTS

We are grateful to Dr. Julia Laube, Rachel Hillabrand, and Michael Matiu for their help and statistical advice as well as local institutions for providing sampling permissions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00598/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 13 June 2017
doi: 10.3389/fpls.2017.01016

Moderate Drought Stress Induces Increased Foliar Dimethylsulphoniopropionate (DMSP) Concentration and Isoprene Emission in Two Contrasting Ecotypes of *Arundo donax*

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The function of dimethylsulphoniopropionate (DMSP) in plants is unclear. It has been proposed as an antioxidant, osmolyte and overflow for excess energy under stress conditions. The formation of DMSP is part of the methionine (MET) pathway that is involved in plant stress responses. We used a new analytical approach to accurately quantify the changes in DMSP concentration that occurred in two ecotypes of the biomass crop *Arundo donax* subject to moderate drought stress under field conditions. The ecotypes of *A. donax* were from a hot semi-arid habitat in Morocco and a warm-humid environment in Central Italy. The Moroccan ecotype showed more pronounced reductions in photosynthesis, stomatal conductance and photochemical electron transport than the Italian ecotype. An increase in isoprene emission occurred in both ecotypes alongside enhanced foliar concentrations of DMSP, indicative of a protective function of these two metabolites in the amelioration of the deleterious effects of excess energy and oxidative stress. This is consistent with the modification of carbon within the methyl-erythritol and MET pathways responsible for increased synthesis of isoprene and DMSP under moderate drought. The results of this study indicate that DMSP is an important adaptive component of the stress response regulated via the MET pathway in *A. donax*. DMSP is likely a multifunctional molecule playing a number of roles in the response of *A. donax* to reduced water availability.

Keywords: photosynthesis, stomatal conductance, methionine pathway, chlorophyll fluorescence, dimethylsulphide, giant reed, biomass crop

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Marian Brestic, Slovak University of Agriculture, Slovakia
Cyril Douthe, University of the Balearic Islands, Spain

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *01 March 2017*
Accepted: *26 May 2017*
Published: *13 June 2017*

Citation:
Haworth M, Catola S, Marino G, Brunetti C, Michelozzi M, Riggi E, Avola G, Cosentino SL, Loreto F and Centritto M (2017) Moderate Drought Stress Induces Increased Foliar Dimethylsulphoniopropionate (DMSP) Concentration and Isoprene Emission in Two Contrasting Ecotypes of Arundo donax. Front. Plant Sci. 8:1016. doi: 10.3389/fpls.2017.01016

INTRODUCTION

Dimethylsulphoniopropionate (DMSP) is an important part of sulfur metabolism in photosynthetic organisms (Giovanelli, 1987; Stefels, 2000) and the precursor of the volatile organic compound (VOC) dimethyl sulphide (DMS) (Bentley and Chasteen, 2004). Approximately half of the global flux of sulfur to the atmosphere occurs in the form of DMS (Malin et al., 1992), and DMS plays a key role in the climate system by acting as a condensation nucleus (Keller et al., 2012). Despite the central role played by DMSP in the global sulfur cycle, comparatively little is known about its role in plant growth or potential dynamics in response to plant stress. Previous analyses of DMSP have focused on marine phytoplankton and plants where it is found in higher concentrations. This has led to a hypothesis that DMSP formation competes with the emission of isoprene during stress (Dani and Loreto, 2017). If replicated in vascular plants, this would have major implications for our understanding of the regulation VOC emissions during stress. However, analysis of DMSP in higher plants, particularly under drought stress, has been neglected, and as a consequence the role of DMSP is unclear.

The function of DMSP is not well defined (Stefels, 2000), but it may serve as a metabolically compatible osmolyte (Kirst, 1996; Van Bergeijk et al., 2003), an antioxidant (Sunda et al., 2002; Husband and Kiene, 2007; Husband et al., 2012), a defensive compound against herbivores (Van Alstyne and Houser, 2003), a sink for excess sulfur (Stefels, 2000), a methyl donor in transmethylation reactions (Challenger et al., 1957; Giovanelli, 1987) or an overflow for excess energy (Groene, 1995; Stefels, 2000). DMSP is part of the methionine (MET) pathway (**Figure 1A**; James et al., 1995; Trossat et al., 1996). The MET pathway is an important component in the regulation of plant metabolism linked to the production of the antioxidant glutathione (Leustek and Saito, 1999), regulation of iron metabolism via the action of the amino acid nicotianamine (Curie et al., 2009; Klatte et al., 2009), the influence of polyamine on cellular signaling of abiotic stress (Alcázar et al., 2010) and the production of the stress hormone ethylene (Sato and Theologis, 1989). Given the importance of the MET pathway to metabolic adaptation to environmental stress, improved analysis of the dynamics of DMSP would contribute to our understanding of this critical component of plant metabolism.

During drought events the availability of soil water declines and plants close stomatal pores to reduce transpirative water-loss. The decrease in stomatal conductance (G_s) reduces the availability of CO_2 at the active site of carboxylation in the

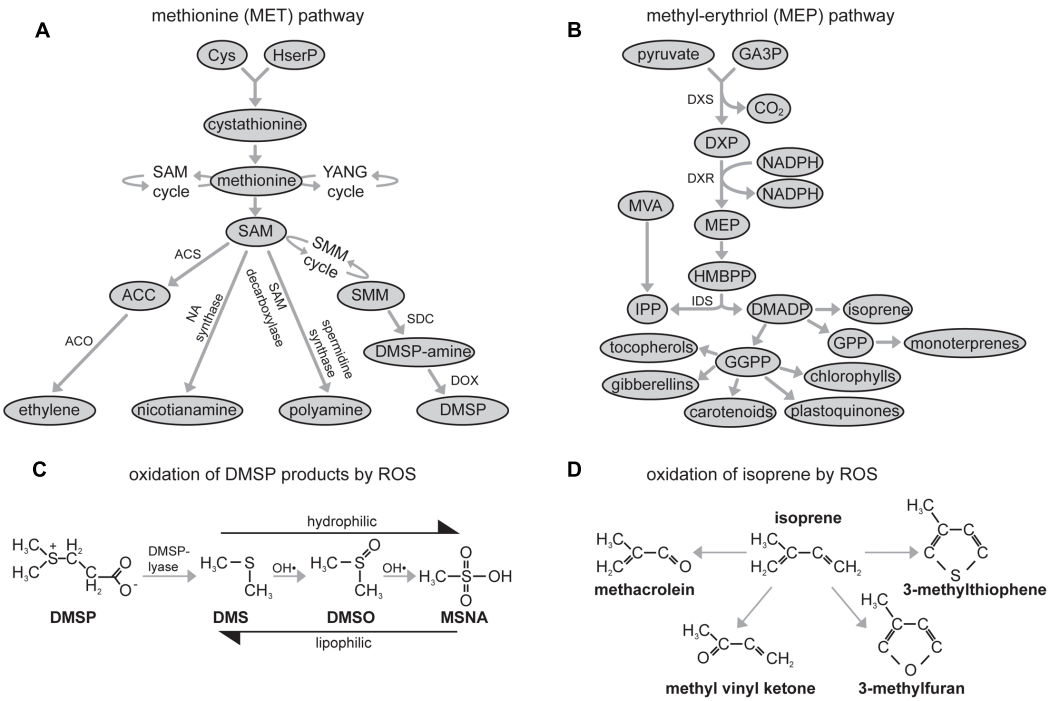

FIGURE 1 | Schematic diagrams of: **(A)** the methionine (MET) pathway (DMSP, dimethylsulphoniopropionate; SAM, *S*-adenosylmethionine; SMM, *S*-methylmethionine; NA, nicotianamine; PA, polyamine; ACS, Aminocyclopropane-1-carboxylic acid synthase (ACC synthase); ACO, ACC oxidase; SDC, SMM decarboxylase; DOX, DMSP-amine oxidase; HserP, O-phosphohomoserine), the stages involving synthesis of SMM and DMSP-amine occur outside the chloroplast envelope before DMSP-amine is transported into the chloroplast envelope where DMSP is formed (Amir et al., 2002); **(B)** oxidation of DMSP products by ROS (DMS, dimethylsulphide; DMSO, dimethylsuphoxide; MSNA, methane sulphinic acid) (Stefels, 2000; Sunda et al., 2002; Bentley and Chasteen, 2004); **(C)** the methyl-erythritol (MEP) pathway (GA3P, glyceraldehyde 3-phosphate; DMADP, dimethylallyl diphosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; GPP, geranyl diphosphate; GGPP, geranylgeranyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxyxylulose 5-phosphate synthase; HMBPP, (*E*)-4-hydroxy-3-methylbutyl-2-enyl pyrophosphate; MVA, mevalonate synthesized via the mevalonate pathway contributing to IPP) (Lichtenthaler et al., 1997), all stages other than MVA and the formation of gibberellins occur within the chloroplast, and; **(D)** oxidation of isoprene by ROS (Jardine et al., 2012).

chloroplast envelope and lowers rates of photosynthesis (P_N) (Centritto et al., 2011b; Lauteri et al., 2014; Dbara et al., 2016; Killi et al., 2017). Plant stress during drought is often the result of excess of energy as the amount of intercepted radiation utilized in photochemistry declines, and increasing amounts of energy instead induce oxygen photoreduction forming dangerous reactive oxygen species (ROS) (Pinheiro and Chaves, 2011; Zivcak et al., 2013). During the initial stages of drought, plants may utilize the emission of isoprene derived from the plastidic 2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP) (**Figure 1B**) to neutralize ROS (**Figure 1C**). Isoprene can strengthen thylakoid membranes (Velikova et al., 2011) and act directly as an antioxidant (**Figure 1D**) quenching biochemical reactions with ROS and reactive nitrogen species (Vickers et al., 2009a). The carotenoid xanthophylls are also part of the MEP pathway and are involved in the dissipation of excess energy via non-photochemical quenching (NPQ) (Cousins et al., 2002). DMSP may also exert an antioxidant function (Sunda et al., 2002; Husband and Kiene, 2007; Husband et al., 2012). *Solanum lycopersicum* leaves subject to severe drought exhibited a 75% increase in foliar concentrations of DMSP (Catola et al., 2016), consistent with antioxidant (Sunda et al., 2002; Husband and Kiene, 2007) or overflow (Stefels, 2000) functions for DMSP. Dimethlysulphide has been proposed to act as a delocalised antioxidant, stabilizing the lipid phase of photosynthetic and cellular membranes during oxidative stress (Catola et al., 2016) in a manner similar to isoprene (Velikova and Loreto, 2005). The subsequent oxidation products of DMS by ROS, dimethylsuphoxide (DMSO) and methane sulphinic acid (MSNA), are increasingly hydrophilic. Therefore, the degradation products of DMSP have differential partitioning between lipid and aqueous phases, and may consequently protect different cellular compartments from oxidative stress (Sunda et al., 2002). Acrylate, a by-product of DMSP cleavage by DMSP-lyase (**Figure 1B**), is also a precursor for ethylene, and increased concentrations of acrylate stimulate ethylene release (Plettner et al., 2005). During drought emissions of ethylene generally decline (Habben et al., 2014), possibly indicating reduced availability of DMSP due to oxidation by ROS (**Figure 1B**).

A new analytical technique developed by Catola et al. (2016) now allows rapid and accurate analysis of low concentrations of DMSP. We utilized this approach to investigate the response of DMSP in the giant reed (*Arundo donax*) subject to moderate drought stress in field conditions. *Arundo donax* is a fast growing isoprene emitting (Haworth et al., 2017b) member of the Poaceae family that shows potential as a biomass crop (Mantineo et al., 2009). The rapid biomass accumulation of *A. donax* is sustained by high rates of CO_2 assimilation (Haworth et al., 2017b). However, these levels of P_N are accompanied by high rates of stomatal conductance (G_s) and transpirative water-loss that require a high level of water availability (Haworth et al., 2017b). In comparison to other biomass crops such as *Populus* (e.g., Kauter et al., 2003; Centritto et al., 2011a), little is known regarding the physiological and metabolic response of *A. donax* to drought, and the potential for varietal differences in this response that may be exploited in the commercial development of *A. donax* as a biomass crop. Information regarding the dynamics of DMSP may allow the modification of the MET pathway in *A. donax* to optimize the physiological response to drought stress and maximize growth. We selected two *A. donax* ecotypes from a temperate and a more arid habitat (Sesto Fiorentino, Central Italy, and Marrakesh, Morocco, respectively) that had shown contrasting responses to severe drought in a previous study (Haworth et al., 2017a). These *A. donax* ecotypes were planted in a common garden field trial and subjected to moderate drought to: (i) investigate the impact on leaf gas exchange characteristics; (ii) quantify the effect on photochemical and non-photochemical energy usage using chlorophyll fluorescence; (iii) examine any change in the emission of the VOC isoprene, (iv) study the response of DMSP to drought, and; (v) propose a hypothesis regarding the possible role of DMSP in the drought response of *A. donax* and the potential to utilize this response, and any varietal differences found in this study, to optimize biomass production of *A. donax* in drought prone semi-arid marginal lands. We hypothesize that the MET pathway plays a key role in the drought response of *A. donax* via increased production of DMSP (e.g., Catola et al., 2016).

MATERIALS AND METHODS

Field Site and Experimental Design

Rhizomes were collected from clonal populations from a warm sub-Mediterranean humid region, Sesto Fiorentino, Florence, in Central Italy, that receives 800 mm of precipitation each year and has a mean summer (June to August) temperature of 23°C, and from an arid pre-desert area of Marrakesh, Morocco, that receives 200 mm of precipitation each year and has a mean summer temperature of 30°C. The same ecotypes were exposed to severe drought in another study and exhibited differences in xylem morphology associated with the response to soil drying (Haworth et al., 2017a). The rhizomes were cut into equal portions of 20 cm in length (at least one bud was visible on each portion) and planted in a common garden experiment at the experimental farm of the University of Catania, Sicily (37° 25′N 15° 03′E), in March 2015. The rhizomes were planted at a depth of 15 cm every 0.5 m in rows spaced 0.8 m apart in 4.0 m × 3.0 m sized plots. Six plots of each of the Italian and Moroccan *A. donax* ecotypes were planted (12 plots in total), and to avoid edge effects a 2.4 m border of three rows of *A. donax* was placed around the field. The field was unfertilised and all plants were irrigated equally to field capacity until July 2015 when the rhizomes had established and the stems were roughly 1.5 m in height. On the 7th July irrigation was ceased to half of the field; three plots each of the Italian and Moroccan ecotypes were rain-fed, and the remaining three plots for each ecotype continued to receive irrigation. Supplementary irrigation was equivalent to 100% of potential evapotranspiration (ETc) during July to August, calculated each day as:

$$ETc = E_o * K_p * K_c$$

Where E_o is the evaporation of water from a class-A pan (mm); K_p is the pan coefficient, and; K_c, the crop growth stage (between 0.4 and 1.9) (Triana et al., 2015). Daily rain-fall was subtracted

from the daily calculation of water to be supplied as irrigation (Allen et al., 1998). Soil samples were collected on the same day as the leaf samples were collected from 0 to 90 cm depth and the soil water content determined gravimetrically (Klute, 1986; Killi et al., 2014). Full details of the site and soil conditions at the experimental farm are given in Cosentino et al. (2006).

Leaf Gas Exchange and Chlorophyll Fluorescence

Six weeks after the cessation of supplementary irrigation to half of the *A. donax* plants, analyses of leaf gas exchange and chlorophyll fluorescence parameters were performed. Measurements of P_N, G_s, the sub-stomatal concentration of [CO_2] (C_i) and electron transport rate (ETR) were performed on the mid-section of the second newest fully expanded leaf using a LiCor Li6400XT fitted with a 2 cm^2 leaf cuvette (Li-Cor, Inc., Lincoln, NE, United States). Two replicate plants from the centre of three plots were analyzed for each ecotype and treatment ($n = 6$). Environmental parameters were controlled and the following settings were used: 400 ppm [CO_2], 2000 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR, 10% blue and 90% red light), and leaf temperature of 30°C. To reduce diffusive leaks through the chamber gasket, a supplementary gasket was added and the IRGA exhaust air was fed into the interspace between the chamber and the supplementary gaskets. The ETR was calculated as:

$$\mathrm{ETR} = \Phi\mathrm{PSII} \times \mathrm{PPFD} \times \alpha \times \beta$$

where: PPFD is the photosynthetic photon flux density (μmol m^{-2} s^{-1}); ΦPSII is the actual quantum yield of photosystem II; α is the leaf absorbance (a standard value of 0.85 was used), and; β is the partitioning of electrons between photosystem I and II (assumed to be 0.5) (Genty et al., 1989). The respiration in the dark (R_N) was estimated 10 min after switching off the light unit in the cuvette, when CO_2 emission from the leaf had stabilized (e.g., Shi et al., 2015). Gas-exchange measurements were performed between 10.00 and 12.00 each day, when the plants exhibited the highest levels of P_N and G_s. The maximum (F_v/F_m) and the actual quantum yield of photosystem II (ΦPSII: ΔF/F'm), and the dissipation of light energy as NPQ were recorded using a Hansatech FMS-2 (Hansatech, King's Lynn, United Kingdom) after 30 min of dark adaptation (Genty et al., 1989).

Isoprene Emission

The emission of isoprene was measured in the field from the same leaves of *A. donax* used for gas-exchange analysis, under the same environmental settings, but a LiCor Li6400 fitted with a 6 cm^2 cuvette and LED light unit was used. When monitoring isoprene emission, air from the cuvette with the enclosed leaf passed through a biphasic adsorbent trap containing 30 mg of Tenax and 20 mg of Carboxen (GERSTEL GmbH & Co.KG, Germany). A pump (Elite 5, A.P. Buck, Orlando, FL, United States) was used to pass 2 L of air through each trap at a rate of 200 ml min^{-1}. Measurements of the concentration of isoprene in the ambient air (blanks) were performed using an empty leaf cuvette before and after each measurement. The traps were then stored at 4°C prior to analysis in the laboratory. Isoprene was first desorbed from traps at high temperature and then measured using a gas chromatograph – mass spectrometer (GC-MS) with an Agilent HP-INNOWAX (30 m × 0.32 mm × 0.15 μm) GC column. A 5977A mass selective detector with electron ionization operating at 70eV was used for analysis. Isoprene was identified by matching the spectrum peak with a library spectral database (NIST 11.L) and through comparison of the retention time and mass spectrum with an isoprene analytical standard (Sigma Aldrich, St. Louis, MO, United States) injected into the GC-MS at different concentrations. The isoprene analytical standard was also used to construct a calibration curve by injecting known concentrations of isoprene into the GC-MS. The data was analyzed using Agilent MassHunter Workstation software (Agilent 7890A, Agilent Technologies, Santa Clara, CA, United States). The concentration of isoprene within the leaf was calculated using the approach of Singsaas et al. (1997).

Analysis of Dimethylsulphoniopropionate (DMSP) and Relative Water Content (RWC)

Leaf samples were collected after completion of the leaf gas exchange and chlorophyll fluorescence measurements. The first fully most expanded leaf was collected from the same two plants in each plot ($n = 6$ for each ecotype and treatment) adjacent to the leaf that had been used for physiological analysis. The lower 4–5 cm of each leaf was removed to be used for determination of foliar relative water content (RWC) following the protocol of Diaz-Pérez et al. (1995). The remainder of the leaf was flash frozen in liquid nitrogen before being stored at −80°C prior to analysis of DMSP by solid phase micro extraction from head-space (HS-SPME). An in-depth description of the method is provided by Catola et al. (2016). Briefly, the leaf samples were ground in liquid nitrogen. An aliquot (0.2 g) of each sample was then placed inside a 20 ml screw-cap head-space vial (Agilent Technologies, Santa Clara, CA, United States), together with 250 μl 0.5 M NaOH, 2 g of NaCl and sufficient distilled water to make up a total 5 ml volume. Teflon coated silicon septa (Agilent Technologies) were used to seal the head-space vials, which were then incubated at 60°C for 12 h, to allow complete hydrolysis of DMSP to DMS. A three-phase divinylbenzene/carboxen/polydimethylsiloxane (Supelco, Bellafonte, PA, United States) 75 μm width, 2 cm long, solid phase micro-extraction fiber was placed in the head-space of the vials for 10 min at 40°C. To ensure consistent sampling and mixing of DMS in the head-space of the vials, a Gerstel MPS2 XL auto-sampler (Gerstel GmbH & Co. KG, Mülheim an der Ruhr, Germany) was used. The VOCs adsorbed by the fiber were analyzed using an Agilent 7820 GC-chromatograph with a 5977A M5D with electron ionization running at 70 eV and a HP-Innowax column (50 m, 0.2 mm, ID 0.4 μm DF). Dimethyl sulfide was identified via comparison with a spectral database library (NIST11.L) and injection of a known standard of DMS into the GC-MS (Sigma Aldrich). An example chromatograph of DMS is given in the Supplementary Data. A calibration curve was constructed by injecting increasing concentrations of DMS

into the GC-MS and the amount of DMS used to infer the concentration of DMSP within the leaves.

Statistical Analysis

Statistical analyses were performed using SPSS 20 (IBM, Armonk, New York, NY, United States). To test the effect of water deficit on the Moroccan and Italian *A. donax* ecotypes we used a two-way ANOVA (Supplementary Information) and a one-way ANOVA with an LSD *post hoc* test to assess differences in variance between samples associated with either ecotype or treatment effects.

RESULTS

Growth under rain-fed field conditions resulted in a reduced RWC of *A. donax* leaves (particularly in the Italian ecotype), but the difference in comparison with leaves of irrigated plots was not statistically significant (**Figure 2**). The maximum (F_v/F_m) (**Figure 3A**) and actual (ΦPSII) (**Figure 3B**) quantum yields of PSII were also not significantly affected by the cessation of supplementary irrigation, although the two parameters were more reduced again in the Italian than in the Moroccan ecotype. Under rain-fed conditions, the Italian ecotype exhibited a significant 90.0% increase in the dissipation of energy as heat (as shown by the parameter NPQ), while the Moroccan ecotype showed no response (**Figure 3C**). Photosynthesis (**Figure 4A**) and G_s (**Figure 4B**) showed no difference between the *A. donax* ecotypes under irrigated conditions. However, under moderate drought stress the Moroccan ecotype exhibited more pronounced reductions in P_N and G_s (52.0 and 75.5%), in comparison to the Italian ecotype (33.0 and 64.5%). Reductions in G_s resulted in lower C_i in both *A. donax* ecotypes, especially in the Moroccan (**Figure 4C**). The drought stressed Moroccan ecotype also exhibited an ETR/P_N ratio higher than the Italian. However, the ETR/P_N ratio was not different in the ecotypes under irrigated conditions. No ecotype or treatment effect was observed in R_N (**Figure 4D**).

No difference was observed in the rate of isoprene emission between the two *A. donax* ecotypes under irrigated conditions. However, the emission of isoprene was significantly enhanced under rain-fed conditions, by 236.4% in the Moroccan ecotype, and to a lesser extent (76.4%) in the Italian ecotype (**Figure 5A**).

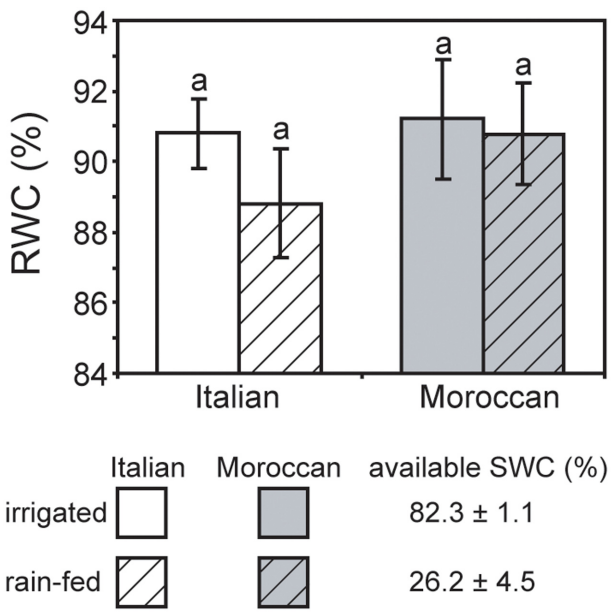

FIGURE 2 | Relative water content of leaves of Moroccan (gray) and Italian (white) *Arundo donax* ecotypes grown in the field under irrigated control (open) and rain-fed drought (hatched) conditions. Error bars indicate one standard error either side of the mean (n = 6). Letters indicate homogenous groups determined using a one-way ANOVA and LSD *post hoc* test.

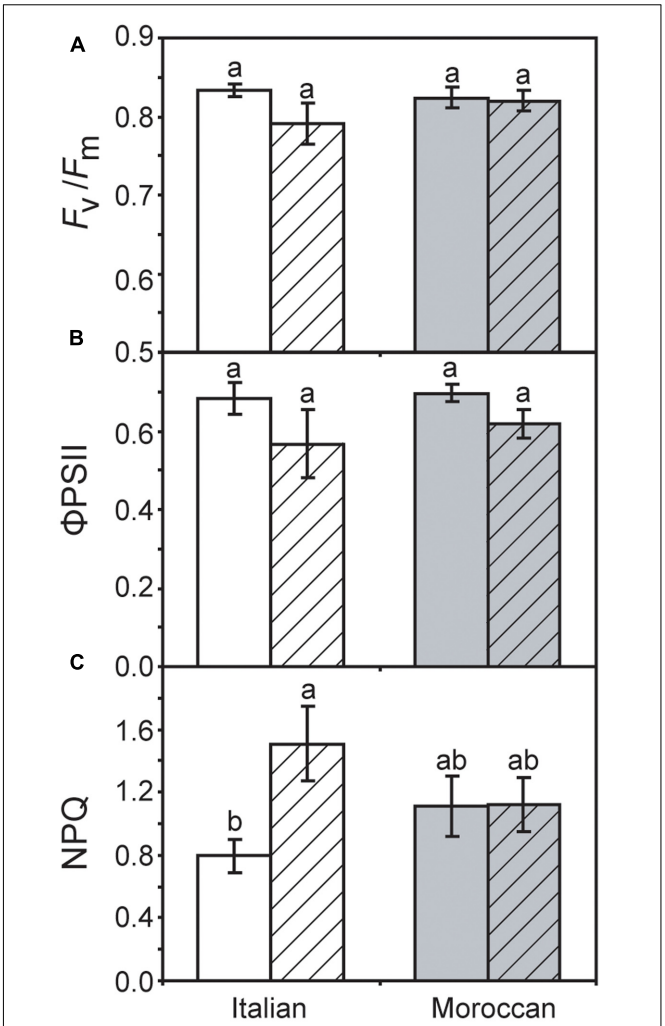

FIGURE 3 | Chlorophyll fluorescence measurements of Moroccan (gray) and Italian (white) *A. donax* ecotypes grown in the field under irrigated control (open) and rain-fed drought (hatched) conditions: **(A)** maximum quantum efficiency of PSII (F_v/F_m); **(B)** actual quantum efficiency of PSII (ΦPSII), and; **(C)** non-photochemical quenching (NPQ). Error bars indicate one standard error either side of the mean (n = 6). Letters indicate homogenous groups determined using a one-way ANOVA and LSD *post hoc* test.

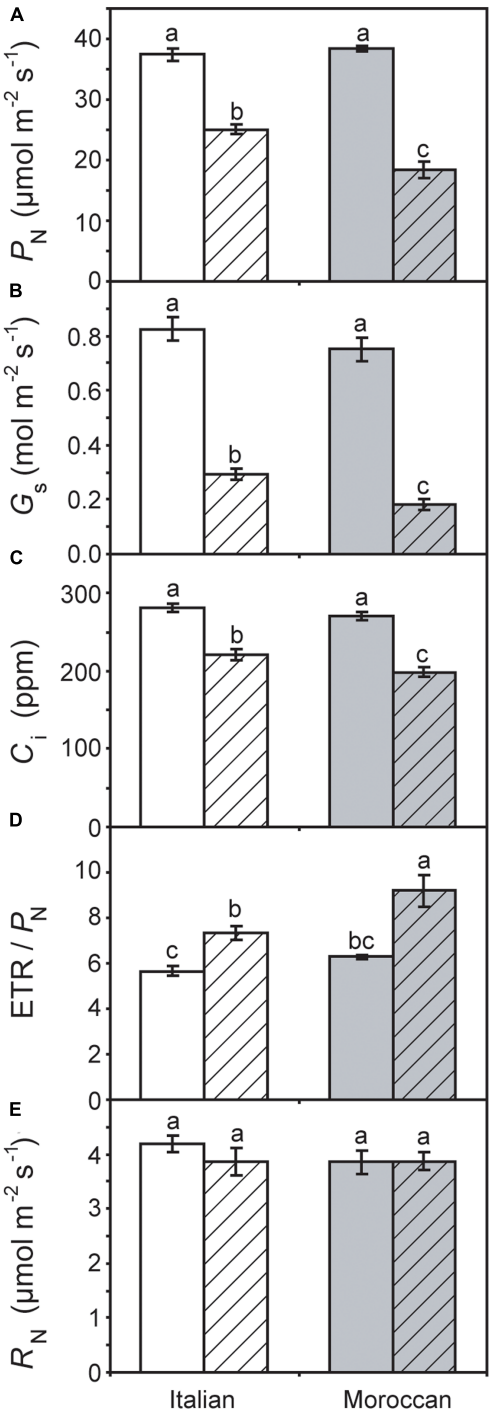

FIGURE 4 | Leaf gas exchange and simultaneous chlorophyll fluorescence parameters of Moroccan (gray) and Italian (white) *A. donax* ecotypes grown in the field under irrigated control (open) and rain-fed drought (hatched) conditions: **(A)** photosynthesis (P_N); **(B)** stomatal conductance (G_s); **(C)** internal sub-stomatal concentration of CO_2 (C_i); **(D)** the ratio of electron transport rate (ETR) to P_N, and; **(E)** respiration in the dark (R_N). Error bars indicate one standard error either side of the mean ($n = 6$). Letters indicate homogenous groups determined using a one-way ANOVA and LSD *post hoc* test.

A similar pattern was also observed in the concentration of isoprene within the leaf (**Figure 5B**). The amount of DMSP when determined on a leaf area basis was significantly greater in rain-fed than irrigated plants. This increase was 98.4 and 67.3% in the Italian and Moroccan ecotypes, respectively (**Figure 5C**). A similar increase in the concentration of DMSP was also recorded in both *A. donax* ecotypes under rain-fed conditions, when a dry weight basis was used (**Figure 5D**). No significant difference in the response of DMSP was observed between the two *A. donax* ecotypes, both when irrigated and rain-fed. The results of a two-way ANOVA of gas exchange, chlorophyll fluorescence, DMSP and isoprene parameters are presented in the Supplementary Information.

DISCUSSION

Arundo donax has potential to be a productive biomass crop in many warm to hot regions (Angelini et al., 2009; Mantineo et al., 2009), which are, however, often exposed to recurrent drought. The *A. donax* plants examined in this study showed no effect of drought on foliar RWC, and comparatively minor reductions in leaf gas exchange parameters in comparison to other studies (e.g., Cosentino et al., 2016; Haworth et al., 2017b); therefore the drought stress experienced by the plants in this study was considered to be moderate for *A. donax*. A number of studies have examined the effect of severe drought on *A. donax* (e.g., Sánchez et al., 2015; Haworth et al., 2017b), but comparatively few have investigated the response of this species to moderate drought over a sustained period, which may be more representative of the majority of situations where water availability limits growth in the field (Farooq et al., 2009; Yan et al., 2016). As such, the results of this study provide valuable physiological and biochemical insights into the response of *A. donax* to moderate drought under field conditions.

Impact of Moderate Drought Stress on Photosynthesis

Previous studies have reported comparatively little ecotypic variation in the leaf gas exchange and chlorophyll fluorescence parameters of contrasting *A. donax* ecotypes when subjected to severe drought (i.e., where G_s falls below 10% of control values) (Sánchez et al., 2015; Haworth et al., 2017a). In contrast, we have observed statistically significant ecotypic differences, albeit comparatively minor, in P_N, G_s, C_i and ETR/P_N responses of the Italian and Moroccan *A. donax* ecotypes to moderate drought (**Figure 4**). Despite both ecotypes showing no significant differences in RWC (**Figure 2**), stem height or stem number per rhizome (data not shown), the Moroccan ecotype generally exhibited more pronounced reductions in leaf gas exchange parameters under the moderate drought stress in the rain-fed treatment. The Moroccan ecotype has been shown to increase xylem vessel size during drought, while the Italian ecotype reduced xylem vessel size (Haworth et al., 2017a). This contrast in the morphological response of the water transport systems

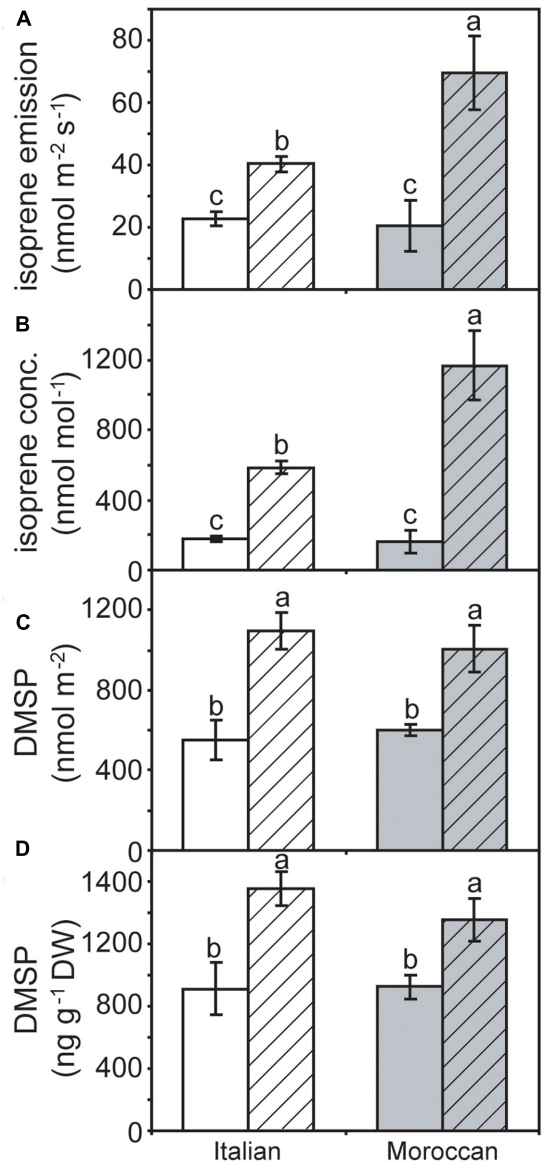

FIGURE 5 | (A) Isoprene emission, **(B)** the concentration of isoprene within the sub-stomatal air-spaces inside the leaf, **(C)** dimethylsulphoniopropionate (DMSP) as a function of leaf area, and **(D)** concentration of DMSP per unit dry mass of leaf matter in Moroccan (gray) and Italian (white) *A. donax* ecotypes grown in the field under irrigated control (open) and rain-fed drought (hatched) conditions. Error bars indicate one standard error either side of the mean (n = 6). Letters indicate homogenous groups determined using a one-way ANOVA and LSD *post hoc* test.

could be related to the adaptation of the *A. donax* ecotypes to their respective environments. The increase in xylem vessel size found in the Moroccan *A. donax* may promote the loss of above ground photosynthetic tissues via enhanced xylem embolism (e.g., Cochard, 2002; Cochard et al., 2002) to preserve the viability of the rhizome in an environment where drought is more severe and its onset more rapid. In contrast, the reduction in the xylem vessel diameter in the Italian ecotype would favor increased resistance to xylem embolism and the retention of leaves and stems (Tyree and Sperry, 1989). The lower G_s observed in the Moroccan ecotype under moderate drought stress (**Figure 4B**) may be due to a higher rate of xylem embolism promoting stomatal closure than in the Italian *A. donax* (Cochard, 2002). The Moroccan ecotype also exhibited a higher ETR/P_N ratio under drought, indicative of a greater proportion of electrons being utilized in photorespiration (Sun et al., 2014). However, no difference was observed in F_v/F_m values of both ecotypes under irrigated and rain-fed conditions (**Figure 3A**) and alongside reduced G_s (**Figure 4B**) suggest that the limitations to P_N under moderate drought were mostly diffusive (Centritto et al., 2003; Aganchich et al., 2009). However, the Italian ecotype did exhibit increased dissipation of heat as NPQ alongside a lower increase in isoprene emission under rain-fed conditions, in comparison to the Moroccan ecotype. Increased isoprene formation during drought might have helped maintain P_N limitations only at the diffusion level (limited by stomatal closure and low C_i; **Figure 4**), avoiding the increase of NPQ and the probable resultant photochemical damage. Indeed, isoprene emitters show lower NPQ than non-emitters under physiological and stress conditions, especially under drought (Beckett et al., 2012; Pollastri et al., 2014). This possibly indicates lower oxidative stress at the chloroplast thylakoid level and enhanced protection of photochemistry of photosynthesis (Velikova et al., 2011) associated with high isoprene emission in the Moroccan ecotype. Severe drought stress has been shown to induce an increase in the proportion of photorespiration and R_N relative to P_N, with absolute values of photorespiration and R_N declining as drought progresses (Centritto et al., 2011a; Sun et al., 2014; Killi et al., 2017). The moderate drought stress imposed on the *A. donax* did not appear sufficient to induce any alteration in R_N values (**Figure 4E**).

Dynamics of Dimethylsulphoniopropionate and Isoprene Concentrations under Moderate Drought Stress

Under severe drought stress in the field the rate of isoprene emission in *A. donax* did not increase (Haworth et al., 2017b). However, in many other experiments including this study, isoprene emission and concentration are stimulated by moderate stress conditions, which uncouple isoprene from photosynthesis, its main source of carbon (**Figure 1A**). Isoprene has been proposed to act as a mobile diffusive antioxidant stabilizing chloroplast membranes under stress conditions (Velikova and Loreto, 2005; Vickers et al., 2009b). As already mentioned, the higher synthesis and emission of isoprene in the drought-stressed Moroccan ecotype, in comparison to the Italian ecotype, might be related to avoidance of photochemical damage in leaves where photosynthesis is impaired by diffusive limitations, or due to enhanced xanthophyll function in the Italian ecotype to promote NPQ. The increased emission of isoprene might be more suitable for ecotypes that frequently endure prolonged drought in their natural habitats. Exploitation of differences in isoprene emission in commercial

A. donax clones would require consideration of the benefits of enhanced protective capacity balanced against increased losses of assimilated carbon.

The results of this study are consistent with the observations of Catola et al. (2016) of stimulated DMSP formation under drought. This enhanced foliar concentrations of DMSP occurred alongside the increase in isoprene emission in the *A. donax* plants in the rain-fed treatment when compared to irrigated plants (**Figure 5D**). This is not consistent with competition between DMSP and isoprene synthesis within the chloroplast proposed to take place in marine phytoplankton (Dani and Loreto, 2017). A previous study at the same site found no difference in isoprene emission rates between irrigated and rain-fed *A. donax* plants (Haworth et al., 2017b). However, the previously analyzed *A. donax* experienced a longer more severe drought stress, while the *A. donax* in the present study experienced a shorter less severe drought that allowed the maintenance of P_N (**Figure 4A**) and the likely existence of a pool of labile carbon sufficient to sustain enhanced isoprene emission (Barta and Loreto, 2006). The synthesis of isoprene (Banerjee et al., 2013) and DMSP (James et al., 1995; Trossat et al., 1996) reveal activation of two chloroplastic pathways (MEP and MET, respectively). As largely demonstrated for the MEP pathway (Loreto and Schnitzler, 2010), also the MET pathway possibly plays a protective role in moderate drought stress conditions. The increase in DMSP concentration would be consistent with a role as an overflow for excess energy (Stefels, 2000). The increased NPQ in the Italian *A. donax* (**Figure 3C**) is indicative of a reduction in the usage of energy for photochemistry (Haworth et al., 2017b). DMSP is considered to act more strongly as a protective overflow under conditions of low nitrogen availability, facilitating the redistribution of nitrogen to other amino acids via the MET pathway (Groene and Kirst, 1992). As the field where the *A. donax* rhizomes were planted was unfertilised prior to the field trial, this may have promoted the increase in DMSP levels observed during growth under moderate drought stress, when the energy partitioning to photochemistry became unbalanced (Stefels, 2000). The increase in foliar DMSP concentration found in the *A. donax* plants subject to moderate drought in this study may have also acted as an osmolyte reducing the leaf water potential and allowing the maintenance of RWC (Kirst, 1996; Van Bergeijk et al., 2003). This is consistent with observations in the salt-marsh grass *Spartina alterniflora* where the concentration of DMSP did not adjust in response to an increase in oxidative stress (Husband et al., 2012), indicating that the role of DMSP may not be protective.

The MET pathway leads to the formation of many compounds that have well-defined roles in abiotic tolerance. However, it is unclear whether DMSP in higher plants is a by-product of the synthesis of these other MET derived compounds. However, a protective role for DMSP is consistent with its synthesis within the chloroplast. Greater availability of DMSP may also promote enhanced emission of DMS (Husband and Kiene, 2007) to act as a mobile antioxidant protecting thylakoid membranes in a manner similar to isoprene (Velikova and Loreto, 2005), via the oxidation of DMS to DMSO (Husband and Kiene, 2007). The rate of DMS emission is often correlated to the availability of DMSP (Groene, 1995; Catola et al., 2016); however, the rate of release of DMS is also dependent upon the activity of DMSP-lyase (Stefels et al., 1995) and of bacterial degradation of DMS (Carini, 2016). It is not possible to estimate potential rates of DMS emission based upon the concentrations of DMSP observed in the present study due to the low affinity of DMSP-lyase for DMSP (Stefels et al., 2007; Reisch et al., 2011) Further analytical advances are required to directly determine accurate foliar DMS release at the low levels likely to occur in *A. donax* before more definitive conclusions may be drawn as to the role of DMS and its precursor DMSP as aqueous and gaseous antioxidants.

CONCLUSION

Arundo donax has typically shown little ecotypic variation in physiological responses to severe drought. However, our analysis of the responses to moderate drought stress under field conditions in *A. donax* ecotypes from warm sub-humid (Central Italy) and hot semi-arid (Morocco) habitats indicated a degree of ecotypic variation, with the Moroccan ecotype exhibiting more pronounced reductions in P_N and G_s. This may reflect selective pressures experienced by the Moroccan ecotype to preserve the viability of the rhizome in a habitat where droughts develop more rapidly, are more severe and longer in duration. As synthesis of isoprene and DMSP increased significantly under moderate stress, we suggest that the underlying MEP and MET pathways, play an important role in adapting to moderate drought and preserving photosynthetic capacity once the stress is relieved. Modification of MEP and MET pathways may potentially assist in the development of stress resistant and climate-adapted *A. donax* biomass crops.

AUTHOR CONTRIBUTIONS

MH, GM, ER, GA, SLC, CB, SC, and MM conducted the experiment. MH, MC, and FL wrote the manuscript.

FUNDING

This work was funded by the EU FP7 project WATBIO (Development of improved perennial non-food biomass and bioproduct crops for water stressed environments – no.311929).

ACKNOWLEDGMENTS

We are grateful for the assistance of the staff of the Di3A Experimental Field (University of Catania), Alessandra Pellegrino and Salvatore La Rosa (CNR-IVALSA). Luca Calamai

(University of Florence) and Gabriele Cencetti (CNR – IBBR) assisted with GC-MS measurements. We thank Said Wahbi (Université Cadi Ayyad, Marrakech) for providing rhizomes. The comments of two reviewers significantly improved this work.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01016/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 02 November 2016
doi: 10.3389/fpls.2016.01629

Timing Effects of Heat-Stress on Plant Ecophysiological Characteristics and Growth

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Heat-waves with higher intensity and frequency and longer durations are expected in the future due to global warming, which could have dramatic impacts in agriculture, economy and ecology. This field study examined how plant responded to heat-stress (HS) treatment at different timing in naturally occurring vegetation. HS treatment (5 days at 40.5°C) were applied to 12 1 m^2 plots in restored prairie vegetation dominated by a warm-season C_4 grass, *Andropogon gerardii,* and a warm-season C_3 forb, *Solidago canadensis,* at different growing stages. During and after each heat stress (HS) treatment, temperature were monitored for air, canopy, and soil; net CO_2 assimilation (A_{net}), quantum yield of photosystem II (Φ_{PSII}), stomatal conductance (g_s), and internal CO_2 level (C_i), specific leaf area (SLA), and chlorophyll content of the dominant species were measured. One week after the last HS treatment, all plots were harvested and the biomass of above-ground tissue and flower weight of the two dominant species were determined. HS decreased physiological performance and growth for both species, with *S. canadensis* being affected more than *A. gerardii*, indicated by negative HS effect on both physiological and growth responses for *S. canadensis*. There were significant timing effect of HS on the two species, with greater reductions in the net photosynthetic rate and productivity occurred when HS was applied at later-growing season. The reduction in aboveground productivity in *S. canadensis* but not *A. gerardii* could have important implications for plant community structure by increasing the competitive advantage of *A. gerardii* in this grassland. The present experiment showed that HS, though ephemeral, may promote long-term effects on plant community structure, vegetation dynamics, biodiversity, and ecosystem functioning of terrestrial biomes when more frequent and severe HS occur in the future.

Keywords: global climate change, photosynthesis, aboveground productivity, *Solidago canadensis*, *Andropogon gerardii*

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Rubén Retuerto, University of Santiago de Compostela, Spain
Bartosz Adamczyk, University of Helsinki, Finland

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *25 July 2016*
Accepted: *17 October 2016*
Published: *02 November 2016*

Citation:
Wang D, Heckathorn SA, Mainali K and Tripathee R (2016) Timing Effects of Heat-Stress on Plant Ecophysiological Characteristics and Growth. Front. Plant Sci. 7:1629. doi: 10.3389/fpls.2016.01629

Abbreviations: A_{net}, net photosynthetic rate ($\mu mol\ m^{-2}\ s^{-1}$); ANPP, Aboveground net primary production (g); g_s, stomatal conductance to water vapor ($mol\ m^{-2}\ s^{-1}$); HS, heat stress; LAI, leaf area index ($m^2\ m^{-2}$); LWC, (leaf water content, %); SLA, specific leaf area ($m^2\ kg^{-1}$); W_a, aboveground biomass (g); W_f, biomass of flowers (g); $_i$WUE, intrinsic water use efficiency (the ratio of A_{net} to g_s); Φ_{PSII}, quantum yield of electron transport of photoystem II.

INTRODUCTION

The increased concentration of CO_2 and other greenhouse gasses in atmosphere is causing a future climate with higher temperatures and dramatic changes in rainfall patterns (IPCC, 2013). In addition to rising mean annual temperatures, the frequency, duration, and severity of periods with exceptionally high temperatures are also increasing (Easterling et al., 2000; Tripathi et al., 2016). HS events with a trend of high frequency and extremity have already been reported in different parts of the world (Henderson and Muller, 1997; Gaffen and Ross, 1998; Yan, 2002). Thus, plants in the future will be exposed to both higher mean temperatures, and likely more extreme HS. The World Meteorological Organization (WMO) defines HS events as episodes of 5 or more continuous days with air temperatures over 5°C above daily maximum temperatures (Frich et al., 2002). During extreme climate events the acclimatory capacities of an organism are substantially exceeded (Gutschick and BassiriRad, 2003) and the impact of extreme climate events can be significantly greater than those associated with mean temperature increases (Karl et al., 1997). Combining the climatological and biological definitions, Smith (2011) stated that an extreme climate event is an episode in which a statistically rare climatic period could cause community responses, with loss of key species, invasion by novel species, and alteration ecosystem structure and/or function outside the bounds of normal variability. Therefore, extreme climate events, in spite of their ephemeral nature, can potentially cause shifts in the structure of plant communities (Smith, 2011) and greatly impact ecosystem productivity (Ciais et al., 2005) and biodiversity (Thomas et al., 2004). It is, however, difficult to determine whether the ecological response is explicitly attributable to an extreme climate event, since it may not be extreme enough to cause ecological consequences (Niu et al., 2014).

Accordingly, research has started to not only focus on the impact of the trend of gradual increases in mean temperatures but also on the effects of increasing extreme HS events (Brown et al., 2004; Jentsch et al., 2011; Sentis et al., 2013). However, due to the difficulties in conducting experiments and simulating extreme heat events under field conditions, they are most frequently conducted under controlled conditions in the laboratory (Wang et al., 2008a, 2012). Therefore, the effects of extreme heat events on the vegetation structure and dynamics remained less well understood than effects of climate warming and atmospheric CO_2 enrichment, especially on crops photosynthesis, respiration, and growth (Long et al., 2004; Ainsworth et al., 2008). To date, only a few experiments with HS treatment have been conducted in plant communities, and these studies focused on recolonization, competition, invasion, and the role of species richness during extreme events in community processes. HS manipulations were conducted mostly on grassland (White et al., 2001; Van Peer et al., 2004) or arctic species (Marchand et al., 2005, 2006). In this study, we will apply short-term HS treatment in a restored prairie and concentrate on the ecophysiological and growth responses of two dominant warm-season tall-grass prairie species with contrasting photosynthetic pathways (a C_4 grass and a C_3 forb) to HS.

The negative effects of HS on plants growth and crop yield mainly was caused through its negative effects on photosynthetic process, which is among the most thermosensitive aspects of plant functions (Wang et al., 2008a). Due to inter-annual variations in the timing and duration of hot days, HS events may affect plant physiological processes and community structure differently. Hence, the response of plants photosynthetic activity to HS will depend on the season and growing stage when HS events occur (Xu and Baldocchi, 2003; Yu et al., 2003; Richardson et al., 2010). Although the research of the effects of the timing of the extreme events is urgently needed, there is still a lack of studies in this regard (Jentsch et al., 2011; Smith, 2011). We urgently need to advance research on the effect of the timing of extreme events and their consequences by collecting evidence from experimental studies in natural field conditions. Therefore, this study will simulate HS events in the key phenological stages of the dominant species in an old prairie to investigate how variation in the timing of HS events during the growing season influences physiological processes and growth of individual species and community dynamics.

The optimal temperature for photosynthesis is typically higher for C_4 species than for C_3 species because C_4 species usually have higher water use efficiency and lower photorespiration due to its CO_2-accumulating mechanisms in the leaf (Sage and Monson, 1999). This may contribute to greater tolerance to HS for C_4 species than co-occurring C_3 species (Coleman and Bazzaz, 1992; Ehleringer et al., 1997; Wang et al., 2008b). Differential sensitivities to HS among different species may lead to divergent responses in these dominant species, particularly if the stress exceeds species-specific physiological thresholds (Gutschick and BassiriRad, 2003). In natural systems, the significance of climate warming for C_4 vegetation can depend less on the mean increase in global temperature and more on the spatial and temporal variation of the temperature increase (Sage and Kubien, 2003). In New Zealand, for example, episodic heat events inhibit C_3 plants more than C_4 grasses, and as a result, facilitate C_4 grass invasion of C_3-dominated grasslands (White et al., 2000, 2001). However, whether the timing of HS events impacts differently on C_3 vs. C_4 species remains to be determined and the differences in the responses to HS applied at different growing stages will have a bearing on the relative impact of global environmental change on the abundance, productivity and distribution of C_3 and C_4 species and therefore community structure.

To examine the influence of HS on plants ecophysiological and growth response in naturally occurring mixed C_3–C_4 vegetation, we conducted a field study and aimed with the following two major objectives: (1) to determine how HS affects the ecophysiological and morphological characteristics of a C_4 and C_3 species which co-dominate a restored prairie community; (2) to determine the effect of timing of HS on each species growth and physiology. Our specific hypotheses were as follows: (1) HS will have a less pronounced negative effect on the C_4 than the C_3 species; (2) differences in the responses to HS applied at different

growing stage (HS timing effect) will lead to differences in plants ecophysiological responses and growth.

MATERIALS AND METHODS

Field Site and Experimental Treatments

The experiment site was located within a restored prairie vegetation at the University of Toledo's Stranahan Arboretum (Toledo, OH, USA), within the oak-savannah glacial-sand ecosystem referred to as "Oak Openings" region[1]. *Andropogon gerardii* (big bluestem), a warm-season C_4 perennial grass, and *Solidago canadensis* (goldenrod), a warm-season C_3 perennial herbaceous dicot, together account for almost 95% plant canopy cover and the majority of total aboveground productivity in this ecosystem. Top-vented 1 m^3-chambers made with transparent plastic attached to a wooden frame was used to simulate HS treatment. Heat treatment was applied *in situ* from June 21 to June 25, July 22 to July 26, and August 28 to September 1 in 2007 (as in Wang et al., 2008b). There was no obvious drought situation before each heat treatment. For each heat treatment, eight 1 m × 1 m plots were selected randomly for use; four were untreated controls and four were heated to 39–41°C daytime temperature. A portable electric heater (Heat Runner model 33551, 1500 W), suspended near a corner of the chamber, was used to increase and regulate chamber temperature, and a fan was used to distribute warm air inside the chamber. Spatial variation in temperature within chambers was found to be minimal. The temperature in the central chamber was 0.5 ± 0.3 (standard deviation)°C higher than the edge of the chamber. Plants were not watered during the heat treatment. This experimental design did not allow for determination of chamber effects on plants (increased humidity, decreased wind, and slightly decreased light levels), but such effects would only serve to minimize the negative effects of HS, and make detection of heat effects more difficult. Air temperature was monitored continuously, with either a temperature probe and data logger (HOBO8, Onset Computer Corp, Bourne, MA, USA) or a fine-wire thermocouple and data logger (LI-1000, LiCOR, Lincoln, NE, USA). Leaf temperature was measured with an IR thermometer (cross-checked against the probes above). Soil temperature (10 cm) during midday and at the end of the HS treatment was monitored with a temperature probe and a thermometer (Wang et al., 2008b; Mainali et al., 2014). Heat-treatments in this study were chosen to represent those HS events encountered by vegetation in the Toledo region (northwest Ohio, USA) during summer months. On average, there is about 10 days of HS in July and August during which day time maximal temperatures are higher than 32°C in Toledo. The recorded daytime maximal temperatures for June, July, and August were 40, 41, and 39°C, respectively, and the mean daytime maximal temperatures for June, July, and August were 28, 30, and 29°C, respectively[2]. Therefore, the target HS treatment temperature was set at 40, 41, and 39°C for June, July, and August, respectively, in this experiment.

Gas Exchange and Leaf Trait Measurements

Photosynthetic measurements were conducted during and after each HS treatment in order to determine the timing effect of HS on foliar gas exchange. During and after each heat treatment, one fully expanded leaves were chosen randomly from each plot and net photosynthetic rate, stomatal conductance to water vapor, and internal CO_2 level were measured daily with a portable infrared gas analyzer (LI-COR 6400LCF; LI-COR, Lincoln, NE, USA). During measurements, CO_2 concentration of 380 μmol mol^{-1}, leaf temperature of 25°C, photosynthetic photon flux (PPFD) of 1500 μmol m^{-2} s^{-1} and airflow through the chamber of 250 μmol s^{-1} were set in the leaf chamber. Net photosynthetic rate (A_{net}) was taken as the rate of photosynthesis at a PPFD of 1500 μmol m^{-2} s^{-1}. The parameters including stomatal conductance (g_s) and intercellular CO_2 concentration (C_i) were recorded during the photosynthetic measurement. Intrinsic water use efficiency ($_i$WUE) was calculated as the ratio of net photosynthetic rate to stomatal conductance. Quantum yield of PSII electron transport (Φ_{PSII}) was measured with a pulse-amplitude-modulated (PAM) fluorometer with a saturating pulse of 3000 μmol photons m^{-2} s^{-1} (Model PAM 101/103, Walz, Germany) on ambient light-adapted (∼800 μmol photons m^{-2} s^{-1}) plants, as in Wang et al. (2008b). Leaf area index (LAI) was measured once per week, using LAI-2000 (LI-COR Biosciences, Lincoln, NE, USA). After gas-exchange measurements of last heat-stress treatment, ten 0.5 cm^2 leaf punches from each leaf were taken and oven-dried at 65°C for 2 weeks for measurement of SLA (m^2 kg^{-1}) and LWC (%). An index of the total leaf chlorophyll content was measured using a chlorophyll meter (SPAD-502, Konica Minolta, Japan). Readings were taken along the middle section of the four leaves of one plant and the mean value was used for analysis. The measurements were made on five plants from each treatment before, during and after the HS.

Biomass and *C*, *N* Measurements

Four-week after the last HS treatment, 40 cm × 50 cm of each plot was harvested. The clipped plants were sorted into different categories (species, green and senescent leaves, stems and flowers), oven-dried at 65°C for 1 week and weighed.

Statistical Analysis

All statistics were tested in the R statistical language[3]. The normality of the residuals of all the variables was tested using the Shapiro–Wilk test. Fixed effects of species, heating stress at different time and their interactions on the morphological, biochemical, and physiological parameters were tested by a linear mixed-effects model, using the lme4 package[4]. The measuring time were specified as a random factor to control for their associated intra-class correlation. Linear mixed-effects models also tolerate the necessarily unequal number of responses and unbalanced sample sizes for each treatment. We obtained *p*-values for regression coefficients using the nlme package. For

[1] http://oakopen.org/

[2] http://www.ncdc.noaa.gov

[3] http://www.r-project.org/

[4] http://cran.r-project.org/web/packages/lme4/index.html

the sake of brevity, we present only the *F* tests from the LMER results here (type III Wald F tests with Kenward–Roger degrees of freedom approximation). A *Post hoc* Tukey HSD tests were made on specific contrasts to examine significant treatment effects among groups (step function in the nlme package, R). End of season measurements of aboveground primary production, flower weight and leaf morphological parameters were analyzed via *t*-tests to account for heat-stress timing effect. All statistical tests were considered significant at $P \leq 0.05$. Mean values of each variable were expressed with their standard error (SE).

RESULTS

Air temperature in the heated plots increased on average to 40.5 ± 2.8°C during HS treatment (data not shown, as in Wang et al., 2008b; Mainali et al., 2014). During the 5-days HS treatment, leaf temperature of *A. gerardii* and *S. canadensis* in heated plots was higher than that in control plots, but returned to control levels right after the end of HS (**Figure 1**; **Table 1**). For HS applied during early-, peak-, and reproductive- growing season, leaf temperature reached 34.9, 34.3, and 33.2 for *A. gerardii* and 34.1, 34.1, and 33.8°C for *S. canadensis* in the heated plots, respectively (**Figure 1**).

Aboveground net primary production at the end of growing season differed significantly among different treatments (**Figure 2A**). ANPP of the plots heat-stressed at reproductive-growing season was significantly lower than that of the control plots. The productivity of *S. canadensis*, but not *A. gerardii*, was significantly reduced by HS. The flower weight of *S. canadensis* was higher than that of *A. gerardii*, but neither was affected by HS (**Figure 2B**). LAI was significantly lower at the plots heat-stressed at reproductive growing stage than the control plots (**Figure 2C**). LAI was highest at the plots heat-stressed at early-growing season and lowest at the plots heat-stressed at reproductive-growing stage (**Figure 2C**).

Specific leaf area of *S. canadensis* was higher than that of *A. gerardii*. Compared with control plots, HS at peak-growing stage significantly increased SLA for both *A. gerardii* and *S. canadensis* (**Figure 3A**). SLA of plants heat-stressed at peak- and reproductive- stages was significantly higher than that heat-stressed at early-growing stages. LWC of *S. canadensis* was higher than that of *A. gerardii*. Compared with control plots, HS at reproductive-growing stages significantly decreased LWC for *S. canadensis*. And for *A. gerardii*, LWC of plants at the plots heat-stressed at peak-growing stage was significantly lower than that at control plots and plots heat-stressed at early-growing stages (**Figure 3B**).

Heat stress treatment reduced net CO_2 assimilation rate and stomatal conductance in heat-stressed plants. A_{net} (net photosynthetic rate) was higher for *A. gerardii* than that of *S. canadensis*. A_{net} was significantly lower in heated plots than in control plots during HS for *A. gerardii* and *S. canadensis* (statistical results not shown). A_{net} remained depressed for at least 1 week after HS in heated plants, relative to unheated plants. Throughout the experimental duration, A_{net} was significantly decreased by heat-stress at peak and reproductive growing stages, compared with control plots. A_{net} was lowest for the plots heat-stressed at reproductive stage, followed by the plots heat-stressed at peak-growing and early-growing stage (**Figure 4**). Stomatal conductance to water vapor (g_s) varied among different species and treatment. For *A. gerardii* and *S. canadensis*, g_s was lower in heated plots. There was also a similar significant timing effect of HS on g_s as on A_{net}, with lowest g_s achieved at plots heat-stressed at reproductive stage (**Figure 4**; **Table 1**). Variation in internal CO_2 (C_i) was also a function of species and treatment. For *A. gerardii* and *S. canadensis*, C_i was higher in heated plots. There was also a significant timing effect of HS on C_i, with

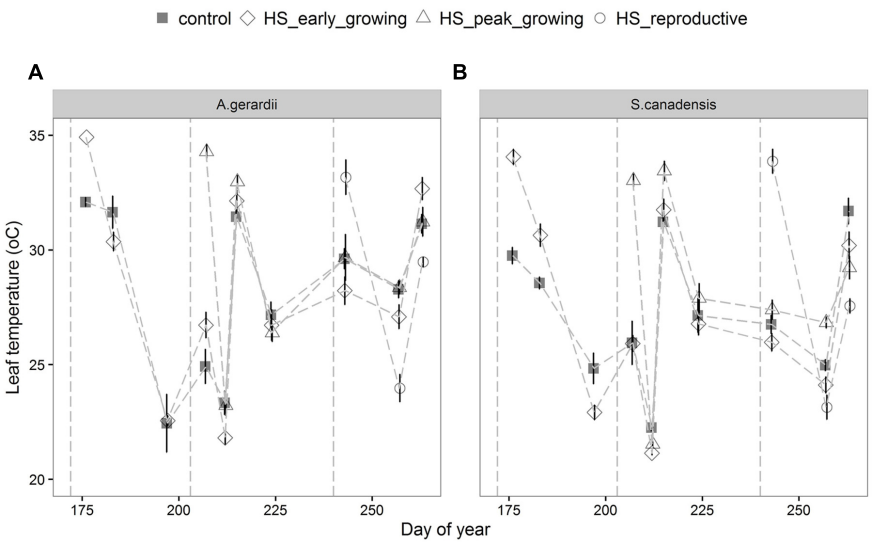

FIGURE 1 | Effects of HS applied at different growing stage on leaf temperature of (A) *Andropogon Gerardii* and (B) *Solidago Canadensis*. Measurements were taken during and after each heat-stress treatment. Values are means ± 1 SD; $n = 4$.

TABLE 1 | Degrees of freedom (numerator, denominator) and *F*-statistics from the linear mixed effect model on the fixed effect of treatment on the morphological, biochemical, and physiological parameters.

Factors	LAI	ANPP	Flower weight	Leaf Temp	Φ_{PSII}	A_{net}	g_s	C_i	T_r	iWUE	SLA	LWC	*Chl*
Species			1,70	1,590	1,583	1,274	1,274	1,274	1,274	1,274	1,123	1,123	1,1526
			35.6**	16.3**	526.78**	232.8**	69.4**	738.1**	17.0**	656.1**	73.86**	25.50**	29.0**
Treatment	3,122	3,16	3,70	3,591	3,583	3,278	3,276	3,275	3,278	3,278	3,123	3,123	3,1505
	10.9**	2.88*	1.2	13.5**	15.15**	15.1**	2.6*	7.9**	0.6	24.6**	3.22**	11.41**	24.6**
Species			3,70	3,590	3,583	3,274	3,274	3,274	3,274	3,274	3,123	3,123	3,1526
*Treatment			1.0	0.1	1.41	2.3*	0.2	1.6	0.7	3.6*	1.69	0.39	1.6

**Denotes significance at $P < 0.05$; **Denotes significance at $P < 0.01$. The full name and explanation for the symbols were provided in the abbreviation list.*

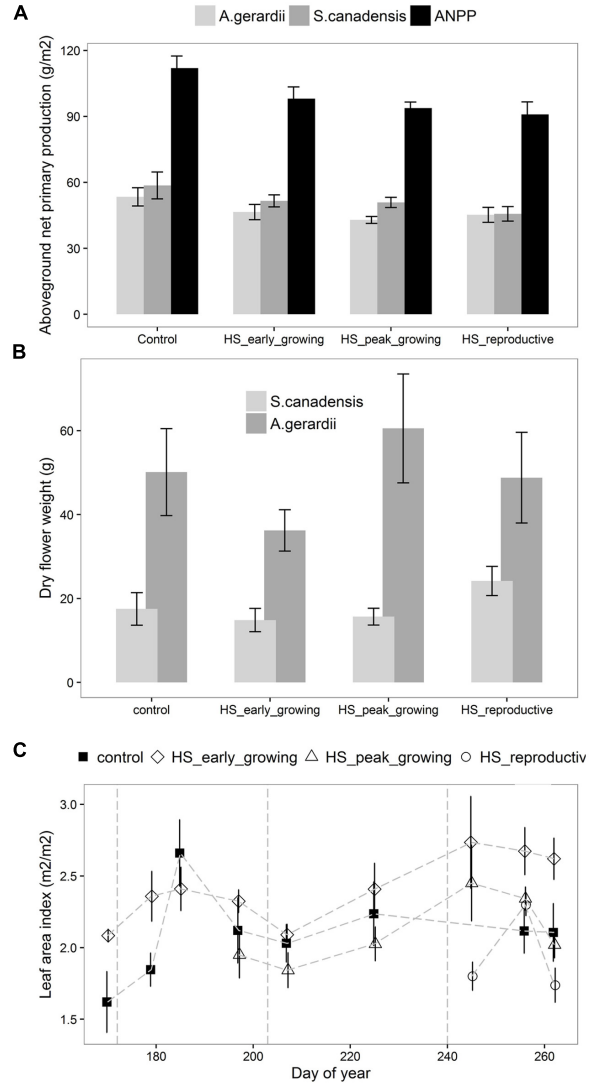

FIGURE 2 | Effects of HS applied at different growing stage on (A) the aboveground net primary productivity, productivity of *A. gerardii* and *S. Canadensis*; (B) the flower weight of *A. gerardii* and *S. Canadensis*; (C) leaf area index (LAI). LAI was measured once during and twice after each heat-stress treatment. Values are means ± 1 SD; $n = 4$.

highest C_i achieved at plots heat-stressed at reproductive stage than that of control plots and plots heat-stressed at early- and peak- growing stages (**Figure 4**; **Table 1**). The intrinsic water use efficiency ($_i$WUE) was lower for the plants heat-stressed at the reproductive stages than control plants. There was no significant difference between control plots and plots heat-stressed at early- and peak- growing season (**Figure 4**).

Quantum yield of PSII electron transport (Φ_{PSII}) was higher for *S. canadensis* than for *A. gerardii*. HS at different growing stages played a significant role in affecting Φ_{PSII}. HS decreased Φ_{PSII} significantly when it was applied at the peak-growing season, compared with control plots (**Figure 5**). The content of chlorophyll (chlorophyll a + chlorophyll b) was

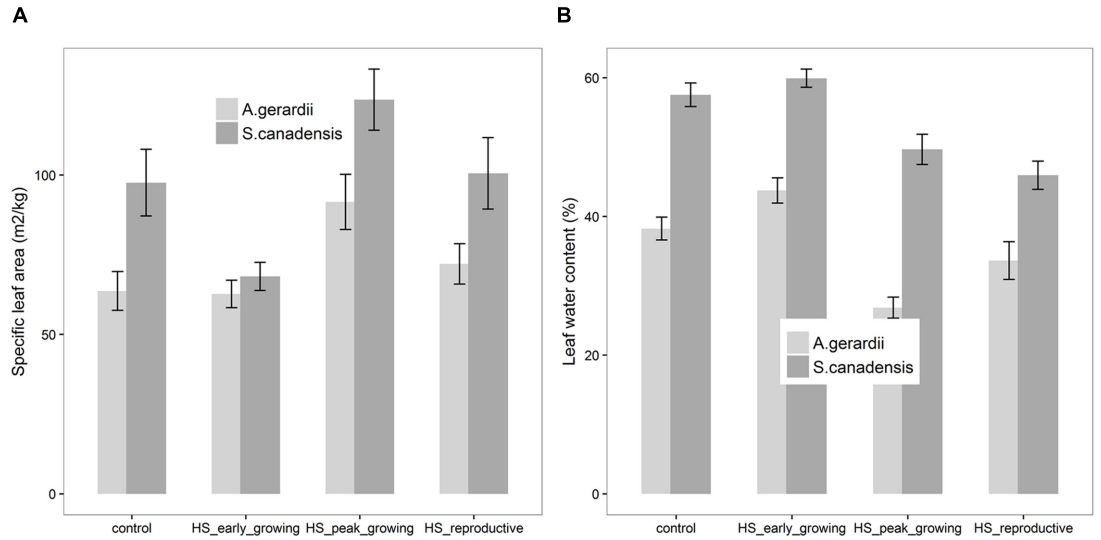

FIGURE 3 | Effects of HS applied at different growing stage on (A) specific leaf area (SLA) and (B) relative leaf water content of *A. gerardii* and *S. canadensis*. Values are means ± 1 SD; $n = 4$.

significantly different among different treatments. For both species, chlorophyll content in newly developed leaves was significantly lower than that in the fully developed and senescent leaves (statistical not shown). The chlorophyll content was affected by heat-stress at different growing stages significantly. HS applied at different growing stages all lowered chlorophyll content significantly compared with control plots for both species. Chlorophyll content of the two species heat stressed at peak and reproductive growing stage was lower than that heat-stressed at early growing stages (**Figure 6**).

DISCUSSION

Extreme climate events have long been acknowledged as a universal phenomenon in recent years and caused great agricultural, economic and ecological consequences (IPCC, 2013). However, in natural field conditions, comprehensive investigations of the effect of HS occurring at different growing stage on plants ecophysiology and growth are still scarce. In this study, we simulated HS events in a tallgrass prairie and collected plant ecophysiological and growth data throughout a growing season. These *in situ* physiological and whole-plant responses of the two dominant species showed different sensitivity to temperature manipulations exposed at different growing stages. Overall, we found that (1) the physiology of both species and growth of *S. canadensis* were affected by HS treatment; (2) the degree of HS effect varied when it applied at different growing stages, with greater negative effect associated with HS applied at later-growing season; (3) the physiology and growth of the two dominant species showed differential sensitivity to HS, with *S. canadensis* being affected more than *A. gerardii*.

Both *A. gerardii* (C_4) and *S. canadensis* (C_3) experienced decreased A_{net} and intrinsic water use efficiency during HS. The decreases in A_{net} were still evident 1 week after heat treatment ended and the recovery to the control level took at least 1 week, which indicates that under moderate HS conditions (most commonly reported at temperatures between 35 and 40°C), photosynthesis can be reversibly reduced (Sharkey and Zhang, 2010; Huve et al., 2011). The direct effects of HS could have led to thermal damage to the photosynthetic machinery. We detected differences in A_{net} among the HS treatments persisting after the treatments ended, as well as a negative response in end of season aboveground productivity for *S. canadensis*, so there could have been significant thermal damage to the photosynthetic capacity of the two species. Photosynthesis can be reduced directly through non-stomatal limitations or indirectly through stomatal limitations under HS conditions (Sage et al., 2008; Bussotti et al., 2014). In this study, the decrease in A_{net} was associated with either reduced stomatal conductance for *S. canadensis* or down-regulation of quantum yield photosystem II (PSII) for both species (Rennenberg et al., 2006; Sage et al., 2008). Preventing excessive water loss and hydraulic failure through stomatal closure can also limit evaporative cooling and restrict CO_2 input into the leaf, which is a strategy to save water before further damages happen due to increase in the temperature and/or drought stress (Bauweraerts et al., 2014; Teskey et al., 2014). The restrictions on CO_2 input to the leaf due to stomatal closure resulted in reduced carbon assimilation (McDowell et al., 2008). In contrast to heat-induced stomatal closure, *A. geraidii* kept stomata relatively open under HS conditions which could enable effective transpirational cooling and prevent leaf from overheating (McDowell et al., 2008). Also, reduced intercellular CO_2 concentration suggested that CO_2 concentration also had negative effects on carbon assimilation, as observed previously in other species (Wang et al., 2008a,b, 2012).

When absorbed light are not dissipated efficiently as heat or used in the photosynthetic process, stomatal closure and

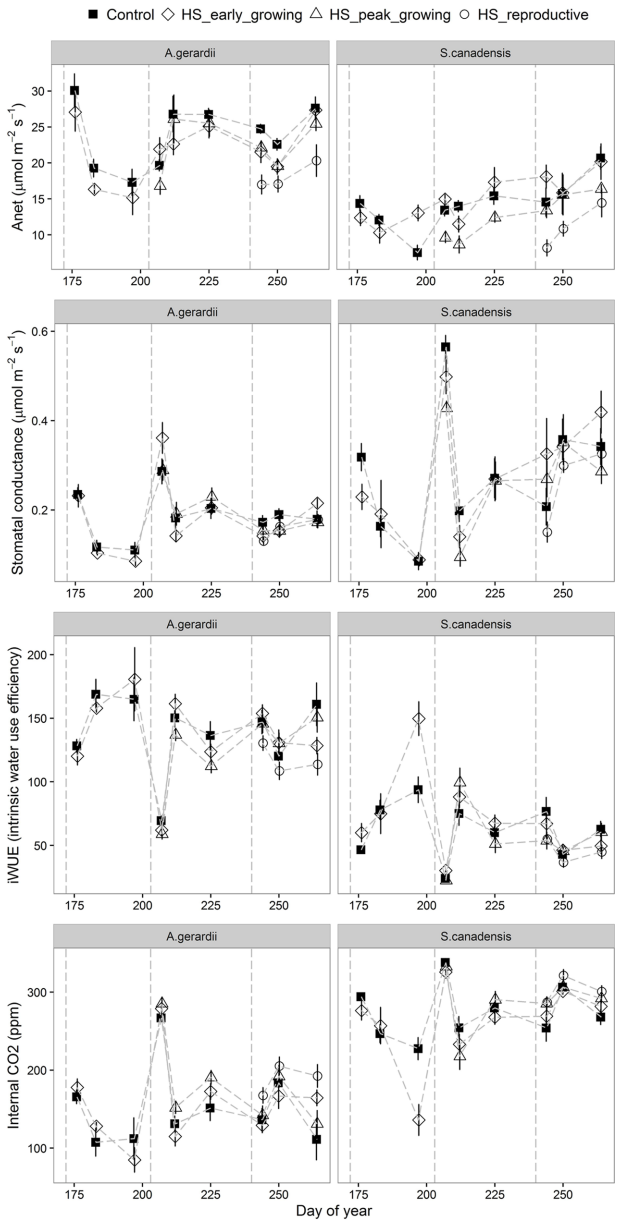

FIGURE 4 | Effects of HS applied at different growing stage on net photosynthesis (A_{net}), stomatal conductance (g_s), leaf internal CO_2 (C_i) and intrinsic water use efficiency of *A. gerardii* and *S. canadensis*. Measurements were taken once during and twice after each heat-stress treatment. Values are means ± 1 SD; $n = 4$.

reduced CO_2 uptake can lead to the photo-oxidative stress (Demmig-Adams et al., 2012; Foyer et al., 2012). The quantum yield of PSII (Φ_{PSII}) measures the proportion of light absorbed by chlorophyll associated with PSII system that is used in photochemistry (Baker and Rosenqvist, 2004). In this study, *S. canadensis* exhibited higher Φ_{PSII} than *A. gerardii* during and after HS, but the decrease of Φ_{PSII} compared to control samples during HS for *A. gerardii* was not significantly different from *S. canadensis* (**Figure 4**; **Table 1**). The significantly decreased Φ_{PSII} suggested that both species engaged flexible heat dissipation in response to HS, presumably because the activation of Rubisco was inhibited at higher temperatures (Feller et al., 1998). The observed reduction of Φ_{PSII} was indicative of acclimation responses or repair processes rather than sustained damages to PSII, because Φ_{PSII} recovered after HS treatment ended. The relative chlorophyll content of the leaves in the two species decreased significantly after HS and most of them did not recover completely, which is more evident in *S. canadensis* and when HS was applied during the later-growing season. HS have also been found to decrease total chlorophyll content significantly in eight Australian wheat varieties when the temperature increased from 28 to 36°C during 6 days (Balouchi, 2010). Efeoglu and Terzioglu (2009) reported that the total chlorophyll content in two wheat cultivars did not change during an 8 h HS treatment of 37°C, but significantly decreased during an 8 h HS treatment of 45°C. The high chlorophyll contents have been associated with heat tolerance in some wheat varieties (Reynolds et al., 1997).

Specific leaf area of both species in this study increased due to HS treatment applied in the peak-growing season. Alterations in leaf structure are an important mode of acclimation in many species (Wright et al., 2005). Higher SLA is beneficial for obtaining higher potential evaporative demand and a more extensive foliar display that captures more light for constant biomass investment (Schuepp, 1993; Niinemets, 1999; Wright et al., 2004). SLA was reported to be higher in higher growth temperatures (Williams and Black, 1993; Loveys et al., 2002), while others reported no systematic temperature-induced change in SLA of five deciduous and evergreen tree species grown at five temperatures (Tjoelker et al., 1999). The impact of temperature on SLA therefore depends on which species is being investigated and the temperature regimes at which the plants are grown and treated.

The optimal temperature for photosynthesis ranges between 20 and 35°C for most plant species (Rennenberg et al., 2006; Sage et al., 2008). However, thermotolerance of photosynthesis to HS differs in different species (Berry and Björkman, 1980) and foliage types (Dreyer et al., 2001; Duan et al., 2014). The responses of photosynthesis to HS depends on adaptation strategies to habitat conditions (Knight and Ackerly, 2002; Cunningham and Read, 2006; Weston and Bauerle, 2007; Gunderson et al., 2010) and climate change scenarios such as CO_2 elevation (Wang et al., 2008a, 2012). However, the species differences were not always found (Ghannoum et al., 2010) or the thermotolerance of species was reported to be unrelated to the temperature at their site of origin (Lin et al., 2013). In contrast with our hypothesis, both species showed reduced A_{net} during HS and the sensitivity of A_{net} of the two species responding to the HS did not vary significantly (**Figure 5**; **Table 1**). However, the C_3 species, *S. canadensis*, tended to close stomata in response to HS, leading to reduced transpiration (and therefore reduced transpirational cooling upon HS). In contrast, the C_4 species, *A. gerardii*, tended to keep stomata relatively open and maintained high transpiration rates which would limit negative temperature effects on the foliage.

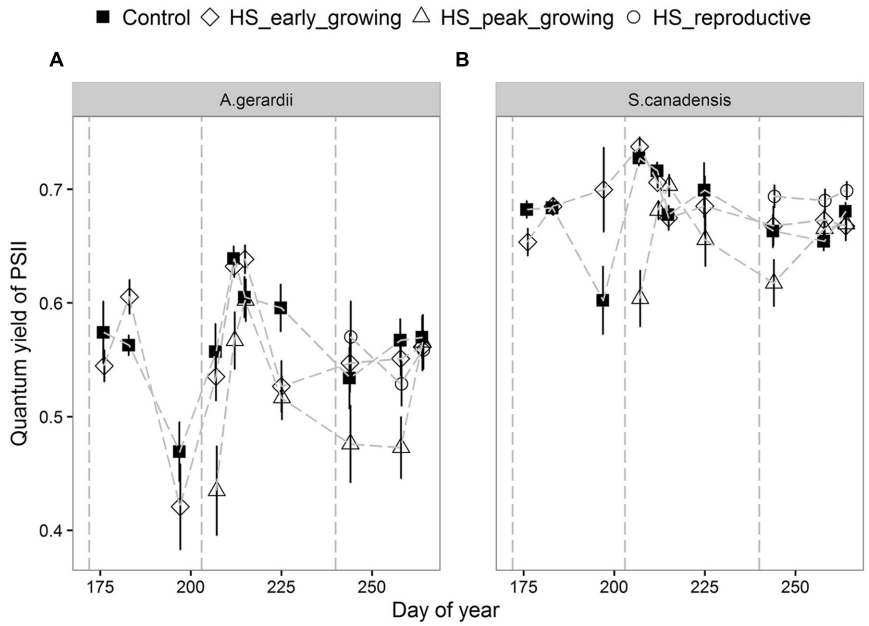

FIGURE 5 | Effects of HS applied at different growing stage on quantum yield of photosystem II (Φ_{PSII}) of (A) *A. gerardii* and (B) *S. canadensis*. Measurements were taken during and after each heat-stress treatment. Values are means ± 1 SD; $n = 4$.

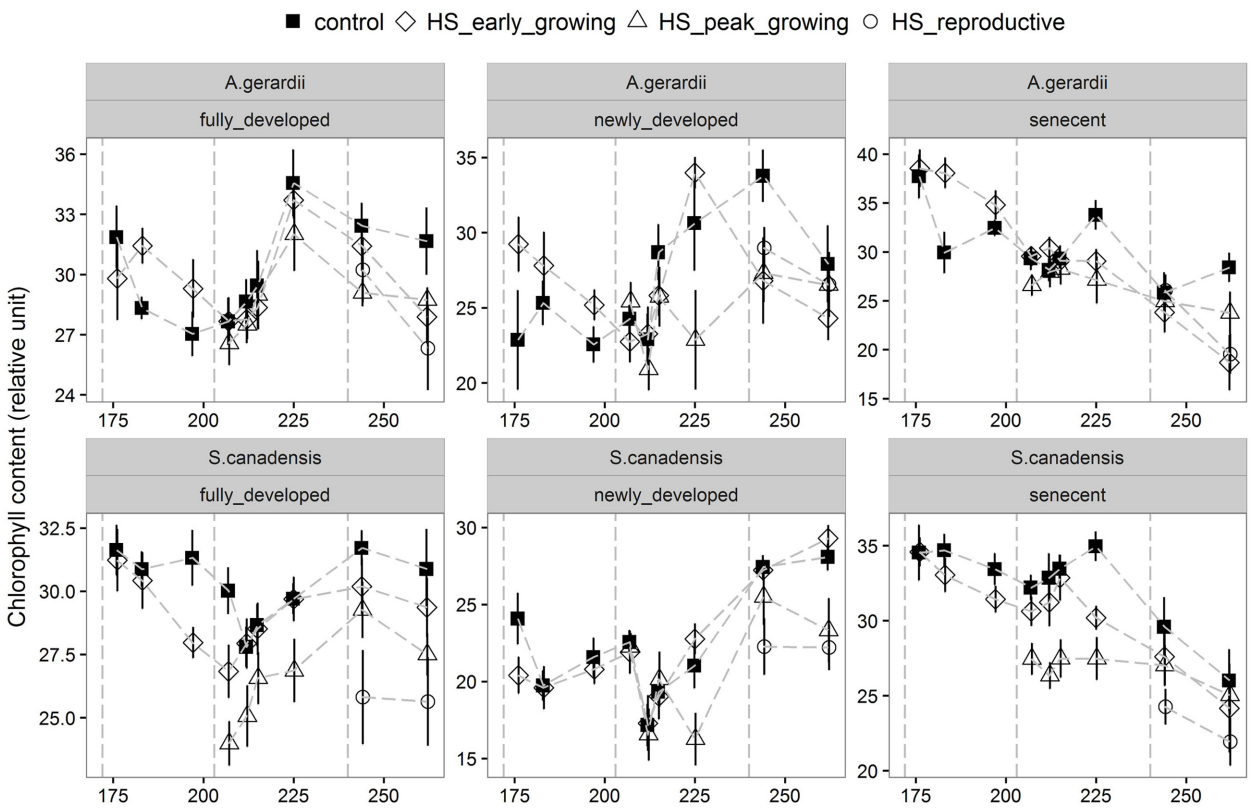

FIGURE 6 | Effects of HS applied at different growing stage on relative chlorophyll content of the newly develop, fully developed and senescent leaves of *A. gerardii* and *S. canadensis*. Measurements were taken during and after each heat-stress treatment. Values are means ± 1 SD; $n = 4$.

The response of plants to HS was variable depending on the season or life stage during which the HS event happened. Plants were reported to be more susceptible to HS during later reproductive developmental stages (Cross et al., 2003). Early-growing or peak-growing season HS had neutral effects on plants growth for both species. In contrast, later-growing season HS significantly decreased the productivity of *S. canadensis*. Mid- or late- summer heat event was associated with strong physiological stress (De Boeck et al., 2011). In this study, ANPP and LAI of the plots heat-stressed at reproductive season was significantly lower than that of the control plots and the reduction was mostly caused by the negative HS effect on *S. canadensis*. The reduction in ANPP was mostly due to the experimental treatment, as the ratio between the two species in each treatment was not significantly different (**Figure 2A**). Consistently, the physiological performance of A_{net}, g_s, and Φ_{PSII} were all decreased more during HS applied at peak- or reproductive- growing stages.

Notably, in this study, the flower weight of the two species was not affected by HS (**Figure 2B**), which is contrary to what have reported that heat-stressed plants decreased flower production and produced later flowers on existing inflorescences (Sato et al., 2000; Cross et al., 2003). HS applied at different growing stage had no effect on mortality for the two species (data not shown). *S. canadensis* had similar mortality rate, while *A. gerardii* showed no mortality among different treatments. Andrello et al. (2012) reported increased mortality of juvenile plants in the endangered *Eryngium alpinum* L. during HS, while adult plants were less affected. Similarly, mortality of the Tenerife endemic *Helianthemum juliae* seedling reached nearly 100% in years of extreme drought (Marrero-Gómez et al., 2007).

CONCLUSION

Understanding the responses of dominant species to climate extremes is essential to predict future ecosystem dynamics and is particularly important when these species experience similar growing phenology but differ in their sensitivities to the climate factors. In this study, we examined the timing effects of HS on two dominant species in a tallgrass prairie ecosystem. There were two main conclusions drawn from this research. First, the photosynthetic and growth responses of these two species to HS were different, with *S. canadensis* being affected more than *A. gerardii*, indicated by the negative HS effect on both physiological and growth responses for *S. canadensis*; secondly, there were significant timing effect of HS on the two species, with greater reductions in photosynthesis and productivity occurred when HS was applied at later-growing season. The reduction in aboveground productivity in *S. canadensis* but not *A. gerardii* can increase the competitive advantage of *A. gerardii*, which therefore could have dramatic implications for species abundance, distribution and community structure. The present experiment showed that ephemeral HS may promote stochastic successions at the community level (Kreyling et al., 2011) or promote long-term effects on deterministic trajectories at the ecosystem scale (Allen and Breshears, 1998). It is worth pointing out that the negative HS effect in this study may be smaller than likely to occur, as HS treatment applied in this experiment was a single HS event and plants in Northwest Ohio experience multiple HS events per summer. Thus, the negative HS effect could be underestimated in this study and special caution should be paid when to predict long-term heat-stress consequences and differences between C_3 and C_4 plants. Furthermore, this study focused on plants ecophysiological processes and only examined short-term plant responses to HS within one generation of perennial plants. The results suggest that long-term effect of HS on plant communities and ecosystems dynamics should be studied more extensively and with longer experimental durations, particularly in combination with other potentially interactive aspects of global environmental change including increases in atmospheric CO_2 and O_3 concentration and altered precipitation pattern (Wang et al., 2014a,b).

AUTHOR CONTRIBUTIONS

DW and SH came up with the research idea. DW led the experiment and writing. KM and RT assisted conducting the experiment and revising the manuscript.

FUNDING

Funding for this research was provided by grants from the Jiangsu Distinguished Professorship Award, National Science Foundation to SH and Nanjing University of Information Science and Technology, Jiangsu Natural Science Foundation (BK20150894), national natural science foundation of China (31500503) and the International S&T Cooperation Program of China (2012DFA60830) to DW.

ACKNOWLEDGMENTS

We thank Dr. Daryl Moorhead, Sandra Stutzenstein, and Walter Schulisch for providing access to experimental field sites, as well as for logistical support and assistance conducting the experiment. We thank Dr. Jiquan Chen for providing us vehicles and experimental equipments. We appreciate the reviewers for giving their advice for the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 21 June 2017
doi: 10.3389/fpls.2017.01052

Differential Response of Floating and Submerged Leaves of Longleaf Pondweed to Silver Ions

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In this study, we have investigated variations in the potential of floating and submerged leaves of longleaf pondweed (*Potamogeton nodosus*) to withstand silver ion (Ag^+)-toxicity. Both floating and submerged leaves changed clear colorless $AgNO_3$ solutions to colloidal brown in the presence of light. Transmission electron microscopy revealed the presence of distinct crystalline Ag-nanoparticles (Ag-NPs) in these brown solutions. Powder X-ray diffraction pattern showed that Ag-NPs were composed of Ag^0 and Ag_2O. Photosystem (PS) II efficiency of leaves declined upon exposure to Ag^+ with a significantly higher decline in the submerged leaves than in the floating leaves. Similarly, Ag^+ treatment caused a significant reduction in the carboxylase activity of the ribulose bisphosphate carboxylase/oxygenase in leaves. The reduction in this carboxylase activity was significantly higher in the submerged than in the floating leaves. Ag^+ treatment also resulted in a significant decline in the levels of non-enzymatic and enzymatic antioxidants; the decline was significantly lower in the floating than in submerged leaves. X-ray photoelectron spectroscopy revealed the presence of Ag_2O in these leaves. Inductively coupled plasma mass spectrometry analysis revealed a three-fold higher Ag content in the submerged than in floating leaves. Our study demonstrates that floating leaves of longleaf pondweed have a superior potential to counter Ag^+-toxicity compared with submerged leaves, which could be due to superior potential of floating leaves to reduce Ag^+ to less/non-toxic Ag^0/Ag_2O-nanoparticles/nanocomplexes. We suggest that modulating the genotype of longleaf pondweed to bear higher proportion of floating leaves would help in cleaning fresh water bodies contaminated with ionic forms of heavy metals.

Keywords: antioxidants, Ag-nanoparticles, ecophysiological adaptation, heterophyllous aquatic plant, photosystem II, *Potamogeton nodosus*

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Carmen Arena, University of Naples Federico II, Italy
Amy S. Verhoeven, University of St. Thomas, United States

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *04 January 2017*
Accepted: *31 May 2017*
Published: *21 June 2017*

Citation:
Shabnam N, Sharmila P, Govindjee, Kim H and Pardha-Saradhi P (2017) Differential Response of Floating and Submerged Leaves of Longleaf Pondweed to Silver Ions. Front. Plant Sci. 8:1052. doi: 10.3389/fpls.2017.01052

Abbreviations: Chl, chlorophyll; DHA, dehydroascorbate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylene diamine tetra acetic acid; EDX, energy dispersive X-ray; fcc, face cubic centered; F_o, minimum chlorophyll *a* fluorescence; F_m, maximum chlorophyll *a* fluorescence; F_v, variable chlorophyll *a* fluorescence ($F_m - F_o$); GAE, gallic acid equivalent (s); GPX, guaiacol peroxidase; GR, glutathione reductase; GSH, reduced glutathione; MDA, malondialdehyde; MDHAR, monodehydroascorbate reductase; $NAD(P)^+$, oxidized nicotinamide adenine dinucleotide (phosphate); NAD(P)H, reduced nicotinamide adenine dinucleotide (phosphate); NEM, *N*-ethylmaleimide; NP, nanoparticle; PS, photosystem; PVP, polyvinylpyrrolidone; PXRD, powder X-ray diffraction; ROS, reactive oxygen species; Rubisco, ribulose 1,5-biphosphate carboxylase/oxygenase; SAED, selected area electron diffraction pattern; SOD, superoxide dismutase; TCA, trichloroacetic acid; TEM, transmission electron microscope; Tris, tris-(hydroxymethyl)-aminomethane.

INTRODUCTION

Human activities, in particular, industrialization and urbanization, have led to a drastic acceleration in heavy metal pollution of our surroundings and ecosystems (Nriagu, 1996). Negative impact of heavy metal(s) on the health of living beings (including the humans) and ecosystems is of serious concern; this effect is being increasingly felt over the past few decades. In view of its ecofriendly nature, bioremediation (i.e., use of living beings or their components for detoxification of pollutants through, e.g., transformation, and degradation) is being used as one of the key approaches to decrease the level of heavy metals in the surroundings (Dhir et al., 2009; Rai, 2009). Microbial-assisted removal of heavy metals has been a popular bioremediation process. However, due to difficulties in harnessing the microbes from soils or water, plant-based biosorption of heavy metals is now receiving greater attention across the world. A large number of plants are hyper-accumulators of heavy metals, so many researchers are now trying to understand hyper-accumulating strategies in these plants (Kamal et al., 2004; Dhir et al., 2009; Sharma and Dietz, 2009). Pardha-Saradhi et al. (2014a,b,c) have shown that terrestrial plants have the potential to biotransform precious heavy metal ions (e.g., Au^{3+} and Ag^+) and essential metal ions (e.g., Fe^{3+}) into less/non-toxic nanoparticles (NPs)/nanocomplexes. Shabnam et al. (2017) have recently demonstrated that Ag-NPs are significantly less toxic than ionic Ag.

Heavy metals released from industries and other sources often find their way into water bodies, e.g., lakes, rivers, and oceans (Rai, 2009). Phytoplanktons contribute to over 50% of the organic material produced through photosynthetic CO_2 fixation (Arrigo, 2005). However, research has, thus far, been focused mainly on the macrophytes simply because of the ease with which they can be handled and harvested. Amongst the macrophytes, attention has been given mostly to homophyllous aquatic plants. In spite of being better adapted to the fluctuating climatic conditions compared to homophyllous aquatic macrophytes, heterophyllous aquatic plants have received less attention from the researchers (Iida et al., 2009).

Pardha-Saradhi et al. (2014a) have used silver as an ideal model heavy metal, since response of plants to Ag^+ can be visually recorded and easily characterized. Anthropogenic activities such as mining, electroplating and photographic industry are responsible for the release of silver into our surroundings (Purcell and Peters, 1998; Ratte, 1999). No attempt has, thus far, been made to evaluate the impact of silver on any heterophyllous aquatic macrophyte. Longleaf pondweed has floating and submerged leaves. While floating leaves are present on the surface, submerged leaves are under water. Previously, we reported that the floating leaves have superior photosynthetic efficiency and antioxidant system compared to the submerged ones (Shabnam et al., 2015; Shabnam and Pardha-Saradhi, 2016). Therefore, in this study, we chose this plant to evaluate differences in the tolerance of these types of leaves to Ag^+ toxicity. We have evaluated the impact of silver on photosynthesis and antioxidant system. Our findings revealed that these leaves possess potential to generate Ag-NPs on exposure to Ag^+. We believe that this potential of leaves to generate Ag-NPs is a mechanism to restrict the uptake of Ag^+ and thus, counter its toxic effects.

MATERIALS AND METHODS

Experimental Procedure

Longleaf pondweed (*Potamogeton nodosus*, Potamogetonaceae) was grown at the University of Delhi, as described by Shabnam et al. (2015). For studying the impact of Ag^+ on floating and submerged leaves of longleaf pondweed, fully expanded mature leaves were used. Leaves, after washing three times with double-distilled water, were acclimatized under laboratory conditions for 3 h. Silver nitrate ($AgNO_3$) was used to impose silver ion (Ag^+)-toxicity. The leaves were exposed to different levels (0, 5, 10, 50, 100, 250, and 500 μM) of $AgNO_3$ in Borosil dishes (190 mm diameter × 100 mm height) under continuous white light (120 μmol photons m^{-2} s^{-1}) for 24 h. Understandably, in this experimental setup floating leaves float and submerged leaves get submerged in test solution during experimental exposure.

Impact of Ag^+ on floating and submerged leaves of longleaf pondweed was evaluated by measuring (i) photosystem (PS) II efficiency; (ii) carboxylase activity of ribulose bisphosphate carboxylase/oxygenase (Rubisco); and (iii) enzymatic and non-enzymatic antioxidants according to the protocols of Shabnam and Pardha-Saradhi (2016).

Analytical Methods

Photosystem II Efficiency

For determining PS II efficiency, leaves were dark-adapted for 40 min and Chl *a* fluorescence induction measurements were made on 8–10 different portions of leaves. Fluorescence transient, from 10 μs to 1 s, was measured using a plant efficiency analyzer (PEA) (Handy PEA; Hansatech Ltd, Norfolk, United Kingdom); leaves were excited with red light (peak at 650 nm) at an intensity of 3,500 μmol photons m^{-2} s^{-1}, provided by an array of six light-emitting diodes (LEDs). At least five leaves were used for each treatment. Biolyzer software HP 3 (Bioenergetics Laboratory, University of Geneva, Geneva, Switzerland) was used to plot Chl *a* fluorescence data. For details on measurement of Chl *a* fluorescence, see Shabnam et al. (2015). Quantum efficiency of PS II activity was inferred from the ratio of variable (F_v) to maximum (F_m) Chl *a* fluorescence, where $F_v = F_m - F_o$, F_o being the minimum fluorescence (see Govindjee, 2004). Chl *a* and Chl *b* levels of leaves were quantified according to the method and equations used by Arnon (1949).

Carboxylase Activity of Rubisco

Carboxylase activity of Rubisco (EC 4.1.1.39) was measured as described earlier (Shabnam and Pardha-Saradhi, 2016). Leaves were homogenized in chilled 50 mM Tris–HCl buffer (pH 7.6) containing 1 mM DTT, 5 mM EDTA and 5% PVP with acid washed sand in pre-chilled mortar and pestle. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4°C, and the supernatant was used as a crude enzyme. The carboxylase

activity of Rubisco was measured, at 25 ± 2°C, using an assay mixture containing crude enzyme extract, Tris-HCl buffer (200 mM, pH 8.5), 1 mM RuBP, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 0.1 mM DTT, 1 mM ATP, 5 units of phosphoglycerate kinase, 5 units of glyceraldehyde-3-phosphate dehydrogenase, and 0.2 mM NADH. Oxidation of NADH was recorded as decrease in absorbance at 340 nm; the carboxylase activity of Rubisco was initially calculated in terms of nmoles of NADH oxidized min^{-1} g^{-1} fresh weight. Subsequently carboxylase activity of Rubisco was extrapolated and expressed in terms of CO_2 fixed min^{-1} g^{-1} fresh weight.

Determination of Silver in Leaves

Silver content of leaves was measured using inductively coupled plasma mass spectrometry (ICP-MS) (NexION 300D, Perkin Elmer, Waltham, MA, United States) and expressed as mg silver g^{-1} dry weight. Leaves exposed to Ag^+ were also analyzed by X-ray photoelectron spectroscopy (XPS; Phi 5000 VersaProbe, Ulvac-Phi, Chigasaki, Japan).

Determination of Non-enzymatic Antioxidants

Levels of non-enzymatic antioxidants (i.e., phenolics, thiols, and ascorbate) were measured as described earlier (Shabnam and Pardha-Saradhi, 2016). Leaves were homogenized in chilled 5% TCA with mortar and pestle. The homogenate was centrifuged at 20,000 × *g* for 15 min at 4°C, and the supernatant was used for determining the levels of total ascorbate, total phenolics and thiols, as described below.

Total ascorbate

The reaction mixture consisted of 200 µl supernatant, 100 µl DTT (10 mM), 100 µl NEM (0.5%), 500 µl TCA (10%), 400 µl orthophosphoric acid (43%), 400 µl α-α′-bipyridyl (4%) and 200 µl $FeCl_3$ (3%); it was immediately vortexed to avoid the formation of any precipitate. This reaction mixture was incubated at 37°C for 1 h and the absorbance was measured at 525 nm. The amount of total ascorbate was expressed as nmoles g^{-1} fresh weight.

Total phenolics

One ml of supernatant was incubated with a mixture of 1 ml Folin–Ciocalteu reagent and 2 ml Na_2CO_3 (700 mM) for 1 h in dark at room temperature. Subsequently, absorbance of the reaction mixture was measured at 765 nm. Total phenolic content was expressed as nmoles of GAE g^{-1} fresh weight, using a standard curve obtained with gallic acid.

Thiols

To 200 µl supernatant, 775 µl K_2HPO_4 (500 mM) and 25 µl 5,5′-Dithiobis (2-nitrobenzoic acid) (DTNB) (10 mM in 100 mM phosphate buffer, pH 7.0) were added. Absorbance of the samples was measured at 412 nm and corrected against the absorbance of a sample without added DTNB. Thiol content was expressed as nmoles g^{-1} fresh weight, using an extinction coefficient of 13.6 mM^{-1} cm^{-1} at 412 nm.

Determination of Activities of Enzymatic Antioxidants

The activities of antioxidant enzymes, such as SOD (EC 1.15.1.1), catalase (EC 1.11.1.6), GPX (EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11), MDHAR (EC 1.6.5.4), DHA reductase (DHAR, EC 1.8.5.1), and GR (EC 1.6.4.2) were measured as described earlier (Shabnam and Pardha-Saradhi, 2016). Leaves were homogenized in chilled 50 mM Tris–HCl buffer (pH 7.6) containing 1 mM DTT, 5 mM EDTA and 5% PVP with acid washed sand in chilled mortar and pestle. The homogenate was centrifuged at 15,000 × *g* for 20 min at 4°C. The supernatant was taken as a crude enzyme extract and was used for estimating activities of various antioxidant enzymes as briefly described below.

Superoxide dismutase

To 4 ml of 200 mM Tris-HCl buffer (pH 7.6), we added 200 µl L-methionine (20 mM), 200 µl EDTA (0.1 mM), 100 µl hydroxylamine, 100 µl Triton X (0.1%), 200 µl riboflavin (0.5 mM) and the enzyme extract. Tubes containing the resultant reaction mixture were exposed to 120 µmol photons m^{-2} s^{-1} of white light, using an incandescent lamp, at 25 ± 2°C. After exposure to light for 45 min, 2 ml of freshly prepared Greiss reagent [containing equal volumes of 0.1% naphthylethylenediamine dihydrochloride (NED) and 1% sulphanilamide dissolved in 5% orthophosphoric acid] was added to the reaction mixture and absorbance was measured at 543 nm. SOD activity was expressed in terms of nmoles of O_2^- consumed min^{-1} g^{-1} fresh weight.

Catalase

Activity of catalase (CAT, EC 1.11.1.6) was determined by measuring the rate of oxygen evolution in a reaction mixture containing the enzyme extract in 200 mM phosphate buffer (pH 6.5) and 20 mM H_2O_2 at 25 ± 2°C, using a Clark-type liquid phase O_2 electrode (Hansatech, United Kingdom). The enzyme activity was expressed as nmoles of oxygen evolved min^{-1} g^{-1} fresh weight.

Guaiacol peroxidase

The reaction mixture for determining activity of guaiacol peroxidase activity consisted of a reaction mixture consisting of 200 mM phosphate buffer (pH 6.5), 2 mM guaiacol and 20 mM H_2O_2 incubated with enzyme extract, at 25 ± 2°C. Enzyme activity was measured by recording increase in absorbance at 470 nm with time. The enzyme activity was expressed as nmoles of tetraguaiacol formed min^{-1} g^{-1} fresh weight, using an extinction coefficient of 26.6 mM^{-1} cm^{-1} at 470 nm.

Ascorbate peroxidase

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was determined by estimating the rate of oxidation of ascorbate at 290 nm in a reaction mixture consisting of 200 mM Tris-HCl buffer (pH 7.6), 20 mM H_2O_2, 1 mM sodium azide, 2 mM ascorbate and the enzyme extract, at 25 ± 2°C. The activity of APX was expressed as nmoles of ascorbate oxidized min^{-1} g^{-1} fresh weight, using an extinction coefficient of 2.8 mM^{-1} cm^{-1}, at 290 nm.

Monodehydroascorbate reductase

For measuring activity of MDHAR (EC 1.6.5.4), the reaction mixture consisted of 200 mM Tris-HCl buffer

(pH 7.6), the enzyme extract, 2 mM ascorbate, 10 units of ascorbate oxidase and 0.2 mM NADH. Decrease in absorbance at 340 nm, due to the oxidation of NADH, was measured at 25 ± 2°C. Extinction coefficient of 6.2 mM^{-1} cm^{-1} (at 340 nm) was used to express the activity of MDHAR as nmoles of NADH oxidized min^{-1} g^{-1} fresh weight.

Dehydroascorbate reductase

The reaction mixture for the determination of dehydroascorbate reductase (DHAR, EC 1.8.5.1) activity included 200 mM Tris-HCl buffer (pH 7.6), enzyme extract, 1 mM reduced glutathione (GSH), and 1 mM dehydroascorbate. An increase in absorbance at 265 nm, due to the formation of ascorbate, from DHA by DHAR, in the presence of GSH, was measured at 25 ± 2°C. Enzyme activity was expressed in terms of nmoles of ascorbate formed min^{-1} g^{-1} fresh weight, using an extinction coefficient of 14 mM^{-1} cm^{-1}at 265 nm.

Glutathione reductase

The reaction mixture for measuring GR (EC 1.6.4.2) activity consisted of 200 mM Tris-HCl buffer (pH 7.6), enzyme extract, 1 mM oxidized glutathione (GSSG) and 0.2 mM NADH. Decrease in absorbance at 340 nm, due to the oxidation of NADH, was measured at 25 ± 2°C. The activity of GR was expressed as nmoles of NADH oxidized min^{-1} g^{-1} fresh weight, using extinction coefficient of 6.2 mM^{-1} cm^{-1} (at 340 nm).

Characterization of Ag-NPs

For TEM studies, 10 μl of colloidal solution was drop-coated on a 200 mesh copper grid with an ultrathin continuous carbon film, and allowed to dry in a desiccator at room temperature. Grids were viewed under a TEM (Technai G2 T30, Lonate Pozzolo, Italy) at a voltage of 300 KV. The hardware associated with the instrument allowed us to obtain (i) the EDX analysis to measure the elemental composition of the particle sample; and (ii) the SAED analysis to determine the crystalline/amorphous nature of NPs.

For PXRD studies, colloidal solutions were centrifuged. The pellet obtained was re-suspended in distilled water, drop-coated on silica surface, dried in a desiccator, and then used for collecting PXRD pattern, using Rigaku Rotaflex RAD-B with copper target CuK(α)1 radiation, with a tube voltage of 40 kV and a current of 60 mA in 2 theta (θ) range of 30–80°.

Statistical Analysis

All the experiments were carried out independently six times. The data obtained were statistically tested with ANOVA using the general linear model. The variations between the means of treatments were compared using Duncan's multiple range test (at $P \leq 0.05$). All these statistical analyses were performed using IBM-SPSS statistical software, version 22.0 (IBM Corporation, Armonk, NY, United States).

RESULTS

Potential of Floating and Submerged Leaves to Generate Ag-NPs

We observed alteration of clear colorless $AgNO_3$ solutions to colloidal brown when incubated with floating and submerged leaves of longleaf pondweed within 24 h (**Figures 1A,B**). Clear colorless $AgNO_3$ solutions turned colloidal brown due to the formation of Ag-NPs (Shabnam et al., 2016). $AgNO_3$ solutions incubated in the absence of leaves did not show any alteration in color, thus confirming that leaves were responsible for the observed color change. Floating leaves turned $AgNO_3$ solutions colloidal brown more intensively compared to the submerged leaves, although only one side of floating leaves was in contact with test solution. Supplementary Figure 1 shows experimental setup revealing that the floating leaves possess superior potential to turn clear colorless $AgNO_3$ (500 μM) solutions colloidal brown compared to submerged leaves. For depicting the gradation in color with better clarity the test solutions (i.e., different concentrations of $AgNO_3$) incubated with floating and submerged leaves for 24 h were transferred to test tubes along with leaves (**Figures 1A,B**). However, absorption spectra of the brown colloidal solution did not show any Ag-NP specific absorption peak.

Transmission electron microscopy revealed the presence of distinct NPs of varying shapes and sizes (∼10–80 nm) in these colloidal brown solutions (**Figures 1C–F**). EDX of these NPs showed peaks specific to Ag (**Figures 1I,J**). SAED pattern revealed the crystalline nature of these Ag-NPs (**Figures 1G,H**). PXRD patterns showed Bragg reflections (111), (200), and (311), revealing crystalline nature and face centered cubic structure of Ag^0-NPs (**Figures 1K,L**) (Pardha-Saradhi et al., 2014a). Additional peaks observed in the PXRD spectra might be due to Bragg reflections (111)*, (211)*, (220)*, (221)* of cubic Ag_2O (**Figures 1K,L**).

Impact of Ag^+ on Photosynthesis in Floating and Submerged Leaves

In view of large differences in photosynthetic activities between floating and submerged leaves (Shabnam et al., 2015), we examined the effects of Ag^+ on Photosystem II (PS II) efficiency of these leaves. Photosystem II efficiency (maximum quantum yield) is often determined as a ratio of variable Chl *a* fluorescence to maximum Chl *a*, i.e., F_v/F_m. Chl *a* fluorescence of oxygenic organisms shows a rise from a basal level (F_o) (i.e., minimum fluorescence) to the maximum (F_m) (Strasser et al., 1995; Stirbet and Govindjee, 2012; Shabnam et al., 2015, 2017). F_o and F_m values of both floating and submerged leaves declined significantly on exposure to Ag^+; the decline was significantly higher in the submerged leaves than in the floating ones (**Table 1**). Ag^+, like other heavy metal ions, brought about a significant decline in the quantum yield of PS II activity, as inferred from F_v/F_m values, in both floating and submerged leaves (**Figure 2**). However, at any given concentration, the decline in F_v/F_m was significantly higher in the submerged leaves.

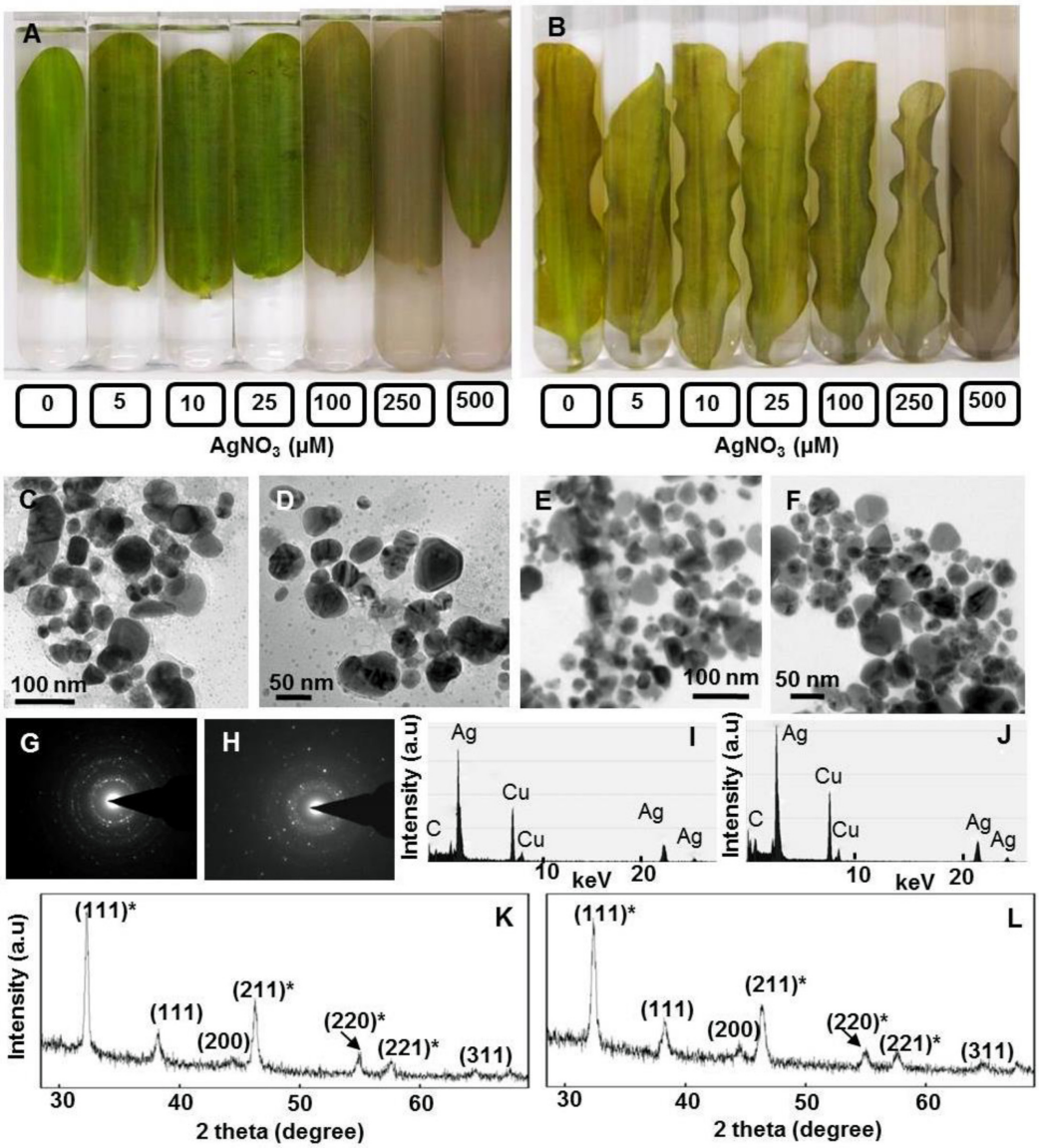

FIGURE 1 | Potential of floating and submerged leaves of longleaf pondweed (*Potamogeton nodosus*) to generate Ag-NPs. Photographs were taken after 24 h exposure of floating **(A)** and submerged **(B)** leaves to varying concentrations of $AgNO_3$ (in μM) in Borosil dishes (190 mm diameter × 100 mm height, in order to ensure that floating leaves remain floating and submerged leaves remain submerged in respective test solution during the course of exposure to $AgNO_3$). TEM **(C–F)**, SAED pattern **(G,H)**, EDX **(I,J)** and PXRD patterns **(K,L)** of Ag^0/Ag_2O-NPs generated by floating **(C,D,G,I)** and submerged leaves **(E,F,J,L)** of longleaf pondweed. PXRD patterns **(K,L)** show Bragg reflections specific to crystalline face-centered cubic structure of Ag^0 (in parenthesis without star) and cubic structure of Ag_2O (in parenthesis with star).

Figure 3 shows the fast (up to a second) polyphasic Chl *a* fluorescence transients of floating and submerged leaves which were exposed to different Ag^+ levels. All oxygen-evolving organisms show polyphasic Chl *a* fluorescence transients (also called the OJIP curves) with distinct O, J, I, and P steps (**Figure 3**). In these curves, "O" is the minimum fluorescence (F_o), "P" is the peak (F_m), and "J" and "I" are intermediate levels. The polyphasic rise of Chl *a* fluorescence transient was severely reduced in both floating and submerged leaves, exposed to Ag^+ (**Figure 3**), which is in agreement with the decline in F_v/F_m. The decline in the amplitude of fluorescence was significantly higher in submerged leaves than in floating leaves, revealing superior potential of the latter to tolerate Ag^+ than the former. Extreme sensitivity of submerged leaves to Ag^+-toxicity was also evident from the loss in the polyphasic nature of Chl *a* fluorescence transients even at concentration as low as 5 μM of Ag^+.

Chlorophyll *a* fluorescence kinetics is affected by such factors as Chl content. Therefore, we evaluated the impact of 24 h Ag^+

TABLE 1 | Variations in F_o (the minimum fluorescence) and F_m (maximum fluorescence) of floating and submerged leaves of longleaf pondweed (*Potamogeton nodosus*) exposed to different concentrations of $AgNO_3$.

Ag^+ (μM)	F_o		F_m	
	Floating	Submerged	Floating	Submerged
0	302 ± 29.6^a (100)	468 ± 26.1^a (100)	1355 ± 87.7^a (100)	1272 ± 101.8^a (100)
5	255 ± 18.5^a (84.4)	257 ± 24.3^b (54.9)	936 ± 68.3^b (69.1)	377 ± 21.1^b (29.6)
10	238 ± 21.6^{ab} (78.8)	253 ± 19.7^b (54.1)	549 ± 39.9^c (40.5)	391 ± 19.8^b (30.7)
50	257 ± 11.7^a (85.1)	250 ± 17.5^b (53.4)	501 ± 42.4^c (36.9)	299 ± 23.7^c (23.5)
100	213 ± 13.3^b (70.5)	248 ± 11.8^b (52.9)	365 ± 24.5^d (26.9)	311 ± 28.5^c (24.4)
250	162 ± 11.3^c (53.6)	258 ± 26.7^b (55.1)	160 ± 11.1^e (11.8)	245 ± 16.9^d (19.3)
500	152 ± 11.1^c (50.3)	142 ± 11.5^c (30.3)	144 ± 8.9^e (10.6)	128 ± 9.6^e (10.1)

Data are a mean of recordings from six independent experiments. Data represent mean ± standard error. Values followed by the same small letter (in superscript) within a column do not differ significantly at $P \leq 0.05$ level (Duncan's multiple range test). Values in parenthesis represent the percent change over respective controls.

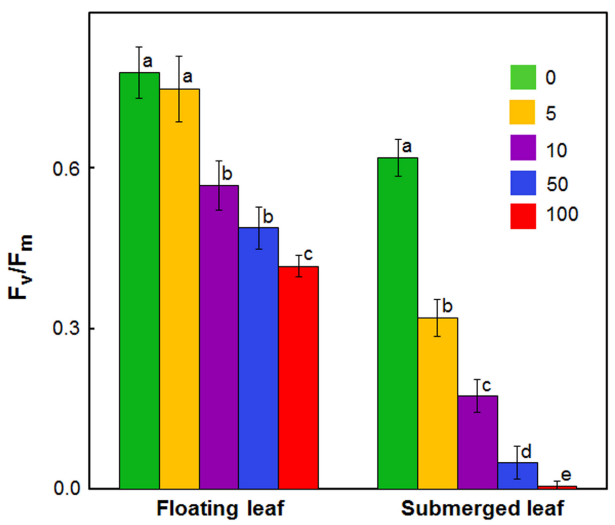

FIGURE 2 | Impact of Ag^+ on the quantum yield of Photosystem II activity (as inferred from F_v/F_m; see text) of floating and submerged leaves of longleaf pondweed (*P. nodosus*) after 24 h exposure to varying concentrations of $AgNO_3$ (μM). Data are a mean of recordings from six independent experiments. Vertical lines on data points represent standard errors. Different small letters over the bars reflects that the values recorded for leaves (floating or submerged) exposed to different concentrations of $AgNO_3$ do not differ significantly at $P \leq 0.05$ level (Duncan's multiple range test).

treatment on both Chl *a* and Chl *b* levels. Decline of these Chls was recorded in both floating and submerged leaves, as a function of silver concentration (**Table 2**). However, the decline in the levels of both Chl *a* and Chl *b* levels were significantly lower in the floating leaves. Interestingly, decline in levels of Chl *a* was significantly higher than that of Chl *b* in both floating and submerged leaves.

Figure 4 depicts impact of 24 h Ag^+ treatment on carboxylase activity of Rubisco in floating and submerged leaves. Upon exposure to 10 μM Ag^+, the carboxylase activity of Rubisco declined by 50% in the submerged leaves, whereas it remained unaltered in the floating ones. However, 100 μM Ag^+ caused ∼50 and ∼90% decline in Rubisco activity in the floating and submerged leaves, respectively (**Figure 4**).

Levels of Ag in Floating and Submerged Leaves

Since there was a significant variation in the impact of Ag^+ on photosynthetic efficiency between floating and submerged leaves, we measured Ag content in these leaves. Both floating and submerged leaves of longleaf pondweed exposed to Ag^+ showed the presence of Ag. The level of Ag in these leaves increased as a function of Ag^+ concentration to which they were exposed (**Figure 5A**). At any Ag^+ concentration, silver content in submerged leaves was ∼3 times higher than that in floating ones. XPS analysis also confirmed the presence of Ag in both leaves. XPS spectra showed two peaks at binding energies of 368 and 374 eV (**Figures 5B,C**), which arise due to the emission of $3d_{5/2}$ and $3d_{3/2}$ photoelectrons, respectively (Adegboyega et al., 2013).

Impact of Ag^+ on Antioxidant System in Floating and Submerged Leaves

Like our earlier findings (Shabnam and Pardha-Saradhi, 2016), we did not observe any significant variation in the levels of ascorbate, phenolics, and thiols amongst floating and submerged leaves which were not exposed to Ag^+. However, both floating and submerged leaves exposed to 10 and 100 μM Ag^+ showed a decline in the levels of all these three non-enzymatic antioxidants (**Figure 6**). Irrespective of the Ag^+ concentrations to which leaves were exposed, the decline in the level of phenolics was significantly higher in the submerged leaves (**Figure 6A**). However, the decline in the levels of ascorbate and thiols was almost similar for both leaves (**Figures 6B,C**).

Antioxidant enzymes, such as SOD, catalase (CAT), GPX, ascorbate peroxidase (APX), MDHAR, dehydroascorbate reductase (DHAR), and GR play an important role in scavenging ROS in plants exposed to heavy metals (Prasad et al., 1999; Dhir et al., 2009). Therefore, we evaluated the impact of Ag^+ on activities of these enzymes in both floating and submerged leaves of longleaf pondweed. Ag^+ treatment caused a significant decline in SOD activity in both floating and submerged leaves, although the decline was significantly higher in the latter compared to the former (**Figure 7A**). Both floating and submerged leaves, with the exception of floating leaves exposed to 10 μM Ag^+, showed a significant decrease in the catalase activity compared to their respective controls (**Figure 7B**). However, the degree of loss in catalase activity was higher in submerged leaves. Contrary to decreased activity of SOD and catalase, activity of GPX increased by 2–2.5-fold in submerged leaves exposed to Ag^+ (**Figure 7C**). However, floating leaves showed a decrease in GPX activity on exposure to Ag^+.

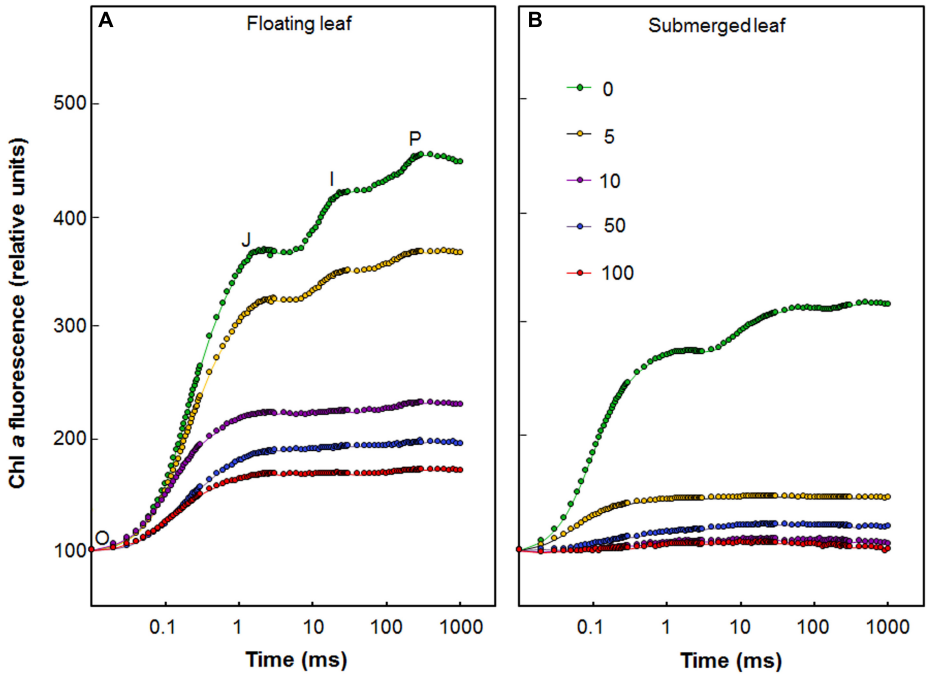

FIGURE 3 | Effect of Ag^+ on chlorophyll *a* fluorescence transient, the OJIP curve (see text), of floating and submerged leaves of longleaf pondweed (*P. nodosus*) after 24 h exposure to varying concentrations of $AgNO_3$ (in μM). Chl *a* fluorescence induction curves of floating **(A)** and submerged **(B)** leaves of longleaf pondweed exposed to varying concentrations of $AgNO_3$ (in μM) for 24 h. Chl *a* fluorescence induction curves were plotted by normalizing data at the F_o values.

TABLE 2 | Variation in Chl *a* and Chl *b* levels (μg g^{-1} fresh weight) in floating and submerged leaves of longleaf pondweed (*P. nodosus*) exposed to different concentrations of $AgNO_3$.

Ag^+ (μM)	Chl *a*		Chl *b*	
	Floating	Submerged	Floating	Submerged
0	626 ± 33.3^a (100)	490 ± 27.3^a (100)	492 ± 21.5^a (100)	471 ± 20.1^a (100)
5	598 ± 28.9^a (95.5)	438 ± 30.3^a (89.4)	486 ± 26.7^a (98.8)	429 ± 25.4^a (91.1)
10	492 ± 31.1^b (78.6)	368 ± 23.7^c (75.1)	391 ± 23.3^b (79.4)	367 ± 26.7^b (77.9)
50	431 ± 23.3^b (68.8)	298 ± 16.9^d (60.8)	381 ± 18.4^b (77.4)	342 ± 19.5^{bc} (72.6)
100	401 ± 23.5^{bc} (64.1)	214 ± 21.1^e (43.7)	368 ± 25.4^{bc} (74.7)	273 ± 20.3^c (57.9)
250	369 ± 19.7^c (58.9)	184 ± 14.5^e (37.6)	340 ± 16.7^c (69.1)	242 ± 14.7^c (51.3)
500	227 ± 20.8^d (36.2)	144 ± 9.1^f (29.4)	298 ± 17.4^d (60.6)	191 ± 16.7^d (40.5)

Data are a mean of recordings from six independent experiments. Data represent mean ± standard error. Values followed by same small letter (in superscript) within a column do not differ significantly at $P \leq 0.05$ level (Duncan's multiple range test). Values in parenthesis represent the percent change over respective controls.

Activities of enzymes of ascorbate-glutathione cycle, e.g., APX, MDHAR, DHAR, and GR, decreased significantly in both floating and submerged leaves as a function of given Ag^+ level (**Figure 8**). In general, irrespective of the concentration of Ag^+ used, the activities of ascorbate-glutathione cycle enzymes were significantly higher in floating leaves. Amongst enzymes of ascorbate-glutathione cycle, Ag^+ induced highest decrease in the activity of GR, followed by those of APX, MDHAR, and DHAR.

DISCUSSION

Leaves of Longleaf Pondweed Generate Ag-NPs as a Defense Mechanism

Earlier, Pardha-Saradhi et al. (2014a,b,c) reported that plants reduce toxic ionic forms of heavy metals into non/less-toxic NPs as a defense mechanism. During our investigations, we found that both floating and submerged leaves of longleaf pondweed could turn clear colorless $AgNO_3$ solutions to colloidal brown (**Figures 1A,B** and Supplementary Figure 1). TEM coupled with SAED and EDX confirmed the presence of distinct crystalline NPs composed of Ag in these colloidal brown solutions (**Figures 1C–F**). However, similar to our earlier findings (Pardha-Saradhi et al., 2014a), these brown colloidal solutions did not show any Ag-NP specific peak in the absorption spectra. In addition to SAED, PXRD confirmed the crystalline nature of Ag-NPs; PXRD pattern showed peaks specific to face centered cubic structured Ag^0 and cubic structured Ag_2O. These PXRD analyses clearly showed that Ag-NPs generated by floating and submerged leaves are composed of both Ag^0 and Ag_2O. XPS analysis confirmed the presence of Ag in both leaves and that the accumulated Ag in these leaves existed predominantly as

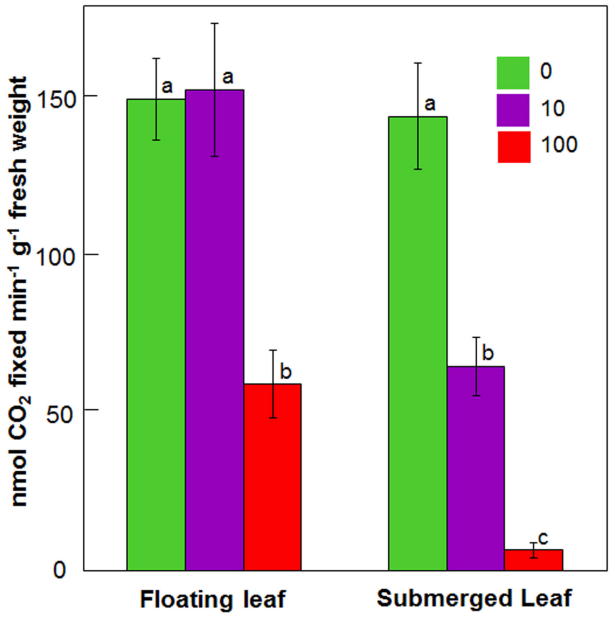

FIGURE 4 | Impact of Ag^+ on the carboxylase activity of Rubisco of floating and submerged leaves of longleaf pondweed (*P. nodosus*) after 24 h exposure to 10 or 100 μM $AgNO_3$. Data are a mean of recordings from six independent experiments. Vertical lines on bars represent standard errors. Different small letters over the bars reflect that the values recorded for leaves (floating or submerged) exposed to different concentrations of $AgNO_3$ do not differ significantly at $P \leq 0.05$ level (Duncan's multiple range test).

Ag_2O state. It is well-known that Ag^0 and Ag^0-NPs are prone to oxidation (Pardha-Saradhi et al., 2014a; Shabnam et al., 2016). Recently, Shabnam et al. (2016) demonstrated the potential of photosynthetic electron transport to reduce Ag^+ to Ag^0 and to generate Ag^0-NPs. They also specifically showed that O_2 released as a byproduct during photosynthetic electron transport promotes oxidation of Ag^0 and/or Ag^0-NPs to generate Ag_2O-NPs. Therefore, we believe that O_2 released by light harvesting photosynthetic machinery of leaves promotes oxidation of Ag^0 and/or Ag^0-NPs to generate Ag_2O-NPs.

As evident from the Supplementary Figure 1, only one side of floating leaves is in immediate contact with test solution and further, overall size (i.e., surface area) of submerged leaves is nearly double of floating leaves (Shabnam and Pardha-Saradhi, 2016). In spite of such a vast variation in the surface area in contact with the test solution, the color intensity of the $AgNO_3$ solutions incubated with floating leaves was higher than the ones incubated with submerged leaves. This reveals superior potential of floating leaves to generate Ag-NPs compared to submerged leaves. Superior potential of floating leaves to generate Ag-NPs might be due to their superior photosynthetic photochemical reactions (Shabnam et al., 2015; Shabnam and Pardha-Saradhi, 2016).

Inductive coupled plasma analysis revealed that submerged leaves exposed to Ag^+ possessed 3–4 fold higher levels of silver than floating leaves, which discloses that the uptake of Ag by the former leaves is significantly higher than the latter leaves. Higher levels of Ag in submerged leaves can be accounted to (i) larger surface area (as detailed above) available for uptake of Ag; and (ii) lower potential to reduce Ag^+ and generate Ag-NPs, compared to floating leaves. We have recently demonstrated that the uptake of Ag by plants of *Spirodela polyrhiza* in the ionic state is 3–4 times higher than in the NP state (Shabnam et al., 2017). Therefore, we believe that superior potential to efficiently reduce Ag^+ and generate Ag-NPs is one of the mechanisms acquired by floating leaves to curb the uptake of Ag.

Floating Leaves Possess Superior Potential to Protect Photosynthetic Machinery Against Ag^+-Toxicity Than Submerged Leaves

Photosystem II plays a vital role in photochemical reactions. Accordingly, overall photosynthetic capacity of plants often relies on PS II activity (Shabnam et al., 2015). Therefore, during the present investigations, we evaluated the impact of Ag^+ on PS II efficiency. F_v/F_m is a commonly used parameter to determine Photosystem II efficiency of plants (Strasser et al., 1995; Stirbet and Govindjee, 2012). PS II efficiency, measured in terms of F_v/F_m as well as through Chl *a* fluorescence kinetics, was significantly higher in floating leaves compared to submerged leaves, just as in our earlier findings (Shabnam et al., 2015).

In this paper, we found that Ag^+ caused a significant decline in PS II efficiency of both floating and submerged leaves of longleaf pondweed. Ag^+-induced decline in the quantum yield of PS II activity has also been reported in submerged *P. crispus*, as well as in free floating *S. polyrhiza* (Xu et al., 2010; Jiang et al., 2012; Shabnam et al., 2017). However, Ag^+-induced suppression in PS II efficiency was significantly lower in the floating leaves compared to that in the submerged leaves. These findings unequivocally demonstrate the prevalence of superior mechanism(s) in floating leaves to counter Ag^+.

As mentioned earlier, all oxygen-evolving organisms show polyphasic Chl *a* fluorescence transients with distinct O-J, J-I, and I-P photochemical phases (**Figure 3**). While O-J rise (0.05–2 ms) involves the reduction of Q_A to Q_A^-, the J-I rise (2–30 ms) denotes reduction of PQ pool and the I-P rise (30–300 ms) implies reduction of the acceptor side of PS I (Strasser et al., 1995; Stirbet and Govindjee, 2012; Hamdani et al., 2015; Shabnam et al., 2015, 2017). The OJIP transient kinetics are highly sensitive to various stresses including heavy metal stress (Appenroth et al., 2001; Oukarroum et al., 2012; Shabnam et al., 2015, 2017). During this study, we observed a drastic negative impact of Ag^+ on the OJIP transients in longleaf pondweed leaves, even at a concentration of 5 μM. Negative impact of Ag^+ on the OJIP transients was significantly higher in the submerged leaves. As shown in **Figure 3**, while floating leaves retain polyphasic nature of the OJIP transients, the submerged leaves showed a complete loss in the polyphasic nature of this transient on exposure to 5 μM Ag^+. Severe loss in fluorescence intensity or polyphasic nature of OJIP transients has also been observed in several algae and plants exposed to heavy metals (Appenroth et al., 2001; Oukarroum et al., 2012; Wang et al., 2014; Shabnam et al., 2017). There are reports

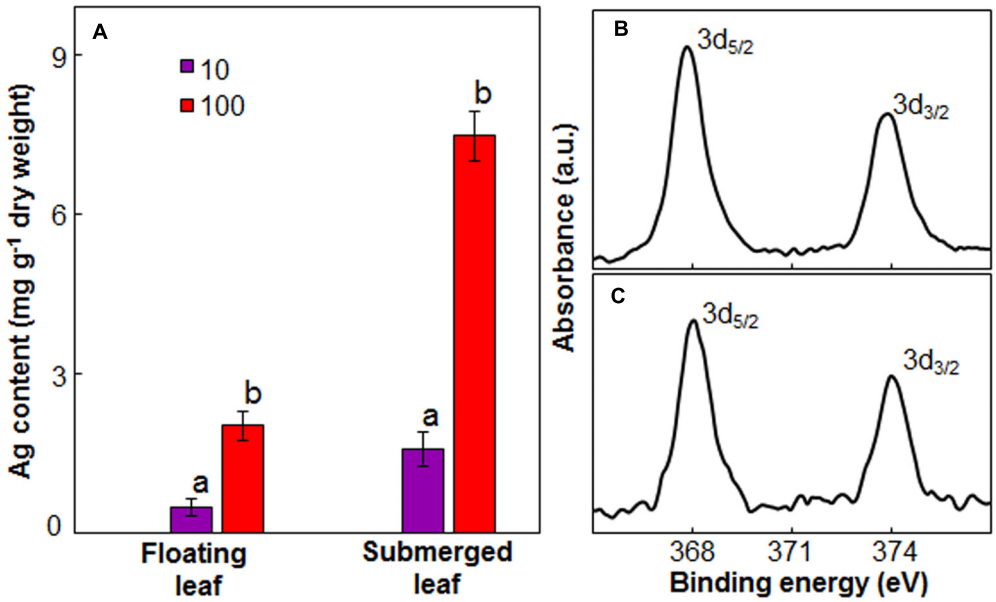

FIGURE 5 | Ag content in floating and submerged leaves of longleaf pondweed (*P. nodosus*) after 24 h exposure to 10 or 100 μM $AgNO_3$. **(A)** Bars are a mean of data from three independent experiments for different treatments. Vertical lines on data points represent standard errors. Different small letters over the bars reflect that the values recorded for leaves (floating or submerged) exposed to different concentrations of $AgNO_3$ do not differ significantly at $P \leq 0.05$ level (Duncan's multiple range test). High resolution XPS **(B,C)** of floating **(B)** and submerged **(C)** leaves showing presence of peaks specific to Ag_2O.

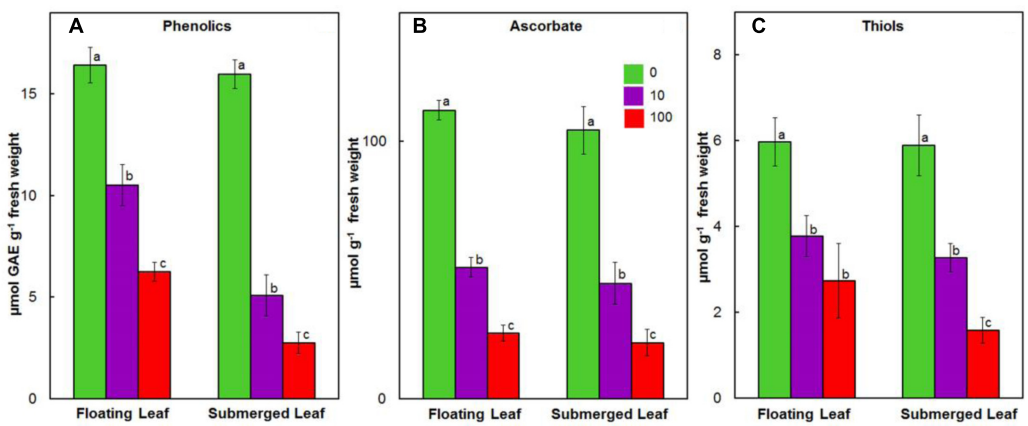

FIGURE 6 | Impact of Ag^+ on the levels of non-enzymatic antioxidants. Levels of phenolics **(A)**; ascorbate **(B)**; and thiols **(C)** in floating and submerged leaves of longleaf pondweed (*P. nodosus*) after 24 h exposure to 10 or 100 μM $AgNO_3$. Bars are a mean of data from six independent experiments for different treatments. Vertical lines on bars represent standard errors. Different small letters over the bars reflect that the values recorded for leaves (floating or submerged) exposed to different concentrations of $AgNO_3$ do not differ significantly at $P \leq 0.05$ level (Duncan's multiple range test).

of inhibition of the oxygen evolving complex (OEC) by metal ions such as Cd and Cr (see e.g., Atal et al., 1991). Severe negative impact of Ag^+ on the OJIP transients during this study suggests that Ag^+ could be inhibiting the OEC of PSII as well as the flow of electrons from Q_A^- to the electron acceptor side of PS I (via the PQ-pool), in both floating and submerged leaves of longleaf pondweed. Higher Ag content in submerged leaves could be responsible for a significantly higher decline in their photosynthetic efficiency, compared to that of floating leaves.

As noted earlier, Chl *a* fluorescence kinetics can be affected by such factors like Chl content. A 24-h Ag^+ treatment caused a decline in the levels of Chl *a* and Chl *b* in a concentration-dependent manner, in both floating and submerged leaves (**Table 2**). However, the decline in the levels of both Chl *a* and Chl *b* were significantly lower in the floating leaves. Our present findings clearly demonstrate that floating leaves are better equipped to protect their photosynthetic machinery against Ag^+-toxicity than submerged leaves. Superior potential of the floating leaves to withstand Ag^+ induced suppression of photosynthetic

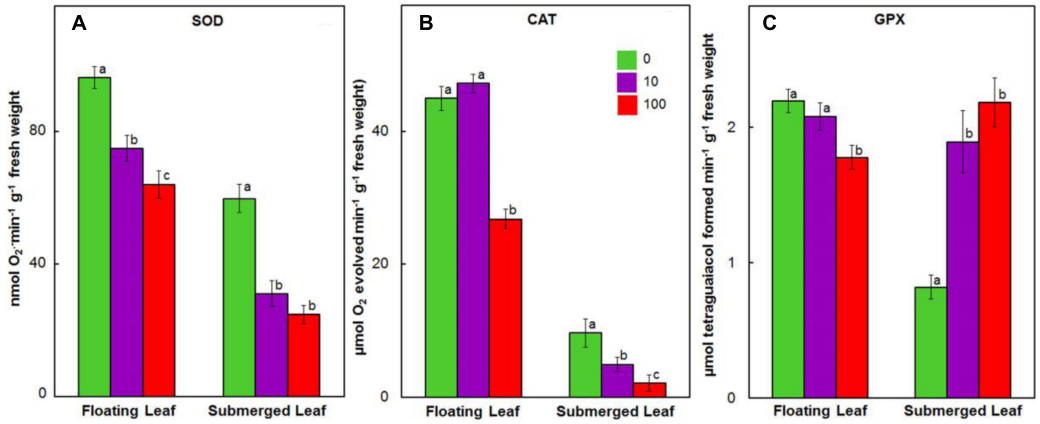

FIGURE 7 | Impact of Ag^{+} on the activities of antioxidant enzymes. Activity of SOD **(A)**; catalase (CAT) **(B)**, and guaiacol peroxidase (GPX) **(C)** in floating and submerged leaves of longleaf pondweed (*P. nodosus*) after 24 h exposure to 10 or 100 µM $AgNO_3$. Bars are a mean of data from six independent experiments for different treatments. Vertical lines on data points represent standard errors. Different small letters over the bars reflect that the values recorded for leaves (floating or submerged) exposed to different concentrations of $AgNO_3$ do not differ significantly at $P \leq 0.05$ level (Duncan's multiple range test).

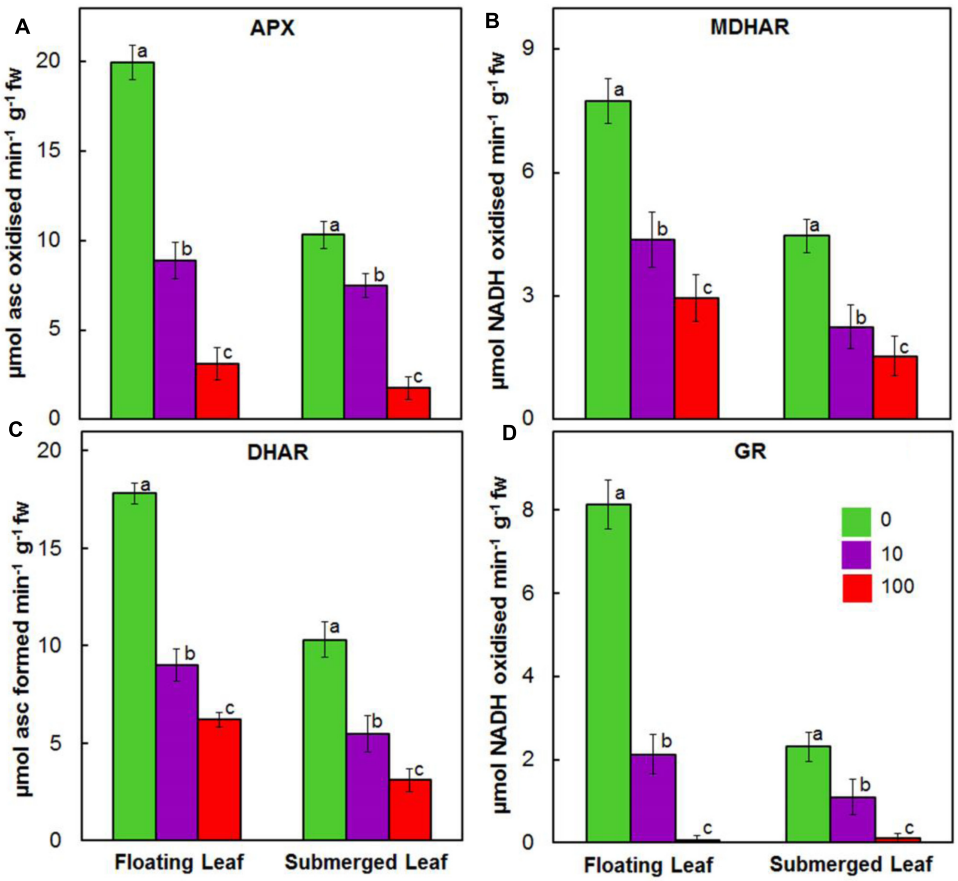

FIGURE 8 | Impact of Ag^{+} on the activities of antioxidant enzymes of ascorbate-glutathione cycle. Activity of ascorbate peroxidase (APX) **(A)**; monodehydroascorbate reductase (MDHAR) **(B)**; dehydroascorbate reductase (DHAR) **(C)**; and glutathione reductase (GR) **(D)** in floating and submerged leaves of longleaf pondweed (*P. nodosus*) after 24 h exposure to 10 or 100 µM $AgNO_3$. Data are a mean of recordings from six independent experiments. Vertical lines on data points represent standard errors. Different small letter over the bars reflects that the values recorded for leaves (floating or submerged) exposed to different concentrations of $AgNO_3$ do not differ significantly at $P \leq 0.05$ level (Duncan's multiple range test).

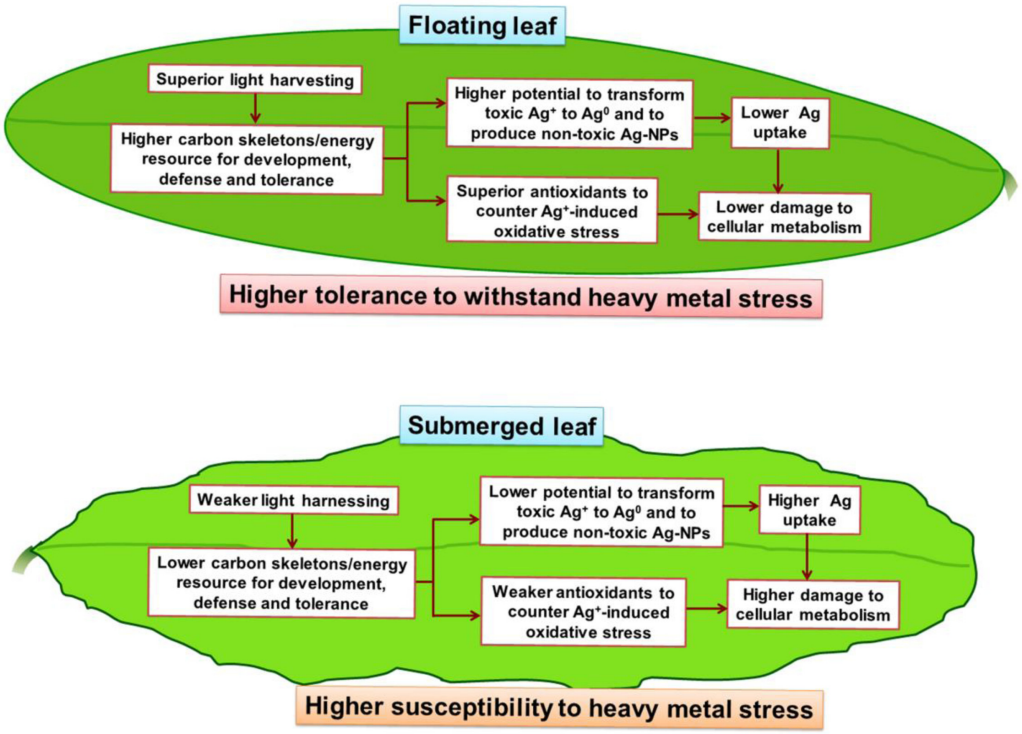

FIGURE 9 | A hypothetical model depicting the key metabolic events in floating and submerged leaves of longleaf pondweed (*P. nodosus*) influenced by Ag^+-stress. Note that the superior photosynthesis enables floating leaf to be better equipped with carbon skeletons and energy resources required to counter heavy metal stress than submerged leaves.

efficiency could be due to (i) higher potential to reduce Ag^+ to Ag-NPs at the surface, and (ii) restricted uptake of Ag compared to submerged leaves.

In spite of having similar carboxylase activity of Rubisco, the control submerged leaves possess lesser carbon skeletons compared to the control floating leaves. Higher carbon skeletons in floating leaves is due to significantly higher and efficient light harvesting photochemical reactions (pivotal for the generation of assimilatory power essential for CO_2 fixation and the synthesis of various carbon skeletons). Lower decline in carboxylase activity of Rubisco in floating leaves revealed that floating leaves are better equipped to protect the carboxylase activity of Rubisco than submerged leaves.

Decline in the carboxylase activity of Rubisco has been reported earlier in terrestrial plants, such as *Phaseolus vulgaris*, *Zea mays*, *Oryza sativa*, and *Citrus grandis,* exposed to Zn, Cd, and Mn (Van Assche and Clijsters, 1986; Krantev et al., 2008; Li et al., 2010; Wang et al., 2014) and aquatic plants, such as *Salvinia natans* and *Ceratopteris pteridoides,* exposed to Cr and Cd (Dhir et al., 2008; Deng et al., 2014). Ag^+ induced decline in PS II efficiency and carboxylase activity of Rubisco of these plants may be due to (i) ROS induced inactivation (Sharma and Dietz, 2009; Foyer and Noctor, 2016), (ii) enhanced proteolytic activity (Hajduch et al., 2001; Gajewska et al., 2013), (iii) interference with enzyme's structure by substitution of native ions (Van Assche and Clijsters, 1986) and/or interaction with SH groups (e.g., by Cu and Cd) (Stiborova, 1998; Stiborova et al., 1998), and (iv) impaired protein biosynthesis (Kremer and Markham, 1982). A simple comparison of the impact of Ag^+ on PSII efficiency with carboxylase activity of Rubisco reveals that the light harvesting photochemical reactions are more sensitive to Ag^+ than carbon fixation reactions.

Superior Antioxidant System of Floating Leaves Counters Ag^+-Toxicity

In general, heavy metals promote generation of ROS by interfering with electron transport and redox reactions (Prasad et al., 1999; Hall, 2002; Metwally et al., 2003; Sharma and Dietz, 2009; Pardha-Saradhi et al., 2014b). Accordingly, plants have evolved antioxidant systems, both non-enzymatic and enzymatic, to counter oxidative stress (Prasad et al., 1999; Shabnam and Pardha-Saradhi, 2016). Amongst the non-enzymatic antioxidants, ascorbate, phenolics and thiols play important roles in scavenging ROS and/or chelating heavy metals (Sakihama et al., 2002; Shabnam et al., 2014; Shabnam and Pardha-Saradhi, 2016). Plants exposed to heavy metal ions, such as Cd, Pb, and Zn, show enhanced levels of non-enzymatic antioxidants (Prasad et al., 1999; Oncel et al., 2000; Mishra et al., 2006). On the contrary, we observed a decline in the levels of all the non-enzymatic antioxidants in both floating and submerged leaves exposed to Ag^+ (**Figure 6**). Significantly lower Ag^+-induced decline in the content of phenolics in floating

leaves could be one of the factors contributing to their superior potential to withstand Ag^+-stress. Posmyk et al. (2009) also reported a decline in the phenolic content in red cabbage under Cu stress. A significant decline in the levels of ascorbate and/or thiols/GSH has been reported in studies with (i) submerged *P. crispus* plants exposed to Ag^+ (Xu et al., 2010); (ii) pigeon pea seedlings exposed to Ni^{2+} and Zn^{2+} (Rao and Sresty, 2000); (iii) roots of soybean exposed to Cd^{2+} (Balestrasse et al., 2001); and (iv) roots and shoots of maize exposed to Cd^{2+} (Tukendorf and Rauser, 1990; Meuwly and Rauser, 1992). Ag^+ has a strong affinity for thiols (Rao and Sresty, 2000; Blaske et al., 2013) like other heavy metals. A positive correlation has been established between the depletion of thiol content and the amount of metal ions (Cu^{2+}, Zn^{2+}) accumulated by plants (Tripathi et al., 2006). Thus, a decline in the levels of thiols, shown in this study, could be due to the binding of thiols to Ag^+. In general, decrease in the levels of non-enzymatic antioxidants could be due to (i) enhanced catabolic degradation, (ii) alteration in their structure via chelation or reduction of metal ions, and/or (iii) decreased biosynthesis.

Significantly higher activities of antioxidant enzymes in floating leaves impart superior potential to counter oxidative damage compared to submerged leaves. Ag^+ induced a significant decline in activity of SOD and catalase in both floating and submerged leaves; the decline was significantly higher in the latter (**Figure 7A**). Contrary to the decreased activity of SOD and catalase, the activity of GPX increased by 2–2.5-fold in the submerged leaves exposed to Ag^+ (**Figure 7C**). However, the floating leaves showed a decrease in GPX activity on exposure to Ag^+. In contrast to the decline in the activities of SOD and catalase, and an increase in GPX activity recorded in the submerged leaves of longleaf pondweed (present study), Xu et al. (2010) noted increase in activities of SOD and catalase, and a decline in the activity of GPX in submerged *P. crispus*, exposed to Ag^+. *Salvinia natans* exposed to Cr-rich water, however, showed an increase in GPX activity, without any significant alteration in CAT activity (Dhir et al., 2009). Interestingly, Shah et al. (2001) also noted enhancement of GPX activity accompanied with a decline in CAT activity in rice exposed to cadmium. Peroxidases play a significant role in the synthesis of lignin, which is impermeable to metal ions (Hegedüs et al., 2001; Parrotta et al., 2015). Therefore, we believe that an increased GPX activity in submerged leaves might be a strategy to restrict the uptake of Ag^+.

Silver ions also suppressed activities of enzymes of the ascorbate glutathione cycle in both types of the leaves. Ag^+ induced decline in the activities of antioxidant enzymes in both floating and submerged leaves, except GPX in the submerged leaves, which is in agreement with those measured in the roots of soybean and poplar exposed to Cd^{2+} (Balestrasse et al., 2001; Schutzendubel et al., 2002). A similar decline in the activities of several antioxidant enzymes was observed in cotyledons and leaves of sunflower seedlings under Cd^{2+}, Fe^{2+}, and Cu^{2+} stress (Gallego et al., 1996a,b). In addition, the potential of Ag^+ to displace native metal cations from their usual binding sites in enzymes has been reported (Ghandour et al., 1988). Ag^+ induced decline in the activity of the antioxidant enzymes might be due to the effect of Ag^+ on expression of the relevant genes at the transcriptional/translational level by binding with DNA/RNA. Further, this effect might be at the post-translational level. The binding of Ag^+ to SH and other active groups might alter 3-D structure of these antioxidant enzymes affecting the catalytic/active site(s) vital for their activities (Ghandour et al., 1988).

A summary of the differential impacts of Ag^+ on the floating and the submerged leaves of longleaf pondweed is presented in a hypothetical model (**Figure 9**). Superior photosynthesis in floating leaves leads to production of more carbon skeletons and energy resources compared to that in submerged leaves. Accordingly, floating leaves are better equipped to counter/tolerate stress imposed by heavy metals, such as Ag^+. This includes (i) a superior capacity to biotransform toxic ionic state of heavy metals (such as Ag^+) into less/non-toxic NPs (such as Ag^0/Ag_2O-NPs); and (ii) a better capacity to counter oxidative stress through a superior antioxidant system. In addition, significantly higher levels of Ag accumulated in submerged leaves would also directly interfere with their cellular metabolism.

CONCLUSION

In this paper, we have demonstrated for the first time that the floating leaves of longleaf pondweed possess a significantly higher potential to withstand Ag^+-toxicity compared to that in the submerged leaves due to (i) superior photosynthetic machinery and an antioxidant system, (ii) superior potential to reduce Ag^+ to Ag^0 and generate Ag-NPs (Ag^0/Ag_2O-NPs) on their surface; and (iii) superior potential to restrict uptake of Ag. Our findings suggest that any effort made to increase the proportion of floating leaves to the submerged leaves in longleaf pondweed would be beneficial for apt detoxification of water bodies contaminated with heavy metal ions.

AUTHOR CONTRIBUTIONS

The conception or design of the work: PP-S, NS, and HK. The acquisition, analysis: NS, PS, PP-S, and HK. Interpretation of data for the work: NS, PS, PP-S, HK, and G. Drafting the work: NS, PS, and PP-S. Revising it critically for important intellectual content: NS, PS, PP-S, HK, and G. Final approval of the version to be published: NS, PS, PP-S, HK, and G. Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved: NS, PS, PP-S, HK, and G.

FUNDING

This study was supported by Department of Biotechnology, Ministry of Science and Technology [Award Number: BT/Bio-CARe/02/604/2011-12 (PS)]; University of Delhi [Award

Number: RC/2015/9677 (PP-S)]; Korea Environmental Industry and Technology Institute [Award Number: 2015001790002 (HK)].

ACKNOWLEDGMENTS

Financial support from the Department of Biotechnology (Government of India), University of Delhi and the Korea Environmental Industry and Technology Institute is gratefully acknowledged. G thanks the Departments of Biochemistry and Plant Biology, University of Illinois at Urbana-Champaign for support.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.01052/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

PERSPECTIVE
published: 19 May 2017
doi: 10.3389/fpls.2017.00832

Nanoparticles Alter Secondary Metabolism in Plants via ROS Burst

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The particles within the size range of 1 and 100 nm are known as nanoparticles (NPs). NP-containing wastes released from household, industrial and medical products are emerging as a new threat to the environment. Plants, being fixed to the two major environmental sinks where NPs accumulate — namely water and soil, cannot escape the impact of nanopollution. Recent studies have shown that plant growth, development and physiology are significantly affected by NPs. But, the effect of NPs on plant secondary metabolism is still obscure. The induction of reactive oxygen species (ROS) following interactions with NPs has been observed consistently across plant species. Taking into account the existing link between ROS and secondary signaling messengers that lead to transcriptional regulation of secondary metabolism, in this perspective we put forward the argument that ROS induced in plants upon their interaction with NPs will likely interfere with plant secondary metabolism. As plant secondary metabolites play vital roles in plant performance, communication, and adaptation, a comprehensive understanding of plant secondary metabolism in response to NPs is an utmost priority.

Keywords: nanoparticles, nanopollution, reactive oxygen species, antioxidant enzymes, signaling pathways, plant secondary metabolism

OPEN ACCESS

Edited by:
Raquel Esteban, University of the Basque Country, Spain

Reviewed by:
Oren Shelef, University of Nevada, Reno, United States
Basil J. Nikolau, Iowa State University, United States

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *29 January 2017*
Accepted: *03 May 2017*
Published: *19 May 2017*

Citation:
Marslin G, Sheeba CJ and Franklin G (2017) Nanoparticles Alter Secondary Metabolism in Plants via ROS Burst. Front. Plant Sci. 8:832. doi: 10.3389/fpls.2017.00832

INTRODUCTION

The National Science Foundation (NSF) projects that the global market for products incorporating nanotechnology could amount to three trillion USD by 2020 (Roco, 2011). Currently, more than 1000 commercial products containing nanoparticles (NPs) are available in the market (Vance et al., 2015). The NPs commonly found in household, industrial and healthcare products are Au (Gold), Ag (silver), ZnO (zinc oxide), CuO (copper oxide), TiO_2 (titanium dioxide), Fe_3O_4/Fe_2O_3 (iron oxides), and CeO_2 (cerium oxide). Similarly, incorporation of Ag, ZnO, TiO_2, and SiO_2 (silicon dioxide) NPs into agrochemicals (pesticides, fungicides, herbicides, fertilizers, etc.) is expected to have great potential in nanotechnology-driven smart agriculture (DeRosa et al., 2010; Khot et al., 2012; Parisi et al., 2015; Boxi et al., 2016; Fraceto et al., 2016). The expanding applications of nanotechnology in domestic, industrial and agricultural sectors are also increasing the possibilities of NPs reaching the environment as nanomaterial-containing wastes. As the consequences of NP pollutants reaching the environment in significant quantities are unknown, understanding the plant's response to NPs is an intensive area of research.

Most studies with NPs indicated a certain degree of phytotoxicity, especially at high concentrations (Miralles et al., 2012). Depending on their size, NPs can enter plant cells from the apoplast, crossing the plasma membrane via endocytosis; subsequently they can be translocated from one part to another through symplastic flow (Rico et al., 2011). There is also evidence for the transport of NPs into subcellular organelles such as the nucleus, plastids, and vacuoles (Chichiriccó and Poma, 2015; Da Costa and Sharma, 2016).

Arabidopsis thaliana (L.) Heynh seedlings grown on soil treated with ZnONPs were observed to have reduced growth, chlorophyll content and rates of photosynthesis (Wang et al., 2016). These effects were concentration dependent with growth compromised 20 and 80%, respectively, with 200 and 300 mg/L treatments. At 300 mg/L, the chlorophyll content, net rate of photosynthesis, leaf stomatal conductance, intercellular CO_2 concentration and transpiration rate were all reduced more than 50%. Similarly, an increasing concentration (0, 2.5, 10, 50, 100, and 1,000 mg/L) of CuONPs negatively affected *Oryza sativa* L. seedling growth in a hydroponic system (Da Costa and Sharma, 2016). Photosynthetic rate, transpiration rate, stomatal conductance, maximal quantum yield of PSII photochemistry, and photosynthetic pigment contents declined, with a complete loss of PSII photochemical quenching at 1,000 mg/L. ZnONPs inhibited the expression of genes involved in chlorophyll synthesis and photosystem structure (Wang et al., 2016). Accumulation of CuONPs in the chloroplasts was accompanied by a lower number of thylakoids per granum (Da Costa and Sharma, 2016). AgNPs inhibited Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and the photo-protective capacity of PSII in the model aquatic higher plant *Spirodela polyrhiza* (L.) Schleid (Jiang et al., 2017).

In addition to reduced photosynthetic rates the growth inhibition caused by NPs has also been associated with increased oxidative stress (Da Costa and Sharma, 2016; Li et al., 2016; Jiang et al., 2017). However, whether the arrest of photosynthesis or the induction of oxidative stress is the dominant impact of NPs is a subject of debate, since both of them go hand in hand (Aarti et al., 2006). Although the accumulation of NPs in chloroplasts and damage to the photosynthetic apparatus (Da Costa and Sharma, 2016; Jiang et al., 2017) supports the former, the fact that to reach the chloroplast NPs must cross the plasma membrane, where they can induce reactive oxygen species (ROS) via NADPH oxidases (Sosan et al., 2016) argues the reverse. ROS production, damage to the membrane structure and function, and fluctuation in antioxidant enzymatic activities are documented across plant species as common responses to NPs (Thwala et al., 2013; Vannini et al., 2013; Fu et al., 2014; Mirzajani et al., 2014; Hossain et al., 2015; Xia et al., 2015; Jiang et al., 2017; Tripathi et al., 2017). A few studies have also demonstrated that treatment of plants and photosynthetic microorganisms with NPs resulted in increased production of phenolics (Comotto et al., 2014; Ghorbanpour and Hadian, 2015; Večeřová et al., 2016), which might act as antioxidants to scavenge the ROS (Dixon and Paiva, 1995; Franklin et al., 2009).

The possibility of NP-induced disturbance in ROS homeostasis and associated signaling pathways as a major factor underlying the changes in plant secondary metabolism is explored in this perspective.

"OXIDATIVE STRESS"- A COMMON RESPONSE OF PLANT TO NPs TREATMENT

Oxidative burst has been consistently reported in plants exposed to toxic levels of NPs (Thwala et al., 2013; Hossain et al., 2015; Xia et al., 2015). Exposure to various NPs, for example Ag, ZnO, and Al_2O_3 (aluminum oxide), also induced reactive nitrogen species (*NO, nitric oxide) and H_2O_2 in duckweed (Thwala et al., 2013), corn (Zhao et al., 2012) and tobacco bright yellow (BY2) cells (Poborilova et al., 2013). In tobacco BY2 cells, Al_2O_3NPs also induced the production of superoxide anion ($O\cdot_2^-$), one of the highly reactive forms of ROS. Although it is debated whether ROS activation stems, actually, from intact particles or, rather, from ions released from NPs, recent studies supports the latter. In *S. polyrhiza*, internalized Ag, regardless of whether the exposure was Ag^+ ions or AgNPs, had the same capacity to generate ROS supporting the hypothesis that intracellular AgNPs dissociate into highly toxic Ag^+ ions (Jiang et al., 2017). Similarly, dissolution of ZnO, CuO, and CeO_2 (cerium oxide) into their respective ions (Zn^{2+}, Cu^{2+}, or Ce^{4+}) has been established in other studies (Ebbs et al., 2016; Bradfield et al., 2017).

The mechanisms through which NPs induce ROS production and trigger oxidative stress at the cellular level have also been investigated. AgNPs triggered Ca^{2+} and ROS signaling through the induction of Ca^{2+}-permeable pores and direct oxidation of apoplastic L-ascorbic acid (Sosan et al., 2016). *A. thaliana root hair defective 2* (*rhd2*) mutant lacking NADPH oxidase RBOHC showed a significantly lower level of ROS generation in response to AgNPs compared with wild type plants (Sosan et al., 2016), indicating that the accumulation of ROS in cells is mediated by plasma membrane-bound NADPH oxidases (RBOH) enzymes that produce ROS at the apoplast (Mittler, 2017). On the other hand, chloroplastic ROS generation was observed in *S. polyrhiza*, based on the ability of AgNPs to inhibit Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and the photo-protective capacity of PSII (Jiang et al., 2017).

A common consequence of harmful levels of ROS is the damage to cellular macromolecules including membrane lipids that leads to cell death (Van Breusegem and Dat, 2006). Growth inhibition coupled with lipid peroxidation has been reported in *O. sativa* seedlings treated with 0.5, 1.0, and 1.5 mM CuONPs (Shaw and Hossain, 2013) and in 5 mg/L TiO_2NPs treated *Nitzschia closterium* (Xia et al., 2015). NPs could also damage other macromolecules like DNA. AgNPs and AuNPs affected cell division in *Allium cepa* L. root tip cells (Kumari et al., 2009; Rajeshwari et al., 2016), the former causing chromatin bridge, chromosomal stickiness, disturbed metaphase, multiple chromosomal breaks, and cell disintegration (Kumari et al., 2009). DNA damage, mitochondrial dysfunction, and cell

apoptosis were also observed in eggplant, as a consequence of oxidative stress induced by Co_3O_4 (Faisal et al., 2016).

In order to mitigate the effects of oxidative stress plants activate both enzymatic and non-enzymatic antioxidant defense machinery to scavenge excess ROS (Sewelam et al., 2016). Correspondingly, NP-mediated stress also activates plant's antioxidant machinery/enzymes. Briefly, superoxide dismutase (SOD) that catalyzes detoxification of $O\cdot_2^-$ into either ordinary molecular oxygen (O_2) or H_2O_2 and ascorbate peroxidase (APX), which detoxifies peroxides such as H_2O_2 using ascorbic acid (Asc) as a substrate, were up-regulated in plants upon treatment with NPs (Fu et al., 2014). Whereas, dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDAR) enzymes that regulate the cellular Asc redox state were downregulated (Fu et al., 2014). Proteomic analysis of AgNPs treated *O. sativa* roots revealed an increased abundance of SOD, APX, and glutathione-*S*-transferase (GST) (Mirzajani et al., 2014). These NPs also stimulated the activities of SOD and APX significantly, while inhibiting glutathione reductase (GR) and DHAR in *Pisum sativum* L. seedlings (Tripathi et al., 2017). Catalase (CAT), another enzyme that protects the cells from oxidative damage, was significantly elevated upon treatment of wheat roots with 500 mg/kg CuONPs (Dimkpa et al., 2012). Maize plants germinated and grown on soil amended with 0, 400, and 800 mg/kg CeO_2NPs showed a concentration dependent increase in the accumulation of H_2O_2 when tested after 10 days, but on day 20 did not show any difference (Zhao et al., 2012). A similar pattern in the increase of CAT and APX activities protected CeO_2NP treated maize seedlings from lipid peroxidation (Zhao et al., 2012).

As disruption of ROS homeostasis impairs plant growth and development, whereas maintenance of ROS levels within appropriate parameters promotes plant health (Mittler, 2017), it is emerging that the induction of antioxidant machinery by NPs might promote plant growth as reported in a few studies (Sharma et al., 2012; Burman et al., 2013; Kumar et al., 2013) as long as a harmful level of ROS is not reached in the cells, whereas, once breached, this may lead to impaired organelle function, membrane damage, and eventually phytotoxicity.

"NP-INDUCED ROS"- CAN IT BE AN INDUCTIVE SIGNAL FOR PLANT SECONDARY METABOLISM?

So far, a handful of studies have showed that NPs could affect microbial and plant secondary metabolism. For example, the concentration of phenolic compounds secreted to an extracellular medium was increased 127.5 and 22.1%, respectively, in *Arthrospira platensis* Gomont (cyanobacterium) and *Haematococcus pluvialis* Flotow (microalga) after treating with 100 mg/L TiO_2NPs (Comotto et al., 2014). Artemisinin content was increased 3.9-fold in *Artemisia annua* L. hairy root cultures after 900 mg/L AgNPs treatment for 20 days (Zhang et al., 2013). This increase was associated with oxidative stress (H_2O_2 production), lipid peroxidation and CAT activity. A substantial increase in plant growth and diosgenin concentration was observed in fenugreek after 2 μg/kg AgNP treatment (Jasim et al., 2017). Ferulic acid and isovitexin were increased in barley plants exposed to CdO (cadmium oxide) NPs in air for 3 weeks at a concentration of $2.03 \pm 0.45 \times 10^5$ particles cm^{-3} (Večeřová et al., 2016). In *A. thaliana*, anthocyanin and flavonoid biosynthetic genes were upregulated in response to AgNPs (Garcia-Sanchez et al., 2015).

Although all the studies discussed above provide evidence for NP-mediated modulation of plant secondary metabolism, the following studies provide an indirect link between ROS and secondary metabolism. *Satureja khuzestanica* Jamzad calli growth improved significantly with increasing concentrations of carbon nanotubes (CNTs) in culture medium up to 50 mg/L, and then began to decrease at 500 mg/L (Ghorbanpour and Hadian, 2015). At this toxic concentration (500 mg/L), the highest level of H_2O_2 was observed together with significantly higher polyphenol oxidase (PPO), peroxidase (POD), and secondary metabolic activities. Similarly, when *A. thaliana* was exposed to 250 and 1000 mg/L CeO_2 and indium oxide (In_2O_3) NPs, in addition to excessive ROS production, the activities of phenylalanine ammonia lyase (PAL) and PPO were greatly induced (Ma et al., 2016) revealing a possible role of secondary metabolism in protection against oxidative stress. Furthermore, PAL is the first enzyme of the general phenylpropanoid pathway that catalyses the deamination of phenylalanine to cinnamic acid and play a key role in diverting aromatic amino acids from primary metabolism to phenylpropanoid pathway.

There are several lines of evidence available in the literature implicating ROS-mediated signaling events as inductive cues for plant secondary metabolism. ROS themselves are signaling molecules, capable of inducing plant secondary metabolism (Simon et al., 2010). This could be observed during the wound-induced activation of secondary metabolism where ROS plays a key role as signaling molecule (Jacobo-Velazquez et al., 2015). In addition, ROS can also serve as signals for other messengers like jasmonic acid (JA) (Wu and Ge, 2004), salicylic acid (SA) (Maruta et al., 2012; Noshi et al., 2012; Wrzaczek et al., 2013; Baxter et al., 2014), ethylene (ET) (Zhang et al., 2016a,b), NO (Wang et al., 2013; Lindermayr and Durner, 2015), brassinosteroids (BRs) (Xia et al., 2009), etc., which are capable of modulating secondary metabolisms directly or indirectly.

To support the notion that ROS induced by NPs acts as signals for secondary metabolism, many indirect lines of evidence are available. ZnONP treatment induced SA, whereas it suppressed JA in *A. thaliana* (Vankova et al., 2017). Moreover, SA-mediated systemic acquired resistance (SAR) against microbial pathogens was compromised in *A. thaliana* after treatment with Ag, TiO_2NPs, and CNTs, resulting in an increased colonization by *Pseudomonas syringae* pv. tomato, *Pst* (Garcia-Sanchez et al., 2015). These authors further suggested that SA pathway repression is a common feature of NP exposure, as an inducible kinase in the pathway that activates basal immune response upon perception of bacterial flagellin namely FLG22-induced receptor-like kinase 1 (FRK1) was downregulated in

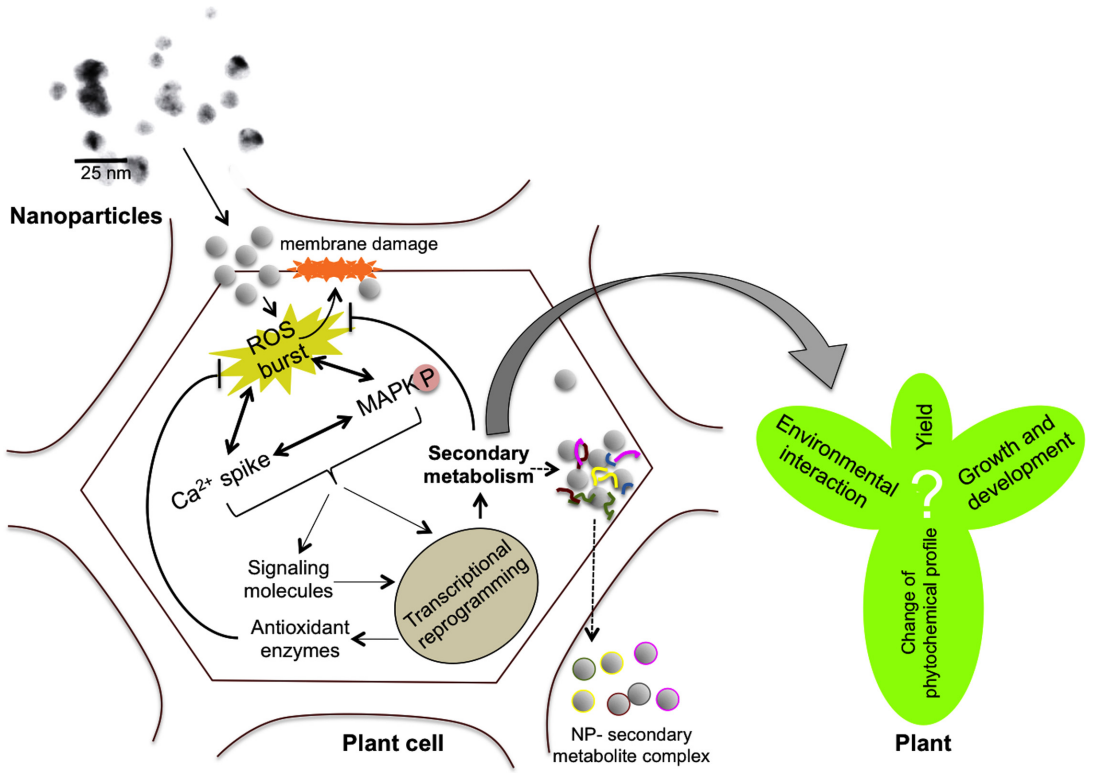

FIGURE 1 | Scheme describing the possible mechanisms involved in nanoparticle (NP)-mediated modulation of plant secondary metabolism. NPs could enter plant cells through plasmodesmata causing physical damage to the plasma membrane. NPs can induce reactive oxygen species (ROS) production, calcium spikes, antioxidant machinery activation, mitogen-activated protein kinase (MAPK) cascades, etc., which could lead to transcriptional reprogramming of secondary metabolism. Activation of enzymatic antioxidant and non-enzymatic machineries including secondary metabolism might scavenge the ROS and protect the cells from oxidative damage. However, the exact consequences of changes in plant secondary metabolism on plant's performance, environmental interaction, growth and yield are still unknown. On the other hand, the ability of NPs (e.g., anatase TiO_2) to enter plant cells and exit as NP-secondary metabolite complexes could possibly be exploited for molecular pharming (dotted arrows).

response to NPs (Garcia-Sanchez et al., 2015). In addition to SA-mediated SAR, other signaling pathways such as ET, BRs, and NO were also affected by NPs. In *A. thaliana* plants treated with AgNPs expression of ET biosynthetic components 1-aminocyclopropane-1-carboxylate synthase ACC and ACC oxidase 2 was reduced (Syu et al., 2014), suggesting that these NPs could inhibit ET perception and affect its biosynthesis. ET is an important signaling molecule mediating sesquiterpenoid biosynthesis in the *Atractylodes lancea* (Thunb.) endophytic fungi *Gilmaniella* sp. AL12 interaction (Yuan et al., 2016). BRs, the steroidal phytohormones that play important role in plant growth, secondary metabolite accumulation, stress responses and adaptation (Çoban and Göktürk Baydar, 2016) could ameliorate ZnONP-induced oxidative stress by improving antioxidant potential and redox homeostasis in tomato seedlings (Li et al., 2016). NO, another universal signaling molecule that plays a central role in secondary metabolite production in plant cells (Zhang et al., 2012; Zeng et al., 2014), is also involved in plant–NP interactions. For instance, AgNP-induced phytotoxicity could be alleviated by NO in *P. sativum* seedlings (Tripathi et al., 2017). Correspondingly, *O. sativa* NO excess mutant (noe1) plants were tolerant to ZnONP treatment, whereas OsNOA1-silenced (noa1) plants were susceptible to ZnONP-induced phytotoxicity (Chen et al., 2015).

POSSIBLE MECHANISMS OF MODULATION OF PLANT SECONDARY METABOLISM BY NPs

Although the aforementioned reports suggest that NPs are interfering with various signaling pathways and capable of modulating plant secondary metabolism, the exact mechanism through which this modulation could occur is not understood. We believe that the initial responses of plants to NPs might include elevated levels of ROS, cytoplasmic Ca^{2+} and upregulation of mitogen-activated protein kinase (MAPK) cascades similar to other abiotic stresses (**Figure 1**) because of the following reasons. Recognition of AgNPs by plasma membrane bound receptors triggered a Ca^{2+} burst and ROS induction in *A. thaliana* (Sosan et al., 2016). Ca^{2+} levels and associated signaling pathway proteins were found to be upregulated in the

proteomic analysis of AgNP treated *O. sativa* roots (Mirzajani et al., 2014). These authors hypothesized that AgNPs, or ions released thereof, impede cell metabolism by binding to Ca^{2+} receptors, Ca^{2+} channels, and Ca^{2+}/Na^{+} ATPases. As sensed by calcium binding proteins (CaBPs) or other NP-specific proteins, NPs either mimic Ca^{2+} or signaling molecules in the cytosol (Khan et al., 2017). MAPK phosphorylation, and activation of downstream transcription factors generally lead to the transcriptional reprogramming of secondary metabolism in plants (Vasconsuelo and Boland, 2007; Schluttenhofer and Yuan, 2015; Phukan et al., 2016). Although no direct evidence for the involvement of MAPK pathways in plant-NP interactions is available, animal and human cell line studies revealed that analogous pathways are involved in AgNP-induced signaling (Eom and Choi, 2010; Lim et al., 2012), and it has been postulated that plants may also utilize MAPK cascade upon exposure to Ag NPs (Kohan-Baghkheirati and Geisler-Lee, 2015).

CONCLUSION

As discussed in this article, exposure to NPs has the potential to alter plant secondary metabolism. Secondary metabolites can act as phytoalexins/phytoanticipins to protect plants from herbivores and pathogenic microbes, as signals for plant symbiotic interactions with beneficial microbes and as allelopathic agents to protect plants from rhizosphere competitors (Abdel-Lateif et al., 2012). In addition, they also serve as physical and chemical barriers to abiotic stressors and as antioxidants to scavenge ROS (Franklin et al., 2009; Ramakrishna and Ravishankar, 2011). Although NP-mediated changes in plant secondary metabolism would affect the optimal interaction of plants with their surrounding environment and possibly growth and productivity, substantial research is needed to understand the exact impact.

The presence of NPs in the environment might affect the pharmacological properties of medicinal plants, as many phytomedicines exert their beneficial effects through additive or synergistic actions of several compounds acting on single or multiple target sites associated with a physiological process (Briskin, 2000). While it is necessary to tackle these adverse effects, NP-mediated changes in secondary metabolism could also be beneficial if harnessed in such a way that NPs are used as elicitors in molecular pharming to enhance the production of desired secondary metabolites. For example, the content of important drugs like artemisinin (Zhang et al., 2013) and diosgenin (Jasim et al., 2017) were enhanced in plants treated with NPs. The ability of NPs to adsorb secondary metabolites (Kurepa et al., 2014) could be exploited for purification of precious compounds from plants via nanotrapping, if harnessed properly. Similarly, *in vitro* green synthesis of NPs using plant extracts can be further extended to develop high throughput tools to purify specific classes of compounds, as green synthesized NPs are often found as conjugates of secondary metabolites (Marslin et al., 2015).

Paucity of knowledge on the exact consequences of NP accumulation in the environment on plant metabolism is exacerbated by the fact that most of the studies have been conducted under controlled laboratory conditions and typically at much higher concentrations than what could be expected in the environment (Gottschalk et al., 2009; Baalousha et al., 2016). For instance, to induce statistically significant changes in the growth characteristics of *A. thaliana* plants, the minimum concentration of AgNPs was 300 mg/L under laboratory conditions (Sosan et al., 2016), a value much higher compared to the predicted environmental concentration of AgNPs in different environmental compartments: e.g., 1.3–4.4 mg/kg in sewage sludge (Gottschalk et al., 2009; Choi et al., 2017). Moreover, the ecologically relevant concentration of NPs largely depends on their environmental fate, plant species, characteristics of NPs, the medium through which it reaches the plant, etc. (Yin et al., 2012; Syu et al., 2014; Goswami et al., 2017), in addition to other, yet unknown, parameters. Although a recent study showed that ecologically relevant size and concentration of CdONPs could activate secondary metabolism in barley plants (Večeřová et al., 2016), it is difficult to generalize the impact of NPs on plant secondary metabolism in the environmental perspective. However, it is necessary to improve our understanding on the environmental fate of NPs and their hazards/risks, testing ecologically relevant conditions and concentrations in the context of plant secondary metabolism. Considering that plant secondary metabolism includes a vast array of compounds that are tightly controlled by signaling events and environmental cues, a case-by-case analysis might be necessary to have a deeper understanding.

AUTHOR CONTRIBUTIONS

GM collected information on NPs and secondary metabolism. CS prepared the possible mechanisms of plant secondary metabolism induction by NPs. GF conceived the idea of this perspective and collected all other information on ROS, singling pathways, and phytotoxicity in response to NPs. All the authors participated in writing and approved the manuscript for publication.

ACKNOWLEDGMENTS

This work has received funding from the National Science Center, Poland (2016/21/B/NZ9/01980). GM is supported by the Construction Project of Shaanxi Collaborative Innovation Center (Qinling-Bashan Mountains Bioresources Comprehensive Development and Collaborative Innovation) and university grant for the construction of Chinese–German Joint Laboratory for Natural Product Research. GF is supported by European Union's 7th Framework Programme for research, technological development and demonstration under grant agreement n°621321 and co-financed by funds allocated for education through project no W26/7.PR/2015 [GA 3413/7.PR/2015/2] for the years 2015–2019. We thank Dr. William Truman for his critical reading of our manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 20 September 2017
doi: 10.3389/fpls.2017.01575

A Streamlined Approach by a Combination of Bioindication and Geostatistical Methods for Assessing Air Contaminants and Their Effects on Human Health in Industrialized Areas: A Case Study in Southern Brazil

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OPEN ACCESS

Edited by:
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Reviewed by:
Bernd Markert, Environmental Institute of Scientific Networks, Germany
Alessandra Francini, Sant'Anna School of Advanced Studies, Italy

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *26 February 2017*
Accepted: *28 August 2017*
Published: *20 September 2017*

Citation:
Ferreira AB, Ribeiro AP, Ferreira ML, Kniess CT, Quaresma CC, Lafortezza R, Santos JO, Saiki M and Saldiva PH (2017) A Streamlined Approach by a Combination of Bioindication and Geostatistical Methods for Assessing Air Contaminants and Their Effects on Human Health in Industrialized Areas: A Case Study in Southern Brazil. Front. Plant Sci. 8:1575. doi: 10.3389/fpls.2017.01575

Industrialization in developing countries associated with urban growth results in a number of economic benefits, especially in small or medium-sized cities, but leads to a number of environmental and public health consequences. This problem is further aggravated when adequate infrastructure is lacking to monitor the environmental impacts left by industries and refineries. In this study, a new protocol was designed combining biomonitoring and geostatistics to evaluate the possible effects of shale industry emissions on human health and wellbeing. Futhermore, the traditional and expensive air quality method based on $PM_{2.5}$ measuring was also used to validate the low-cost geostatistical approach. Chemical analysis was performed using Energy Dispersive X-ray Fluorescence Spectrometer (EDXRF) to measure inorganic elements in tree bark and shale retorted samples in São Mateus do Sul city, Southern Brazil. Fe, S, and Si were considered potential pollutants in the study area. Distribution maps of element concentrations were generated from the dataset and used to estimate the spatial behavior of Fe, S, and Si and the range from their hot spot(s), highlighting the regions sorrounding the shale refinery. This evidence was also demonstrated in the measurements of $PM_{2.5}$ concentrations, which are in agreement with the information obtained from the biomonitoring and geostatistical model. Factor and descriptive analyses performed on the concentrations of tree bark contaminants suggest that Fe, S, and Si might be used as indicators of industrial emissions. The number of cases of respiratory diseases obtained from local basic health unit were used to assess a possible correlation between shale refinery emissions and cases of repiratory disease.

These data are public and may be accessed on the website of the the Brazilian Ministry of Health. Significant associations were found between the health data and refinery activities. The combination of the spatial characterization of air pollution and clinical health data revealed that adverse effects were significant for individuals over 38 years of age. These results also suggest that a protocol designed to monitor urban air quality may be an effective and low-cost strategy in environmentally contaminated cities, especially in low- and middle-income countries.

Keywords: air pollution, environmental monitoring, geostatistical approach, industrial pollutants, urban impact

INTRODUCTION

A robust air quality management system is vital for protecting public health in the face of technological and climate change impacts. However, despite efforts from the United States Environmental Protection Agency (USEPA) to provide guidance on the use of sensors and data interpretation, in addition to promoting workshops with a focus on streamlining technologies, traditional networks with stationary facilities are still costly and complex (Snyder et al., 2013). Accordingly, the World Health Organization (World Health Organization [WHO], 2012) has encouraged the development of environmental studies to verify the feasibility of adopting simplified techniques such as bioindication/biomonitoring for measuring air pollution.

Both biological methods have been considered as low-cost and effective tools for identifying the impacts regarding external factors on ecosystems, by comparing unpolluted areas with polluted ones, or their consequences over the long term, in a specific location (Markert et al., 1997, 2003). Based on the receptor responses to environmental stress, some premises may be raised on the risks for human being (Mulgrew and Williams, 2009; Markert et al., 2011).

The difference between the methods lies in the fact that bioiondication approach supplys information on the quality of the environment, whereas quantitative aspects from environmental stresses, particularly due to chemical substances, are obtained by the biomonitoring approach (Markert et al., 1997, 2003).

Living organisms, such as plant leaves, lichens, moss and tree bark, are receptors of atmospheric contaminants (Markert et al., 2003; Schelle et al., 2008; Ferreira et al., 2012; Norouzi et al., 2015). With respect to tree barks, their suitability as bioindicator have been considered in critical areas for assessing air quality (Kuang et al., 2007; Schelle et al., 2008; Sawidis et al., 2011).

In this study, an atmospheric quality assessment of São Mateus do Sul City, Brazil, was performed adopting tree bark as a pollution bioindicator together with geographic and health datasets, since this city hosts a refinery that extracts shale from the soil for the production of oil, gas and sulfur by heating organic material.

During the cooling process observed in shale refineries, gas and vapors are emitted into the atmosphere releasing organic and inorganic chemical compounds that are harmful to the environment and human health. In all the stages of shale extraction (mining, transportation, and residue stockpiling), particulate matter is produced and carried by the wind reaching neighboring areas. Therefore, the type and level of pollutants determined in tree bark sample will enable to supply information on the air quality (Kuang et al., 2007; Sawidis et al., 2011) with reference to São Mateus do Sul.

The precise geographical coordinates of the sampling site allow verifying the spatial variation of the pollutants (Hoek et al., 2008). As a consequence of the economic benefits of the shale oil industry, São Mateus do Sul has geographically expanded toward the shale plant subjecting the surrounding population to industrial emissions. However, the area affected by the shale industry and the related health effects were not verified because air pollution measurements are not available in this area. Indeed, the potential sources of air pollution in areas without a qualified structure for measuring contaminants is quite a common phenomenon in developing countries (Norouzi et al., 2015; Moreira et al., 2016).

The overarching goal of our study is to devise a streamlined approach to assess air contaminants and their effects on human health in the industrialized area of São Mateus do Sul. We used the bioindincation method combined with a geostatistical model to trace elements of air pollution and identifying hot spots of contamination at large scale. Finally, we investigated the relationship between the spatial distribution of the hot spots and the health data related to respiratory disease in people living in the study area.

Our research features a novel approach – the "Attenuation of the Concentration Model" – (Wasserman and Queiroz, 2004; Ribeiro et al., 2013) that provides data similar to those obtained through conventional methods for monitoring air pollution based on measuring the composition of fine particulate matter (Brown et al., 2007).

MATERIALS AND METHODS

Area of Study

São Mateus do Sul is located in the southern region of Paraná State (latitude 25°44′S- 26°08′S, longitude 50°09′W-50°39′W) about 150 km from Curitiba, the State capital (**Figure 1**), in Southern Brazil. Since its foundation in 1912, the city's economy has passed from being agricultural to industrial. It occupies an area of 1,343 km^2 and has a population of 41,257 inhabitants; population density is 30.75 inhabitants/km^2. São Mateus do Sul is characterized by a subtropical climate given its altitude

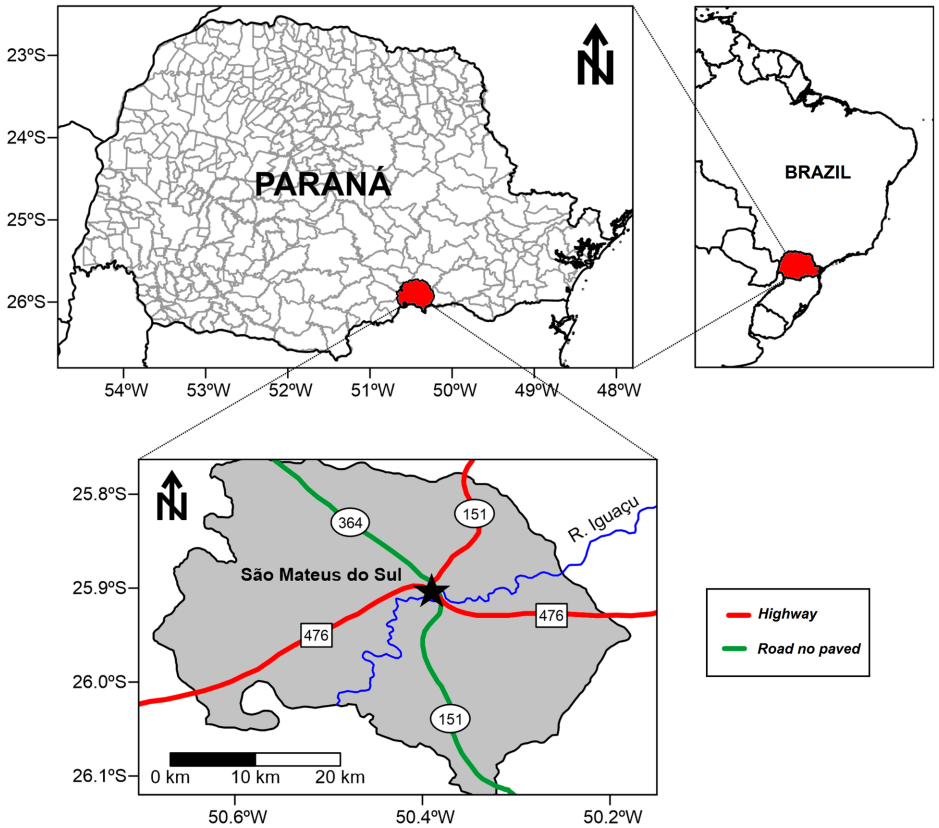

FIGURE 1 | Location of the study area, São Mateus do Sul in the State of Paraná, Southern Brazil.

of 835 m a.s.l. Apart from the two most important industrial plants (shale oil and ceramic), 100 small industries account for 60% of the local economy, while agriculture and services represent 40% (Instituto Brasileiro de Geografia e Estatística [IBGE], 2010).

Tree Bark and Shale Sampling

Approximately 60 sampling sites were selected across the city of São Mateus do Sul (**Figure 2**). For each sampling site, the precise coordinates were recorded using a Global Position System for the Universal Transverse Mercator (UTM) coordinate system. The strategy for collecting was based on the main wind direction (W-E) around the shale plant. Rough tree barks from *Araucaria angustifolia* (commonly known as Parana-pine or Brazilian-pine) were taken for the chemical analysis. This is the predominant tree species in São Mateus do Sul (Barbieri and Heiden, 2012).

Diameter at Breast Height (DBH) method was used to ensure homogeneity of the sampling. Since bark contamination from soil inputs is limited to 1.5 m the length of the trunk, it has been assumed that above this level, air becomes the main source of pollutants in bark (Wolterbeek and Bode, 1995; Schelle et al., 2008). For this reason, a special attention was given in order to all the bark samples were taken from trees with average perimeter of 2.0 and 1.5 m above ground, instead of 1.30 m, that is commonly used for DBH (Clark et al., 2001).

Further, some rough bark trees from Caucaia do Alto city, located in a rural area 50 km from São Paulo city, have been analyzed given that the region is considered a control area for evaluating the levels of the potential pollutants under sudy. According to Guimarães et al. (2000), the level air cotaminants does not represent a health risk for the inhabitants of Caucaia do Alto.

Some samples of retorted shale ($n = 5$), a solid residue obtained from the thermal transformation of oil shale, were taken from the area surrounding the refinery to be analyzed and compared with the elemental data of the tree bark.

Particulate Matter Sampling

Fine particulate matter was sampled and collected from five georeferenced sampling sites with an aerodynamic diameter below 2.5 μm ($PM_{2.5}$); wind direction (W-E) was also considered (**Figure 2**). The $PM_{2.5}$ samples were collected on 0.8-μm and 37-mm polycarbonate filters (Isopore^{TM} Membrane Filters Polycarbonate, Millipore, United States) using Harvard Impactors (Air Diagnostics, Harrison, ME, United States) operating at 10 L min^{-1} (Mauad et al., 2008) over a 24-h sampling period of 5 days. The $PM_{2.5}$ facilities used in São Mateus do Sul belong to the Faculty of Medicine, University of São Paulo.

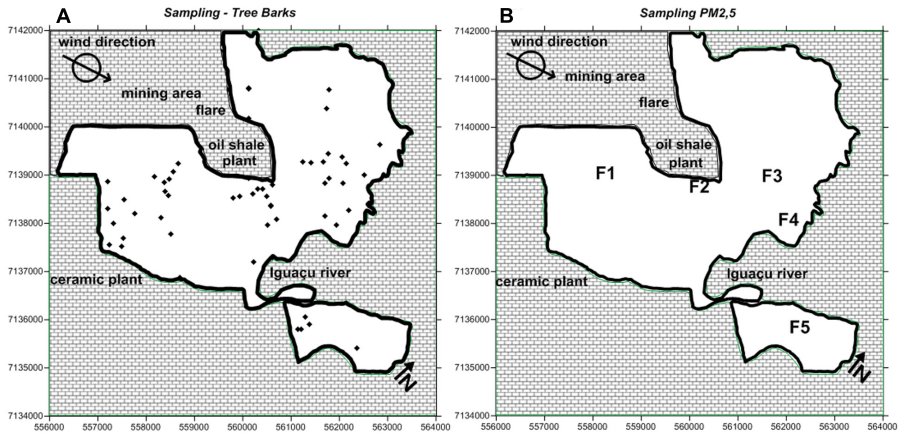

FIGURE 2 | (A) Distribution of 60 sampling sites for tree bark collection; **(B)** representation of wind direction and the five georeferenced sites (F1–F5) for $PM_{2.5}$ sampling.

Although $PM_{2.5}$ measuring is an expensive method, its application was also necessary to validate the geostatistical approach (Wasserman and Queiroz, 2004; Ribeiro et al., 2013) and enabling its utilization in future low-cost air pollution studies (Brown et al., 2007). Accordingly, given that $PM_{2.5}$ measuring makes use of facilities and staff to ensure the safety of the equipment and reliability of the data generated, which make it a costly procedure as mentioned earlier, the financial resources and trained staff are not yet available in the study area. As a result, the $PM_{2.5}$ measuring was carried out by using only the five sample sites in São Mateus do Sul, emphasizing the regions closer to the shale refinery and ceramic plant and others more distant from the industrial areas.

Preparation and EDXRF Analysis

Tree bark samples were removed using a sharp knife and stored in sealed brown paper envelopes. The green layer of lichens and mosses was removed from some of the bark samples, and the outer layer of the bark (∼3 mm thick) was analyzed (Schelle et al., 2008). The samples were not washed for the purpose of measuring the elements that were physically trapped on the surface of the bark. Both tree bark and retorted shale samples were cleaned using a soft nylon toothbrush. Afterward, they were grated using a titanium grater, ground and sieved to obtain small-sized grains (maximum 0.2 mm). About 0.5 g of each sample was weighed for the analysis. The powdered material was pressed (4 t/load) with approximately 2.5 g of boric acid (reagent with analytical purity grade) to produce a pellet that was analyzed using an Energy Dispersive X-ray Fluorescence Spectrometer (EDXRF-720/800HS, Shimadzu Corporation, Japan). The measurement parameters were: time (180 s), target (Rh, 50 kV × 100 μA) and Si (Li) detector. The calibration curves were adjusted by linear regression using specific parameters of the equipment to correct the matrix effects. EDXRF was employed to determine the composition of the tree bark, retorted shale and particulate matter ($PM_{2.5}$), according to Richardson et al. (1995).

Attenuation of the Concentration Model

The USEPA defines natural attenuation as "the naturally occurring processes in soil and groundwater environments that act without human intervention to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in those media" (United States Environmental Protection Agency [USEPA], 1987).

Although this phenomenon is preferred over human intervention to restore polluted areas, it rarely happens. Therefore, several approaches have been conceived to assess environmental impacts caused by anthropogenic activity (Nowak and Crane, 2000; Fenn et al., 2009).

In this work, we used the attenuation of the concentration model of the element based on the studies by Wasserman and Queiroz (2004) and Ribeiro et al. (2013). The model evaluates the spatial distribution of elements on the natural surface and generates values that describe the reduction in levels of elements from a "hot spot" (point of high concentration) in different directions, thereby simulating the destination of movement of the element in the study area. Accordingly, from the pollutant's concentration data it is possible to assess the element's behavior in a particular ecosystem and estimate pollutant mobility. Attenuation (A) values are given according to the following equation (Ribeiro et al., 2013):

$$A = \text{grad } F = \left(\frac{\partial E}{\partial x}\right) + \left(\frac{\partial E}{\partial y}\right)$$

where

A = attenuation of concentration in (μg/g)/m
E = concentration of an element E in (μg/g)/m
grad F = concentration gradient of an element E in (μg/g)/m

$\left(\frac{\partial E}{\partial x}\right)$ = longitudinal derivative of the concentration of an element E in (μg/g)/m

$\left(\frac{\partial E}{\partial y}\right)$ = latitudinal derivative of the concentration of an element E in (μg/g)/m.

From the A values, it is possible to elaborate a map that indicates the spatial behavior of an element and its range from the hot spot(s). This, in turn, indicates the element's mobility and retention areas (higher values of A), which may be associated with singular characteristics (chemical, geological or physical variables) at the investigated area and highlight the main source of the element (Ribeiro et al., 2013).

By using this model in the study area, for the elements investigated, it was expected that the maps could more effectively exhibit the main source of air contaminants and the local sites characterized by their marked retention in conformity with $PM_{2.5}$ maps, World Health Organization [WHO]se information has commonly been accepted in studies evaluating air pollution.

Surfer® 8.0 (GoldenSoftware) was used to create all the maps in this study. The contour lines were interpolated by the simple Kriging method (Matheron, 1971). Excel software (Microsoft, version 2010) was used in the attenuation of the concentration model as well.

Statistical Analyses

To compare the concentrations of elements between tree bark samples from São Mateus do Sul and samples from Caucaia do Alto, the statistical *t*-test was applied using the STATISTICA® 8.0 software for Windows. To identify the possible source of pollution, the multivariate statistical analysis – factor analysis (FA) with Extraction with Principal Components – was applied (Yeomans and Golder, 1982; Johnson and Wichern, 1992), using STATISTICA® 8.0 for Windows.

The possible association between gradients of pollutants in tree bark and the frequency of respiratory diseases was evaluated using one-way ANOVA across four categories (quartiles) of element accumulation in the barks. ANOVA was followed by Tukey's and Bonferroni's *post hoc* tests (Calado and Montgomery, 2003).

Health Outcomes

The health records were obtained under supervision of a nurse assistant, World Health Organization [WHO] belonged to the technical staff of the Basic Health Unit of São Mateus do Sul. The ratio between respiratory/non-respiratory (disease) was calculated for the period between 1997 and 2006. Of the 3000 records, only 245 patients had provided their residential address, which was a key parameter to plot the maps. By using a tool available in the SURFER program, the geographical coordinates (UTM) for each patient address were identified on the city map.

This database is public and can also be obtained from the Information Technology Department of the Public Health Care System -SUS (DATASUS), Brazilian Ministry of Health (Brasil - Ministério da Saúde, 2007). According to the Brazilian laws, researches based on public information, without possibility of individual identification, can be developed with no approval of the National Committee for Research Ethics (Brasil, 2011, 2016).

RESULTS AND DISCUSSION

Quality Assurance

The accuracy and precision of the tree bark analyses were checked by analyzing the elements that were found in retorted shale, which is the main solid waste surrounding a shale refinery. These elements are also found in standard reference materials (SRM): NIST 1547 Peach Leaves (**Table 1**), provided by the National Institute of Standards and Technology, USA, and Basalt Geological reference material - JB2 (**Table 1**) from the Geological Survey of Japan.

The accuracy with relative errors for Cu, Fe, Mn, Pb, and Si was lower than 3.4% while the precision with relative standard deviations (RSD) was lower than 6.4%, except for Mn and Pb with RSD around 11 and 24%, respectively. The accuracy for S and Cr was not calculated, since these elements present only information values in the SRM. Considering that only the levels of Fe, S, and Si in the retorted shale samples were significantly higher than those found in tree barks from Caucaia do Alto, the precision and accuracy of the results for the particulate matter were verified solely for these elements. The results for SRM-NIST 2783 Air Particulate Matter on Filter Media were considered satisfactory and are presented in **Table 1**. The relative errors for Fe, S, and Si were lower than 3.7%, and RSD were lower than 2.8, except for Fe with an RSD around 17.4%.

Retorted Shale, Tree Bark and $PM_{2.5}$ Results

The main chemical elements found in the retorted shale samples were Cr, Cu, Fe, Mn, Pb, S, Si, and Zn; their average concentrations ($\mu g\ g^{-1}$) and standard deviations are shown in **Table 2**.

Table 3 reports the concentrations of the elements (i.e., range, average, median and standard deviation) for about 60 tree bark samples from São Mateus do Sul and Caucaia do Alto.

By comparing the concentrations of the elements found in the retorted shale with those in tree bark from São Mateus do Sul, it is possible to observe that only the Fe, S, and Si levels in retorted shale were higher than their levels in the tree bark samples (**Figure 3**). Also, the Fe, S, and Si levels in the tree bark samples from the same study area were much higher than their levels in the tree bark samples from Caucaia do Alto, which is considered the control region for this biomonitoring study. Student's *t*-test (with a significance level ≤ 0.05) indicated that the concentrations of these elements are statistically different. In contrast, the Cr, Cu, Mn, Pb, and Zn concentration levels in the study site are in agreement with the levels determined in the samples from the control region (**Figure 3**); i.e., their results were not relevant to suggest an anthropogenic impact in São Mateus do Sul, but they will be used for the multivariate statistical approach. Therefore, based on the results it seems that the shale refinery is the main source of Fe, S, and Si contents in São Mateus do Sul.

A comparison of the concentration levels, for Fe and S, obtained in this study with those of similar studies in other cities is reported in **Table 4**. The concentrations for both elements are much higher in São Mateus do Sul than in large cities around the

TABLE 1 | Concentrations of elements obtained from NIST 1547 Peach Leaves, NIST 2783 Air Particulate Matter on Filter Media and from Basalt Geological JB2 and standard reference materials (SRM).

SRM	Element	Mean ± SD[a]	RSD[b] (%)	RE[c] (%)	Values of certificate
Peach Leaves NIST 1547 ($\mu g\ g^{-1}$)	Cd	n.d[d]			0.026 ± 0.003
	Cr	1.00 ± 0.06	6.0		1[e]
	Cu	3.7 ± 0.2	5.4	1.0	3.7 ± 0.4
	Fe	219.7 ± 14.0	6.4	0.8	218 ± 14
	Mn	97.9 ± 10.6	10.8	0.1	98 ± 3
	Ni	n.d[d]			0.69 ± 0.09
	Pb	0.84 ± 0.16	23.8	3.4	0.87 ± 0.03
	S (%)	0.20 ± 0.08			0.2[e]
	V	n.d[d]			0.37 ± 0.03
	Zn	18.0 ± 1.0	5.6	0.6	17.9 ± 4
Air Particulate Matter NIST 2783 ($ng\ cm^{-3}$)	Fe	27500 ± 4800	17.4	3.7	26500 ± 1600
	S	1050 ± 30	2.8	2.1	1050 ± 260
	Si	58500 ± 315	0.5	0.2	58600 ± 1600
Basalt JB2 (%)	SiO_2	53.3 ± 0.4	0.7	1.5	52.54 ± 0.03

[a]*Arithmetic mean and standard deviation;* [b]*relative standard deviation;* [c]*relative error,* [d]*not determined;* [e]*information value.*

world. For instance, Schulz et al. (1999) conducted a temporal (1987 and 1996) study in East Germany on Scots pine bark to monitor several pollutants, including Fe. The Fe contents were approximately five times lower (3490 $\mu g\ g^{-1}$) than some Fe levels (16528 $\mu g\ g^{-1}$) observed for the barks collected surrounding the shale refinery. Schulz et al. (1999) analyzed tree bark collected in the industrial regions around the cities of Leipzig, Halle, and Bitterfeld, which (unlike São Mateus do Sul) comprise several huge industrial facilities of the steel, metallurgical and chemical sectors.

The comparison of our study results with the levels of pollutants with $PM_{2.5}$ results from other studies also emphasizes how allarming the issue of air pollution is in São Mateus do Sul. **Table 5** presents the analytical results for $PM_{2.5}$ from the five sampling sites around the city. The levels of Fe and Si are in the same order of magnitude as those found in Barcelona, Mexico, and Seoul (Querol et al., 2001; Chow et al., 2002; Kang et al., 2004). The *S* values are slightly lower than those in Seoul (Kang et al., 2004), which is one of the most densely urbanized areas in the world with 52500 inhabitants per square mile (Jun et al., 2013). Therefore, these element concentrations point out that although São Mateus do Sul is a small city, where agricultural activities are also economically relevant, it has expanded rapidly with inhabitants settling around the refinery facilities. As a result of this unplanned urbanization, the entire city has been suffering from the same adverse effects that can be observed in megacities around the world.

TABLE 2 | Concentrations of elements in the retorted shale samples.

Elements ($\mu g\ g^{-1}$)	Mean ± SD
Cr	21 ± 1
Cu	32 ± 1
Fe	21445 ± 15
Mn	130.03 ± 0.02
Pb	15.1 ± 0.1
S	11335 ± 10
Si	118422 ± 54
Zn	28 ± 1

Distribution Maps of Fe, S, and Si Concentrations in Tree Bark and $PM_{2.5}$

The map of São Mateus do Sul was divided into quadrants labeled AQ, BQ, CQ and DQ to view the city in regions. As shown in **Figure 4A**, Fe concentrations vary across the city and tend to exhibit higher concentrations at the downwind borders of the shale plant. The chemical composition of shale particles consists partly of Fe (Costa-Neto, 1983; Pimentel et al., 2006).

Figure 4B shows the distribution of S concentrations in tree bark collected in São Mateus do Sul. The influence of emissions from the shale oil company seems to be more evident when S is considered as its tracer. Likewise Fe, S, and Si are also chemical constituents of shale residues (Costa-Neto, 1983; Pimentel et al., 2006).

Figure 4C shows the distribution of Si concentrations in tree bark collected in the study area. The area of influence of Si is located downwind of the mining area, with a more restricted spatial distribution than that of S and Fe, probably reflecting the higher granulometry of particles generated during the drilling process or the reduced height of the emission source (ground level at the mine vs. chimney in the case of S).

The $PM_{2.5}$ maps for the Fe, S and Si concentrations are illustrated in **Figure 5**. Because of the weakness in the number of sampling sites, the spatial distribution extrapolated by the Kriging method may lead to uncertain findings, which should be analyzed to evaluate the application of the attenuation of the concentration model. In general, all portions of the map seem to have been affected by the air contaminants. Even so, the highest Fe contents were observed mainly at the map quadrants AQ and BQ, and slightly at CQ (**Figure 5A**). The AQ quadrant seems to be more affected by S derived from the atmosphere (**Figure 5B**).

TABLE 3 | Concentrations of elements in tree bark from São Mateus do Sul and Caucaia do Alto.

Elements μg g^{-1})	Sampling Site					
	São Mateus do Sul, PR*			Caucaia do Alto, SP**		
	Mean ± SD	Median	Min-Max	Mean ± SD	Median	Min-Max
Cr	17 ± 12	14	7–83	17 ± 14	12	6–49
Cu	32 ± 10	31	16–60	31 ± 10	27	21–46
Fe	4177 ± 3175	2909	525–16528	888 ± 337	704	618–1553
Mn	335 ± 176	287	135–839	294 ± 160	219	146–548
Pb	13 ± 7	11	3–48	10 ± 4	9	6–15
S	2429 ± 664	2382	1469–3760	1202 ± 64	1210	1077–1270
Si	14890 ± 2698	11012	558–71738	722 ± 450	632	174–1168
Zn	29 ± 14	26	3–86	23 ± 14	19	9–48

*PR, Paraná State; **SP, São Paulo State.*

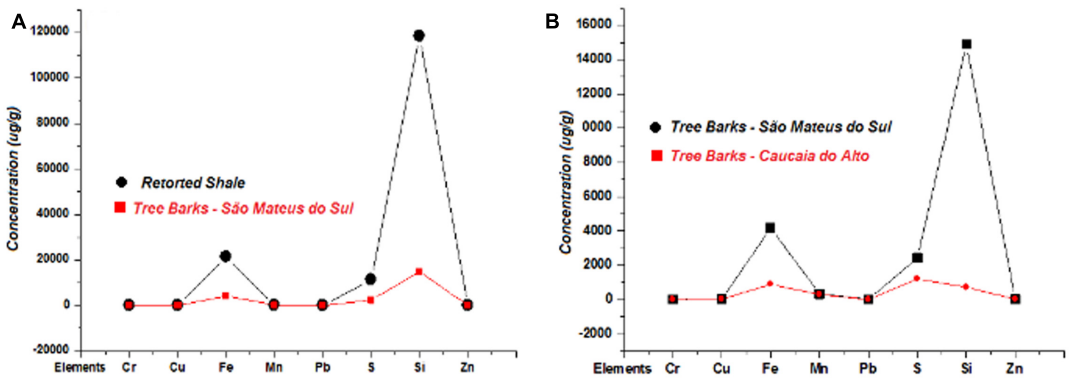

FIGURE 3 | (A) Comparison of element concentrations found in tree bark in the study area and in retorted shale; **(B)** comparison of element concentrations found in tree bark in the study area and in Caucaia do Alto, São Paulo State.

TABLE 4 | Literature data for Fe and S concentrations (μg g^{-1}) in tree bark.

Other countries (large cities)	Fe (Mean ± SD or Range)	S (Mean ± SD or range)
Czech Republic (Böhm et al., 1998)	2917	1035
Northern Finland and the Kola Peninsula (Poikolainen, 1997)	102 ± 67	373 ± 71
Germany (Schulz et al., 1999)	3490 ± 210	#
United Kingdom (Schelle et al., 2002)	147 – 3570	#
Argentina (Fujiwara et al., 2011)	454.5 – 1230	#
Bosnia and Herzegovina (Škrbiæ et al., 2012)	184 – 1648	#

#*Data not available.*

The assessment of Si contamination reveals that its highest values were found at BQ and CQ. The dataset obtained from the $PM_{2.5}$ analysis indicates that the regions in the vicinities of the two largest industries (shale refinery and ceramic plant) of São Mateus do Sul (**Figure 5**) were more affected by anthropogenic activity.

Maps of Attenuation of Fe, S, and Si Concentrations in Tree Bark Samples

From the results of the Fe, S, and Si concentrations in tree bark samples, it was possible to apply the attenuation of the concentration model. The attenuation maps obtained for Fe, S, and Si, respectively, are shown in **Figure 6**. The maps of Fe concentration in tree bark (**Figure 4A**) and in $PM_{2.5}$ (**Figure 5A**) provided a general impression of the high concentrations of this metal around the refinery facilities.

By applying the geostatiscal model, it is possible to observe the point where Fe was strongly retained (**Figure 6A**). The map showed that the hot spot of Fe attenuation was found close to the main source (shale refinery), in accordance with the highest Fe concentration shown in **Figures 4A**, **5A**. Data from the attenuation maps indicate that Fe had low mobility precisely in a lower region in São Mateus do Sul, suggesting that low altitude acts as a geological barrier for the metal.

TABLE 5 | Concentrations of Fe, S, and Si in $PM_{2.5}$ samples.

Local	Fe (ng m^{-3})	S (ng m^{-3})	Si (ng m^{-3})
Site F1 in SMS	327	430	798
Site F2 in SMS	549	900	718
Site F3 in SMS	111	733	171
Site F4 in SMS Site F5 in SMS	136 104	568 397	247 329
Los Angeles (Chow et al., 1994)	99	#	52
Ch'ongyu (Kang et al., 1997)	146	#	360
Barcelona (Querol et al., 2001)	260	#	490
México City (Chow et al., 2002)	560	#	#
Seoul (Kang et al., 2004)	555	3163	1361

SMS São Mateus do Sul - this study; #data not available.

The attenuation map of S emphasized two hot spots in the AQ quadrant (**Figure 6B**): a slight hot spot, also located in the valley region, and an intense hot spot found in the northern portion. In this region the flare device is located, which functions as a continuous source of S emissions into the environment. During the refining process, shale is heated at high temperatures producing oil, gas, and sulfur. Therefore, although the movement of air is intense, it is not enough to dissipate the sulfur. As a result, the element is deposited close to the shale plant.

The highest values from the $PM_{2.5}$ measurements (**Table 5**) corroborate the data obtained from the attenuation of concentration model. Furthermore, despite their weakness, $PM_{2.5}$ maps could provide an overview of the highest concentrations of S (**Figure 5B**), World Health Organization [WHO]se regions include the highest attenuation values (hot spots).

For Si, the attenuation map indicates a hot spot in the same valley of the city (**Figure 6C**), but only with a larger coverage than Fe retention. The prevailing wind direction in the region is West to East (W-E), i.e., Si emissions from the ceramic industry and the mining process should be carried to the eastern part of the city. However, due to the topographic depression (natural barrier), the air remains trapped. The attenuation map of Si is consistent with the information obtained from the distribution of Si concentrations in the $PM_{2.5}$ map (**Figure 5C**). Because

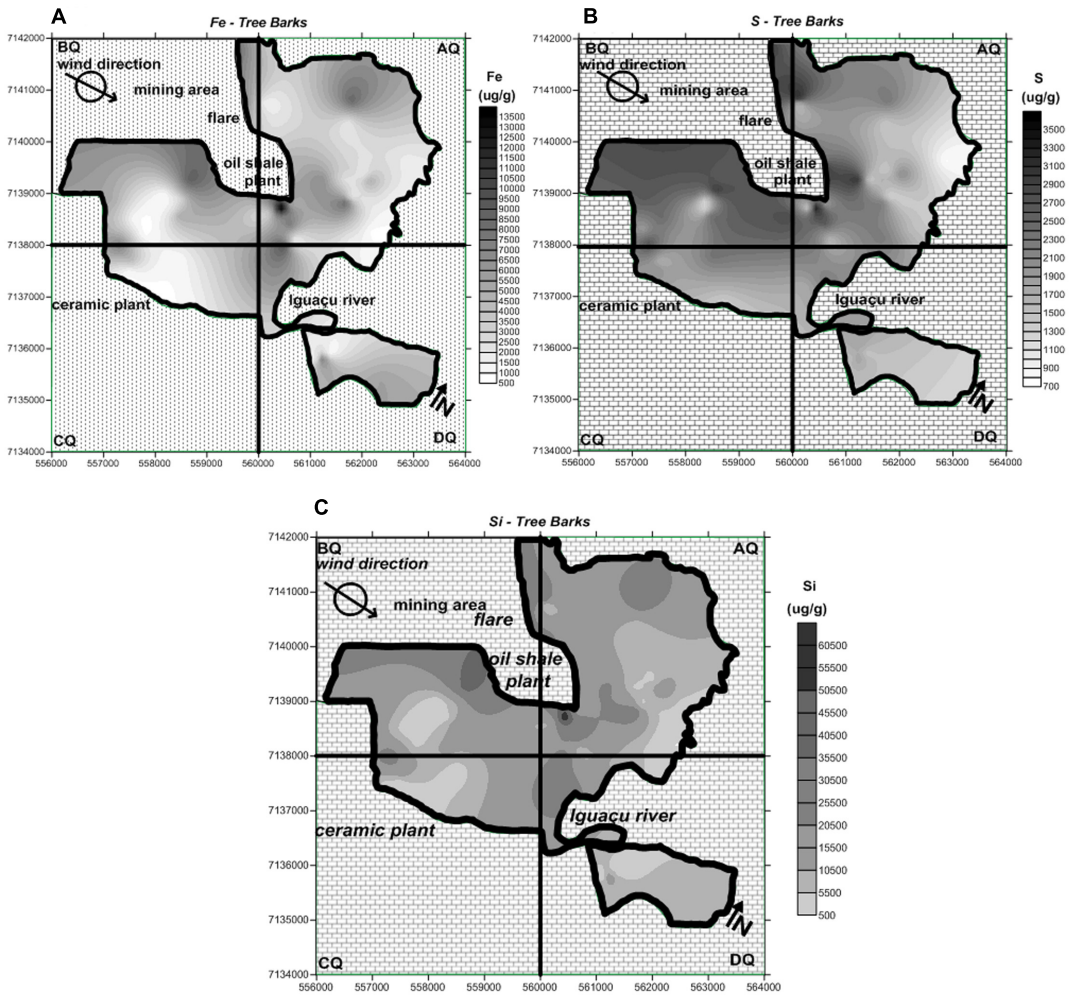

FIGURE 4 | Distribution map of the **(A)** Fe, **(B)** S, and **(C)** Si concentrations found in tree bark in the study area.

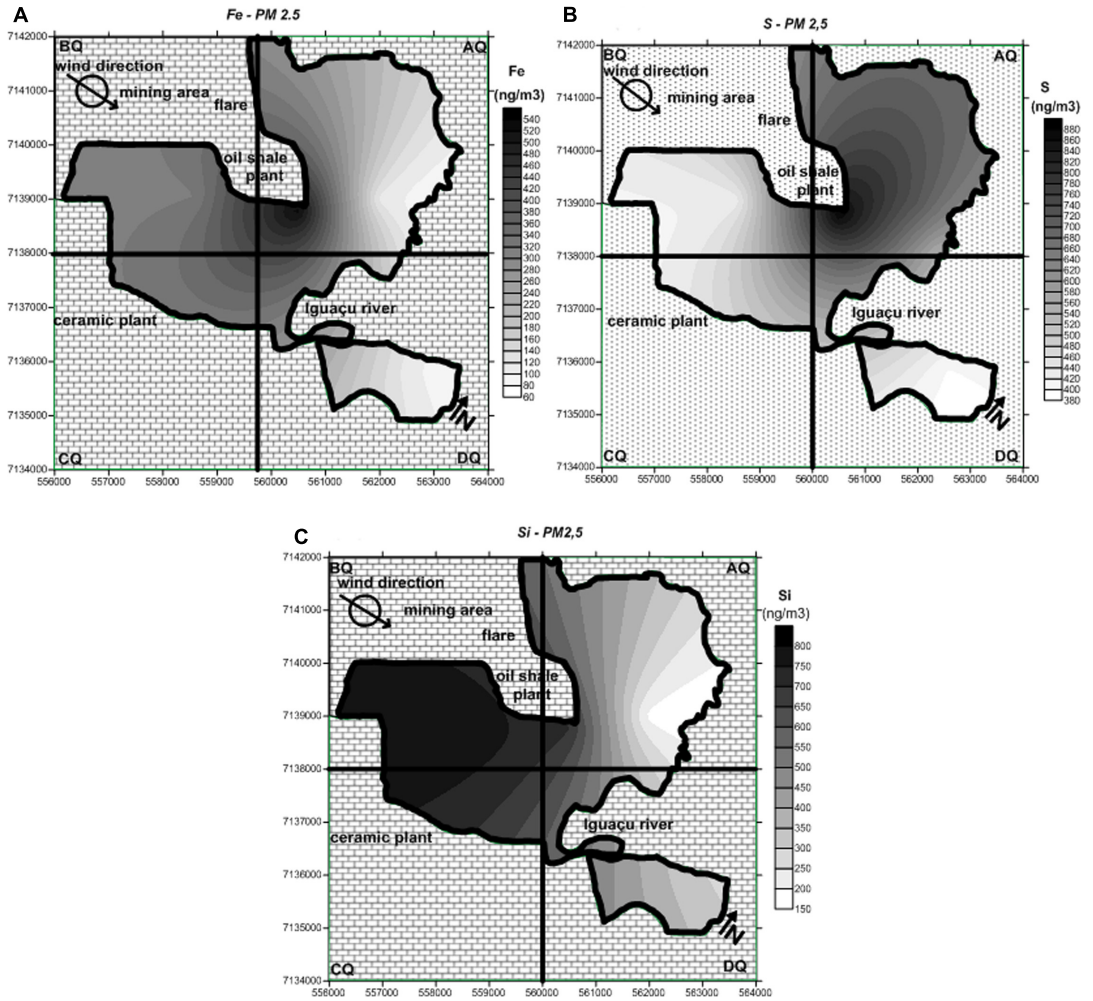

FIGURE 5 | Distribution map of the **(A)** Fe, **(B)** S, and **(C)** Si concentrations found in in $PM_{2.5}$ in the study area.

geological features prevent the diffusion of the contaminants, neighboring dwellers may be more affected by the air pollution.

Multivariate Statistical Analysis

Multivariate statistical analysis, Factorial Analysis (FA) with extraction by principal components, is a powerful pattern recognition technique that attempts to explain the variance of a dataset of inter-correlated variables with a smaller set of independent variables (Yeomans and Golder, 1982; Johnson and Wichern, 1992). Mathematically, FA searches for such joint variations in response to unobserved latent variables through the description of the measured variables according to the linear combination of potential factors, plus error terms. Therefore, FA indirectly determines the association between elements that correspond to the main elements observed and generated by diverse emissions sources (Johnson and Wichern, 1992).

Twelve elements formed two groups of factor correlations (**Table 6**). According to the Kaiser criterion (Yeomans and Golder, 1982) used to assess the results, two main components were considered which accounted for 71% of the total variance (**Table 6**).

The matrix of the components for the dataset indicated that Cr, Cu, Pb, and Zn presented good correlations and were associated with the first component (F1), with a total variance of 49%. Fe, Mn, S, and Si were grouped with the second factor (F2), with a total variance of 22%. F1 grouped the metals that in the pattern of soil geochemistry are termed trace elements and are also commonly related to anthropogenic activities, such as vehicular emissions (Oliveira et al., 2002; Wang et al., 2003; Assunção, 2004; Ravindra et al., 2004; Bergamini et al., 2006). Nevertheless, the Cr, Cu, Pb, and Zn concentrations in the bark samples from São Mateus do Sul were similar with those found in the bark samples from Caucaia do Alto (**Table 3** and **Figure 3**), suggesting that these metals are associated with natural sources in the study region. F2 grouped the elements considered, together with others, as major components of the soil matrix (Osman, 2012). In reality, the soil matrix consists of soil fractions which include organic soil, inorganic non-crystalline material and inorganic crystalline material. These latter two

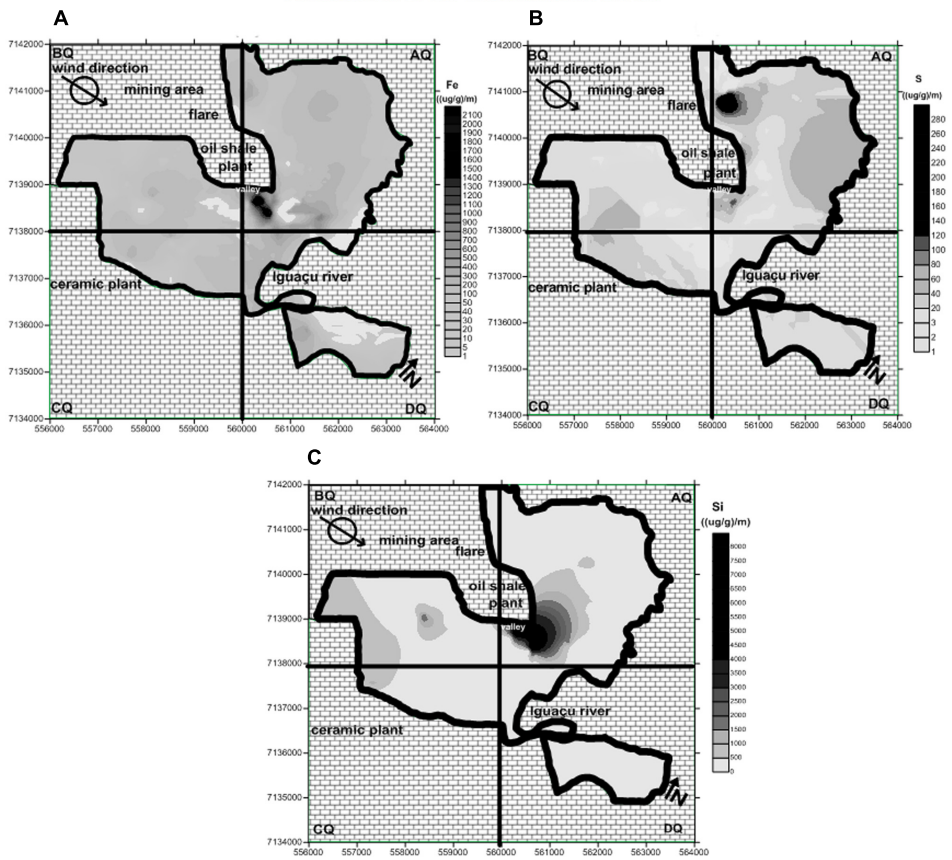

FIGURE 6 | Maps of the attenuation model showing hot spots for the **(A)** Fe, **(B)** S, and **(C)** Si concentrations in the study area.

include components like the oxides and hydrous-oxides Fe, Al, Mn, and Si, primary and clay minerals, carbonates, sulfates, phosphates and sulfides (Yong and Mulligan, 2003). Thus, the Fe, Mn, S, and Si found in particulate matter deposited on tree bark could also be associated with natural sources. However, except for Mn, the other elements presented the highest concentrations in the shale and tree bark samples and exceeded their levels in the samples from Caucaia do Alto (**Table 3** and **Figure 3**). Moreover, the Fe, S, and Si levels in $PM_{2.5}$ were in conformity with their levels in polluted areas (**Table 5**). According to Ying-Mei et al. (2009), in regions where there is industrial mining the main source of Fe, S, and Si may be associated with burning ore. Ots and Reisner (2007) also reported that S is produced during the burning of tailings in mining industry. Therefore, although statistical analysis grouped the major elements of the soil matrix in F2, it seems that the main sources of Fe, S, and Si are from the two most important industries (ceramic and shale mining) of São Mateus do Sul.

Health Outcomes

The size of the PM is the main factor responsible to the intensity of the health problem, although the exposure effectiveness to PM also depends on the physical characteristics of each individual (Brown et al., 2013). The most impact on human health are associated to PM with an aerodynamic diameter below 10 μm, because of their capapability to penetrate deeply into respiratory tract, causing a greater inflammatory response (Nemmar et al., 2001). For $PM_{2.5}$, they are able to reach the respiratory bronchioles and the alveoli where gas exchange occurs (Löndahl et al., 2006).

TABLE 6 | Factor loadings eigenvalues and total variance.

Variables	F1	F2
Cr	0.862575	#
Cu	0.599942	#
Fe	#	0.757039
Mn	#	0.620149
Pb	0.902466	#
S	#	0.828164
Si	#	0.895939
Zn	0.927540	#
Eigenvalues	3.901023	1.785852
Total variance (%)	48.76279	22.32315

#*Data not available.*

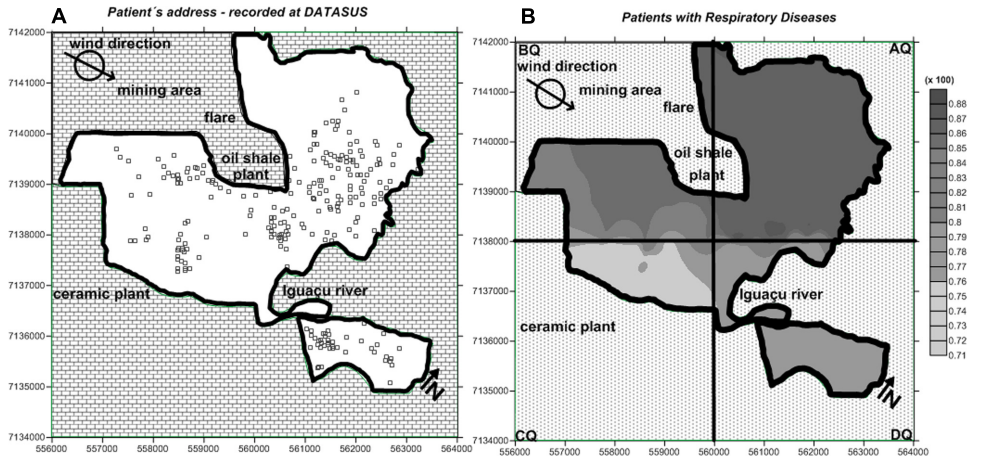

FIGURE 7 | (A) Residential location of the 245 patients selected in the present study; **(B)** distribution map of respiratory diseases.

Figure 7A shows the residential sites of the 245 patients in the study. For each quadrant, the relative number of patients with respiratory disease was calculated (**Table 7**).

Bioassay on redox activity in particulate matter toxicology indicated that the production of reactive oxygen species (ROS) was significantly positively correlated with the contributions of three main sources: Fe, soil dust and water-soluble carbon. ROS is constantly produced in the human body as the natural consequence of aerobic metabolism, and is integral for maintaining tissue oxygen homeostasis (Zhang et al., 2008). An overproduction of ROS caused by Fe was responsible to promote some kind of damage to living cells and tissues, which was enough to initiate an inflammatory process in living organism (Kadiiska et al., 1997).

In the case of air pollution in São Mateus do Sul, the regions with the highest prevalence of respiratory diseases are located in quadrants AQ and BQ (**Figure 7B**). Nonetheless, the number of cases observed in the CQ and DQ quadrants was slightly lower, suggesting that the population of the entire city was experiencing respiratory distress.

A study was performed in Alvarez City (located in the dry area of Iran) to evaluate the number of hospital admission for respiratory disease (HARD) from human exposure to sulfur dioxide. The authors also observed that the number of hospitalization was maximum among the citizens World Health Organization [WHO] lived close to heavy industry (steel, oil, and gas) areas with high sulfur dioxide emitters. An increase of 10 $\mu g\ m^{-3}$ in sulfur dioxide level led to an increase of 3.4% in the HARD (Goudarzi et al., 2016).

Although the complexity of epidemiological studies focused on air pollution, there is no doubt that chemical contaminants are carried in the atmosphere as gases, aerosols, and particulate matter and may be transported over large distances causing local, regional or global impacts, even in pristine areas such as the polar regions (Boutron et al., 1994; Kim et al., 2015). Even so, the map generated using the health data was consistent with the information obtained from the Fe, S, and Si distribution maps for tree bark, the $PM_{2.5}$ samples and attenuation of the concentration modelattenuation of the concentration model (**Figures 4–6**, respectively).

By applying ANOVA, the coefficients of Factors 1 and 2, obtained during FA for each sampling site, were categorized in levels (tertiles). The frequency of respiratory disease (respiratory/total) was considered the dependent variable of tertiles of each factor and the four age categories (1–6, 7–13, 14–36, and 38–93 years of age). F1 did not exhibit any significant statistical association with health outcomes. On the other hand, the frequency of patients older than 38 years increased with increasing contributions of F2 at their residency site ($p < 0.05$). The correlation between F2 and age is shown in **Figure 8**.

The combination of spatial characterization of pollution, clinical data and the inferential statistics approach revealed adverse effects only in the older population. This finding may be the result of two non-exclusive events. One possibility is that respiratory events are quite common in children, and thus the influence of air pollution is masked by the "high noise" in the signal. On the other hand, it may be suggested that it takes shale oil emissions longer to induce adverse effects, as the result of cumulative exposure and decreased respiratory defense with age.

TABLE 7 | Number of patients with respiratory disease in regions per quadrants (Q) of the study area.

Quadrant	Total number of patients per quadrant	Absolute number of patients with respiratory disease	Relative number of patients with respiratory disease (%)
AQ	115	100	87
BQ	48	41	85
CQ	23	17	74
DQ	59	46	78

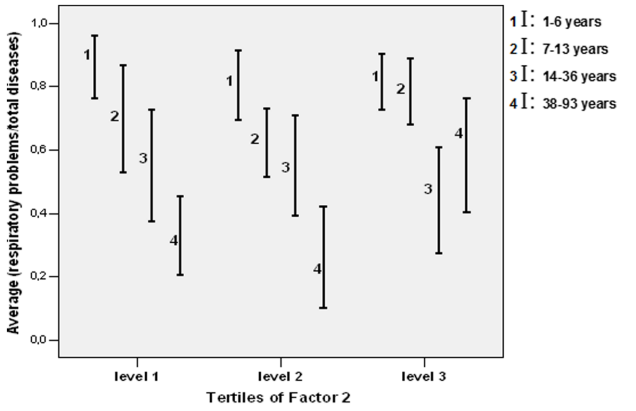

FIGURE 8 | Ratio values of respiratory disease/total diseases (standard error) according to the categories of Factor 2 (Fe, S, and Si) disaggregated by quartiles of age-groups.

The exposure to $PM_{2.5}$ is statistically significant to cause cardiovascular and respiratory hospitalizations, reducing the life expectancy of the population by about 8.6 months on average. The elderly people are more susceptible to these illness (Dominici et al., 2006; Krewski, 2009).

Moreover, it sould bear in mind that the economic impacts related to airborn epidemic diseases are also significant. For the year 2009, the economic loss related to the Chinese exposure to air pollution was estimated in US$ 106.5 billion (Hou et al., 2012).

From these perspectives, a possible alternative to remedy the problem would be the introduction of green infrastructure, which could filter out some atmospheric contaminants (Sanesi et al., 2016). The role of vegetation cover in reducing $PM_{2,5}$ concentrations in Nanjing, China was also discussed by Chen et al. (2016).

Accordingly, for the scenario observed in São Mateus do Sul it is plausible to consider older individuals as the better bioindicators for the adverse effects caused by shale industry emissions. However, the health outcomes of São Mateus do Sul are limited, since epidemiological studies must take into account pre-existing illnesses, human exposure to contaminants from air, soil, drinking-water and food, and in-depth knowledge about the principle of causality (Kundi, 2006), which could not be considered in this study.

CONCLUSION

The bioindication method (using tree barks) to assess the air quality can be considered a comprehensive, precise, inexpensive, and easy strategy to handle and implement. It is important that developed alternatives to estimate the level of contamination do not depend on sophisticated instrumental methods that require substantial resources for their purchase and, above all, long-term maintenance.

Further, the results from the spatial distribution in $PM_{2.5}$ enabled to validate the concentration attenuation model, which highlighted the hot spot retention of Fe, S, and Si, also indicating the presence of a valley region which acts as an orographic barrier and obstructs the spread of these air pollutants throughout the city.

According to Hopke (2009), the atmosphere is a very complex system and the air quality managements are usually based on computer models which take into account information on the levels of local potential contaminants. Alternatively, the majority of air quality models uses chemical composition in particulate matter, which indicates the main polluting source surrounding the investigated area. Therefore, the attenuation of the concentration model is in agreement with this premise.

Further, the findings from the attenuation of the concentration model may explain why the cases of respiratory disease were observed mainly in those patients World Health Organization [WHO] live in areas contiguous to the geochemical barrier. Therefore, the protocol designed to monitor the air quality of São Mateus do Sul using the combined biomonitoring/geostatistical approach and health data highlighted the main sources of atmospheric contaminants in the city and neighboring regions. For this reason, it may be useful to conduct environmental screening in areas with aerial emissions (pollutants as oxides, dioxides, particulate matters and others) and lacking the conventional networks for air pollution monitoring. Moreover, Lafortezza et al. (2013) stress the importance of urban green infrastructure, which may supply ecosystem services and promote human health and wellbeing. In this sense, trees in the urban enviroment might be used not only as bioindicators of air pollution but also as important tools for filtering pollution and cooling.

The rapid and unplanned growth observed in São Mateus do Sul could be characterized as a process of land development. This would imply the causes and consequences of particular land-use behaviors, which in the future may lead to urban sprawl as pointed out by Galster et al. (2001).

However, since the early 2000s, the population of São Mateus do Sul has taken a firm stand to ensure that the city's growth is guided by sustainable planning and management. Because of the benefits generated by the industrial sector, its activities normally affect urban planning policies creating a conflict of interests and divergent moral and political viewpoints. Thus, in the last 10 years the authorities have made a special effort to form partnerships with different stakeholders (e.g., public administration/university) to ensure that industries meet their social and environmental responsibilities for securing quality of life for the city's inhabitants.

This concern of local residents with their well being has caused an improvement to the São Mateus Master Plan, which might lead to sustainable development for the city. Furthermore, based on the information from our study, the Paraná authorities could request the shale refinery to bear the costs of a more detailed environmental study focusing on the toxicity of pollutants and human exposure and indicating remediation measures to minimize the effects of emissions produced by human activity surrounding industrial sites and bordering São Mateus do Sul. In light of the above, and despite the lack of evidence on the adverse effects of mining activities on human health, the results of this

air pollution study provide scientific support for sound decision making.

AUTHOR CONTRIBUTIONS

AF: design and planning of the research; sample collection, analysis and interpretation of the dataset. AR: analysis and interpretation of the dataset, elaboration of the draft, critical review of the content and construction of the final version of the manuscript. MF: analysis and interpretation of the dataset, elaboration of the draft, critical review of the content and construction of the final version of the manuscript. CK: critical review of the content and elaboration of the manuscript draft. CQ: analysis and interpretation of the dataset, elaboration of the draft, critical review of the content and construction of the final version of the manuscript. RL: analysis and interpretation of the dataset, elaboration of the draft, critical review of the content and construction of the final version of the manuscript. JS: analysis and interpretation of the dataset, elaborating of the draft, critical review of the content and construction of the final version of the manuscript. MS: design and planning of the research, analysis and interpretation of the dataset. PS: design and planning of the research, analysis and interpretation of the dataset.

FUNDING

Coordination for the Improvement of Higher Education Personnel (CAPES).

ACKNOWLEDGMENT

The authors wish to thank CAPES for the doctoral fellowship.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 07 March 2017
doi: 10.3389/fpls.2017.00178

Electromagnetic Field Seems to Not Influence Transcription via CTCT Motif in Three Plant Promoters

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It was proposed that magnetic fields (MFs) can influence gene transcription via CTCT motif located in human HSP70 promoter. To check the universality of this mechanism, we estimated the potential role of this motif on plant gene transcription in response to MFs using both bioinformatics and experimental studies. We searched potential promoter sequences (1000 bp upstream) in the potato *Solanum tuberosum* and thale cress *Arabidopsis thaliana* genomes for the CTCT sequence. The motif was found, on average, 3.6 and 4.3 times per promoter (148,487 and 134,361 motifs in total) in these two species, respectively; however, the CTCT sequences were not randomly distributed in the promoter regions but were preferentially located near the transcription initiation site and were closely packed. The closer these CTCT sequences to the transcription initiation site, the smaller distance between them in both plants. One can assume that genes with many CTCT motifs in their promoter regions can be potentially regulated by MFs. To check this assumption, we tested the influence of MFs on gene expression in a transgenic potato with three promoters (16R, 20R, and 5UGT) containing from 3 to 12 CTCT sequences and starting expression of β-glucuronidase as a reported gene. The potatoes were exposed to a 50 Hz 60–70 A/m MF for 30 min and the reporter gene activity was measured for up to 24 h. Although other factors induced the reporter gene activity, the MF did not. It implies the CTCT motif does not mediate in response to MF in the tested plant promoters.

Keywords: *Arabidopsis thaliana*, electromagnetic field responsive element, CTCT, *Solanum tuberosum*, 50 Hz magnetic field

OPEN ACCESS

Edited by:
Alessio Fini,
University of Florence, Italy

Reviewed by:
Anca Macovei,
University of Pavia, Italy
Myrtill Simkó,
Austrian Institute of Technology, Austria

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *23 September 2016*
Accepted: *27 January 2017*
Published: *07 March 2017*

Citation:
Sztafrowski D, Aksamit-Stachurska A, Kostyn K, Mackiewicz P and Łukaszewicz M (2017) Electromagnetic Field Seems to Not Influence Transcription via CTCT Motif in Three Plant Promoters. Front. Plant Sci. 8:178. doi: 10.3389/fpls.2017.00178

INTRODUCTION

During the life cycle organisms are continually exposed to various external stimuli, which requires adequate responses to maintain homeostasis; this process is often called a stress response. The stress factors cause changes in gene expression resulting in adaptive responses at the proteome (synthesis of relevant proteins) or metabolome levels (production of appropriate metabolites, e.g., antioxidants) (Łukaszewicz and Szopa, 2005).

One of such factors could be extremely low-frequency magnetic fields (ELF-MFs), which influence living organisms are supported by the increasing number of evidences. However, the failure to produce repeatable effects (Heredia-Rojas et al., 2010; Buchachenko, 2016) has made this study difficult and the subject questionable (Blank and Goodman, 2011a; Foster, 2011). Despite

many studies, the mechanisms of MF influence are still at the stage of hypotheses rather than well-documented scientific models (Zaporozhan and Ponomarenko, 2010; D'Angelo et al., 2015). Among the various proposed mechanisms, the influence of ELF-MFs on DNA via gene expression is a challenge to test. The interaction could be direct, e.g., DNA can act as fractal antennae (Blank and Goodman, 2011b), or indirect, e.g., free radicals, the circadian clock, or calcium-related pathways can participate in the response (Sztafrowski et al., 2011; Manzella et al., 2015). The influence of ELF-MFs on gene transcription could be mediated by specific sequences, which were found in promoter regions in animals. Indeed, a hypothesis has been proposed that CTCT sequences might act as electromagnetic field response elements (EMREs) in the human HSP70 promoter (Lin et al., 2001). However, it was criticized by other researches (e.g., Alfieri et al., 2006).

Several regulatory mechanisms are similar in animals and plants, especially general stress responses to factors like heat shock or heavy metals (Aksamit et al., 2005). Some data indicate that plants, like animals, perceive and respond to varying MFs by altering their gene expression and phenotypes (Maffei, 2014). However, the influence of MFs on CTCT was studied only in animals and never in plants. It would be interesting to check if these motifs are universal and also mediate responses to ELF-MFs in plants. Therefore, to further examine the hypothesis about CTCT acting as EMRE motifs, we selected a plant model. In particular, we tested if the CTCT motif can regulate gene transcription in response to MF stress in plants. For this purpose, putative promoter regions of all annotated protein-coding nuclear genes from *Solanum tuberosum* and *Arabidopsis thaliana* were analyzed *in silico*. Subsequently, this hypothesis was experimentally evaluated using three promoters that contained CTCT motifs. We selected 16R, 20R and 5UGT promoters, which are involved in flavonoid biosynthesis regulation and responses to free radical stress. The free radicals influence the regulation of genes related to the flavonoid biosynthetic pathway, such as glucose transferase (Lorenc-Kukuła et al., 2004; Korobczak et al., 2005), or regulatory genes encoding 14-3-3 proteins (Szopa et al., 2003a,b; Łukaszewicz et al., 2004). To study the MFs influence, we measured β-glucuronidase (GUS) activity driven by the promoters in a transgenic potato with and without exposure to a 60–70 A/m MF.

MATERIALS AND METHODS

Plant Material and Bacterial Strains

To transform the potato plants (*S. tuberosum L.* cv. *Desiree*) obtained from Saatzucht Fritz Lange KG (Bad Schwartau, Germany), three promoters were used: 20R (EMBL/GenBank database acc. no. AY518222), 5UGT (EMBL/GenBank database acc. no. AY033489), and 16R (EMBL/GenBank database acc. no. AY070220). Each promoter regulated transcription of the reporter gene *uidA* (coding for β-glucuronidase). For plants transformation binary vector pBI101 (Clontech, Mountain View, CA, USA) and *Agrobacterium tumefaciens* strain C58C1 were used. Plants were grown in tissue culture under 16-h light (23 mmol/s/m^2) – 8-h dark regime in MS medium (Murashige and Skoog, 1962) containing 0.8% sucrose. Plants in the greenhouse were cultivated in soil under 16-h light (in 22°C temperature) – 8-h dark (in 15°C) regime. Plants were grown in individual pots and watered daily.

Fluorometric GUS Assay

Transcriptional activity of the tested promoters in transgenic plants was measured by GUS reporter gene activity (Łukaszewicz et al., 1998). Briefly, samples were extracted with 50 mM Tris buffer (pH 8.0) containing 10 mM β-mercaptoethanol and 10 mM EDTA, and centrifuged for 10 min at 13 000 rpm. Aliquots of the supernatant were used for an enzyme assay, with 4-methylumbelliferyl β-D-glucuronide as a substrate (Jefferson et al., 1987), and for protein determination with the Bradford reagent (Bradford, 1976). The reaction product, 4-methylumbelliferone, was measured fluorometrically (SFM 25 Fluorescence Spectrophotometer, Kontron Instrument, Hamburg, Germany).

Bioinformatic analyses of tested promoters 16R, 20R and 5UGT identified several regulatory motifs, which could be recognized by various transcription regulation factors (Szopa et al., 2003b; Lorenc-Kukuła et al., 2004; Aksamit et al., 2005). Based on these results, several putative regulatory factors were experimentally tested for each promoter. In this study, we used the following factors inducing the highest and the fastest promoter expression as positive controls: IAA for 16R promoter (Szopa et al., 2003b), $ZnSO_4$ for 20R promoter (Aksamit et al., 2005) and ABA for 5UGT promoter (Lorenc-Kukuła et al., 2004). GUS activity was measured in leaves (about 8–12 plastochrons old) incubated on the MS medium supplemented with 2.5% sucrose and 100 μM of the inducing factors.

Western Blot Analysis

An assessment of the GT protein level was conducted by means of western blot analysis using the rabbit anti-GT IgG and *Solanum sogarandinum* plants. Briefly, the solubilized protein was run on 12% SDS-polyacrylamide gels and blotted electrophoretically onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). After the transfer, the membrane was sequentially incubated with a blocking buffer (5% dry milk) and then with antibodies directed against the GT protein (1:500 dilution). Alkaline phosphatase-conjugated goat anti-rabbit IgG served as the second antibody and was used at the dilution of 1:1500.

Polymerase Chain Reaction (PCR)

Pooled samples from at least three samples were used for the total RNA extraction. cDNA was synthesized from 5 μg of the total RNA using High Capacity cDNA reverse transcription kit (Applied Biosystems, Poland). cDNA was added to 5 μl of SYBR Green PCR mix (A&A Biotechnology, Gdynia, Poland) and 0.5 μl of each primer (0.5 μm) in triplicate. Polymerase chain reaction (PCR) was carried out with the use of specific primers for glycosyltransferase gene (forward, GTCCTCTTGGTGACATTTCCCACAC and reverse, TGAGGAAATGCCACCACAGGTACAC). Amplification and

detection were performed using LightCycler 2.0 instrument and lightcycler software version 4.0 (Roche, Warszawa, Poland).

Exposure System

Plant material was exposed to 50 Hz 76–88 μT (60–70 A/m) MF for 30 min in the air-conditioned room with the temperature of 19°C. Taking into account the air flow and the layout of exposition with the minimum distance from the plant of several centimeters there was no significant effect of temperature on the experimental results with the applied MF.

The EMF exposure system was composed of two Helmholtz coils with the inside diameter of 400 mm, external diameter of 462 mm, and 40-mm width with 216 mm spacing. Each of the Helmholtz coils was made of copper wire (2.15 mm in diameter) coiled for 223 winding turns. Both coils were positioned vertically to ensure that the magnetic flux was generated in the horizontal plane. The output of the autotransformer (ZWE Eltra, Bydgoszcz, Poland) connected to the electric energy supply was an electric source for a sinusoidal 50-Hz alternating current (sinusoidal 50-Hz MF) in the experiment. The difference of the potential applied to the coils could be regulated. The amplitude of the current intensity was controlled by the ammeter (Multimeter Fluke 8846A; Fluke, Cleveland, OH, USA). MF strength in the center of the Helmholtz coils can be calculated from the formula derived from Biot–Savart's law:

$$H_{calculated} = 0,7156 \cdot \frac{N * I}{r} = 0,7156 \cdot \frac{223 * I}{0,2155} = 740 \cdot I$$

where: I – *value of current*

r — *radius of coils*

N — *number of winding turns*.

Higher harmonic waves in the current were monitored during the experiments (Power Quality analyzer Fluke 43, Fluke, Cleveland, OH, USA) and did not exceed 2%. Magnetic induction was measured with EPRI – Emdex II meter (Patterson, CA, USA) and with Holaday HI-3627 meter (Eden Prairie, MN, USA) for traceability. MF strength was kept in our experiments within the range of 60–70 A/m.

Exposure was carried out on potato leaves with 25 mm long and 15 mm width. Orientation of leaf's stem was parallel to the force lines of MF generated in the exposure system (**Supplementary Figure S1**). During the exposure, the leaves were put on Petri dish (35 mm × 10 mm) positioned in the geometrical center of Helmholtz coils. Additionally, for transgenic plants with 16R promoter, the whole plants in jars were subjected to exposition.

In the case of frequency 50 Hz, the relevant wave length is about 6000 km, therefore the field produced in the exposure system can be considered quasi stationary, i.e., slowly variable in time. It has been proposed that the field produced by alternating 50 Hz current can be described by Biot–Savart formula though it is relevant for direct current (DC). Then, the replacement of alternated current with its root mean square

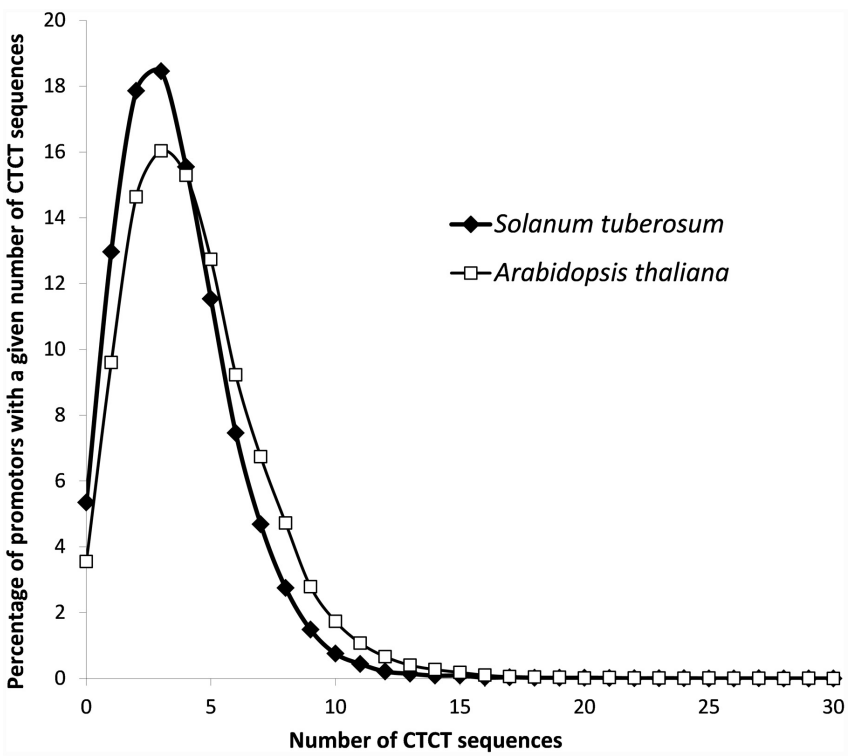

FIGURE 1 | Distribution of CTCT sequences in the set of potential *S. tuberosum* and *Arabidopsis thaliana* promoters (1000 nucleotides upstream of the transcription start site).

(RMS) value allows for determination of the equivalent strength of MF.

Statistical Analyses of Experimental Results

t-test and confidence intervals (CIs) were calculated using Excel (Microsoft, Warszawa, Poland) to assess statistical significance of the obtained results. *p*-values less than 0.05 were considered statistically significant.

Searching and Analysis of Potential EMREs in Plant Promoter Regions

Potential defined EMREs, i.e., CTCT sequences (Lin et al., 2001), were searched in 41,036 *S. tuberosum* and 31,036 *A. thaliana* potential promoter sequences, which were defined as regions of 1000 nucleotides upstream of the transcription initiation site of protein-coding nuclear genes; we excluded sequences with unidentified nucleotides in this study. The scanned promoter sequences and gene annotations were obtained from *S. tuberosum* Group Phureja DM1-3 516R44 (CIP801092) genome annotation v3.4 (Xu et al., 2011), deposited in Phytozome v10.2 (Goodstein et al., 2012), and the *Arabidopsis* Information Resource (TAIR) database, release 9 (Lamesch et al., 2012). For each promoter region, the observed number of EMRE sequences was compared with its expected number, which was calculated according to the nucleotide composition of the given promoter sequence. The statistical significance of this comparison was assessed in the test of proportion with the Benjamini-Hochberg multiple comparisons procedure for controlling false discovery rate (Benjamini and Hochberg, 1995) as implemented in R package 3.1.1 (R Development Core Team, 2014). In the putative EMRE search and analysis, in-house written Perl scripts were used.

RESULTS

In Poland, according to the Regulation of the Minister of Environment of 30 October 2003 (Dz.U. 2003, Nr 192, poz. 1883), 60 A/m is the upper limit intensity for unlimited exposure of humans to 50-Hz MFs. The nCTCTn motif may be responsible for the regulation of gene expression in response to 50-Hz MFs in animal cells (Lin et al., 2001). Therefore, we decided to investigate the potential influence of MFs on plant gene expression involving the EMRE motif.

Frequency and Distribution of Potential EMREs in Plant Promoter Regions

To analyze the frequency and distribution of the potential EMRE motif within gene promoters in plants, we included two plant representatives, *S. tuberosum,* the subject of the

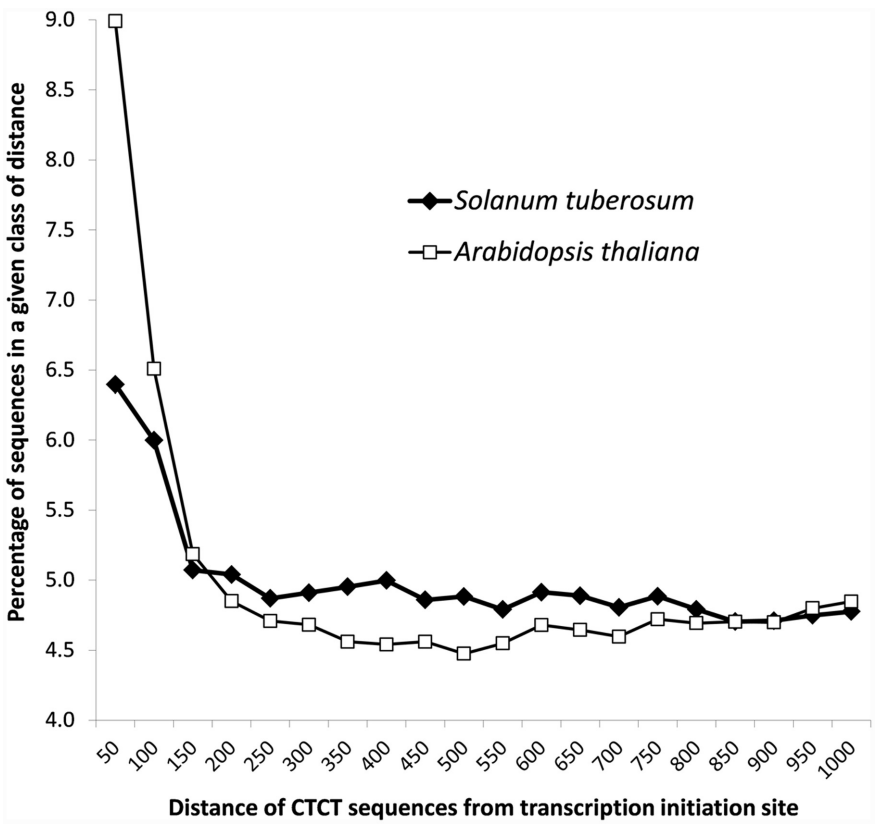

FIGURE 2 | Distribution of the distance of CTCT sequences from the transcription start site in *S. tuberosum* and *A. thaliana*.

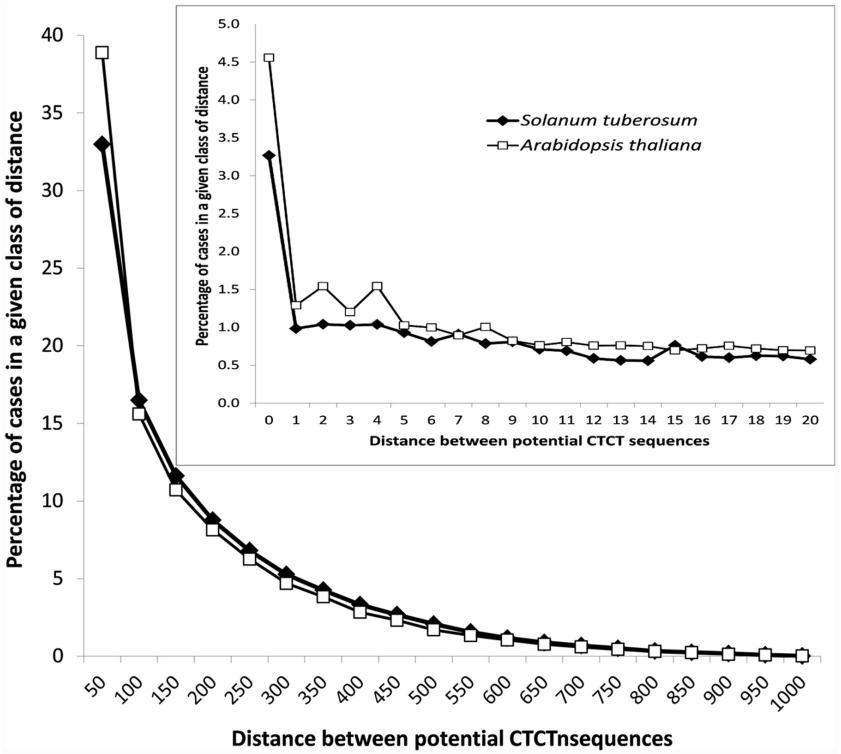

FIGURE 3 | Distribution of the distances between CTCT sequences found in promoter regions of *S. tuberosum* and *A. thaliana*. The inset shows the distribution in the narrower range from 0 to 20 nucleotides.

experimental studies in this paper, and a popular model plant organism, *A. thaliana*. The genome of *S. tuberosum* has the total length of ∼800 Mb that is arranged into 12 chromosomes (Xu et al., 2011), whereas *A. thaliana* has the ∼135 Mb genome that is organized into five chromosomes (Lamesch et al., 2012). We searched potential 1000 bp-promoter sequences from these two genomes. The analysis identified 148,487 in *S. tuberosum* and 134,361 in *A. thaliana* putative EMRE motifs, which gives respective averages of 3.6 and 4.3 such sequences per promoter. In both plants, promoters that contained three CTCT sequences were most abundant (**Figure 1**). They constituted more than 18% (7573) in *S. tuberosum* and 16% (4977) in *A. thaliana* of cases. More than 15% of the promoters (i.e., 6380 in *S. tuberosum* and 4745 *A. thaliana* cases) included four potential EMRE sequences, whereas 10 or more motifs were present in 783 *S. tuberosum* and 1449 and *A. thaliana* promoter regions (**Figure 1**). The motifs were absent from only 2195 and 1103 promoter regions, respectively.

Detailed analyses revealed that CTCT sequences were not randomly distributed in the promoter regions, but rather located close to the transcription initiation site (**Figure 2**). Almost 6.5% and 9% of these sequences (9,500 from *S. tuberosum* and 12,080 *A. thaliana*) were found in less than 50 nucleotides from the transcription start site, whereas more than 12% and 15% of these sequences (18,407 and 20,825 cases) were present in 100 nucleotides upstream of the site, respectively. The contribution

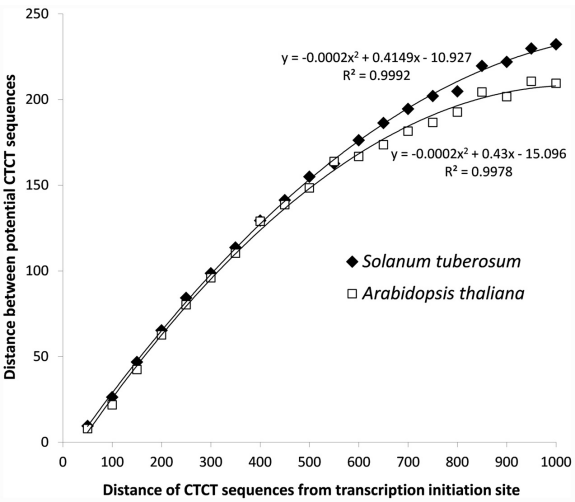

FIGURE 4 | Relationship of the distance between potential CTCT sequences with their distance from the transcription initiation site for *S. tuberosum* and *A. thaliana*.

of these motifs decreases rapidly at 150 bp from the start transcription site (**Figure 2**).

In addition to that, the analysis of distances between potential EMRE sequences that were found in promoter regions indicated

that many of these sequences are closely packed (**Figure 3**). The average distance between these motifs is 162 and 146 bp for *S. tuberosum* and *A. thaliana*, whereas between these motifs randomly distributed across the promoters much larger, i.e., 271 and 226 bp, respectively. In 36,165 and 40,610 cases (33 and 39%) in *S. tuberosum* and *A. thaliana*, the distance was less than 50 nucleotides, whereas in 3582 and 4757 cases (3.3 and 4.5%), respectively, the sequences were adjacent to each other. If we randomized position of these motifs within their promoter regions, only 0.11% and 0.15% of distances between these motifs were shorter than 50 bp for *S. tuberosum* and *A. thaliana*, and none sequences were adjacent. About 19% and 23% distances up to 20 bp were found for the *Solanum* and *Arabidopsis* genomes, whereas 0.04% and 0.01% for the corresponding randomized data (**Figure 3**, inset). Interestingly, the densely packed CTCT motifs accumulated close to the site of transcription initiation. A clear positive but non-linear relationship can be observed between the distance of potential EMRE sequences and the distance of these sequences from the transcription initiation site (**Figure 4**); the closer the motifs to the transcription initiation site, the smaller distances between these motifs in the studied plants.

To check if the potential EMRE motifs occurred in these promoters by chance, we calculated the expected number of these motifs for each promoter region based on the nucleotide composition of the given promoter sequence and compared the expected number with the observed number of CTCT sequences in this promoter. These sequences occurred four times more often than expected in 126 *S. tuberosum* and 159 *A. thaliana* promoters and three times more often than expected in 728 *S. tuberosum* and 975 *A. thaliana* promoters. However, the excess of the found motifs was statistically significant, with a nominal p-value < 0.05, only in 131 for potato and 236 thale cress promoters. When the Benjamini-Hochberg correction for multiple testing was applied, the difference was statistically significant, with an adjusted p-value < 0.05 only in 12 potato and four thale cress cases.

The top genes with the largest (>20) number of potential EMRE motifs found in their promoters are listed in **Tables 1** and **2**. If the MF response is positively correlated with the number of these motifs, then the expression of these genes would be potentially regulated by MFs. In *S. tuberosum*, there are some genes with extremely large numbers of the motifs but

TABLE 1 | Top *Solanum tuberosum* genes with the largest number of CTCT motifs found in their promoter regions.

Motifs' number	O/E	Nominal p-value	Adjusted p-value	Locus identifier PGSC0003DMG	Gene model description
195	4.8	2.7×10^{-26}	1.1×10^{-21}	402023823	Conserved gene of unknown function
162	5.6	1.1×10^{-23}	2.2×10^{-19}	400036968	Conserved gene of unknown function
147	6.0	3.4×10^{-22}	4.7×10^{-18}	400008659	RHO-related protein from plants 9 (ROP)
126	6.2	2.8×10^{-19}	2.9×10^{-15}	400036183	Conserved gene of unknown function
94	6.0	3.4×10^{-14}	2.8×10^{-10}	400006442	Chlorophyll A-B binding family protein; early light inducible
75	3.7	1.6×10^{-08}	8.3×10^{-05}	400011588	RING/FYVE/PHD zinc finger superfamily protein; inhibitor of growth
69	3.6	1.1×10^{-07}	0.0004	400013655	Zinc finger (CCCH-type/C3HC4-type RING finger) family protein, 113A
65	3.9	9.3×10^{-08}	0.0003	400042564	Gene of unknown function
59	6.7	1.2×10^{-09}	7.3×10^{-06}	400032782	S-domain-2 5; S-receptor kinase
57	8.1	4.8×10^{-10}	3.3×10^{-06}	400029702	Telomerase activating protein Est1; Smg-7
52	5.5	7.7×10^{-08}	0.0003	400006299	Gene of unknown function
42	9.9	4.5×10^{-08}	0.0002	400004251	Stress responsive alpha-beta barrel domain protein
27	2.7	0.0074	1.0000	400009231	Transducin/WD40 repeat-like superfamily protein; eukaryotic translation initiation factor 3 subunit
24	3.1	0.0065	1.0000	400027515	Rhodanese/cell cycle control phosphatase superfamily protein; Cdc25
24	2.1	0.0485	1.0000	400030089	Heat-shock protein 70T-2; 70kD
23	3.4	0.0046	1.0000	400012860	Extracellular ligand-gated ion channel
23	3.1	0.0074	1.0000	400001100	Electron transfer flavoprotein alpha; oxidoreductase
23	3.0	0.0087	1.0000	400025959	Pentatricopeptide (PPR) repeat-containing protein
22	4.2	0.0024	1.0000	400013826	Early flowering 3
21	3.3	0.0083	1.0000	400041921	Gene of unknown function
21	3.2	0.0093	1.0000	400030537	60S ribosomal protein L31e family protein
21	3.1	0.0122	1.0000	400035125	Gene of unknown function
21	2.9	0.0148	1.0000	400044565	Gene of unknown function
21	2.6	0.0244	1.0000	400023145	Pentatricopeptide (PPR) repeat-containing protein
21	2.6	0.0256	1.0000	400029893	Gene of unknown function

O/E is the ratio of the observed to the expected number of motifs in their promoters.

their function is unknown, whereas in the case of *A. thaliana*, more than one-third of these genes constitute transposable elements. The promoter for the potato gene encoding RHO-related protein is also rich in CTCT sequences. Such proteins transmit a variety of extracellular and intracellular signals by regulating downstream pathways and signaling cascades. These proteins are involved in diverse cellular processes, such as cytoskeletal organization, pollen and vegetative cell growth, hormone responses, stress responses, and pathogen resistance. Moreover, the motif-rich genes also encode putative transcription factors, proteins interacting with DNA, protein kinases and phosphatases, as well as others, which could regulate the expression of other genes and coded proteins under the influence of MFs. Both plants also express pentatricopeptide (PPR) repeat-containing proteins, which are expressed in mitochondria and plastids. In these organelles, the PPR repeat-containing proteins bind organellar transcripts and influence their expression by RNA editing, turnover, processing, or translation (Barkan and Small, 2014); consequently, they have profound effects on organelle biogenesis and function, including photosynthesis, respiration, plant development, and environmental responses.

Interestingly, among the *S. tuberosum* genes with the large numbers of potential EMRE motifs are those that code for stress-responsive alpha-beta barrel domain protein and heat-shock protein 70T-2 (**Table 1**). In fact, human HSP70 promoters containing these motifs respond to MFs (Lin et al., 1998, 1999); similarly, we found at least one CTCT motif in the promoters of almost all genes encoding HSP70 in potato (**Table 3**). More than half of the HSP70 genes have more observed motifs than expected. In the case of *A. thaliana* promoters for HSP70 genes, all contained at least one this motif and almost 90% of them had more observed these motifs than expected (**Table 4**).

TABLE 2 | Top *A. thaliana* genes with the largest number of CTCT motifs found in their promoter regions.

Motifs' number	O/E	Nominal *p*-value	Adjusted *p*-value	Locus identifier	Gene model description
50	5.6	1.1×10^{-7}	0.0035	AT2G30740	Serine/threonine protein kinase
43	6.1	5.4×10^{-7}	0.0083	AT3G31406	Transposable element gene
42	4.7	5.6×10^{-6}	0.0438	AT4G04590	Transposable element gene; CACTA-like transposase family
35	5.0	2.5×10^{-5}	0.1317	AT2G12510	Transposable element gene; gypsy-like retrotransposon family
33	7.6	4.7×10^{-6}	0.0438	AT2G13175	Transposable element gene; CACTA-like transposase family
32	4.7	8.7×10^{-5}	0.3872	AT5G28410	Unknown protein
31	3.1	0.0×017	1.0000	AT1G27870	Nucleic acid binding
29	8.2	1.5×10^{-5}	0.0913	AT1G33350	Pentatricopeptide (PPR) repeat-containing protein
27	4.1	0.0007	1.0000	AT3G42060	Myosin heavy chain-related
26	6.3	0.0001	0.4803	AT3G47600	Putative transcription factor (MYB94)
25	5.0	0.0005	1.0000	AT3G29610	Transposable element gene
25	4.9	0.0005	1.0000	AT3G55960	NLI interacting factor (NIF) family protein
25	3.5	0.0028	1.0000	AT1G44060	Transposable element gene; CACTA-like transposase family
24	3.5	0.0035	1.0000	AT3G13140	Hydroxyproline-rich glycoprotein family protein
23	5.6	0.0005	1.0000	AT1G63480	DNA-binding family protein
23	5.0	0.0009	1.0000	AT5G58550	Paralog of ETO1, a negative regulator of ACS5 involved in ethylene biosynthesis pathway
23	4.2	0.0019	1.0000	AT1G36403	Transposable element gene; mutator-like transposase family
23	2.5	0.0256	1.0000	AT2G11620	Unknown protein
22	4.0	0.0029	1.0000	AT3G51390	Zinc finger (DHHC type) family protein
22	3.6	0.0050	1.0000	AT5G35066	Unknown protein
22	2.7	0.0189	1.0000	AT3G30837	Transposable element gene; CACTA-like transposase family
22	2.0	0.0827	1.0000	AT1G10330	Pentatricopeptide (PPR) repeat-containing protein
21	5.5	0.0011	1.0000	AT2G28350	Involved in root cap cell differentiation
21	4.3	0.0028	1.0000	AT1G50620	PHD finger family protein
21	4.2	0.0031	1.0000	AT3G42130	Glycine-rich protein
21	2.9	0.0152	1.0000	AT5G28320	Unknown protein
21	2.5	0.0329	1.0000	AT3G43154	Transposable element gene; pseudogene, hypothetical protein
21	2.2	0.0610	1.0000	AT2G34130	Transposable element gene; CACTA-like transposase family
21	2.1	0.0703	1.0000	AT5G30762	Transposable element gene; pseudogene, hypothetical protein
21	1.6	0.2117	1.0000	AT5G32511	Transposable element gene; pseudogene, hypothetical protein

O/E is the ratio of the observed to the expected number of motifs in their promoters.

TABLE 3 | *Solanum tuberosum* HSP70-encoding genes with the number of CTCT motifs found in their promoter regions.

Motifs' number	O/E	Locus identifier PGSC0003DMG
24	2.1	400030089
12	3.1	402031379
10	2.4	400012254
7	1.6	400015920
5	2.4	400024707
5	1.7	400018544
5	1.5	400028634
5	1.2	401031379
4	1.9	400030089
4	1.7	400000398
4	1.6	400024707
4	1.5	400003246
4	1.1	400003122
3	1.5	400008917
3	1.0	400003246
3	0.9	400014212
3	0.9	400014212
2	0.8	400010677
2	0.8	400024887
2	0.6	400010677
2	0.6	400044451
2	0.5	400011197
1	0.5	400028634
1	0.4	400008698
1	0.3	401031379
0	0.0	400003122

O/E is the ratio of observed to expected number of motifs in their promoters.

TABLE 4 | *Arabidopsis thaliana* HSP70-encoding genes with the number of CTCT motifs found in their promoter regions.

Motifs' number	O/E	Locus identifier
11	3.0	AT1G79920
9	2.1	AT4G16660
9	2.6	AT5G02500
8	2.0	AT4G24280
6	1.5	AT3G09440
6	1.5	AT5G09590
5	2.3	AT1G79930
5	1.3	AT1G11660
5	1.8	AT2G32120
5	1.6	AT3G12580
5	1.5	AT4G17750
5	1.3	AT5G02490
4	1.7	AT1G16030
3	1.0	AT4G37910
3	1.7	AT4G32208
2	0.7	AT1G09080
1	0.3	AT5G49910

O/E is the ratio of observed to expected number of motifs in their promoters.

To look for the elements that could modulate transcription in response to ELF-MF, we further analyzed the sequences of three potato promoters, namely 16R, 20R, and 5UGT. These promoters are well-studied and validated expression models in plants (Korobczak et al., 2005; Łukaszewicz and Szopa, 2005).

14-3-3 Protein 16R Promoter

Within the 16R promoter (972 nucleotides in length), the transcription initiation site is located 89 nucleotides upstream of the translation initiation site. This promoter region contains a typical CCAAT box, located at position -147, but lacks a typical TATA box. Among many others, the following putative transcription factor binding sites were found in this promoter: ARF (Auxin Response Factor), light-regulated element GATA, I-box, GT1, elicitor responsive element (ElRE) regulated upon response to infection (TTGACC), and a frequent motif, AATAGAAAA, present in promoters of genes regulated by sucrose levels. Three CTCT motifs were found, which could potentially regulate gene expression after exposure to MFs.

Sucrose and plant hormones (such as IAA, ABA, and salicylic acid) regulated the expression of GUS under control of the 16R promoter (Szopa et al., 2003b). The fastest-acting factor was salicylic acid (2- to 3-fold increase in 6 h after stimulation). The strongest influence on 16R promoter activity was observed in the case of IAA (after 24 h, sevenfold induction), while there was no statistically significant effect of the 50-Hz MF with 60–70 A/m intensity (**Figure 5**).

14-3-3 Protein 20R Promoter

The 20R promoter (1239 nucleotides in length) contains a number of motifs that potentially respond to light, amylase boxes, and sequences regulated by ABA and cold (Aksamit et al., 2005). Fewer sequences are potentially responsible for regulation by auxins, salicylic acid, pathogens, sucrose, or ethylene. Within the analyzed promoter, a transcriptional activator involved in flavonoid biosynthesis regulation was also found. Finally, a motif regulated by metals in the mouse gene encoding the murine metallothionein (Koizumi et al., 1999) was identified. Twelve CTCT motifs were also recognized in the promoter.

Different factors were tested using this promoter: ABA, ethylene, auxin, salicylic acid, pathogen infections, metals (cadmium, zinc, and copper), salt, glycol, light, wounding, low temperature, and sucrose. Auxin, ethylene, salicylic acid, pathogen infection, and sucrose did not affect GUS activity (Aksamit et al., 2005); however, ABA, cold, light, and heavy metals did regulate GUS expression. The strongest influence on the 20R promoter's activity (eightfold induction) was observed in the case of zinc stress after 24 h; the 50-Hz MF had no statistically significant effect on the activity of the 20R promoter (**Figure 6**). Additionally, to exclude impact of mechanical stress an experiment was performed on 14-days whole potato plants (**Figure 7**). In the leaves of plants exposed to EMF, no statistically significant increase in GUS activity was observed 6 h after the exposition. The activity decreased in the following time-points and was similar to the control.

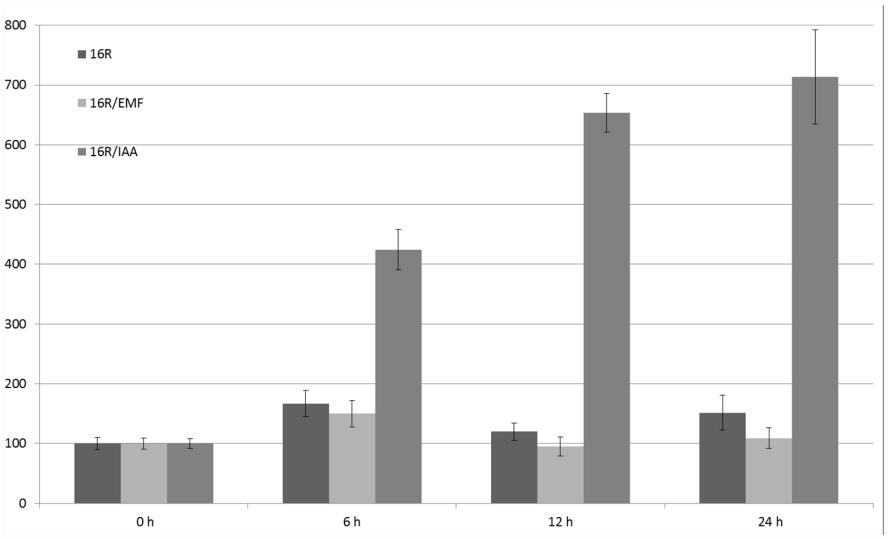

FIGURE 5 | Regulation of expression of 16R promoter under the influence of extremely low-frequency magnetic field (EMF) and IAA. The activity of β-glucuronidase (GUS) in the leaves was measured at the start of the experiment (0 h) and after 6, 12, and 24 h. Young leaves of potatoes grown in the greenhouse were cut (in four parts) and incubated in MS medium (control), under MF 61–69 A/m, or with 100 μM IAA. The graph shows the mean values and confidence intervals obtained from 31 replicates. GUS activity in the control was assumed as 100%.

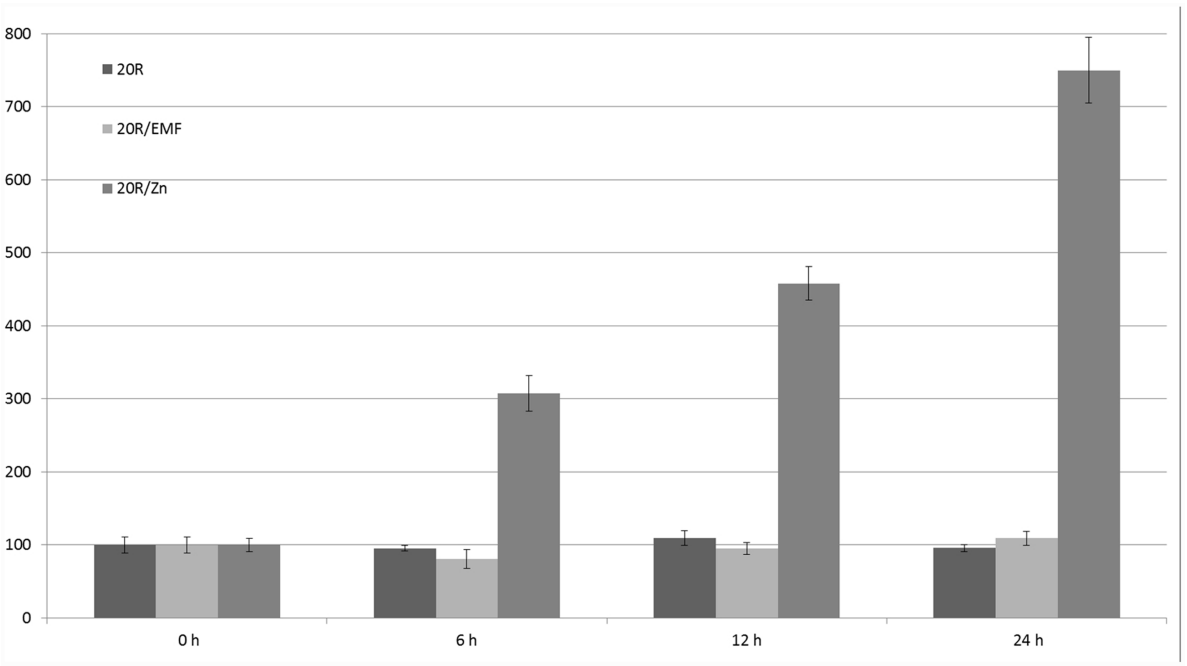

FIGURE 6 | Regulation of expression of 20R promoter under the influence of EMF and zinc ions (Zn). GUS activity in the studied leaves was measured at the start of the experiment (0 h) and after 6, 12, and 24 h. Young leaves of potatoes grown in the greenhouse were cut (in four parts) and incubated with 100 μM zinc or exposed to 62–67 A/m MF. The graph represents the mean values and confidence intervals obtained from 34 replicates. GUS activity in the control was assumed as 100%.

Glucosyltransferase (5UGT) Promoter

The glucosyltransferase promoter (1625 nucleotides in length) was isolated from the wild potato *S. sogarandinum*. Within its entire sequence, several motifs were found upstream of the translation start site that are potentially recognized by transcription factors and involved in the regulation of responses to UV light, ABA, light, sucrose, and potentially MF because five CTCT motifs were identified. Using heterologous expression in

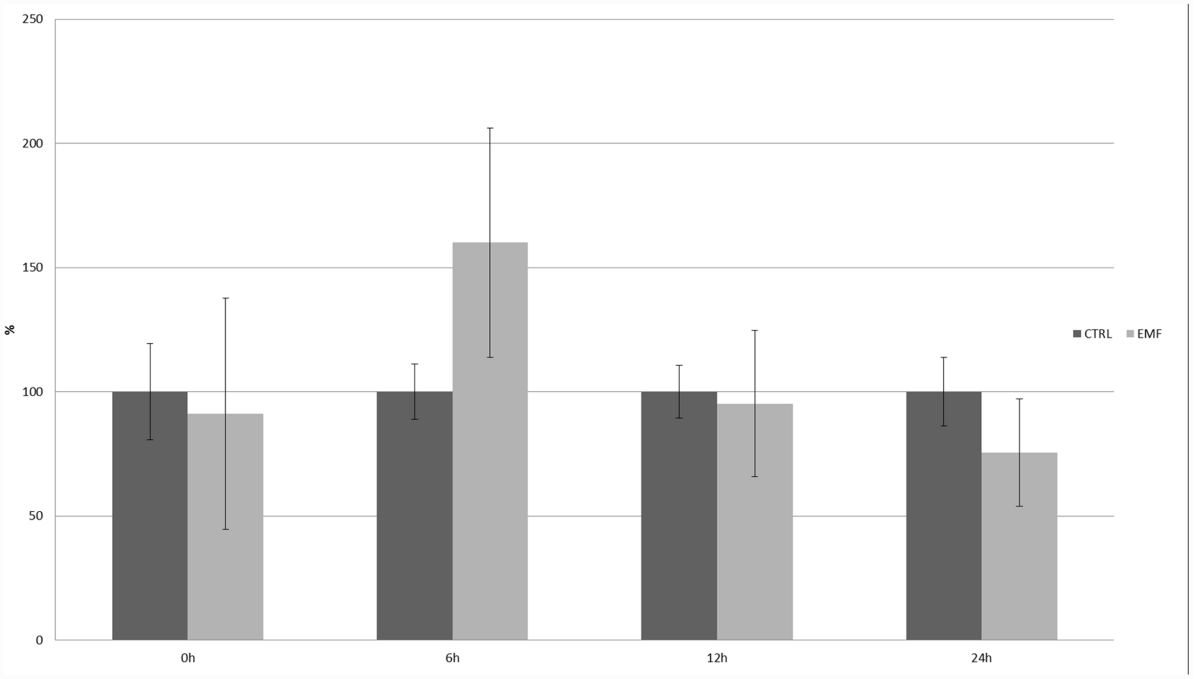

FIGURE 7 | Expression of 20R promoter under the influence of EMF after 30 min exposition of whole 14 day old potato plants. GUS activity in the studied leaves was measured at the start of the experiment (0 h) and after 6, 12, and 24 h. The graph represents the mean values and confidence intervals obtained from three replicates. GUS activity at the start of the experiment (0 h) in the control was assumed as 100%.

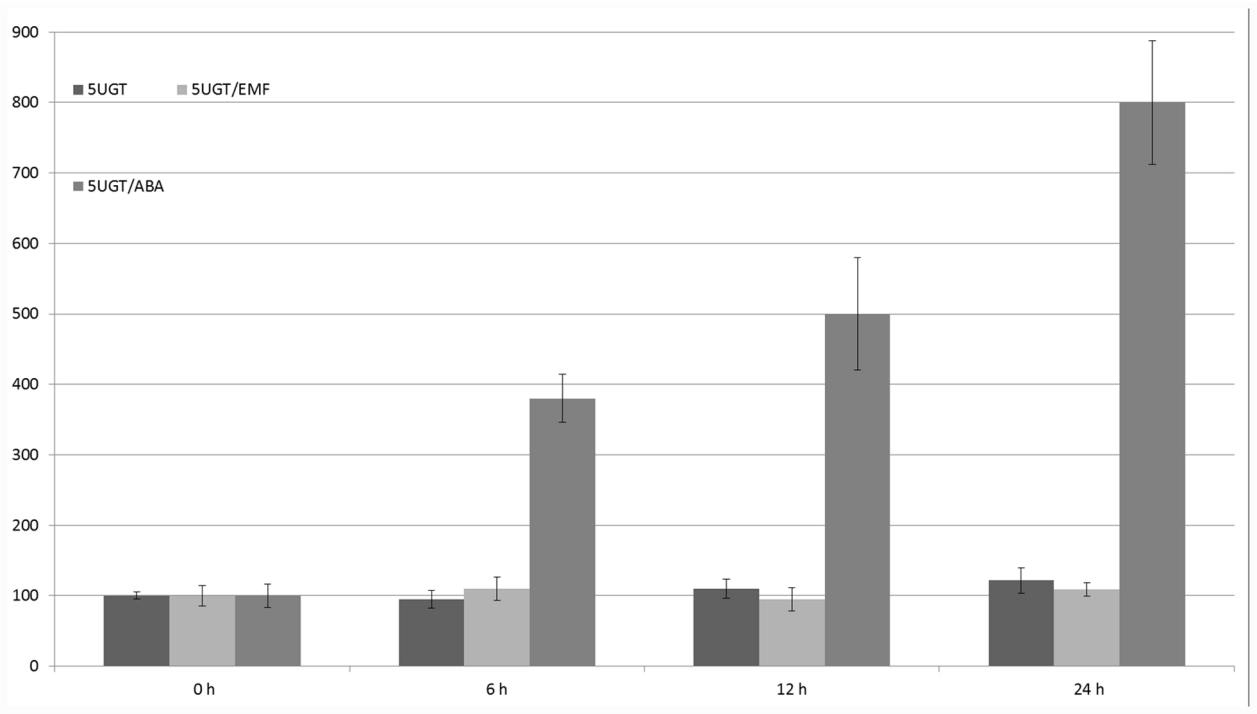

FIGURE 8 | Regulation of expression of the 5UGT promoter under the influence of EMF and ABA. GUS activity in the studied leaves was measured at the start of the experiment (0 h) and after 6, 12, and 24 h. Young leaves of potatoes grown in the greenhouse were cut (in four parts) and incubated with 100 μM ABA or 62–67 A/m MF. The graph represents the mean values and confidence intervals obtained from 32 replicates. GUS activity in the control was assumed as 100%.

S. tuberosum, the 5UGT promoter was induced by UV light, cold, light, ABA, and salt (NaCl). The effect of cooling and light, as well as ABA and cold, were synergistic. The 5UGT promoter's activity was inhibited by sugar concentrations above 2% (Lorenc-Kukuła et al., 2004). The 5UGT promoter's activity increased with time while under the influence of ABA was the highest (eightfold) after 24 h of incubation. However, there was no statistically significant effect of the 50-Hz MF with 60–70 A/m intensity (**Figure 8**). This result was also confirmed in *S. sogarandinum* by mRNA (qPCR) and protein (Western Blot) quantification (**Supplementary Figures S2**, **S3**).

DISCUSSION

It was postulated that MFs may affect DNA by moving charges (Blank and Goodman, 1999). In agreement with that, the movement of electrons in DNA was observed (Wan et al., 1999; Porath et al., 2000). Moreover, it was suggested that conduction in DNA depends on its structure (Meggers et al., 1998). Therefore, it is reasonable to assume that MFs could interact preferentially with some specific DNA sequences. It was assumed that, three CTCT sequences (EMREs) in the human *hsp70* and *c-myc* promoters regulate expression in response to MF exposure for 30 min at 60 Hz and 8 μT (Lin et al., 1997, 1998, 1999, 2001). Although, such response to magnetic flux density (8 μT = 10 A/m) is below the upper limit (60 A/m) regarded as safe (Zmyslony et al., 2005), these values are frequently measured in households, especially those under overhead power lines (Vulevic and Osmokrovic, 2011). We have previously shown that some transcriptionally active motifs within promoters could be conserved across plant and animal taxa, for example, those induced by metals (Aksamit et al., 2005).

Thus, the purpose of this study was to evaluate the hypothesis that 60 A/m alternating MF may regulate plant gene expression through CTCT motifs. First, we analyzed how often this motif occurred in promoters throughout the genome of *S. tuberosum* and the model plant *A. thaliana*. After examining more than 40,000 in *S. tuberosum* and 30,000 in *A. thaliana* potential promoters and, with lengths of 1000 nucleotides, we found, about 150,000 in *S. tuberosum* and 130,000 in *A. thaliana* putative EMRE motifs appearing within the analyzed promoters, about four times on average. Such a high frequency of potential EMRE motif would suggest that this element alone could not be responsible for the precise regulation of gene expression in response to the given factor (e.g., a 50-Hz MF). This could partially explain previous failures to obtain repeatable effects (Heredia-Rojas et al., 2010; Buchachenko, 2016) and supports the hypothesis that additional elements should assist in changing expression in response to MFs.

Although our searches revealed that the CTCT motifs are widely distributed in the studied plant genomes, the conserved test verifying their presence by chance in promoter regions based on their general nucleotide composition showed only 12 and 4 significant cases for *S. tuberosum* and *A. thaliana*, respectively. However, this statistical procedure tests only the presence CTCT motifs but not their arrangements. Our detailed analyses demonstrated that the CTCT sequences are not randomly distributed within promoter regions but are usually located near the transcription initiation site, where they form clusters, i.e., are laid in a very close distance to each other. The majority of these motifs (about 3582 in *S. tuberosum* and 4757 in *A. thaliana*) are adjoining. The organization of these motifs in clusters may suggest some regulation of gene expression, involving the cooperative binding of transcription factors to DNA. Moreover, it cannot be excluded that some of the neighboring stretches of CTCT were generated by CT dinucleotide repeat expansion, e.g., by DNA polymerase slippage mutations. The distribution of CTCT sequences may be also influenced by genome rearrangements and amplifications mediated by transposable elements (Bennetzen, 2000; Fedoroff, 2000; Feschotte and Pritham, 2007), because these motifs were often found near such elements (**Tables 1** and **2**).

However, the non-random distribution and composition of these motifs suggests that many of them overlap or are placed within so-called GAGA elements, which consist of dinucleotide repeats with the pattern $(GA)_n/(TC)_n$. These elements are a target for specific protein complexes, replacing nucleosomes to create a local chromatin environment, which enables a variety of regulatory responses (Lehmann, 2004). GAGA elements can also be related to the epigenetic regulation of homeotic genes and may influence the promoter-proximal pausing of RNA polymerase II (Lehmann, 2004; Fujioka et al., 2008; Lee et al., 2008; Vaquero et al., 2008). Although the GAGA elements were usually studied in *Drosophila melanogaster* in the context of the regulation of numerous developmental genes (Lehmann, 2004), such motifs and the proteins interacting with them were also characterized in plants (Sangwan and O'Brian, 2002; Santi et al., 2003; Meister et al., 2004; Roig et al., 2004; Kooiker et al., 2005; Wanke et al., 2011). Because the expression pattern of these proteins, designated as BBR/BPC, is widespread and their potential target DNA motifs are also numerous in plant genomes, it seems that these factors may influence the expression of various genes involved in different plant processes, in addition to homeobox genes.

The transcriptional activation by GAGA factors and the presence of GAGA elements in promoter regions were also reported for *hsp70* genes (Granok et al., 1995; Wilkins and Lis, 1997; Georgel, 2005). Interestingly, human HSP70 promoters include CTCT motifs that respond to MFs (Lin et al., 1998, 1999). Our analysis of potential promoters in almost all *S. tuberosum* and all *A. thaliana hsp70* genes also revealed the presence of such motifs, which would suggest that the expression of these genes could be regulated by MFs (**Table 2**). Other genes (e.g., those encoding putative transcription factors, pentatricopeptide repeat-containing proteins, or RHO-related protein) may be also considered MF-sensitive because their promoters contain a significant number of CTCT motifs (**Tables 1** and **2**).

It would be worthwhile to reproduce an easy experimental system to study the impact of MFs on living organisms, including plants. Therefore, in addition to the statistical evaluation of plant promoters, we tested the impact of MFs on stress-related promoters in *S. tuberosum*; however, in the studied experimental conditions, there was no significant regulatory effect of the 50-Hz

MF in the range of 60–70 A/m on the expression of 14-3-3 protein gene promoters (16R and 20R) or the glucose 5UGT transferase promoter. Besides the assumption that the EMRE does exist but plants do not respond to MFs because of inappropriate parameters used in the experiments, there are several other possible explanations. One of them is that the CTCT sequences that might act as EMREs should be separated by specific distances. It is also possible that the presence of other motifs and interactions between various transcription factors are necessary for ELF-MF perception, which is very common in many regulators of gene expression. In addition to other uncontrolled parameters that lead to cell line-dependent construct expression, there are clear experimental differences between animal (Jin et al., 1997) and plant (Michelet et al., 1994) cells. In the animal cell experiments, EMF stimuli of less than 1 μT elicited transcripts within 5 min, stress proteins within 20 min, and synthesis gradually decreased after about 3 h. In this work the 30 min EMF stimuli was evaluated after 6, 12, and 24 h. Plants are usually grown in laboratories in temperature about 20°C lower than that in animal cells. Therefore, cellular responses, according to the Arrhenius equation, would be roughly fourfold slower in plant cells incubated in 18°C than in animal cells in 37°C. Consequently, stress responses in plants are often much slower than in animals, with the highest level of reporter proteins occurring often after 24 h (**Figures 5–7**). Indeed, in plant cells, the production of proteins on once-produced mRNA template may be constant for at least 6 h, resulting in steady accumulation of a reporter protein. Once produced and accumulated, the reporter protein, such as GUS, is relatively stable and may be detected for several hours after translation. Consequently, even very short transcriptional activation should be visible in our experimental conditions except for very weak activation, resulting in signal lower than background.

The presented results did not confirm the hypothesis that CTCT motifs are EMREs in the studied plant promoters, which seemed promising because they contain CTCT motifs and are known to response to stress conditions. Although we observed no significant response to MF in the plant model, it cannot be excluded that other promoters or motifs can fulfill such function. Moreover, the response can be realized by a complex regulatory network that escapes the simple promoter studies. The response could be initiated by a subset of genes whose promoters can have some MF-specific motifs. Products of these genes could further serve as transcription factors for many other genes whose promoters may not have such specific motifs. These genes may not give positive response to MF if other set of regulated genes are not sufficiently expressed. This complicated network regulation of gene expression requires a separate approach to particular cases and selection of the promoters to GUS/GFP studies without additional knowledge would be misleading. On the other hand, widespread criticism of the original paper about the presence of EMREs, suggests that such elements do not exists in plants. Therefore, the analysis of promoter regions at the specific genes requires a special and separate consideration.

AUTHOR CONTRIBUTIONS

MŁ, DS: study concept and design, data acquisition, statistical analysis, analysis and interpretation, manuscript drafting, editing and revision, manuscript final version approval, PM: data acquisition, statistical and bioinformatic analyses, analysis and interpretation of results, manuscript drafting, editing and revision, manuscript final version approval, AA-S: data acquisition, analysis and interpretation, manuscript final version approval, KK: data acquisition, analysis, and interpretation.

FUNDING

This work was supported by Wrocław Centre of Biotechnology, program: The Leading National Research Centre (KNOW) for years 2014–2018 (www.know.wroc.pl).

ACKNOWLEDGMENT

Special thanks to Magdalena Żuk for plants maintenance and to Jan Szopa for fruitful discussion.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpls.2017.00178/full#supplementary-material

FIGURE S1 | Exposure system from the top (A) and side **(B)**. Orientation of leaf's stem against the force lines of magnetic field generated in exposure system. C1, C2, coils; L, magnetic field exposed leaf; P-base, structural elements supporting Helmholtz coils; W, force lines of magnetic field; S-Petri dish with the exposed sample.

FIGURE S2 | Western analysis of proteins isolated from leaves of control and treated *S. sogarandinum* plants. 80 μg of protein was applied onto each slot of SDS-polyacrylamide gel electrophoresis and the blot was probed with antibody anti-recombinant GT protein. The treatment conditions are marked over the result of western analysis. C1 and C2- control (untreated sample), samples with 30-min treatment in the magnetic field 62–67 A/m and collected after 6 h (6/1, 6/2, 6/3, 6/4), 12 h (12/1, 12/2, 12/3, 12/4), and 24 h (24/1, 24/2, 24/3, 24/4).

FIGURE S3 | Quantification of GT gene expression by real time PCR in *S. sogaradinum* in response to MF exposition after 0, 6, 12, and 24 h.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

OPINION
published: 25 April 2017
doi: 10.3389/fpls.2017.00629

Observing Climate Change Impacts on European Forests: What Works and What Does Not in Ongoing Long-Term Monitoring Networks

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Keywords: crown dieback, crown defoliation, ICP forests, ecosystem functions, ecosystem services, national forest inventories, tree growth, tree mortality

INTRODUCTION

Ecosystem services of forests are related to specific functions, such as growth, photosynthesis, regeneration, element cycling, soil formation, biodiversity holding, and so on. For this reason, a wide "bouquet" of attributes must be taken into consideration to evaluate the overall functionality of forests and their capacity to provide ecosystem services. These attributes may be considered "proxies" for specific relative ecosystem functions and services. The growth of trees and standing biomass is connected not only to the economic value of the forest (provisional services) but also to carbon sequestration and climate regulation (regulatory services). The overall canopy closure (i.e., leaf area index—LAI) can be considered a proxy for stand productivity (Gower et al., 1999) and stand transpiration (Yan et al., 2012), trees' capacity of uptake and filtration of air pollutants (Janhäll, 2015), and element cycling (Burton et al., 1991) as well for soil conservation through the regulation of heavy rain impacts (Park and Cameron, 2008). Parameters related to stand structure, species composition, and diversity may be considered indicative of the capacity of regeneration, to support game and wildlife and to host beneficial organisms against parasites. Some of these attributes are currently assessed in national forest inventories, and data can be useful to map the extension and level of related ecosystem services.

The ecosystem functions and services of forests can be affected by the harshening of environmental conditions. Climate change impacts on forests is the result of complex interactions between meteorological factors and soil conditions (Barbeta et al., 2015), pathological agents (Wermelinger et al., 2007), forest fires (Flannigan et al., 2000), and environmental pollution (De Vries et al., 2014). The action of these co-occurring factors contributes to tree mortality and the reduction of canopy cover, as well-changes in stand composition and diversity (Millar and Stephenson, 2015). These effects can be evaluated by comparing subsequent forest inventories; however, the long period of time elapsing between two successive surveys can make them scarcely applicable if our purposes are to detect and monitor in time and space the impacts of climate change on forest ecosystem structure, functionalities and services provided.

In Europe and North America are currently implemented programs for forest health assessment. The European ICP Forests program (www.icp-forests.net) consists of two networks of monitoring plots (extensive and intensive) where various monitoring activities are repeated at different temporal bases. These networks were designed to assess the effects of transboundary air pollution and atmospheric depositions (De Vries et al., 2014) and represent the most important tool to assess changes in forest ecosystems health condition at national and European scales. A change of perspective, from atmospheric pollution and deposition to multiple stress pressure related to

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: *13 February 2017*
Accepted: *07 April 2017*
Published: *25 April 2017*

Citation:
Bussotti F and Pollastrini M (2017) Observing Climate Change Impacts on European Forests: What Works and What Does Not in Ongoing Long-Term Monitoring Networks. Front. Plant Sci. 8:629. doi: 10.3389/fpls.2017.00629

climate change, suggests a revaluation of the overall structure of the surveys and of the indicators adopted to observe the impacts on forests. The aim of this study is to verify whether, and to what extent, the current European forest health monitoring program is suitable for assessing the impacts of new global environmental factors, and give suggestions to enhance its informative potential.

IMPACTS AT TREE LEVEL: TREE GROWTH AND CROWN DEFOLIATION

The overall impacts of environmental stress on trees can be summarized with their effects on growth. Tree growth is a key parameter for evaluate the ability of forests to mitigate climate change and provide ecosystem services (Bonan, 2008). Forest health, however, is commonly assessed by means of crown defoliation (Michel and Seidling, 2014). Defoliation is a visual estimate of the relative amount of foliage loss of the target tree compared with that of the reference standard tree. Crown defoliation has been assessed extensively since the 1980s, and the trends recorded are assumed to be correlated with the effects of environmental stress, such as air pollution and climate change (Van Leeuwen et al., 2000). Although no clear relationships were found between defoliation and ecological factors at European level, local studies evidenced the impacts of ozone pollution (Augustatis and Bytnerowicz, 2008); extreme drought and heat weaves (Carnicer et al., 2011) and fluctuating climatic conditions (Ferretti et al., 2014). Intuitively, defoliation may affect growth through the reduction of the photosynthetic surface, but different ecophysiological processes (e.g., better exploitation of sunlight from the foliage in the inner part of the crown, increase of photosynthetic efficiency in the remaining foliage) can compensate for the loss of leaves. Growth reduction has been verified only for the most defoliated pine species (*Pinus sylvestris* L.) in Spain (Sánchez-Salguero et al., 2012), Italy (Castagneri et al., 2015), and Lithuania (Augustatis and Bytnerowicz, 2008).

CHANGES AND DYNAMISMS IN FORESTS SUBJECTED TO CLIMATE CHANGE

According to Millar and Stephenson (2015), forest cover under climatic change undergoes a progressive dieback of the crown and mortality of trees. During the period of decline, a regeneration of trees of different species, or of different genotypes of the same species, will occur. The decline of canopies induced by climate change may trigger a vegetational dynamism, with a temporary success of the early-successional tree species. In a longer time span, the regeneration of a "definitive" (mature) tree cover is supposed to be ensured by more xerophytic species coming from proximal areas (e.g., from lower elevation). This new generation of trees is supposed to be better adapted to the environmental condition of the previous one. The functions and services of forests are assumed to decrease during the canopy-decline period, but they are likely to be restored when the "new forest" is well-established. The equilibrium of the forest, adapted to a new environment, implies probably a different level of ecosystem functions and services, with respect to that which is provided by forests before the action of disturbing factors. Adaptation to drier conditions determines slower growth rates, lower foliar mass, reduced regeneration rate, and so on. These processes are ongoing in some parts of Europe and can be observed, for example, in *P. sylvestris* stands in the Valais (Switzerland) and the Italian Western Alps, where *Quercus pubescens* Willd. is going to replace *P. sylvestris* in the driest conditions (Rigling et al., 2013). The changes in the forest structure and species composition are associated with several events and signals, among which the most significant, due to its consequences, is the drought-induced tree mortality (Allen et al., 2015) and insect attacks in trees weakened by drought (Wermelinger et al., 2007; Anderegg et al., 2015).

Large spatial-scale events of drought-induced tree mortality have been registered in Europe and North America (Anderegg et al., 2013). Tree death is generally preceded by a severe decline of the growth and the dieback of a large part of the crown (Sánchez-Salguero et al., 2012). Dobbertin and Brang (2001) probed that the adoption of "defoliation" improves models to predict the mortality of trees in national forest inventories. Substantial reduction of foliar mass is assumed reflect the loss of photosynthates and compromises carbon allocation and tree growth (Garcia-Forner et al., 2016). According to Grote et al. (2016), big trees are especially subjected to water shortage and more likely to die than small trees, but Ruiz-Benito et al. (2013) and Van Gunst et al. (2016) found that in a "competitive" environment, as far as water resources are concerned, small trees are more likely to die.

A different scenario suggests the persistence of the current tree species, but with changes in the forest structure (i.e., reduction of tree density) to withstand the reduction of water resources. The "local evolution," or adaptation, of dominant tree species is promoted by the selection of more adapted individuals, that are those bearing the most suitable genotypes (Savolainen et al., 2007). The local evolution of species as a result of climate change is achieved by taking advantage of the existing genetic variability in natural populations. Increasing interest is devoted to epigenetic acclimation processes (Barbeta et al., 2013) and the so-called memory effect (Crisp et al., 2016), which may be responsible for the progressive reduction of the negative effects of severe drought on tree physiology and survival (Liu et al., 2015).

Tree diversity enhances growth and stabilizes productivity at stand level and on individual trees (Jucker et al., 2014; Liang et al., 2016), and it is supposed to modify the impacts of stress factors through "plant-to-plant" interactions. Such interactions may be either negative (competition) or positive (facilitation). According to the "stress gradient" hypothesis (He and Bertness, 2014), positive interactions are more frequent than negative ones in the poorest sites, where resources are scarce.

WHAT WORKS AND WHAT DOES NOT

Changes in the health and physiological conditions of trees are assumed to provide insights for the prevision of changes

in ecosystems and may represent an alarm signal to interpret the dynamics of acclimation and/or adaptation processes of forests in new environmental conditions. Moreover, responses at tree and stand levels are influenced by stand structure, tree species composition, genomic features and overall diversity. Therefore, adopting a holistic view in the interpretation of interactions between tree health and all the components and processes of the ecosystem is necessary and more realistic.

The greatest usefulness of the extensive forest monitoring network in Europe is its very existence, as well as the existence of expert groups of scientists and technicians at national and European levels and a set of consolidated methods and manuals that make the assessed parameters (i.e., defoliation) reliable and generally accepted. What it needs is the introduction of concepts and indicators suitable for evaluating the mechanisms and processes performed by trees to withstand new environmental challenges owing to climate change. This integration of the current forest monitoring approach implies a substantial improvement of the diagnostic capacity of tree health indicators, combining the traditional visual assessment with more effective morphological and physiological indicators (Bussotti and Pollastrini, 2015).

The data series of ICP Forests (Level I, Timmermann et al., 2016), available from'90 in the past century, suggest a substantial stability (or slightly increasing defoliation) of crown conditions at the European level of some of the most diffuse tree species (*P. sylvestris*, *Picea abies* (L.) Karst., *Fagus sylvatica* L.), alongside a progressive worsening of crown conditions of Mediterranean species. Local events of mortality (Bussotti et al., 2014, 2015), with impact at the regional level, are scarcely captured in the current forest monitoring activities. Therefore, for management purposes, locally intensifying the network to capture the most critical tree species and/or ecological conditions may be useful. This result can be achieved by involving local communities, as well as appealing to the so-called citizen science (McKinley et al., 2017).

CONCLUSIONS: WHAT WE CAN DO

To make the current terrestrial surveys able to capture the changes in forest ecosystems due to climate change, we need to shift the focus from the conditions of individual trees to the demography of the community. Mortality (including small trees, suckers, and understory woody vegetation) and regeneration are therefore key parameters to predict and interpret changes in tree species composition, local evolution, and possible desertification processes. Before tree death physiological stress conditions occur. These changes in plant health status, can be effectively assessed by foliar parameters, such as carbon isotope composition (that is proxy of drought stress, (Farquhar et al., 1982), and of carbon sequestration strategies of plants), chlorophyll fluorescence analysis, as indicator of overall plant stress conditions (Bussotti et al., 2010), and leaf morphology parameters (e.g., SLA, that indicates photosynthetic acclimation and vulnerability to stress, Bussotti, 2008). Tree ring analysis, moreover, is highly desirable to explore tree growth responses in relation to resilience, mortality and foliage loss (Lloret et al., 2011). A first list of the actions needed to improve the informative potential of extensive monitoring surveys in forest ecosystems is provided in **Table 1**.

TABLE 1 | Summary of the main features of the current extensive terrestrial survey in Europe (ICP Forests, Level I), and proposed additions to make the survey more effective to capture the effects of climate change.

Current surveys	Proposed surveys
GENERAL OBJECTIVES	
Tree-based survey	Stand (forest population/community)-based survey
To assess the conditions of trees in relation to air pollution, atmospheric deposition, and other environmental stress	To assess the changes in the structure and species composition of forests under climate change
SAMPLING DESIGN	
Spatial distribution according to a regular grid	Regular spatial distribution can be locally intensified to capture the most critical situations. Contribute of local communities and citizens
Assessment is done every year.	Assessment may be done at multi-year basis (e.g., every 3–5 years)
INDICATORS	
Mortality of trees with diameter at breast height (DBH)>10 cm	Mortality of trees and woody vegetation, including suckers and understory
Crown conditions (defoliation and symptoms) of trees with DBH>10 cm	Foliar analysis (Chlorophyll content and fluorescence, carbon isotope composition, leaf morphology) combined with crown condition assessment
Measurement of DBH every 5 year	Measurement of DBH combined with tree ring analysis to assess responses and resilience to severe weather events
	Leaf Area Index evolution
	Regeneration
RESULTS REPORTING	
Percent of trees with defoliation over a certain threshold	Temporal and spatial changes in structure and composition of forest in relation to environmental factors
Percent of symptomatic trees	

Establishing close relationships and exchanges between different levels of intensity in monitoring programs is desirable. Intensive monitoring networks, observational comparative plots (Baeten et al., 2013; von Gadow et al., 2016) and experimental plots (ecosystem manipulation, Perry and Troelstrup, 1988) are relevant for the assessment and validation of the feasibility of the proxies to be extensively assessed and their ecological and physiological significance.

AUTHOR CONTRIBUTIONS

FB and MP contributed equally to the discussion of the topic.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Plant Science

ORIGINAL RESEARCH
published: 24 July 2017
doi: 10.3389/fpls.2017.01202

Drought Tolerance in *Pinus halepensis* Seed Sources As Identified by Distinctive Physiological and Molecular Markers

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OPEN ACCESS

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Specialty section:
This article was submitted to Functional Plant Ecology, a section of the journal Frontiers in Plant Science

Received: 10 April 2017
Accepted: 26 June 2017
Published: 24 July 2017

Citation:
Taïbi K, del Campo AD, Vilagrosa A, Bellés JM, López-Gresa MP, Pla D, Calvete JJ, López-Nicolás JM and Mulet JM (2017) Drought Tolerance in Pinus halepensis Seed Sources As Identified by Distinctive Physiological and Molecular Markers. Front. Plant Sci. 8:1202. doi: 10.3389/fpls.2017.01202

Drought is one of the main constraints determining forest species growth, survival and productivity, and therefore one of the main limitations for reforestation or afforestation. The aim of this study is to characterize the drought response at the physiological and molecular level of different *Pinus halepensis* (common name Aleppo pine) seed sources, previously characterized in field trials as drought-sensitive or drought-tolerant. This approach aims to identify different traits capable of predicting the ability of formerly uncharacterized seedlings to cope with drought stress. Gas-exchange, water potential, photosynthetic pigments, soluble sugars, free amino acids, glutathione and proteomic analyses were carried out on control and drought-stressed seedlings in greenhouse conditions. Gas-exchange determinations were also assessed in field-planted seedlings in order to validate the greenhouse experimental conditions. Drought-tolerant seed sources presented higher values of photosynthetic rates, water use efficiency, photosynthetic pigments and soluble carbohydrates concentrations. We observed the same pattern of variation of photosynthesis rate and maximal efficiency of PSII in field. Interestingly drought-tolerant seed sources exhibited increased levels of glutathione, methionine and cysteine. The proteomic profile of drought tolerant seedlings identified two heat shock proteins and an enzyme related to methionine biosynthesis that were not present in drought sensitive seedlings, pointing to the synthesis of sulfur amino acids as a limiting factor for drought tolerance in *Pinus halepensis*. Our results established physiological and molecular traits useful as distinctive markers to predict drought tolerance in *Pinus halepensis* provenances that could be reliably used in reforestation programs in drought prone areas.

Keywords: *Pinus halepensis*, Aleppo pine, drought tolerance, physiological response, soluble sugars, free amino acids, plant proteomics, glutathione

INTRODUCTION

In the context of climate change, the fate of many forest ecosystems depends on their adaptation to such changes (Chen and Jiang, 2010). Interspecific variation in key functional traits along environmental gradients can explain adaptive patterns related to environmental cues (Sánchez-Gómez et al., 2010). This variation can happen at an intraspecific level, which would explain adaptive patterns among different populations throughout the geographical range of individual species (Taïbi et al., 2014, 2015). Hence, characterization of this variation within forest tree species is relevant to understand the interaction and significance of evolutionary forces, and to carry out appropriate, genetically based breeding programs (White et al., 2007). Traditionally, for reforestation and ecological restoration, the common strategy was based on the use of local genotypes or genotypes previously represented. One defect of this approach is that it does not consider forest migration and changes in environmental factors, which may lead to failure in reforestation programs and to an unnecessary waste of resources and time (Williams and Dumroese, 2013).

Regional-scale forest mortality worldwide has been associated to drought and predicted climate change is expected to aggravate the negative impact of extreme drought events (McDowell, 2011). In light of climate change predictions in the Mediterranean region, it is unclear how *Pinus halepensis* (Aleppo pine), an important forest tree in this region, will adapt and persist in reforestation carried out in the most limiting conditions for this species in the future (Maestre and Cortina, 2004). Furthermore, drought is demonstrated to have strong implications on the distribution of forest tree species and is able to limit their geographical distribution (Taïbi et al., 2014).

Marked differences in terms of growth, survival and other adaptive attributes have been reported for different *Pinus halepensis* seed sources originating from most of its climatic and ecologic range in Spain and tested under drought conditions in different trial sites (Taïbi et al., 2014, 2015). These studies revealed the selective role of climate variables in determining each populations' fitness within this species. However, to explain and understand the mechanisms underlying such intraspecific variability, seedling establishment must be analyzed at the molecular and physiological levels (Margolis and Brand, 1990; Grossnickle, 2012). Comparing the physiological and molecular responses among drought-tolerant and drought-sensitive populations and the identification of differential traits can be a useful tool to predict the response of uncharacterized populations before planting them in drought prone areas. This information can be valuable for the conservation of genetic resources, and can facilitate successful assisted migration programs (Taïbi et al., 2014). In fact, the seedling stage is a critical part of the tree's life cycle during which the plant is highly susceptibility to resource limitations that may affect survival, establishment and growth, and thus preventing the success of many reforestation programs (Leck et al., 2008).

Studies addressing integrated ecophysiological response (survival, growth, physiological and molecular responses) of forest trees under stressed conditions could generate important data. However, to the authors' knowledge, no study has incorporated *Pinus halepensis* intraspecific variability as an explicit factor to study such integrated response in plantation establishment. At the physiological level, *Pinus halepensis* is known to prevent water stress damage by stomatal closure before strong changes occur in needle water potential (Baquedano and Castillo, 2006) following an isohydric strategy (Klein et al., 2012). As a consequence of stomatal closure, carbon assimilation can be completely inhibited (Martínez-Ferri et al., 2000), thereby increasing the risk of oxidative stress. The impairment of photosynthetic carbon assimilation by stomatal closure affects the metabolic balance in plants (Pinheiro et al., 2011). Soluble sugars are among the most drought-responsive metabolites, increasing due to starch hydrolysis or impairment of starch production (Rodríguez-Calcerrada et al., 2011). In fact, soluble sugars correlate negatively with photosynthesis (Franck et al., 2006). Moreover, the maintenance of osmotic adjustment is important for normal cell activity and survival (Bartlett et al., 2012). In many forest species, organic solutes, specifically soluble carbohydrates and free amino acids, are the principal compounds involved in osmotic adjustment (Patakas et al., 2002). At the molecular level, most of these responses depend on proteins, therefore comparison of the protein profile of tolerant and sensitive populations can provide valuable information to predict the performance of a given genotype under drought conditions (Hu et al., 2012).

Previously, we have identified drought tolerant and drought sensitive seed sources of *Pinus halepensis* based on a 4-year field experiment (Taïbi et al., 2014, 2015). In the present work, we characterize the physiological and molecular responses of different *Pinus halepensis* seed sources under controlled drought stress. Studies at the molecular level require the use of controlled greenhouse conditions, given that in the field there are many variables such as the presence of pathogens, different light exposure, wound stress caused by strong wind, rain or insect attack or mechanical stimulation that can differentially affect several plants and therefore flaw the results. The objective of this work is to characterize the response of different *Pinus halepensis* seedlings (drought tolerant vs. drought sensitive) at the physiological, biochemical and molecular levels under controlled drought stress to identify patterns and/or features between seed sources previously classified as tolerant or sensitive (Taïbi et al., 2014). We expect that the generated data can be useful to predict if seed sources of *Pinus halepensis* for which no field trials have been carried out will be suitable for planting in areas with either current or predicted drought conditions.

MATERIALS AND METHODS

Plant Material

This study was performed using four *Pinus halepensis* seed sources, covering most of the climatic and ecological regions of the species natural range in Spain and representing considerable phenotypic variation (Taïbi et al., 2014). These seeds have been tested previously in field provenance trials over 3 years (1 year in the nursery+3 years in the field) for survival and growth (height

and stem diameter) under drought stress. During the summer of the second year after planting, additional physiological measurements were taken at the trial sites and will be used here as an independent data set to assess seed source response. Seed sources of 'Levante Interior' (Lev), 'La Mancha' (Man) were considered drought-tolerant while those of 'Ibérico Aragonés' (Arg) and 'Alcarria' (Alc) were considered drought-sensitive based on the results obtained in Taïbi et al. (2014, 2015).

Experimental Conditions and Treatments

Seedlings were grown following common procedures reported in the literature for this species in Forespot© 300 containers. Each 16-cm-deep plastic tray consists of 54 cells providing a density of 360 seedlings m^{-2} (Villar-Salvador et al., 2004) filled with a mixture of sphagnum peat vermiculite-pine bark (3:1:1 v/v) and arranged in a complete random block design with six blocks where the different seed sources were randomized within the block.

Seedlings were grown in a growth chamber under controlled conditions set at a 24°C/16°C day/night temperatures, relative humidity at 70% and a photoperiod of 16 h (200 μmol m^{-2} s^{-1}). Seedlings were watered to full capacity every 2 days alternatively twice by water and once by complete Hoagland's nutrient solution (Hoagland and Arnon, 1950) containing all essential macro and micro-nutrients.

After 25 weeks of growth, healthy plants of similar size from each seed source, accounting for 15 replicates per seed source, were randomly assigned to control and drought treatment; control seedlings were irrigated every 2 days whereas drought conditions were applied through withholding water until seedling weight (plant and container) was reduced to 60% of their initial weight (to a predawn water potential that was 60% of the loss of turgency point), thus avoiding catastrophic xylem cavitation and deleterious associated effects. Measurements were carried out at the end of the duration of the drought treatment after 3 weeks (Vilagrosa et al., 2003; Villar-Salvador et al., 2004).

Measurements

Water Potential

Seedling water potential (Ψ_w, MPa) was measured with a Scholander-type pressure pump (model PMS-1000, PMS Instruments, Corvallis, OR, United States) on five seedlings selected randomly from each seed source per treatment. Measures were carried out at predawn.

Photosynthetic Gas Exchange and Chlorophyll Fluorescence

Instantaneous determinations of net CO_2 assimilation (P_n, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}), transpiration E (mmol H_2O m^{-2} s^{-1}), and instantaneous water use efficiency (WUE_{inst}; μmol CO_2 $mmol^{-1}$ H_2O) calculated as assimilation divided by transpiration P_n/E, were determined in five seedlings per seed source per treatment using a portable photosynthesis open-system (Model LI-6400, LI-COR Biosciences Inc., Lincoln, NE, United States). Gas exchange variables were also estimated under conditions of saturating light (1500 μmol photon m^{-2} s^{-1}), 25°C and environmental CO_2 (390 μmol mol^{-1} CO_2) maintaining the relative humidity in the chamber at approximately 55 ± 5%. All gas-exchange measurements were expressed as a function of needle-projected area.

Maximal photochemical efficiency of PSII (Fv/Fm) was determined at predawn using a chlorophyll fluorometer (PAM 2000, Walz, Effedrich, Germany). Φ_{PSII} (quantum yield of non-cyclic electron transport) was estimated as $(Fm' - Fs')/Fm'$ under steady-state conditions of illumination. It was determined early in the morning by using an open gas exchange system (LI-6400; LI-COR, Inc., Lincoln, NE, United States) with an integrated fluorescence chamber (LI-6400-40 leaf chamber fluorometer; LI-COR). Φ_{PSII} was determined in the same set of needles used for the gas exchange analysis. Maximal efficiency of PSII and Φ_{PSII} were calculated according to Maxwell and Johnson (2008).

Transpiration, net photosynthesis and maximal efficiency of PSII were also measured on the trial sites 'La Hunde' (used as a control, representing the core habitat for the species) and in 'Granja d'Escarp' trial site (used as a dry site, representing a marginal dry habitat of the species). A complete description of these sites can be found in Taïbi et al. (2014). These measurements were used as a control to compare with the greenhouse conditions.

Needle transpiration (E) and photosynthesis rate (Pn) were measured in field conditions on five plants per seed source per site (randomly selected in one block) using an LCpro+ Portable Photosynthesis System with a leaf chamber (ADC Bioscientific Ltd., Hoddesdon, Hertz, EN11 0DB) with a light unit; ambient CO_2 was set at 390 ppm, air humidity at 60–70%, temperature at 25°C and photosynthetic photon flux density (PPFD) at 800 μmol m^{-2} s^{-1}. *In vivo* measurements were performed on intact needles with no visible symptoms of damage. Analysis of leaf area, was performed with a numeric scanner connected to the WinRhizo software (Regent Instruments Inc., Quebec City, QC, Canada). Additionally, the efficiency of photochemical reactions driving photosynthesis was assessed by chlorophyll fluorescence measurements. Minimal fluorescence ($F0$) yield was obtained upon excitation with a weak measuring beam from a pulse light-emitting diode, while maximal fluorescence yield (Fm) was determined after exposure to a 0.8 s saturating pulse [>10,000 μmol (photon) m^{-2} s^{-1}] of white light. The maximal efficiency of PSII was then estimated as the ratio of variable to maximal fluorescence ($Fv/Fm = (Fm - F0)/Fm$; Schreiber et al., 1994).

Metabolite Analysis

Chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids (Car) concentrations were determined spectrophotometrically using the Lichtenthaler method (Lichtenthaler, 1987). Non-structural carbohydrates (NSC) were determined by grinding 0.2 g of needles (fresh weight) in liquid nitrogen with a mortar and pestle, and then the homogenized powder was resuspended in 1 mL water and measured as described in Fayos et al. (2006). Specifically, the samples were incubated at 95°C for 10 min, cooled on ice and centrifuged at 4°C for 5 min to remove debris.

The supernatants were filtered through Sep-Pak Plus C-18 solid phase cartridges (Waters). The soluble sugar fraction (mono and oligosaccharides) was separated by chromatography in a Waters 1525 HPLC system equipped with an evaporative light scattering detector (2424 ELSD). Aliquots (20 μl) were injected into the column ProntoSil 120-amino 3 μm (125 mm × 4.6 mm i.d.) with a Waters 717 autosampler. Elution was carried out at room temperature under isocratic conditions using a mixture of acetonitrile (J.T. Baker) and H_2O (Milli-Q Millipore) (85:15) at a flow rate of 1 mL/min during 25 min. The conditions of the light scattering detector were the following: gain 75, data rate 1 pps, nebulizer heating 60%, drift tube 50°C, and gas pressure 40 psi. Sugars were quantified with the Waters Empower software using glucose, fructose, sucrose, and sorbitol standards calibration curves. Glutathione (GSH) and free amino acids were extracted from 2 grams of needles according to the method described in Mulet et al. (2004). In brief, plant material was pooled and homogenized in liquid nitrogen. Each pooled sample (0.10 g of FW) was heated 12 min at 95°C in 2% isocitrate buffer (pH 2 with HCl). 1/10 dilutions of these extractions were injected in a Beckman Gold amino acid automatic analyser. The analysis was carried out following the protocol provided by the manufacturer, using a sodium citrate system and ninhydrin for detection.

Extraction, Separation and Identification of Proteins by LC-MS/MS Analysis

One gram of plant material obtained from the seedlings growth in chamber (root or needle) was ground to powder with liquid nitrogen with the addition of 0.1 g of polyvinylpolypyrrolidone (PVPP) and extracted with 10 mL of extraction buffer (5% sucrose, 4% SDS and 5% 2-ME) for 10 min at room temperature with gentle stirring, followed by centrifugation at 10.000 *g*. The clear supernatant was heated at 100°C for 3 min and then cooled at room temperature. Proteins were precipitated by adding eight volumes of cold acetone. After at least 1 h at −20°C, the mixture was centrifuged at 10.000 *g*. The pellet was re-suspended in 5 mL of extraction buffer and centrifuged at 10.000 *g* and washed twice with 80% cold acetone, and precipitated by adding four volumes of cold acetone, and was then lyophilized and stored at 0°C (He et al., 2007). The concentrations of extracted proteins were determined using the Bradford method (Bradford, 1976). Proteins were separated by denaturing polyacrylamide gel electrophoresis (SDS–PAGE) using a 5–20% gradient gel. Gels were visualized by staining with Coomassie Brilliant Blue with some modifications as described in Mulet et al. (2006). Rubisco content was used as a visual loading control, as we did not found significant differences in rubisco content among different species. Proteins were identified using standard protein mass spectrometry techniques.

Statistical Analysis

Data were subjected to analysis of variance with tolerance/sensitivity and seed sources as variables, seed source was nested to tolerance/sensitivity to determine differences among drought-tolerant and drought-sensitive seed sources separately, under watered conditions, and then under drought stressed conditions. Additional analysis of variance was carried out to determine significant differences between means at $P < 0.05$ level. Homogeneous groups were separated using the Duncan's test. In all cases, data were examined for normality and homogeneity of variances and assessed for any violations of assumptions using the Duncan's test (Duncan, 1955).

RESULTS

Water Potential and Gas Exchange

The first aim of our study was to confirm if our greenhouse conditions were indeed reflecting real drought conditions and to observe if there were any differences among seedlings from different provenances. Under controlled drought stress, water potential (Ψ_w) decreased significantly by 3.5-fold in both drought-tolerant and drought-sensitive seed sources (**Figure 1A**), indicating that the plants were experiencing drought stress. We also observed that stomatal conductance (*gs*) and transpiration (*E*) showed a higher tendency in drought tolerant seedlings under normal conditions (**Figures 1B,C**). In this sense, only net photosynthesis (P_n) was statistically significant between tolerant and sensitive seed sources when analyzed globally (independently of the watering level) but no differences can be found among means values for each seed source. The photosynthetic rates were about twofold higher in the drought-tolerant seed sources when compared among sensitive/tolerant origins (that is averaged values about 0.41 ± 0.06 and 0.89 ± 0.16 μmol CO_2 m^{-2} s^{-1} for sensitive and tolerant, respectively; **Figure 1D**). No statistical differences were observed between control values and drought stressed ones indicating that the response in terms of *P*n was similar in both conditions.

The maximal efficiency of PSII (*F*v/*F*m) was higher in both watered and drought conditions, but also differed significantly among drought-tolerant and drought-sensitive seedlings. Globally higher values were observed in drought-tolerant seed sources under the watered conditions, while this situation was slightly reversed after the observed decrease of PSII under drought stress (**Figure 1E**). Interestingly, the same pattern was observed regarding data obtained from the field (recorded during summer, see below); drought-sensitive seed sources showed higher PSII values than drought-tolerant ones under natural drought conditions at the Granja d'Escarp trial site (**Figures 1E**, **2C**). The increase of quantum yield of non-cyclic electron transport (Φ_{PSII}) showed slightly higher values in drought-sensitive seedlings than in drought-tolerant seedlings under drought stress (**Figure 1F**). Water use efficiency (WUE_{inst}) was higher in the drought-tolerant seed sources under drought conditions (**Figure 1G**). This fact was consequence of greater capacity to maintain higher *P*n rates in drought tolerant seedlings for similar *E*-values in both, drought-sensitive and drought-tolerant.

We confirmed the validity of our experimental design by measuring the mentioned physiological parameters in plants of the same provenances grown in the field. Under field conditions the results obtained in the greenhouse regarding *E*, *P*n and

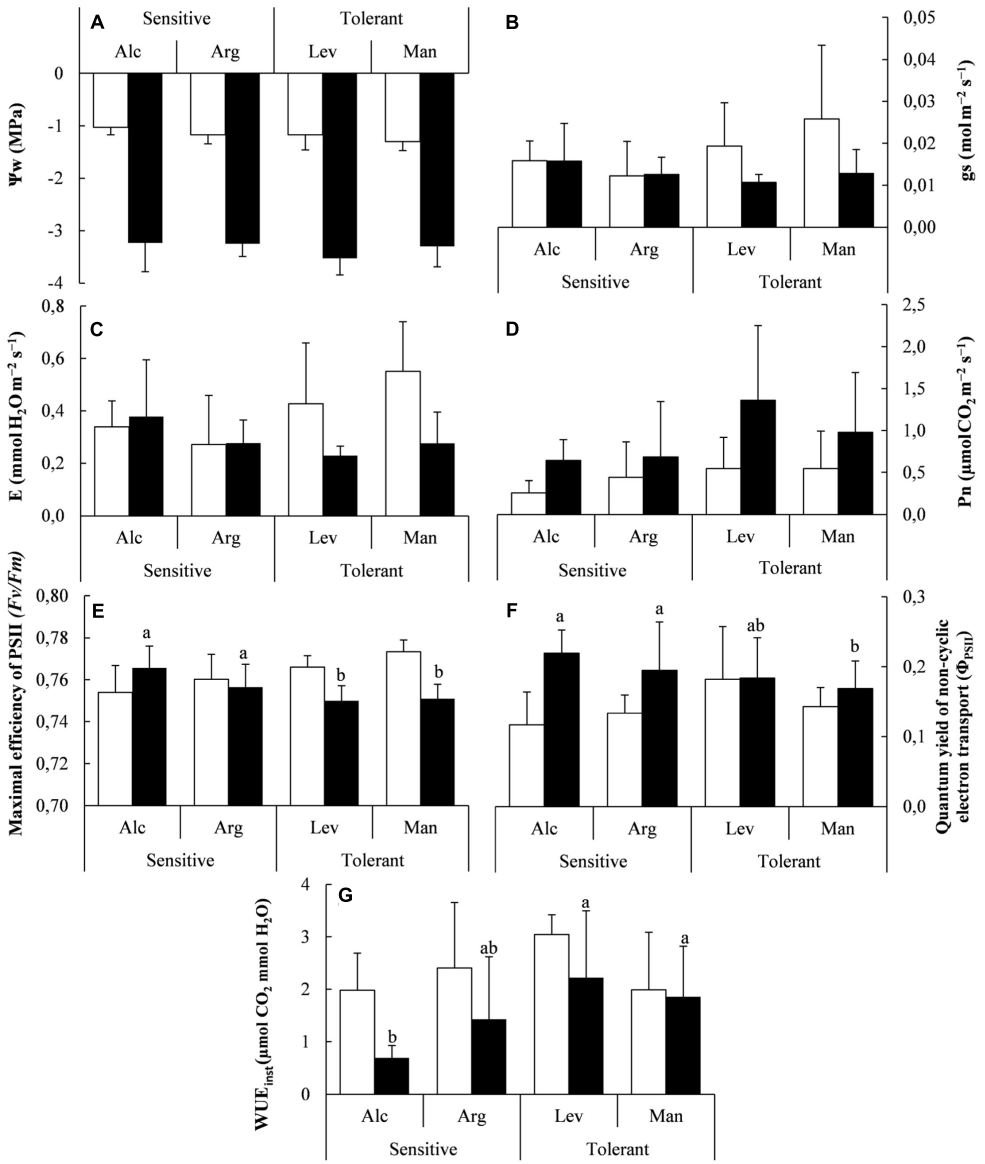

FIGURE 1 | Water potential, gas exchange and photosynthesis measurements under greenhouse conditions. Water potential (Ψw) **(A)**, stomatal conductance (g_s) **(B)**, transpiration (E) **(C)**, Net photosynthesis (*Pn*) **(D)**, Maximal efficiency of PSII **(E)**, quantum yield of non-cyclic electron transport (Φ_{PSII}) **(F)** and instantaneous water use efficiency (WUE_{inst}) **(G)** of drought-sensitive and drought-tolerant seed sources under watered (white bars) and drought stressed (black bars) treatments. The letters above the bars marks the significant difference among the drought-stressed seed sources following the *post hoc* Duncan's test. Scale bars are mean +SE, being the number of samples n = 15 for Ψw and n = 5 for the other variables.

WUE are in agreement with those observed in field under environmental conditions, given that the observed differences are not statistically significant (**Figures 1C,D,G**, **2A,B,D**). However, *Pn* followed the same trend with higher values in tolerant provenances and *Fv*/*Fm* values were also slightly lower, from 0.76 in sensitive seedlings to 0.75 in tolerant.

Photosynthetic Pigments and Soluble Sugars

Once we confirmed the validity of our design, and observed the expected results regarding the physiological parameters related to water use and photosynthesis, we investigated the effect of drought on several photosynthetic pigments. Drought stress induced changes in photosynthetic pigments and differential patterns could be observed among drought-stressed seedlings from different origins. Chl a, Chl b and Car concentrations were less affected by drought in drought-tolerant seedlings and accumulation was 1.5- to 2-fold higher in drought-tolerant seed sources under drought conditions (**Figures 3A–C**).

One of the known physiological strategies to cope with drought stress is the osmotic adjustment through the accumulation of soluble sugars. In our samples, under

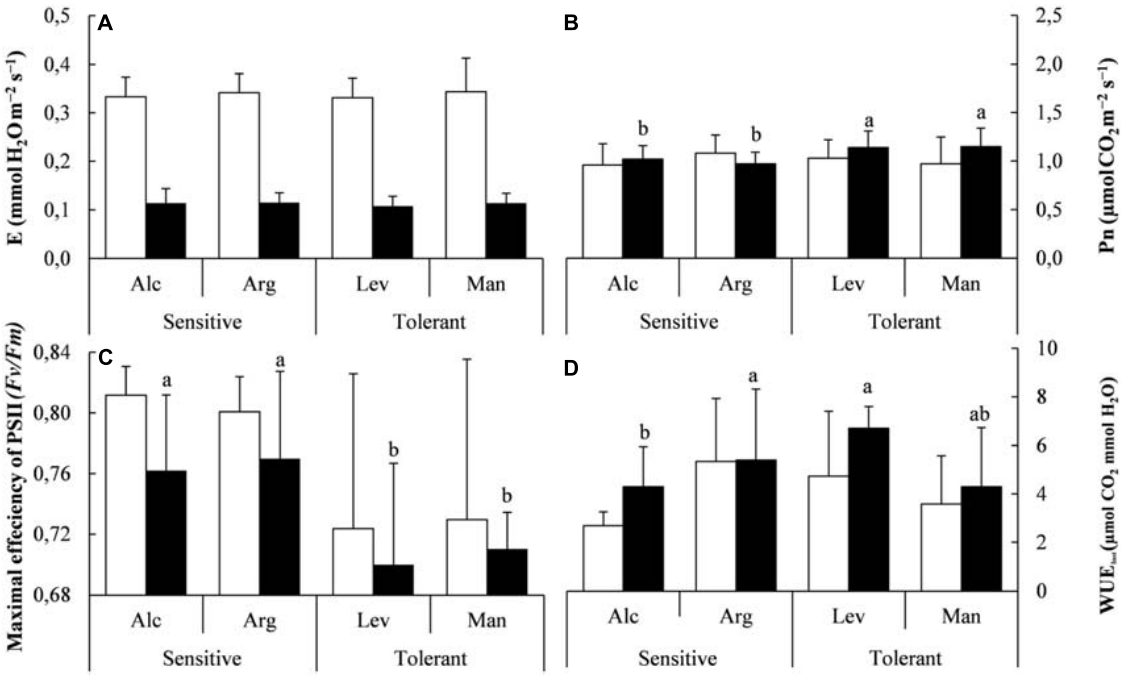

FIGURE 2 | Gas exchange, photosynthesis measurements and water use efficiency under field conditions. Transpiration (E) **(A)**, Net photosynthesis (*Pn*) **(B)**, Maximal efficiency of PSII **(C)** and instantaneous water use efficiency (WUE_{inst}) **(D)** of drought-sensitive and drought-tolerant seed sources under control site(white bars; La Hunde site, considered the core habitat) and the dry site (black bars; La Granja d'Escarp considered the marginal dry habitat for this species). The letters above the bars marks the significant difference among the drought-stressed seed sources following the *post hoc* Duncan's test with $n = 5$. Scale bars are mean +SE.

drought conditions, the concentration of glucose decreased approximately 20 and 35% in drought-tolerant and drought-sensitive seed sources, respectively (**Figure 3D**). We could also observe differences in fructose and sucrose concentration. While fructose remains unchanged in the drought-tolerant seed source 'Man,' it increases about 40% in the drought-tolerant seed source 'Lev.' On the other hand it also increased by 20% in the drought-sensitive seed sources (**Figure 3E**). In addition, sucrose concentration increased about sevenfold in the drought-tolerant seed sources and only twofold in the drought-sensitive ones (**Figure 3F**) showing an enhanced accumulation in tolerant seed sources under conditions of water limitation.

Glutathione and Free Amino Acids

It has been shown that in response to drought stress the biosynthesis of sulfur containing amino acids may become limiting, specifically for the requirement of cysteine for the biosynthesis of glutathione (GSH), which is required to cope with the oxidative stress induced by drought (Mulet et al., 2004). In our study, GSH accumulated approximately 20% more in drought-tolerant seedlings upon drought stress (**Figure 4A**). Drought-tolerant seedlings contained less cysteine under control conditions but maintained the cysteine pool upon stress (**Figure 4B**). The increase of methionine (Met) concentrations under drought was higher than in sensitive seedlings (**Figure 4C**). Serine is required for cysteine and methionine biosynthesis. The concentration of this amino acid was higher in drought-tolerant seedlings both under watered and drought-stressed conditions (**Figure 4D**).

There is no description available in the literature regarding the behavior of the free amino acid pools under drought stress in *Pinus halepensis*. Given that some of these amino acids can act as precursors for osmolytes, act as osmolytes themselves or even have previously undescribed functions in stress tolerance we investigated the complete free amino acid profile in our plants under the studied conditions. Proline and glycine are related to osmotic adjustment and, as expected, proline accumulated under drought stress. Interestingly this accumulation was higher in drought-tolerant seed sources (**Figure 5A**). Glycine concentrations were significantly higher in drought-tolerant seed sources under both watered and drought stressed conditions (**Figure 5B**).

Changes in the hydrophobic amino acids Alanine (Ala) and isoleucine/leucine (Ile/Leu) were also noteworthy; they decreased dramatically under drought stress. Alanine was higher in drought-sensitive seedlings while leucine/isoleucine was higher in drought-tolerant ones (**Figures 5C,D**). Histidine (His) and tyrosine (Tyr) concentrations were higher in the drought-sensitive seed sources under watered conditions but under drought stress, drought-tolerant seedlings accumulated higher concentrations (**Figures 5E,F**).

We did not observe differential behavior between drought-tolerant and drought-sensitive seed sources regarding charged amino acids (**Figure 6**). However, it is interesting to note how their concentrations change under drought stress. Arginine

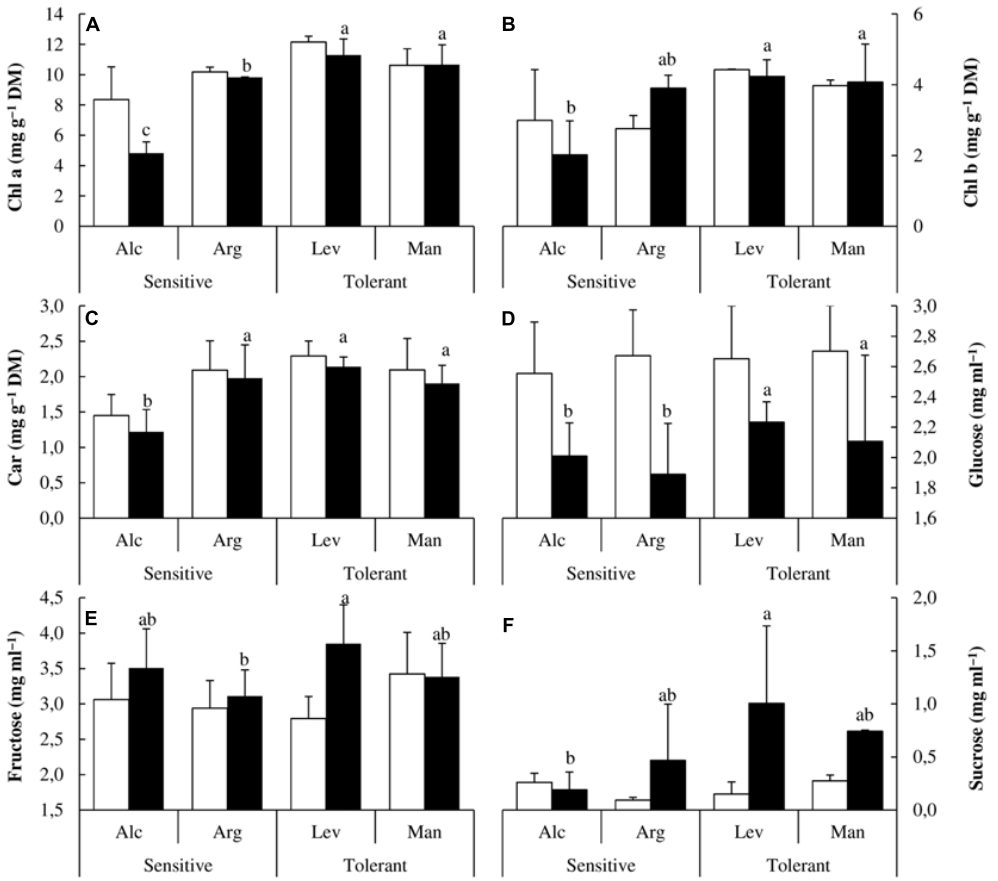

FIGURE 3 | Photosynthetic pigments and soluble sugars content. Chlorophyll a (Chl a) **(A)**, chlorophyll b (Chl b) **(B)**, carotenoid (Car) **(C)**, glucose **(D)**, fructose **(E)** and sucrose **(F)** concentrations of drought-sensitive and drought-tolerant seed sources under watered (white bars) and drought stressed (black bars) treatments. The letters above the bars marks the significant difference among the drought-stressed seed sources following the test of Duncan with $n = 5$. Scale bars are mean +SE.

(Arg), glutamic acid (Glu) and aspartic acid (Asp) concentrations increased significantly (**Figures 6A,E,F**). The change for glutamic acid was about 20-fold (**Figure 6E**). Asparagine (Asn) and lysine/glutamine (Lys/Gln) concentrations decreased twofold (**Figures 6B,D**), and threonine decreased by more than 10-fold under drought stress (**Figure 6C**).

Proteomic Analysis

Changes in the protein profile were also tested to determine which proteins potentially serve as biomarkers for stress tolerance or sensitivity, thus, facilitating the screening of tolerant seed sources in the future. We used 1D gel electrophoresis in order to perform side-by-side comparisons of eight different samples (four drought-sensitive and four drought-tolerant samples). We observed protein profiles of roots and needles under both watered and drought conditions. Under watered conditions we did not observe any significant change in the protein profile from needles. However, in roots under watered conditions we observed five differential bands in drought-sensitive and five in the drought-tolerant seed sources. The proteins identified from these differential bands were related mostly to carbon metabolism or protein translation (data not shown). These kinds of proteins are overrepresented in proteomes, independently of their biological origin, so these data were not considered to be relevant. Under drought stress, we observed the accumulation of proteins in the drought-resistant seed sources that are not present in the drought-sensitive seed sources. These results appear to be more informative since we found several heat shock proteins, a β-pinene synthase and the 5-methyltetrahydropteroyltriglutamate-homocysteinemethyl transferase (EC 2.1.1.14). This enzyme is responsible for the Vitamin-B-12 independent methionine biosynthesis, indicating that its function could be limiting under drought conditions (**Table 1**) and further confirming the increase in methionine in drought tolerant seedlings, as showed in **Figure 4C**.

DISCUSSION

Pinus halepensis, commonly known as Aleppo pine is a useful plant for reforestation due to its ability to adapt to fluctuations in temperature and soil water availability during its life cycle (Baquedano and Castillo, 2006; Baquedano et al., 2008). Here we

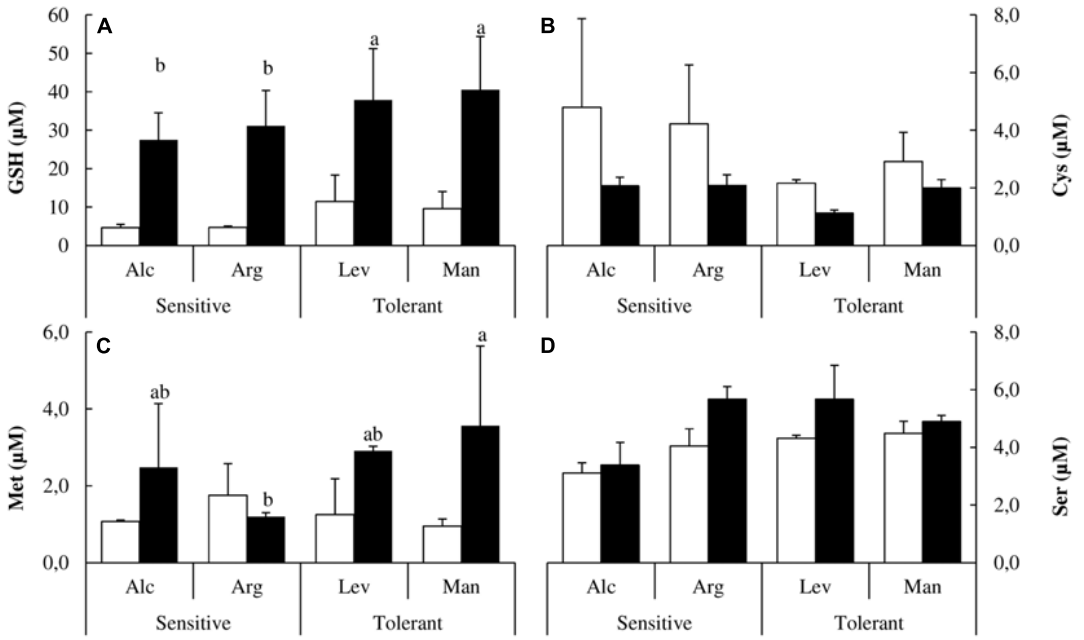

FIGURE 4 | Glutathione, sulfur containing amino acids and serine determination. Glutathione (GSH) **(A)**, cysteine (Cys) **(B)**, methionine (Met) **(C)** and serine (Ser) **(D)** concentrations of drought-sensitive and drought-tolerant seed sources under watered (white bars) and drought stressed (black bars) treatments. The letters above the bars marks the significant difference among the drought-stressed seed sources following the test of Duncan with $n = 5$. Scale bars are mean +SE.

have compared at the molecular and physiological level the effect of drought in different seed provenances.

Throughout this study, the water potential (Ψ_w) exhibited significant reductions in drought-stressed *Pinus halepensis* seedlings, thus, validating our experimental design and confirming that the seedlings in the greenhouse were affected by the drought conditions employed. It is known that one of the main problems caused by drought is oxidative damage (Golldack et al., 2014) affecting several plant processes. One of the strategies to avoid this constraint is to regulate the energy status (Voss et al., 2013) which suggests that drought-tolerant seedlings are more efficient at down-regulating the maximum efficiency of PSII and thus avoiding oxidative stress. Taken together, our data indicate that the most important parameter for drought tolerance is the ability to reduce the efficiency of photosystem II. A recent report has shown that a similar behavior has been observed in other plants, specifically a higher photosynthetic rate in the favorable season was associated with a stronger decline in the unfavorable season (Zhang et al., 2017), which would be similar to what we are observing here for *P. halepensis*. Drought tolerant plants also accumulate more photosynthetic pigments than drought sensitive plants (**Figures 3A–C**) likely diminishing photo-oxidative damage and ROS production (Vilagrosa et al., 2010). In this sense, the pattern of response in the present study in relation to gas exchange and photochemical efficiencies under control and drought conditions follows the results observed in previous studies although our data are located in the lower range of observed values (Baquedano and Castillo, 2006; Cuesta et al., 2010; Klein et al., 2012). This is probably consequence of seedling age as we were using very young seedlings (<1 year) and the water saving strategy followed by this species in small seedlings increases water economy (Baquedano and Castillo, 2006). This effect has been already observed when analyze gas exchange patterns in some Mediterranean species with moderate gas exchange rates (Vilagrosa et al., 2003; Atzmon et al., 2004; Ruiz-Yanetti et al., 2016) and partially can be attributed to xylem characteristics with short and narrow vessels (Vilagrosa, pers. obs). In addition, several studies analyzing plant provenances highlighted the relevance of morphological and functional factors allowing plant acclimation to stress conditions, from hydraulic effects (Peguero-Pina et al., 2014) to changes in mesophyll conductance affecting to photosynthetic parameters (Peguero-Pina et al., 2017).

We extended our research to study the NSC which are among the most drought-responsive metabolites. Their concentrations could increase due to starch hydrolysis or impairment of starch production (Rodríguez-Calcerrada et al., 2011). In *Pinus halepensis* the most distinctive feature of drought-tolerant seed sources was the increase in both sucrose and fructose. Sucrose can act as an osmolyte to prevent water loss and protect cellular structures from dehydration. Therefore; the ability to accumulate sucrose under drought stress could be another key factor to distinguish between drought-tolerant and drought-sensitive seed sources (**Figure 3F**).

Under drought stress, seedlings can down-regulate photosynthesis, accumulate osmolytes or improve the antioxidant response to prevent the deleterious effects caused by ROS. The synthesis of cysteine from serine and the subsequent biosynthesis of GSH is a key aspect of antioxidant defense, especially the activity of the serine acetyl transferase enzyme,

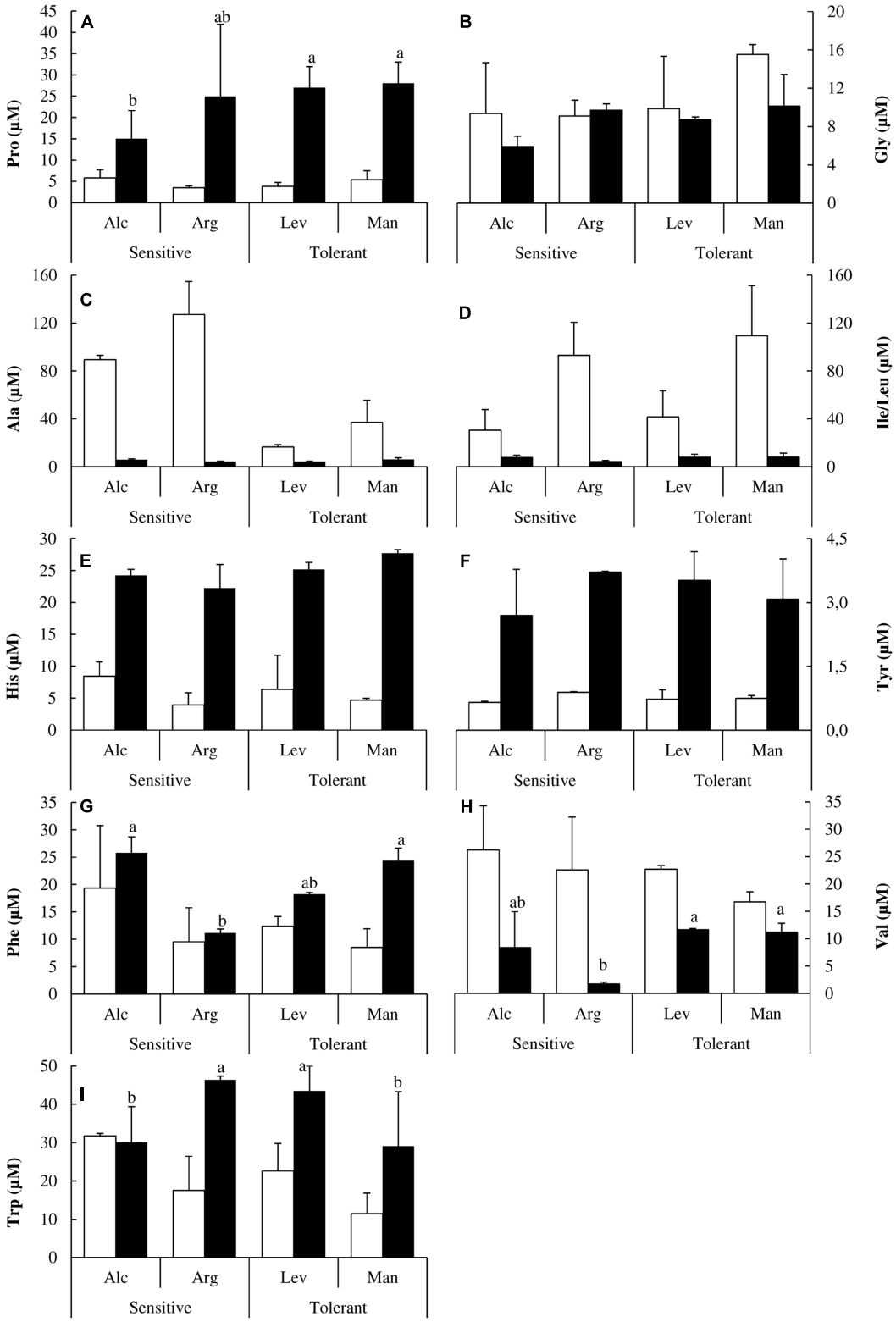

FIGURE 5 | Non-polar amino acids and histidine determination. Proline (Pro) **(A)**, glycine (Gly) **(B)**, alanine (Ala) **(C)**, isoleucine/leucine (Ile/Leu) **(D)**, histidine (His) **(E)**, tyrosine (Tyr) **(F)**, phenylalanine (Phe) **(G)**, valine (Val) **(H)** and tryptophan (Trp) **(I)** concentrations of drought-sensitive and drought-tolerant seed sources under watered (white bars) and drought stressed (black bars) treatments. The letters above the bars marks the significant difference among the drought-stressed seed sources following the test of Duncan with $n = 5$. Scale bars are mean +SE.

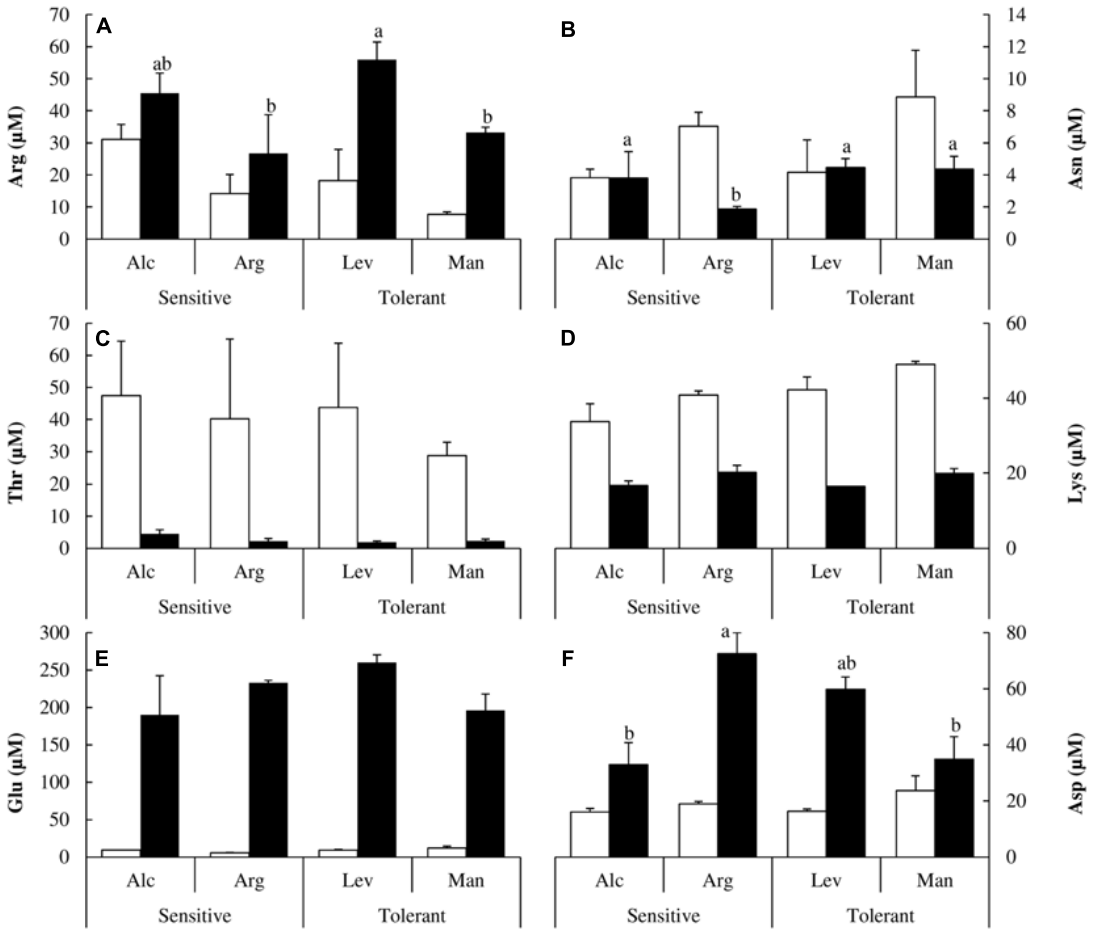

FIGURE 6 | Polar or Charged amino acids determination. Arginine (Arg) **(A)**, asparagine (Asn) **(B)**, threonine (Thr) **(C)**, lysine/glutamine (Lys) **(D)**, glutamic acid (Glu) **(E)** and aspartic acid (Asp) **(F)** concentrations of drought-sensitive and drought-tolerant seed sources under watered (white bars) and drought stressed (black bars) treatments. The letters above the bars marks the significant difference among the drought-stressed seed sources following the test of Duncan with $n = 5$. Scale bars are mean +SE.

which may be considered as the main limiting factor (Freeman et al., 2004; Mulet et al., 2004). Recent reports confirm that GSH is considered to be the most important thiol involved in the prevention of oxidative damage in plants (Labudda and Safiul-Azam, 2013; Pyngrope et al., 2013). We could confirm that GSH accumulation is also a key and a distinctive factor for drought tolerance in woody plants as the drought-tolerant seed sources present a fourfold increase in response to drought stress (**Figure 4A**).

There are several reports in the literature illustrating how the levels of different amino acids change under drought stress. Here, we have performed a complete analysis of free amino acids. This is the first study at the amino acid level comparing different *Pinus halepensis* seed sources under drought stress. We found a distinctive pattern in sulfur amino acids. Drought-tolerant seed sources have less Cys and less Met than the drought-sensitive ones under watered conditions, but they maintain their levels of Cys and increase those of Met under drought stress. However, the drought-sensitive seed sources decrease Cys levels and maintain the levels of Met under drought stress (**Figures 4B,C**). Ser levels remain unchanged under stress, but they are higher in the drought-tolerant seed sources. The complete analysis of free amino acids pools under drought stress showed significant increases in the content of Tyr, His, Pro, Arg, Glu and Asp along with a decrease in Ala, Ile, Val, Thr and Lys concentrations (**Figures 4**, **5**). The increase in Pro (approximately sixfold; **Figure 5A**) can be explained because of its activity as an osmolyte (Serrano et al., 1999; Verbruggen and Hermans, 2008); the Gly content was higher in the drought-tolerant seedlings, but the levels drop under stress (**Figure 5B**), probably due the increase of GSH. The interpretation of the remaining changes goes beyond the scope of this article.

The proteomic study also generated valuable information. Under watered conditions we did not observe any differential bands in needles, but several proteins were differentially expressed in roots. As expected, most of the identified proteins are related to the transcriptional machinery and sugar metabolism. Because proteins participating in these

TABLE 1 | Proteomic analysis.

m/z	Score	NCBI/TrEmbl	Protein family
600,3	252	*Plectranthus scutellarioides Q42662*	5-methyltetrahydropteroyltriglutamate–homocysteine methyltransferase
636,8	127	*Oryza sativa* subsp. indica A2YWQ1	Heat shock protein 82
527,3	95	*Arabidopsis thaliana* P51818	Heat shock protein 81-3
804,4	93	*Zea mays* ACG43057	succinate dehydrogenase flavoprotein subunit, mitochondrial
630,3	166	*Citrus sinensis* XP_006488105	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, chloroplastic-like isoform X1
782,4	55	*Pinus radiate* AEW09360	hypothetical protein UMN_6182_01, partial
589,3	80	*Pinus banksiana* AFU73844	(−)-beta-pinene synthase
950,2	98	*Picea sitchensis* ACN40706	Unknown (~amidase 1-like)
837,4	102	*Picea sitchensis* ABK23116	Unknown (~chloroplast photosystem I subunit)
472,8	83	*Picea sitchensis* ABK27024	Unknown (~histone H2A)

Proteins of differential expression identified among roots of Pinus halepensis plants tested under drought-stressed conditions.

processes are over-represented in proteomes, we considered them irrelevant for the purpose of this study (data not shown). More significant results were obtained in drought stressed roots (**Table 1**) where we detected two different heat shock proteins of the 82 and 81-3 type. The role of these proteins is to act as molecular chaperones that promote the maturation, structural maintenance and proper regulation of specific target proteins in an ATP-dependent manner. Under drought stress, misfolded proteins are likely to accumulate, therefore, the increase in this kind of proteins is surely beneficial for defense against cellular stress. We also found a β-pinene synthase to be up-regulated in roots under drought stress conditions. This protein is regulated in a circadian manner. Given that all samples were collected at the same time and that its enzymatic activity is exerted mainly in leaves, we cannot discard an undescribed role for this enzyme in stress response in roots.

Another interesting finding is the accumulation of the 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (EC 2.1.1.14) in the roots of drought-tolerant seedlings. This enzyme is responsible for the Vitamin-B12-independent methionine biosynthesis. In bacteria, this pathway has been related to abiotic stress response (Mordukhova and Pan, 2013). It is known that this pathway exists in plants (Eichel et al., 1995). This enzyme was identified in a proteomic study as being important for somatic embryogenesis in *Quercus suber* (Gomez-Garay et al., 2013). However, its relation to drought stress has not been postulated before.

Here we report that under drought stress the drought-tolerant seedlings increase the production of Met. As a result, it is possible that the alternative pathway for Met biosynthesis is more active in the drought-tolerant seedlings leading to the increase of Met supply. Met is a substrate for the synthesis of various polyamines with important roles in drought tolerance (Groppa and Benavides, 2008; Alcázar et al., 2010). Sulfur amino-acids metabolism is determinant for drought stress tolerance although the enzyme which becomes limiting could change in different species (Freeman et al., 2004; Mulet et al., 2004). Our results indicate that the alternative biosynthesis of Met, together with the synthesis of GSH are associated to drought tolerance in *Pinus halepensis*.

CONCLUSION

We have used a Greenhouse-based approach to determine differential traits at the physiological and molecular levels between drought-tolerant and drought-sensitive seed sources of *Pinus halepensis*. Our results indicate that the most distinctive trait for drought tolerance in *Pinus halepensis* seedlings are related to oxidative stress. Drought-tolerant seed sources presented a more pronounced down-regulation of PSII and a higher accumulation of photosynthetic pigments including carotenoids which act mainly as antioxidants. In addition, we propose that the photosynthesis rate and the maximal efficiency of PSII could constitute potential distinctive traits among drought-tolerant and drought-sensitive *Pinus halepensis* seedlings.

Analysis of free amino acids indicated that there was a significant increase of GSH and Met concentrations. The increase in Met was corroborated at a different level by the identification of a Met synthase as a differential protein band present in stressed drought-tolerant seed sources.

Taken all together, we propose that the analysis of photosynthetic parameters (photosynthesis rate, the maximal efficiency of PSII and photosynthetic pigments), along with the determination of fructose, Met and GSH concentrations could be a fast and reliable method to screen for *Pinus halepensis* seed sources which are more likely to be drought tolerant.

Our findings are the basis to develop valuable evaluation tools to determine the appropriate seed source for reforestation programs threatened by drought, thus avoiding long field trial tests.

AUTHOR CONTRIBUTIONS

KT planned, performed experiments and wrote the paper. AV assisted in the photosynthetic gas exchange and chlorophyll fluorescence measurements. JB and ML-G helped with the sugar content analysis. DP and JC performed the mass spectrometry identification of the proteins. JL-N undertook the free amino acid analysis. AdC and JM planned, guided and performed experiments and wrote the paper.

FUNDING

This study is a part of the research project: "Application of molecular biology techniques in forest restoration in Mediterranean environments, PAID-05-11" funded by the Universitat Politècnica de València (UPV), program for supporting R&D of new multidisciplinary research lines. The authors are grateful to the Ministerio de Economía y Competitividad AGL2014-57431-P. AV was supported by project Survive-2 (CGL2015-69773-C2-2-P MINECO/FEDER) by the Spanish Government and Prometeo program (DESESTRES-Generalitat Valenciana). CEAM is funded by Generalitat Valenciana.

ACKNOWLEDGMENTS

Jaime Flors and Ana M. Aguado for their collaboration in field measurements. We are indebted to Lynne Yenush for her great help in the elaboration of the manuscript.

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