PLANT-SOIL INTERACTIONS UNDER CHANGING CLIMATE

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PLANT-SOIL INTERACTIONS UNDER CHANGING CLIMATE

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EDITORIAL
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Editorial: Plant-Soil Interactions Under Changing Climate

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Keywords: climate change, greenhouse gas (CH_4, N_2O, CO_2), microbiome, ecosystem, vegetation

Editorial on the Research Topic

Plant-Soil Interactions Under Changing Climate

The health and well-being of plants and soil is crucial for all life on Earth. It is well-known that vegetation cover follows climatic zones, and plants respond to climatic drivers such as temperature and precipitation (Seddon et al., 2016; Kattge et al., 2020). It is also well-known that plant health depends on the properties and health of the soil (Ephrath et al., 2020), and that strong interactions among biota above and belowground dictate the functioning of both realms (Van der Putten et al., 2013). Yet, soils and the processes occurring belowground are often considered a "black box," and are treated very simplistically in our efforts to understand, quantify, and model the future of the planet. Our understanding of the interactions between plants and soils is also far from complete and offers some of the most important research frontiers in community ecology, biogeochemistry, and global change science.

This Research Topic gathers contributions to the growing literature highlighting the importance of interactions between plants and soil to their mutual health and productivity, as well as to their contributions to greenhouse gas emissions and the climate system. The soil in itself is a complex system consisting of the mineral soil matrix mixed with organic materials, mostly of plant origin. Organic matter is decomposed, altered, and modified by the soil microbiome, consisting of a myriad of bacteria, fungi, algae, viruses, and archaea. The products of these processes are typically greenhouse gases such as CO_2, N_2O, or CH_4 that are released to the atmosphere, and dissolved organic carbon (DOC) that remains in the soil or is lost through the hydrologic system (Ontl and Schulte, 2012). These processes also transform key nutrients into forms available for plant use (Jacoby et al., 2017). The rate of transformation and products created depend on the type and amount of organic material, the composition of the microbiome, as well as the chemical and physical environment affected by the soil matrix properties, climate, and weather. These controls, in turn, also influence the composition of the soil-, rhizosphere-, and plant-microbiome. In addition to producing organic materials for decomposition through litter, plants add a layer of complexity to the system by exuding relatively simple carbohydrates to feed their preferred microbiome, which helps the plant to thrive through improved access to water and nutrients (Jacoby et al., 2020) and possibly releasing plant growth-promoting chemical signals (van Dam and Bouwmeester, 2016). The complexity of plant-soil interactions, and the lack of effective methods to analyze microbiome composition and function until recent years, mean that the field is open for discoveries. Recent technical developments are revolutionizing the field across multiple scales and for numerous components of the plant-soil system, including exposing the dynamics of interactions, identifying differences in microbial communities and how the environment influences both (Sergaki et al., 2018).

This Research Topic includes both experimental and review studies addressing many of the key aspects of plant-soil interactions, using novel approaches and innovative perspectives. Soil, plants,

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and microorganisms form a triangle of six possible interactions; all of which are represented in the diverse studies in this collection. The atmosphere—its gas composition and climate—makes for a fourth player, also studied here. Taken as a whole, readers will find updated information on burning questions such as (1) how plants and the environment influence soil microbial communities (Deng J. et al.; Gehring et al.; Karlowsky et al.; Liu et al.; Mandrubia et al.; Na et al.); (2) how soil and the rhizosphere microbiome affect the function of plants (Egamberdieva et al.; Ulrich et al.; Vargas et al.); (3) how plant-microbiome interactions influence nutrient availability and soil chemistry (Salmon et al.; Wei et al.); and (4) how plants, soil, and the microbiome influence greenhouse gas emissions (Bréchet et al.; Deng N. et al.).

This collection of studies covers a wide variety of environments from agricultural systems (Egamberdieva et al.; Na et al.), to temperate forests (Deng J. et al.; Deng N. et al.; Gehring et al.; Liu et al.; Ulrich et al.), subalpine forests (Wei et al.) tropical forests (Bréchet et al.), grasslands (Karlowsky et al.; Vargas et al.; Wei et al.), and the Arctic (Salmon et al.). The Topic also includes a study addressing the effects of plant invasions and range expansion across latitudes on the associated soil microbiome (Mandrubia et al.). The work collated here covers a variety of spatial and temporal scales, as well as the effects of different abiotic stressors or recovery from disturbance on the soil and soil microbiome. The variety of topics highlights the state of the art in the field. The complexity and remaining unknowns of the field do not yet allow for final overarching conclusions. Nevertheless, similar to studies in any rapidly growing field, each contribution adds important knowledge to the catalog of information that forms the basis for building theories and conceptual models for understanding the function of the system. Harnessing microbiomes to improve soil health, control greenhouse gas emissions, and improve plant stress tolerance and performance has a huge potential for resolving some of the largest challenges of humanity from controlling and mitigating climate and environmental change to ensuring food security.

We hope that you enjoy this collection of studies and allow it to inspire your research and quest for understanding how plants and soils interact; how they influence the world around us; and how the changing world impacts them.

AUTHOR CONTRIBUTIONS

SS drafted the story line of the editorial. CG, TK, and SR refined the story line and contributed references and insights to the impact of the papers included in this research topic.

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Drought-Induced Accumulation of Root Exudates Supports Post-drought Recovery of Microbes in Mountain Grassland

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Droughts strongly affect carbon and nitrogen cycling in grasslands, with consequences for ecosystem productivity. Therefore, we investigated how experimental grassland communities interact with groups of soil microorganisms. In particular, we explored the mechanisms of the drought-induced decoupling of plant photosynthesis and microbial carbon cycling and its recovery after rewetting. Our aim was to better understand how root exudation during drought is linked to pulses of soil microbial activity and changes in plant nitrogen uptake after rewetting. We set up a mesocosm experiment on a meadow site and used shelters to simulate drought. We performed two ^{13}C-CO_2 pulse labelings, the first at peak drought and the second in the recovery phase, and traced the flow of assimilates into the carbohydrates of plants and the water extractable organic carbon and microorganisms from the soil. Total microbial tracer uptake in the main metabolism was estimated by chloroform fumigation extraction, whereas the lipid biomarkers were used to assess differences between the microbial groups. Drought led to a reduction of aboveground versus belowground plant growth and to an increase of ^{13}C tracer contents in the carbohydrates, particularly in the roots. Newly assimilated ^{13}C tracer unexpectedly accumulated in the water-extractable soil organic carbon, indicating that root exudation continued during the drought. In contrast, drought strongly reduced the amount of ^{13}C tracer assimilated into the soil microorganisms. This reduction was more severe in the growth-related lipid biomarkers than in the metabolic compounds, suggesting a slowdown of microbial processes at peak drought. Shortly after rewetting, the tracer accumulation in the belowground plant carbohydrates and in the water-extractable soil organic carbon disappeared. Interestingly, this disappearance was paralleled by a quick recovery of the carbon uptake into metabolic and growth-related compounds from the rhizospheric microorganisms, which was probably related to the higher nitrogen supply to the plant shoots. We conclude that the decoupling of plant photosynthesis and soil microbial carbon cycling during drought is due to reduced

carbon uptake and metabolic turnover of rhizospheric soil microorganisms. Moreover, our study suggests that the maintenance of root exudation during drought is connected to a fast reinitiation of soil microbial activity after rewetting, supporting plant recovery through increased nitrogen availability.

Keywords: plant-soil (belowground) interactions, stress tolerance, mountain grassland, ^{13}C pulse labeling, carbohydrates, NLFA, PLFA, chloroform fumigation extraction

INTRODUCTION

Climate change threatens the functioning of terrestrial ecosystems, which will very likely suffer from more frequent extreme events induced by the ongoing global warming (IPCC, 2012). A large part of the terrestrial biosphere consists of grassland ecosystems that cover approximately 40% of the vegetated land surface and strongly contribute to soil carbon storage (White et al., 2000). The functioning of grasslands and their role in the global carbon cycle are particularly placed at risk by periods of severe drought (Reichstein et al., 2013; Frank et al., 2015). Grasslands in some areas may experience more severe drought effects, such as, for example, in the European Alps, which are affected by faster temperature increases compared to the global average (Beniston, 2005; Auer et al., 2007).

Extreme droughts typically lead to reduced carbon assimilation in plants (Huang and Fu, 2000; Naudts et al., 2011; Roy et al., 2016; Ingrisch et al., 2018) and reduced carbon transfer to the roots and the rhizosphere (Fuchslueger et al., 2014a, 2016; Hasibeder et al., 2015; Karlowsky et al., 2018), resulting in a lower soil CO_2 efflux (Ruehr et al., 2009; Barthel et al., 2011; Burri et al., 2014). Consequently, the reduced belowground carbon allocation (BCA) weakens plant–microbial interactions (Brüggemann et al., 2011). Because soil microorganisms strongly depend on plant-derived carbon inputs (Wardle et al., 2004; Bardgett et al., 2005), important soil functions, such as the microbial mineralization of nitrogen and phosphorous, are limited during drought (Stark and Firestone, 1995; Borken and Matzner, 2009; Delgado-Baquerizo et al., 2013; Fuchslueger et al., 2014b; Canarini and Dijkstra, 2015; Dijkstra et al., 2015). In addition, symbiotic interactions with arbuscular mycorrhizal (AM) fungi, which strongly increase the drought resistance of plants (Allen, 2007), are affected by severe drought (Karlowsky et al., 2018). So far, whether the weakening of the link between plants and soil microorganisms during drought (i.e., the reduced soil microbial usage of recently assimilated plant-derived carbon) is due to (1) the altered carbon allocation of plants leading to reduced root exudation, (2) the limited substrate mobility in the rhizosphere, or (3) a slowdown of soil microbial metabolism is unknown. Possibly, these three mechanisms appear at the same time and interact with each other.

Drought has been shown to induce a shift of carbon allocation from the aboveground to the belowground plant organs (Palta and Gregory, 1997; Huang and Fu, 2000; Burri et al., 2014) and to increase the amounts of soluble sugars in the roots (Hasibeder et al., 2015; Karlowsky et al., 2018). The latter two studies also showed that drought-induced reductions of storage sugar concentrations are more pronounced in shoots than roots. The increase of soluble root sugars has been attributed either to osmotic regulation to support the survival of root biomass (Sicher et al., 2012; Hasibeder et al., 2015) while maintaining the carbon demand for respiration (Barthel et al., 2011) or to increased fine root growth to enhance plant access to deeper soil water resources (Huang and Fu, 2000; Burri et al., 2014). Until now, whether these drought-reduced changes in plant carbon allocation to stored reserve sugars versus soluble root sugars that are linked to exudation are affecting the carbon released into the rhizosphere has been unknown. In a recent meta-analysis of the scarce existing literature, Preece and Peñuelas (2016) found that drought can have variable effects on the rhizospheric carbon release. Strikingly, the authors of this study reported a trend toward increased root exudation per gram of plant biomass (including either root and shoot biomass or shoot biomass only) under moderate drought. However, the root biomass response to drought strongly varies among the different studies (Kreyling et al., 2008 and references therein), potentially affecting the total amount of carbon released to the rhizosphere. For example, Fuchslueger et al. (2014a) found that a slightly increased root to shoot ratio during drought was mirrored by higher amounts of plant-derived carbon in the extractable organic carbon (EOC) of soil.

The drying of soil itself has major impacts on the exudate transfer from the release site to rhizospheric microorganisms, which might increase the competition for substrates between functionally different microbial groups. In contrast to AM fungi, which are directly connected to the root carbohydrate pool, saprotrophic fungi (SF) and bacteria depend on the diffusion of substrates for their nutrition (Manzoni et al., 2012). As the lower water content during drought conditions limits the diffusion of substrates (Skopp et al., 1990), the uptake of nutrients by SF and bacteria is limited. Moreover, experimental results suggest that the microbial activity in the soil depends on the environmental conditions that affect diffusion pathways between substrate sources and microorganisms (Nunan et al., 2017). Consequently, if root exudation is increased along with root growth during drought, plant-derived solutes likely will accumulate in the rhizosphere due to reduced microbial carbon mineralization. Indeed, increased amounts of dissolved organic carbon immediately after the rewetting of dried soils (Canarini et al., 2017) suggest the existence of such accumulations. These additional carbon sources could further contribute to the pulse of soil respiration, which appears after rewetting and is associated with higher soil microbial activity and nitrogen mineralization (Birch, 1958). The so-called 'Birch effect' is present in planted and unplanted soils (Canarini et al., 2017) and has been suggested to primarily originate from osmolytes, which accumulate in

microbial cells during drought conditions (Fierer and Schimel, 2003). As a stress response to desiccation, the synthesis of microbial osmolytes is increased at the expense of membranes for cell growth (Schimel et al., 2007). To prevent the bursting of cells due to excessive water uptake, accumulated osmolytes need to be rapidly metabolized after rewetting (Warren, 2014). The metabolically active microorganisms are probably also able to use excess plant-derived carbon, which could support plant recovery by further increasing the nitrogen mineralization rate in the soil.

Plant carbon allocation is best analyzed by pulse-labeling of the plant canopy with ^{13}C-enriched CO_2 and tracing of the assimilated ^{13}C by compound specific carbon isotope ($^{13}C/^{12}C$) ratios of plant non-structural carbohydrates (NSCs) (Bahn et al., 2013; Karlowsky et al., 2018). Similarly, root exudation and the subsequent microbial carbon uptake can be determined by combining the K_2SO_4 extraction and chloroform fumigation method (Vance et al., 1987) with ^{13}C analysis (Malik et al., 2013). This allows the flow of plant-derived carbon in EOC and microbial biomass carbon (MBC) from soil to be traced. The water-soluble EOC is mainly a proxy for the exuded plant carbon (**Supplementary Figure S1**), with minor contributions of AM fungi exudation (Drigo et al., 2010; Balasooriya et al., 2012; Kaiser et al., 2015), which is also directly linked to the plant-derived carbon (**Supplementary Figure S1**). To determine the uptake of plant-derived carbon by the different soil microbial groups, compound-specific ^{13}C isotope analysis on phospholipid fatty acid (PLFA) markers from soil can be used (Kramer and Gleixner, 2006). A comparison of the ^{13}C incorporation into MBC and into PLFA markers allows distinctions to be made between the growth and maintenance of soil microorganisms (Malik et al., 2015).

To study the rhizospheric processes, we used a common garden experiment on a mountain meadow using species representing the local meadow community. Our main objective was to assess the effects of drought and rewetting on the response of plant–microbial carbon transfer as a fundamental part of ecosystem functioning (Wardle et al., 2004; Bardgett et al., 2005; Schimel et al., 2007; Brüggemann et al., 2011). We performed two ^{13}C pulse chase campaigns, a first at peak drought and second shortly after rewetting, and studied the response of carbon assimilation, allocation and transfer to soil microbial markers.

Specifically, we hypothesized that the weakening of the link between plant and soil processes during drought is mainly due to decreased transfer of microbial carbon substrates in the rhizosphere and osmotic effects and is not due to decreased carbon release from roots increasing the competition for carbon between microorganisms. Furthermore, we expected that drought would lead to an accumulation of root sugars and easily degradable EOC in soil, which are available for priming plant and soil microbial activity after rewetting.

MATERIALS AND METHODS

Experimental Site

The study site is near Neustift in the Stubai Valley in the Austrian Central Alps (1,820–1,850 m a.s.l.; 47°7′45″N, 11°18′20″E) and is described in Bahn et al. (2009). Briefly, the average annual temperature is 3°C, the annual precipitation is 1,097 mm, and the soil is a dystric cambisol type. The site is a hay meadow that is cut once per year at peak biomass in early August, is lightly manured every 2–3 years, and has a Trisetum flavescentis vegetation type consisting of perennial grasses and forbs (Schmitt et al., 2010). The meadow soil has a loamy sand texture and a bulk density of 0.7 g cm^{-3} (Meyer et al., 2012a). The total soil carbon content in the uppermost 10 cm is 51 g kg^{-1} (Meyer et al., 2012b).

Establishment of Mesocosms

In 2013, a replicated mesocosm experiment with six blocks and eight mesocosms per block was established on the experimental site. For each mesocosm, two dark plastic pots, 45 cm in diameter and 35 cm in height, one inside the other, were used. The external pot was used as water reservoir and the internal one was used to hold the soil and the plants. Each pot was filled with sieved (<5 mm) subsoil (below 10 cm) from the study site and embedded in the soil on the experimental site. To prevent a possible impact from runoff water on the experiment, the upper edge of the mesocosms were raised by 2 cm relative to the soil surface. A representative selection of plant species from the site was chosen, which consisted of grass, forb and legume species. The individual plants (shoots and roots) were excavated at the experimental site in early July 2013 and were pre-incubated for 6–7 weeks in a greenhouse, in the botanical garden of Innsbruck, Austria. Every mesocosm was planted in late August 2013 with three grasses (*Deschampsia cespitosa*, *Festuca rubra,* and *Dactylis glomerata*), two forbs (*Leontodon hispidus* and *Geranium sylvaticum*) and one legume (*Trifolium repens*). At the time of planting, the plant shoots had a height of 5–15 cm. All mesocosms were planted with 36 individuals and with varying relative abundances of the different grass and forb species (**Supplementary Table S1**). The amount of the legume remained constant to exclude a possible nitrogen fertilization effect. The position of individual plants was randomized on a fixed pattern of locations for each mesocosm. All mesocosms were randomized in the block design. In 2014, the plant community was established on the site, and the biomass was harvested according to the common practice on August 22nd, 2014.

Drought Treatment and Pulse Labeling

The experiment began on the 5th of June 2015 by simulating early summer drought (**Supplementary Figure S2A**), similar to the method described by Ingrisch et al. (2018) and Karlowsky et al. (2018) for a common garden experiment with intact vegetation-soil monoliths. In brief, six rain-out shelters (**Supplementary Figure S2B**), with base areas of 3 m × 3.5 m and 2.5 height, covered by light- and UV-B permeable plastic foil (Lumisol clear AF, Folitec, Westerburg, Germany, light transmittance c. 90%), were installed above the mesocosms. Air ventilation was maintained with an opening the bottom (<0.5 m above ground) and at the top of the sides of the rain-out shelters, thereby preventing the entrance of rain water. On a subset of four to five mesocosms per shelter, soil water content (SWC) and temperature were monitored continuously in the main rooting horizon [5TM sensors (n = 17) for combined SWC and temperature measurement and EC-5 sensors (n = 11)

for SWC measurement, connected to Em50 loggers; Decagon Devices, Pullman, WA, United States]. In addition, the SWC was measured manually for each mesocosm with a PR2 Soil Moisture Profile Probe (Delta-T Devices Ltd., Cambridge, United Kingdom) at depths of 5 cm and 15 cm between the 12th of June and the 10th of August (13 times during drought and four times during recovery).

During rain exclusion, the mesocosms of the control treatments were watered manually to SWCs greater than 19% to avoid water limitation. No water was given to drought-treated mesocosms, yielding SWCs of approximately 6 and 10% at depths of 5 and 15 cm, respectively, at peak drought (**Supplementary Figure S3**). Soil moisture at field capacity was estimated on the 1st of June 2018 on the same mesocosms as 38.6% (SD = 6.7%, n = 27) using data (from 5TM and EC-5 sensors) collected when the soil moisture had stabilized a few days after rain. Four weeks after the drought treatment started, the first ^{13}C pulse labeling (peak drought labeling) started on the 4th of July on a subset of 12 mesocosms (six control and six drought treatments). Drought simulation was stopped on the 14th of July 2015, by removing the rain-out shelters and adding water representing 25 mm of precipitation to all mesocosms (control and drought treatments). Because of a natural dry period, from the 15th to the 22nd of July, another 16 and 36 mm of precipitation equivalents were added in total to the control and drought treatments, respectively. On a subset of another 12 mesocosms, after a recovery phase of 10 days, the second ^{13}C pulse labeling (recovery labeling) began on the 24th of July.

Both labeling campaigns were done on three consecutive days (peak drought from the 4th until the 6th of July; recovery from the 24th until the 26th of July) with high radiation. For each labeling campaign, one control and one drought mesocosm were used in each of the six rain-out shelters (**Supplementary Figure S2C**). The ^{13}C pulse labeling was done on 2–6 mesocosms per day. The labeling was always done in parallel on one drought mesocosm and one control mesocosm, with the starting time shifted by 15 min (randomly started with either control or drought mesocosm). Because the plant growth strongly varied between mesocosms from the same planting scheme, we aimed to visually choose pairs of mesocosms that were as similar as possible. Pulse labeling was performed similarly, as described by Bahn et al. (2009, 2013) and Hasibeder et al. (2015). Briefly, a cylindrical and transparent Plexiglas chamber with 45-cm diameter and 50-cm height was placed on the top of the mesocosms with a rubber gasket between the chamber and the mesocosm (**Supplementary Figure S2D**). Elastic bands were used to fix the chamber on external anchor points in order to ensure gas tightness. Air circulation and temperature control were handled by fans and tubes connected to a pump circulating water cooled with ice packs. During the pulse labeling, we monitored the interior air temperature (shaded sensor), CO_2 concentration (Licor 840A, Lincoln, NE, United States) and ^{13}C isotope ratio of CO_2 (Picarro G2201i Analyzer, Picarro Inc., Santa Clara, CA, United States). Solar radiation was measured outside the chamber using a PAR quantum sensor (PQS 1; Kipp & Zonen, Delft, Netherlands). Pulse labeling was done under comparable light conditions on mostly clear days between 10:00 and 15:00 CET. Highly enriched $^{13}CO_2$ (>99 atom% ^{13}C; Sigma-Aldrich, Taufkirchen, Germany) was added pulse-wise to achieve 30–80 atom% ^{13}C in chamber CO_2 over the complete labeling time of 75 min (peak drought labeling) and 30 min (recovery labeling). The CO_2 concentrations were, on average, 568 ± 99 ppm and 671 ± 98 ppm during the peak drought and the recovery labeling campaigns, with some variation caused by the pulse-wise addition of $^{13}CO_2$ (**Supplementary Table S2**). Potential effects of species-specific differences in isotopic fractionation under slightly elevated CO_2 or drought on recovered amounts of ^{13}C can be excluded due to the significant enrichment of ^{13}C from naturally 1.1 to 30–80 atom% during the labeling campaigns.

Sampling

For each mesocosm, plant and soil samples were collected in a time series after the pulse labeling. The time series included samplings at 15 min, 24, 72, and 120 h after the labeling chamber was removed. Because a minimum distance of ∼5 cm had to be kept to the mesocosm edge, to a soil moisture measurement site and to a centrally located soil respiration measurement chamber, the available area for plant and soil sampling was very limited. The first sampling location was randomly chosen in the available area and further samplings were performed either clockwise or counterclockwise in a distance of ∼5 cm. At each sampling, the shoot material, i.e., the leaves and stems, was cut 1 cm above the soil in two 5 cm × 5 cm squares, which included a random selection of plant species from opposite positions in the mesocosm. The shoot material from both squares was pooled together and separated into biomass and necromass. The biomass was immediately treated by microwave to interrupt any metabolic activity (Popp et al., 1996), stored on ice packs for transport and dried at 60°C for 72 h for later analysis of the sugar content and stable carbon isotope composition. For soil samples, soil cores were collected in or next to plant sampling squares on bare soil spots close to plant cover. Sampling was done using a stainless-steel auger with 1.9 cm inner diameter (Eijkelkamp, Giesbeek, Netherlands). At each sampling, four soil cores (two per shoot sampling square) were taken from a depth of 0–7 cm and pooled in a mixed sample. Mixed soil samples were carefully sieved through a 2-mm mesh, and the roots were removed. Soil for EOC and MBC analysis was transported on ice packs, stored at 4°C and extracted/fumigated by no later than 4 days after sampling. Soil for neutral/phospho-lipid fatty acid (NLFA/PLFA) analysis was directly frozen with dry ice and stored at −18°C until further preparation. Subsamples of frozen soil were used prior to the NLFA/PLFA analysis to determine the soil water content gravimetrically, by weighing the soil before and after drying for 48 h at 105°C. Roots were washed from the remaining soil, and the dead as well as coarse roots (diameter > 2 mm) were removed. The total amount of washed fine root samples was divided into two subsamples. One subsample was treated like shoot samples (microwaved), and the other one (not microwaved) was kept moist with wet paper towels and used as quickly as possible for root respiration measurements in the field.

Microwaved shoot and root samples were completely dried in an oven at 60°C for 72 h, starting on the day of harvest. After its

dry weight had been determined, the plant material was carefully ground to a fine powder using a ball mill (MM200, Retsch GmbH, Haan, Germany). This material was then used to analyze the bulk ^{13}C content, the compound-specific ^{13}C isotope composition and the bulk nitrogen concentration. The aboveground biomass of the mesocosms was harvested completely at the end of each labeling/sampling campaign to determine the community shoot biomass. Community root biomass was directly estimated from the dry mass of all root samples for each individual mesocosm. To obtain samples with natural ^{13}C abundance, on the 14th of July, one soil core was taken from each of four unlabeled control mesocosms, and these cores were pooled together. The same was done for the unlabeled drought mesocosms. Similarly, shoot material was collected from all six species of each mesocosm and pooled together for the four control and four drought mesocosms.

Isotopic Composition of Plant Samples and Carbohydrate Analysis

Ground bulk plant material was used to determine ^{13}C contents ($\delta^{13}C$ vs. VPDB) and nitrogen concentrations of shoots and fine roots by elemental analysis (EA) – isotope ratio mass spectrometry (IRMS) (EA - Model NA 1500, Carlo Erba, Milan, Italy; coupled to an IRMS IsoPrime100, Isoprime Ltd., Cheadle, United Kingdom). NSC analysis was done as described by Karlowsky et al. (2018). Briefly, 30 mg of plant powder was weighed, and water-soluble sugars (fructan, sucrose, glucose, and fructose) were extracted using the method of Wild et al. (2010), as modified by Mellado-Vázquez et al. (2016). Analysis was done by high-performance liquid chromatography (HPLC) – IRMS (Dionex UltiMate 3000 UHPLC coupled via a LC-IsoLink system to a Delta V Advantage IRMS, Thermo Fisher Scientific, Bremen, Germany) in a NUCLEOGEL SUGAR 810 Ca^{2+} column (Macherey & Nagel, Düren, Germany) at 80°C, with 0.5 ml/min of bi-distilled water as eluent (Hettmann et al., 2007). In accordance with previous findings from the same study site (Karlowsky et al., 2018), fructan was assigned to one large peak at the beginning of chromatograms, which likely represented fructans with a high degree of polymerization (Benot et al., 2013). For starch analysis, the remaining pellets from the sugar extraction were washed again with a methanol:chloroform:water mixture (12:3:5, by volume) to remove remaining sugars and then digested with heat stable α-amylase (Göttlicher et al., 2006; Richter et al., 2009). The resulting gluco-oligomers were measured by EA-IRMS (EA 1100, CE Elantech, Milan, Italy; coupled to a Delta + IRMS, Finnigan MAT, Bremen, Germany).

Root Respiration Measurements

A subsample (0.2–1.2 mg) of root material, washed from soil and kept moist, was used for root respiration measurement in the field. Fresh roots were placed in a 100-ml Erlenmeyer flask, sealed by a rubber stopper and incubated at 15 ± 1°C in a water bath. The initial CO_2 concentration in the flask was, on average, 491 ± 12 ppm. Root incubation was performed according to Hasibeder et al. (2015), except for the time collection. Specifically, five gas samples were collected: one immediately after closing the flask and the other four after 7, 20, 40, and 60 min, respectively. Gas sampling was performed with a syringe; each time, 15 ml of gas was collected and transferred completely into pre-evacuated 12 ml vials with a rubber septum, to prevent ambient air from entering the vial. After each sampling, 15 ml CO_2-free air was injected into the Erlenmeyer flasks to replace the gas collected. The CO_2 concentration and the ^{13}C isotope composition were analyzed by IRMS coupled with a Multiflow system (IsoPrime100, Isoprime Ltd., Cheadle, United Kingdom). All gas samples were analyzed as soon as possible after sampling and were stored in the laboratory for a maximum of 4 weeks. Root respiration rate and the $^{13}C/^{12}C$ ratio of the CO_2 respired were calculated according to Hasibeder et al. (2015).

Analysis of Soil-Extractable Organic Carbon and Microbial Biomass Carbon

For the determination of the soil EOC and MBC, the method of Vance et al. (1987) with the modifications of Malik et al. (2013), was used. Soil EOC was extracted from a subsample of approximately 5 g of fresh soil with 25 ml of 0.5 M K_2SO_4 solution (distilled water) in a horizontal shaker with 150 rpm for 30 min. The extract was centrifuged at 12,000 × *g* for 5 min and coarse particles were removed using pre-washed (0.5 M K_2SO_4 solution) filter papers (Whatman Grade 1, *d* = 150 mm, 11 μm pore size, GE Healthcare UK Ltd., Buckinghamshire, United Kingdom). The filtrate was frozen and stored at −18°C until further processing for analysis. Total organic carbon (TOC) was extracted and processed in the same way as the EOC, after another subsample of approximately 5 g fresh soil had been fumigated for ≥24 h with chloroform. If necessary, drought-treated soils were rewetted to control levels with distilled water prior to the fumigation to avoid differences in the extraction efficiency (Sparling et al., 1990). For the analysis, ∼1 ml each of the EOC and TOC extracts was filtered with pre-washed (∼0.5 ml of extract) 0.45 μm cellulose membrane filters (MULTOCLEAR 0.45 μm RC 13 mm, CS-Chromatographie Service GmbH, Langerwehe, Germany). To de-gas the samples of inorganic C, filtered extracts were acidified with phosphoric acid to approximately pH 2 and gas-flushed with N_2 for 15 min. The degassed samples were then analyzed as bulk fraction (no column) on an HPLC-IRMS system (see carbohydrate analysis). Each sample was measured in triplicate. Quality was controlled by repeated measurements of citric acid standards ($\delta^{13}C = -18.58$ ‰ vs. VPDB, Fluka Chemie AG, Buchs, Switzerland; $SD = 0.14$‰, $n = 72$). Quantification was performed using a concentration row of the citric acid standard to calibrate the HPLC-IRMS based on CO_2 peak areas. The results for the EOC and TOC were normalized to the used soil dry mass for each fraction, and the concentration of MBC was calculated from the EOC and TOC by the formula: $[MBC] = ([TOC] - [EOC])/k_{MBC}$. For k_{MBC}, a value of 0.45 was used, which is the typical extraction efficiency of MBC after chloroform fumigation (Vance et al., 1987). The $^{13}C/^{12}C$ ratio (i.e., $\delta^{13}C$ or atom% ^{13}C) of MBC was calculated according

to the isotopic mass balance: $^{13}C/^{12}C_{MBC}$ = ($^{13}C/^{12}C_{TOC}$ * [TOC] − $^{13}C/^{12}C_{EOC}$ * [EOC])/([TOC]-[EOC]).

Analysis of Neutral and Phospholipid Fatty Acids

Neutral and phospholipid fatty acid analysis was done according to the method of Bligh and Dyer (1959), as modified by Karlowsky et al. (2018). Briefly, approximately 5 g of frozen bulk soil was extracted with a mixture of methanol, chloroform and 0.05 M K_2HPO_4 buffer (2:1:0.8, by volume; pH 7.4) using pressurized solvent extraction (SpeedExtractor E-916, Büchi Labortechnik AG, Flawil, Switzerland). A recovery standard (1,2-Dinonadecanoyl-sn-Glycero-3-Phosphatidylcholine; Larodan Fine Chemicals AB, Malmö, Sweden) was added (recovery rate: 62 ± 11%, SD, n = 60) to each sample, and the extraction was carried out at 70°C and 120 bar for 3 min × 10 min. Neutral and phospholipid fractions were separated using silica-filled solid-phase extraction (SPE) columns (CHROMABOND SiOH, 2 g, 15 ml, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). Both fractions were hydrolyzed and methylated with methanolic KOH, and the resulting fatty acid methyl esters (FAMEs) were further purified for analysis by using aminopropyl-modified SPE columns (CHROMABOND NH2, 0.5 g, 3 ml, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). The FAME C13:0 (Sigma-Aldrich Chemie GmbH, Munich, Germany) was added as the internal standard to all samples, and quantification was done by gas chromatography–flame ionization detection (GC-FID) on a GC-FID 7890B system with a programmable temperature vaporization (PTV) injector (Agilent Technologies, Palo Alto, CA, United States) using a DB-1MS UI column (30 m × 0.25 mm internal diameter × 0.25 µm film thickness, Agilent Technologies, Palo Alto, CA, United States) and helium as the carrier gas (1.8 ml/min). The temperature program started at 45°C for 1 min, then increased in a first ramp of 60°C/min to 140°C (held for 0.5 min), followed by a second ramp of 2°C/min until 242°C, and finally, by a third ramp to 320°C (held for 3 min). Directly after injection, the PTV was heated up from 55 to 280°C at a rate of 500°C/min. Compound specific ^{13}C isotope analysis of NLFAs and PLFAs was conducted by GC-IRMS (GC 7890A with PTV injector, Agilent Technologies, Palo Alto, CA, United States; coupled via a Conflo IV/GC IsoLink to a Delta V Plus IRMS, Thermo Fisher Scientific, Bremen, Germany) using a DB-1MS UI column (60 m × 0.25 mm internal diameter × 0.25 µm film thickness, Agilent Technologies, Palo Alto, CA, United States) and helium as the carrier gas (1.8 ml/min). Directly after injection, the PTV was heated from 55 to 280°C at a rate of 500°C/min. The GC temperature program started with 45°C for 1 min, then increased in a first ramp of 60°C/min to 140°C (held for 0.5 min), followed by a second ramp of 4°C/min until 283°C (held for 4.9 min) and a third ramp until 320°C (held for 3 min). Concentrations and ^{13}C isotope content of identified FAMEs were corrected for the methyl group introduced during derivatization. We used the sum of the PLFAs i14:0, i15:0, a15:0, i16:0, a17:0, i17:0, and br18:0 for Gram-positive bacteria (Zelles, 1997, 1999); 10-Me16:0 and 10-Me18:0 for actinobacteria (Lechevalier et al., 1977; Zelles, 1999); and 16:1ω7 and 18:1ω7 for Gram-negative bacteria (Zelles, 1997, 1999). The PLFA 18:2ω6,9 was used as the marker for saprotrophic fungi (Frostegård and Bååth, 1996; Zelles, 1997) and the NLFA 16:1ω5 as the marker for arbuscular mycorrhizal (AM) fungi (Olsson, 1999). Although the NLFA 16:1ω5 does not correctly estimate the biomass of AM fungal populations, it has been found to be more of a proxy than the PLFA 16:1ω5 (e.g., Ngosong et al., 2012; Mellado-Vázquez et al., 2016; Paterson et al., 2016).

Calculation of ^{13}C Tracer Concentrations

To determine the relative abundance of ^{13}C tracer in labeled samples, we calculated the atom% $^{13}C_{excess}$ as follows:

$$atom\%\ ^{13}C_{\text{excess}} = atom\%\ ^{13}C_{labeled} - atom\%\ ^{13}C_{unlabeled}$$

with $atom\%\ ^{13}C_{labeled}$ being the atom% ^{13}C of the labeled samples and $atom\%\ ^{13}C_{unlabeled}$ being the atom% ^{13}C of natural abundance samples from unlabeled mesocosms (mixed samples from shoots of all six species were used as reference for the plant community). Values of atom% $^{13}C_{excess}$ are not presented here but can be found in the **Supplementary Figures S9–S12**.

For all plant and soil samples, we expressed the ^{13}C isotope content as incorporated ^{13}C (mg ^{13}C m^{-2}), which refers to the total amount of ^{13}C found in a certain carbon pool on an area basis, and it was calculated as:

$$incorporated^{13}C = \frac{\text{atom\%}^{13}C_{\text{excess}} * C_{\text{pool}}}{100\%}$$

with C_{pool} being the respective carbon pool (mg C m^{-2}).

The roots respired ^{13}C (mg ^{13}C m^{-2} h^{-1}), which corresponds to the amount of ^{13}C released in respired CO_2 from roots during a certain time, was calculated similarly to the incorporated ^{13}C as follows:

$$root\ respired^{13}C = \frac{\text{atom\%}^{13}C_{\text{excess}} * CO2_{\text{resp. rate}}}{100\%}$$

with $CO2_{resp.rate}$ being the respiration rate of CO_2 (mg CO_2 m^{-2} h^{-1}).

Data Analyses

For root biomass and concentration data, the average values were calculated over the different sampling times after pulse labeling: 1 and 3 days after labeling for NLFAs and PLFAs and 15 min, 1 day, 3 days, and 5 days after labeling for all others. For the soil samples, a bulk soil density of 0.7 g cm^{-3} (Meyer et al., 2012a) was used for calculating area-based pool sizes. The total ^{13}C uptake was calculated as the sum of the bulk shoot and bulk root incorporated ^{13}C at the first sampling directly after labeling (15 min). The ^{13}C tracer fluxes were analyzed for drought effects considering the different sampling times (same times as for concentration data). After removing negative ^{13}C incorporation values (defined as below detection limit), the relative ^{13}C allocation to the different pools was calculated for each sampling time as the ratio of ^{13}C incorporation to total ^{13}C uptake. Relative ^{13}C allocation to shoot and root storage pools was calculated as the sum of relative ^{13}C allocation to fructan and

starch in the shoots and roots. For an overview of the drought effects on all pools (including NLFAs and PLFAs), the relative ^{13}C allocation was averaged for 1 and 3 days samples, and the drought to control ratio was calculated. In general, at 1 and 3 days after pulse labeling, the drought effects on relative ^{13}C allocation were comparable (**Supplementary Figure S4**) and high ^{13}C tracer enrichment was found in all pools of interest, making these two times suitable to assess the strongest differences in ^{13}C allocation patterns. For the calculation of drought to control ratios, only labelings with data from both treatments (i.e., control and drought mesocosms that were labeled at the same time,) were considered. First, the drought to control ratio of each labeling pair was calculated, and second, the average value was formed.

All statistical analyses were done using the R 3.3.2 software (R Core Team, 2016). Time series (in hours after pulse labeling) of the ^{13}C tracer data were tested separately for each labeling campaign for the effects of drought and sampling time, as well as their interaction, using linear mixed-effects models from the 'lme4' package (Bates et al., 2015). In the mixed-effects model, the treatment and sampling time (as factor) were set as fixed effects, whereas the rain-out shelter and mesocosm were set as random effects. Drought effects on relative ^{13}C allocation were analyzed similarly, using treatment and sampling time (as factors) as fixed effects, and labeling pair (control and drought mesocosms labeled in parallel) and mesocosm as random effects. All mixed-effects models were assessed for violations of normality, heteroscedasticity and independency. If necessary, ^{13}C tracer data were log (+1) or square root (+1) transformed. For all other data (i.e., biomass, total ^{13}C uptake and concentration data), the drought effects were evaluated for each labeling campaign separately using permutational ANOVA from the 'lmPerm' package (Wheeler and Torchiano, 2016), from which exact *P*-values (P_{aovp}) were obtained. Permutation tests do not require assumptions about the statistical distribution and are powerful with small sample sizes (Ernst, 2004).

RESULTS

Peak Drought Labeling

The 4 weeks of severe drought had strong effects on the plant community and its biomass at peak drought (**Table 1**). Drought significantly reduced the shoot biomass but had no distinct effect on the total plant biomass, since a strong increase of fine root biomass occurred. Consequently, drought led to a significant increase in the root to shoot ratio. According to the reduction in shoot biomass, the photosynthetic rate (**Supplementary Figure S5A**) and total plant ^{13}C uptake (**Table 1**) were strongly reduced by drought as well. Drought did not change the proportion of total ^{13}C (relative ^{13}C allocation) that was allocated belowground at 24 and 72 h from labeling (**Figure 1A**), although it was lower at 15 min and higher at 120 h (**Supplementary Figure S4**). The little effect of drought on overall BCA was also expressed by similar reductions of ^{13}C tracer incorporation into shoots and roots over the 120-h sampling period (**Supplementary Figure S6**). However, drought more strongly affected relative ^{13}C allocation to NSCs (**Figure 1A**) and their tracer dynamics (**Supplementary Figures S6B–D,F–H**). Significantly less ^{13}C was allocated to shoot storage (**Figure 1A**), i.e., to compounds such as fructan and starch (**Supplementary Figures S6C,D**), whereas slightly more ^{13}C was retained in shoot sucrose over time (**Figure 1A** and **Supplementary Figure S4**). This retention was reflected in the higher sucrose concentrations and lower

TABLE 1 | Drought effects on biomass, ^{13}C tracer uptake, root respiration and biomass N contents.

Labeling	Parameter	Unit	Control	Drought	D^a
Peak drought	Total biomass	$g_{dm}\ m^{-2}$	313 ± 23	353 ± 31	n.s.
	Shoot biomass	$g_{dm}\ m^{-2}$	131 ± 12	82 ± 9	***
	Root biomass	$g_{dm}\ m^{-2}$	182 ± 16	271 ± 25	**
	Root:Shoot ratio	–	1.45 ± 0.21	3.44 ± 0.37	***
	^{13}C uptake	$mg_{13C}\ m^{-2}$	366 ± 32	93 ± 6	***
	Root respiration	$\mu mol_{CO2}\ m^{-2}\ s^{-1}$	0.82 ± 0.03	0.88 ± 0.09	n.s.
	Shoot N	$g_N\ m^{-2}$	1.71 ± 0.16	1.14 ± 0.13	**
	Root N	$g_N\ m^{-2}$	1.41 ± 0.10	2.35 ± 0.30	***
	Total N	$g_N\ m^{-2}$	3.12 ± 0.22	3.49 ± 0.38	n.s.
Recovery	Total biomass	$g_{dm}\ m^{-2}$	295 ± 19	267 ± 12	n.s.
	Shoot biomass	$g_{dm}\ m^{-2}$	114 ± 8	102 ± 7	n.s.
	Root biomass	$g_{dm}\ m^{-2}$	181 ± 20	165 ± 8	n.s.
	Root:Shoot ratio	–	1.7 ± 0.3	1.6 ± 0.1	n.s.
	^{13}C uptake	$mg_{13C}\ m^{-2}$	220 ± 29	231 ± 27	n.s.
	Root respiration	$\mu mol_{CO2}\ m^{-2}\ s^{-1}$	0.81 ± 0.06	0.94 ± 0.11	n.s.
	Shoot N	$g_N\ m^{-2}$	1.34 ± 0.09	1.74 ± 0.19	**
	Root N	$g_N\ m^{-2}$	1.46 ± 0.19	1.59 ± 0.03	n.s.
	Total N	$g_N\ m^{-2}$	2.80 ± 0.23	3.33 ± 0.19	*

[a]*Levels of significance for drought effects: ***$P_{aovp} < 0.001$, **$P_{aovp} < 0.01$, *$P_{aovp} < 0.05$, (*)$P_{aovp} < 0.1$; n.s., not significant. Mean values ± SE (n = 6) are shown for control and drought treatments. For root respiration and N concentrations, the data were averaged over the four sampling times for each mesocosm.*

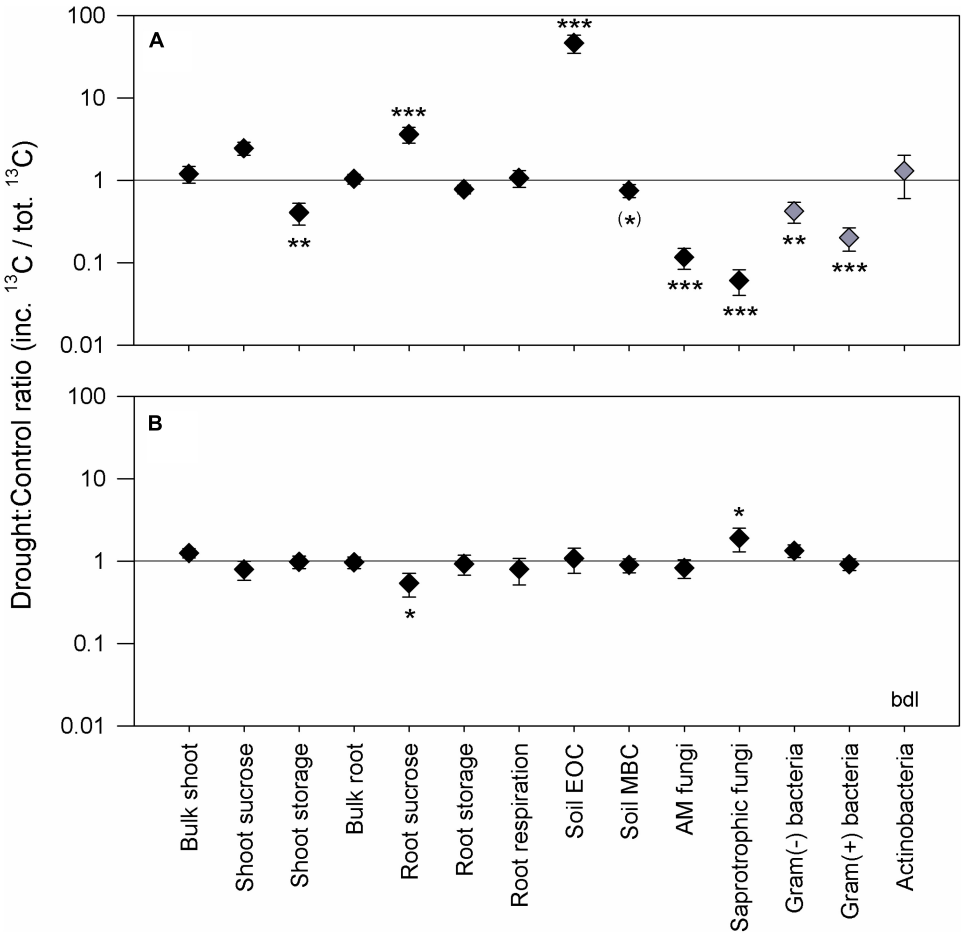

FIGURE 1 | Effects of drought on C allocation patterns at the peak drought **(A)** and recovery **(B)** labeling campaigns. The drought to control ratio of the relative ^{13}C allocation is shown, i.e., the amount of incorporated ^{13}C (inc. ^{13}C) in each pool that was recovered from the total ^{13}C uptake (tot. ^{13}C), averaged for the samplings at 24 and 72 h after pulse labeling. The graph only highlights the strongest effects, and additional data for individual sampling points, including 15 min and 120 h, can be found in **Supplementary Figure S4**. Black symbols represent the mean of $n = 6$ control/drought pairs, and gray symbols the mean of $n = 3$ control/drought pairs. Error bars were obtained by propagating the SE from the replicates of each treatment, control and drought, respectively. Asterisks indicate levels of significance for drought effects (df = 1) from the linear mixed-effects models: $^{***}P_{\chi^2} < 0.001$, $^{**}P_{\chi^2} < 0.01$, $^{*}P_{\chi^2} < 0.05$, and $^{(*)}P_{\chi^2} < 0.1$. The "bdl" notation stands for below detection limit.

fructan and starch concentrations in drought shoots compared to controls (**Table 2**). Drought increased the relative ^{13}C allocation to the root sucrose pool (**Figure 1A**), which showed altered tracer dynamics (**Supplementary Figure S6F**), i.e., lower ^{13}C incorporation until 24 h and higher ^{13}C incorporation. Reduced ^{13}C incorporation was found in fructan and starch from roots (**Supplementary Figures S6G,H**), although their concentrations (**Table 2**) were not affected by drought. Indeed, the relative ^{13}C allocation to root storage was on average only little affected by drought (**Figure 1A**), showing a decrease at 24 h and an increase at 120 h (**Supplementary Figure S4**). Apparently, in root fructan, drought mainly led to slower ^{13}C tracer incorporation over time (**Supplementary Figure S6G**). Moreover, considered the higher fine root biomass, the root fructan pool even increased during drought (Control, 6.1 ± 1.3 $g_C\ m^{-2}$; Drought, 10.2 ± 1.5 $g_C\ m^{-2}$; SE, $n = 6$; $P_{aovp} = 0.009$). Similar to root storage, the drought reduced the amount of root-respired ^{13}C but only at the first two sampling points (**Supplementary Figure S7A**). This reduction led to decreased relative ^{13}C allocation to root respiration at 15 min and 24 h; however, it increased at 72 and 120 h (**Supplementary Figure S4**). This effect was not visible on average for 24 and 72 h (**Supplementary Figure S1**). Consequently, the overall respiration rate was not altered by drought (**Table 1**), despite lower respiration rates at the dry mass level (Control, 4.6 ± 0.3 $nmol_{CO2}\ g^{-1}{}_{dm}\ s^{-1}$; Drought, 3.3 ± 0.6 $nmol_{CO2}\ g^{-1}{}_{dm}\ s^{-1}$; $P_{aovp} < 0.001$). Plant nitrogen concentrations were only little affected by drought and tended to be higher in shoots (Control, 1.31 ± 0.04%$_N$; Drought, 1.40 ± 0.06%$_N$; $P_{aovp} = 0.076$) but not in roots (Control, 0.79 ± 0.05%$_N$; Drought, 0.86 ± 0.06%$_N$; $P_{aovp} = 0.206$). However, if the differences in biomass were considered, drought led to a reduction of shoot nitrogen content and an increase of root nitrogen content per unit area (**Table 1**).

TABLE 2 | Effects of drought on the sizes of plant bulk and carbohydrate pools for the peak drought and the recovery labeling campaigns.

Labeling	Parameter	C content ($mg_C\ g_{dm}^{-1}$)		
		Control	Drought	D^a
Peak drought	Bulk shoot	422 ± 3	423 ± 3	n.s.
	Shoot sucrose	14 ± 0	16 ± 1	***
	Shoot fructan	57 ± 2	41 ± 3	***
	Shoot starch	8.1 ± 0.6	5.1 ± 1.4	**
	Bulk root	345 ± 15	369 ± 15	(*)
	Root sucrose	4.4 ± 0.4	10.8 ± 0.9	***
	Root fructan	32 ± 2	38 ± 6	n.s.
	Root starch	12 ± 4	16 ± 7	n.s.
Recovery	Bulk shoot	421 ± 4	422 ± 4	n.s.
	Shoot sucrose	12 ± 0	13 ± 1	n.s.
	Shoot fructan	47 ± 4	33 ± 3	**
	Shoot starch	9.0 ± 1.3	8.5 ± 0.8	n.s.
	Bulk root	357 ± 7	379 ± 8	(*)
	Root sucrose	4.4 ± 0.6	2.7 ± 0.1	***
	Root fructan	35 ± 6	29 ± 3	n.s.
	Root starch	21 ± 4	14 ± 4	n.s.

*[a]Levels of significance for drought effects: ***$P_{aovp} < 0.001$, **$P_{aovp} < 0.01$, *$P_{aovp} < 0.05$, (*)$P_{aovp} < 0.1$; n.s., not significant. Values represent averages among the mesocosms for each treatment (mean ± SE, n = 6), after averaging over the four sampling times for each mesocosm.*

TABLE 3 | Effects of drought on the sizes of soil carbon and microbial marker lipid pools for the peak drought and the recovery labeling campaigns.

Labeling	Parameter	C content ($\mu g_C\ g_{dm}^{-1}$)		
		Control	Drought	D^a
Peak drought	EOC	34 ± 4	102 ± 8	***
	MBC	402 ± 33	429 ± 20	n.s.
	AM fungi	24 ± 3	17 ± 2	*
	Saprotrophic fungi	1.1 ± 0.1	1.2 ± 0.2	n.s.
	Gram (–) bacteria	5.7 ± 0.4	7.1 ± 0.3	**
	Gram (+) bacteria	4.1 ± 0.3	4.8 ± 0.2	*
	Actinobacteria	2.4 ± 0.2	2.9 ± 0.1	*
Recovery	EOC	32 ± 3	32 ± 1	n.s.
	MBC	393 ± 18	393 ± 15	n.s.
	AM fungi	34 ± 2	19 ± 2	***
	Saprotrophic fungi	0.9 ± 0.1	0.9 ± 0.1	n.s.
	Gram (–) bacteria	6.0 ± 0.4	6.6 ± 0.4	n.s.
	Gram (+) bacteria	4.3 ± 0.3	4.6 ± 0.4	n.s.
	Actinobacteria	2.8 ± 0.2	2.9 ± 0.2	n.s.

*[a]Levels of significance for drought effects: ***$P_{aovp} < 0.001$, **$P_{aovp} < 0.01$, *$P_{aovp} < 0.05$, (*)$P_{aovp} < 0.1$; n.s., not significant. AM, arbuscular mycorrhizal; EOC, extractable organic carbon; MBC microbial biomass carbon. Values represent averages among the mesocosms for each treatment (mean ± SE, n = 6), after averaging over the sampling times (four for EOC and MBC, two for microbial marker lipids) for each mesocosm.*

Regarding the soil, drought led to a threefold increase of water-soluble EOC compared to controls (**Table 3**) but had no effect on the MBC content. Significantly higher relative ^{13}C allocation to the EOC (**Figure 1A** and **Supplementary Figure S4**) resulted from the continuous increase of ^{13}C tracer incorporation into the EOC after the labeling (**Figure 2A**). By contrast, drought consistently reduced the amount of ^{13}C tracer incorporation into MBC over time and delayed the label uptake (**Figure 2B**), leading to lower relative ^{13}C allocation to MBC at 15 min and 24 h (**Supplementary Figure S4**). The reduced microbial ^{13}C incorporation during drought was more pronounced for the individual lipid markers (**Supplementary Figures S8A–D**), yielding significantly decreased relative ^{13}C allocation to AM fungi, saprotrophic fungi, and Gram-negative and Gram-positive bacteria (**Figure 1A**). This effect was not visible for actinobacteria (**Figure 1A**), which, on average, did not incorporate detectable amounts of ^{13}C in control and drought treatments in their lipid markers (**Supplementary Figure S8E**). AM fungi, which took up the largest amount of ^{13}C in the controls, reflected the tracer dynamics of MBC (**Figure 2B** and **Supplementary S8A**). This relation was less pronounced for saprotrophic fungi, whereas bacteria showed a slower label uptake. At the biomass scale, AM fungi were slightly affected by drought, whereas saprotrophic fungi were unaffected, and the bacterial biomass generally increased (**Table 3**).

Recovery Labeling

Ten days after rewetting, drought-treated mesocosms fully recovered their shoot biomass, root:shoot ratio, ^{13}C uptake (**Table 1**), and photosynthetic rate (**Supplementary Figure S5B**). Accordingly, the amount of ^{13}C incorporated in the root and shoot pools mostly recovered (**Supplementary Figures S6I–P**). NSC tracer dynamics partially differed between the control and drought treatments. Drought led to an earlier peak value of ^{13}C incorporation into root sucrose (**Supplementary Figure S6N**) and to faster label decreases in shoot starch and root fructan after peak values were reached (**Supplementary Figures S6L,O**). This also resulted in a lower relative ^{13}C allocation to root sucrose 72 h and 120 h after labeling (**Supplementary Figure S4**), whereas carbon allocation to shoot and root storage was only little affected. Bulk roots mainly reflected the ^{13}C tracer dynamics of root fructan, showing a similar trend over time (**Supplementary Figures S6M,O**), i.e., a decrease of ^{13}C incorporation at 72 h. Despite largely recovered carbon fluxes, the previous drought caused reductions in the concentrations of shoot fructan and root sucrose at the recovery labeling (**Table 2**). The overall root respiration rate was not affected by drought and rewetting (**Table 1**) but was increased at the dry mass level (Control, 4.6 ± 0.8 $nmol_{CO2}\ g^{-1}{}_{dm}\ s^{-1}$; Drought, 5.7 ± 0.6 $nmol_{CO2}\ g^{-1}{}_{dm}\ s^{-1}$; $P_{aovp} = 0.039$). Furthermore, root respiration had similar ^{13}C tracer dynamics like root sucrose, showing an earlier peak of respired ^{13}C in drought-treated mesocosms (**Supplementary Figure S7B**). Rewetting led to significantly higher nitrogen concentrations in the roots (Control, 0.80 ± 0.05%$_N$; Drought, 0.98 ± 0.05%$_N$; $P_{aovp} = 0.006$) and shoots (Control, 1.18 ± 0.05%$_N$; Drought, 1.69 ± 0.11%$_N$; $P_{aovp} < 0.001$), thereby increasing the shoot and total biomass N content per unit area (**Table 1**).

Overall, plant and soil-related parameters recovered from drought at the recovery labeling. Consistently, the concentrations

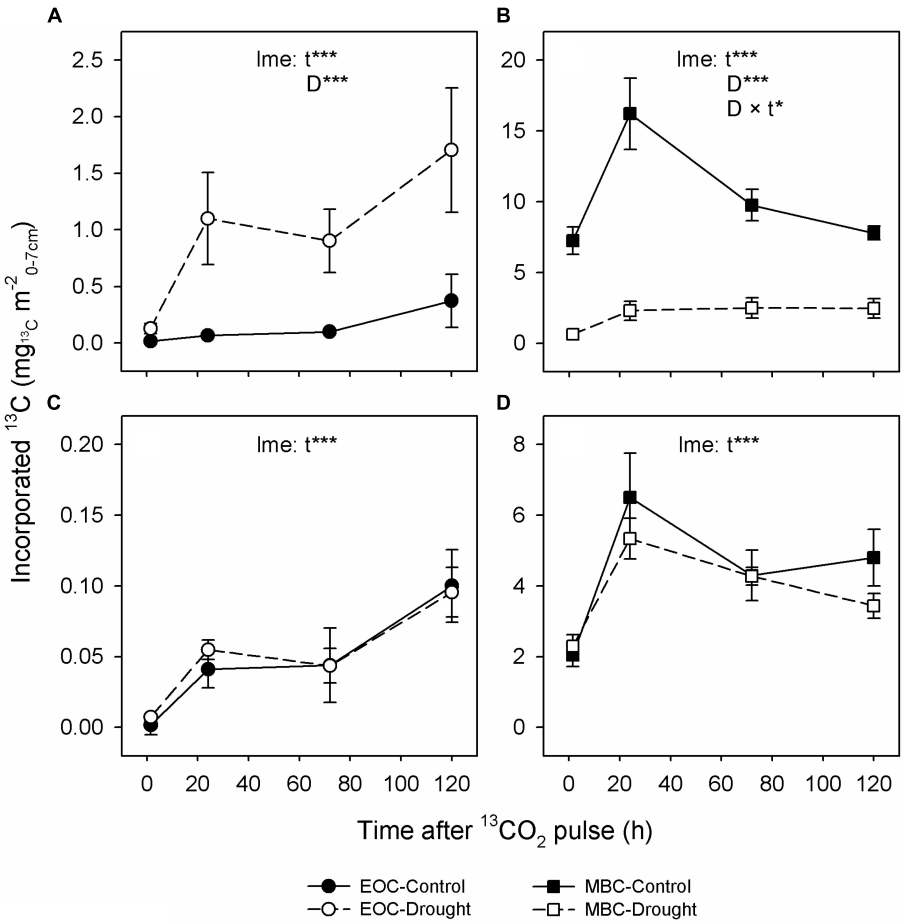

FIGURE 2 | Dynamics of ^{13}C tracer incorporation into extractable organic carbon (EOC; circles; **A,C**) and microbial biomass carbon (MBC; squares; **B,D**) from soil of control (closed symbols and solid lines) and drought-treated (open symbols and dashed lines) mesocosms at the peak drought **(A,B)** and recovery **(C,D)** labeling campaigns. Error bars show the SE of $n = 6$ mesocosms. Levels of significance for time after labeling (t; df = 3), drought treatment (D; df = 1) and the interaction of both (D × t; df = 3) were obtained from linear mixed-effects (lme) models using the R package 'lme4'; $^{***}P_{\chi^2} < 0.001$, $^{**}P_{\chi^2} < 0.01$, $^{*}P_{\chi^2} < 0.05$. Note that the labeling time was 30 min at the recovery labeling compared to 75 min at the peak drought labeling and that the absolute values cannot be compared between the labeling campaigns.

and ^{13}C tracer incorporations of EOC and MBC fully recovered (**Table 3** and **Figures 2C,D**). The ^{13}C uptake in different microbial groups also recovered and showed little variation between the groups (**Supplementary Figures S8F–J**). Only the relative ^{13}C allocation to saprotrophic fungi was significantly increased after rewetting (**Figure 1B**), as visible by the slightly higher ^{13}C incorporation into the saprotrophic fungal marker (**Supplementary Figure S8G**). A similar trend was present for the tracer incorporation into Gram-negative bacterial markers, while no effect was observed on the Gram-positive bacterial markers. In contrast, for the AM fungal marker, a weak trend existed, showing a reduction in the ^{13}C incorporation in drought mesocosms. This trend corresponded to a significantly reduced marker concentration (**Table 3**), which was largely counterbalanced by a higher relative abundance of ^{13}C tracer (atom% $^{13}C_{excess}$) (**Supplementary Figure S9**). For all other microbial groups, the marker concentrations were equal between control and drought treatments.

DISCUSSION

In a previous experiment on intact vegetation-soil monoliths from a managed meadow and an abandoned grassland, we found that drought-induced reductions of plant photosynthetic activity (Ingrisch et al., 2018) were coupled to strong reductions in plant storage NSCs, especially above ground, whereas BCA was maintained at a constant level (abandoned grassland) or even increased (managed meadow) relative to the total carbon uptake (Karlowsky et al., 2018). The carbon allocated to roots was largely recovered in drought-accumulated soluble sugars, whereas the uptake of plant-derived carbon in fatty acid biomarkers of root-associated microorganisms (AM fungi, SF and bacteria) was strongly reduced. Overall, these responses were greater in the managed meadow compared to the abandoned grassland, which likely also profited from enhanced AM fungal growth during drought. Furthermore, we found that after rewetting, the carbon uptake of the SF and bacteria was enhanced in the managed

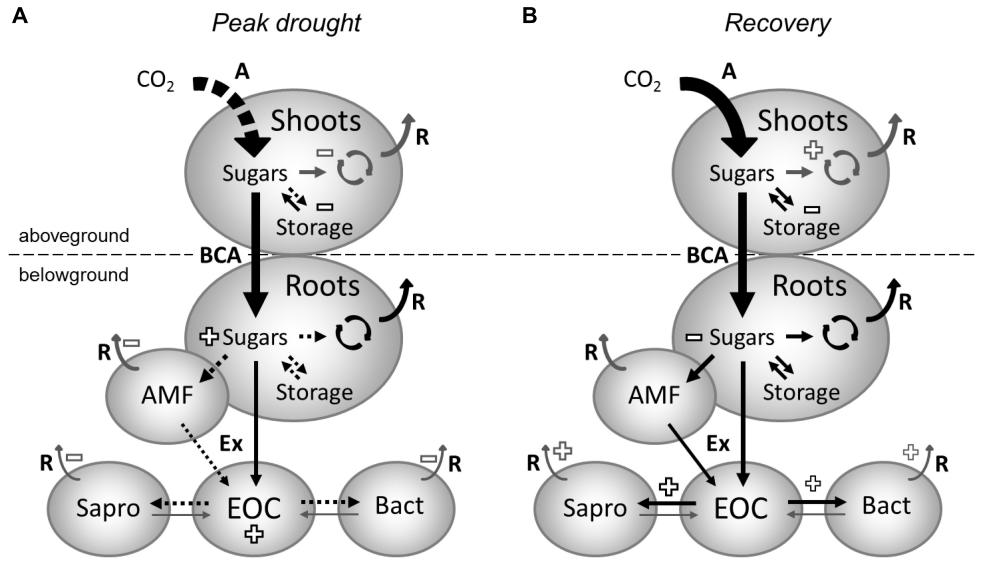

FIGURE 3 | Effects of drought **(A)** and rewetting **(B)** on carbon fluxes and pools in grassland ecosystem. **(A)** During drought, assimilation **(A)** is reduced (reductions shown as dashed arrows). This leads to reduced carbon allocation to aboveground storage decreasing its pool size (effects on pool sizes shown as "+" or "–" signs). Presumably, carbon allocation to shoot growth, maintenance and respiration (R) is also reduced during drought (fluxes that were not determined in this study are represented by gray arrows). Belowground carbon allocation (BCA) is maintained during drought and leads to the accumulation of root sugars because carbon allocation to storage and mycorrhizal interactions are reduced. The size of the root storage pool is unaffected, as its activity is reduced during drought. Root sugars are partially used for root growth and maintenance. Furthermore, there is ongoing exudation (Ex) of new assimilates by roots but not by AM fungi (AMF), leading to an increase of the extractable organic carbon (EOC) in the soil, as the carbon uptake and metabolic activity of saprotrophic fungi (Sapro) and bacteria (Bact) is strongly reduced during drought. Shortly after rewetting **(B)** carbon assimilation and allocation mostly recovers. Because reductions still occur in the shoot storage pool, it is likely that priority is given to shoot re-growth. Accumulations of root sugars and EOC observed during drought rapidly vanish after rewetting and are likely used for priming soil microbial activity. In addition, the root sugar pool is reduced due to a faster carbon turnover, which is associated with increased transfer of newly assimilated carbon to saprotrophic fungi and (by tendency) bacteria in the rhizosphere, indirectly suggesting increased root/mycorrhizal exudation.

meadow (Karlowsky et al., 2018), which was reflected by higher plant nitrogen uptake and a faster recovery of aboveground biomass compared to the abandoned grassland (Ingrisch et al., 2018).

However, we were not able to assess whether the accumulation of root sugars during drought affected the release of carbon to the rhizosphere, nor were we able to determine how the drought-induced shift toward belowground allocation in the meadow might be related to its quick recovery after rewetting. Therefore, the aim of this study was to further elucidate the mechanisms underlying the link between plant photosynthesis and soil microbial carbon cycling during drought and after rewetting.

The Link Between Plant and Soil Microbial Processes at Peak Drought

Surprisingly, drought had no significant effect on the total plant biomass. However, the decrease in shoot biomass and the concurrent increase in fine root biomass indicate that drought led to a shift in plant carbon allocation toward the belowground organs. Similar results have been found before in drought experiments on managed grasslands (Kahmen et al., 2005; Burri et al., 2014) and were attributed by the authors to an adaptation of plants in order to forage the limited water in dry soil. However, the root biomass response to drought can vary (Kahmen et al., 2005) and depends on the severity of the drought (Kreyling et al., 2008). Another root response occurring together with increased BCA is the accumulation of root sugars, especially sucrose (Hasibeder et al., 2015; Karlowsky et al., 2018). Such accumulations of root sugars can indicate an adjustment to dry conditions (Hasibeder et al., 2015) by increasing the concentration of osmolytes that prevent cells from desiccation (Chaves et al., 2003; Chen and Jiang, 2010). In our study, simultaneously increased concentrations of free glucose and fructose in roots (data not shown) further point to osmotic adjustment (Chen and Jiang, 2010).

Independently of its usage, the carbon needed to maintain BCA originates either from recent assimilates or from remobilized aboveground storage compounds. In previous studies, drought increased the proportion of recently assimilated carbon allocated belowground (Palta and Gregory, 1997; Huang and Fu, 2000; Burri et al., 2014; Hasibeder et al., 2015; Karlowsky et al., 2018). Here, we could not identify this effect (**Figure 3A**), suggesting a higher contribution of shoot storage is needed to maintain BCA during drought, as indicated by the depletion of shoot fructan and starch. This might be due to stronger negative effects of drought on carbon assimilation than in the previous studies. Diverging results for the belowground allocation of freshly assimilated carbon have been reported before by Sanaullah et al. (2012) in a lab-based mesocosm experiment with

monocultures and different mixtures of two grasses and one legume, whereas Ruehr et al. (2009) even found that drought increased the residence time of new carbon in leaves from beech trees. Of course, as woody species, trees have additional aboveground storage organs, which likely modify their drought response compared to herbaceous species. As a consequence, the source of the typically observed increase of BCA during drought might vary between fresh assimilates and older reserve carbohydrates, depending on the severity of drought, the timing in the year, as well as the functional composition or type of plants. In general, as previously concluded by Bahn et al. (2013), under reduced carbon supply, BCA in grassland seems to be maintained at the expense of aboveground storage (**Figure 3A**). Furthermore, the increase of nitrogen content in the roots (g_N m^{-2}) of drought-treated plants (**Table 1**) suggests that the disturbance-adapted meadow plants actively preserve their resources belowground during extreme drought, likely to facilitate quick recovery after rewetting (Karlowsky et al., 2018).

Most interestingly, the altered plant resource allocation patterns did not disrupt the release of recently assimilated carbon to the rhizosphere during drought (**Figure 3A**), as visible by the high amount of ^{13}C tracer in the soil EOC fraction, which exceeded control levels shortly after labeling. A similar enrichment of plant-derived carbon in the EOC pool was found by Fuchslueger et al. (2014a) and was attributed by the authors to the role of root exudates in reducing friction resistance in soil and maintaining root-soil connectivity. However, the strong reduction in ^{13}C recovered in the microbial biomass of drought mesocosms points to decreased microbial uptake of recent plant-derived carbon, which probably led to the strong accumulation of carbon in the EOC pool. Nonetheless, increased root exudation during drought, as evidenced by a recent mesocosm study on tree saplings (Preece et al., 2018), could have further contributed to the greater EOC pools in the soil.

Notably, the relative ^{13}C allocation to MBC was much less reduced by drought compared to microbial marker fatty acids (**Figure 1A**). This finding may imply that drought-reduced microbial growth, which can be estimated by the production of new fatty acids, and led to the accumulation of osmotically active compounds in MBC (Schimel et al., 2007). Osmolytes, e.g., amino acids in bacteria and polyols in fungi, are essentially highly water soluble and are more easily recovered than hydrophobic fatty acid-containing lipids in the MBC, which is extracted using aqueous K_2SO_4 solution. Moreover, reduced substrate diffusion, assumed to be the main limiting factor for bacterial activity in dry soil (Skopp et al., 1990; Stark and Firestone, 1995; Nunan et al., 2017), cannot explain the reduced ^{13}C tracer uptake by AM fungi during drought, since mycorrhizal interactions do not depend on substrate diffusion in the soil.

Unexpectedly, bacterial biomass was generally higher in drought-treated mesocosms (**Table 3**). A high resistance to drought was expected for the slow-growing, Gram-positive (actino)bacteria but not for the Gram-negative bacteria with their thin cell wall (Schimel et al., 2007; Lennon et al., 2012). Possibly, Gram-negative bacteria profited from the increased root growth and exudate availability during drought, as the increased amounts of EOC in drought mesocosms at peak drought labeling suggested. If this scenario occurred at earlier stages of drought, when soil moisture conditions were not limiting the bacterial activity, then Gram-negative bacteria could have used the easily consumable carbon from the EOC pool for their growth. Similarly, we did not expect the concentration of AM fungi marker in drought mesocosms to be reduced compared to the controls (**Table 3**). This contrasts previous findings from grassland monoliths (Karlowsky et al., 2018), showing an increase of the (AM + saprotrophic) fungi:bacteria ratio at peak drought. This difference could be due to the use of sieved soil in mesocosms, because the mycorrhizal network strongly interacts with soil structure (Rillig and Mummey, 2006). Other explanations include increased competition for plant carbon between fine roots and AM fungi, or a lower plant dependence on AM fungi due to (a) lower nutrient demand of senescing shoots or (b) higher nutrient availability resulting from decreased competition with soil microorganisms. Additionally, the selected plant species might have interacted differently with AM fungal populations (Legay et al., 2016; Mariotte et al., 2017). Additionally, bacterial foraging of senescing AM fungi structures cannot be excluded and might have contributed to the increase in the Gram-negative bacteria during drought, too.

Carbon Allocation and Plant–Microbial Interactions During Recovery

After rewetting, the mesocosm communities quickly recovered from drought, and both the shoot biomass and the root:shoot ratio were restored to control levels (**Table 1**). The higher fine root growth observed during drought was ceased at recovery labeling, possibly to support the re-growth of shoot biomass. However, the mechanisms behind the change in fine root biomass remain unclear, and thus, we cannot exclude the possibility that this observation was due to initial differences between the mesocosms used for the peak drought labeling and the mesocosms used for the recovery labeling. In general, the root response to drought-rewetting seems to be highly variable because previous studies either found an increase (Fuchslueger et al., 2016; Karlowsky et al., 2018, abandoned grassland) or no change (Karlowsky et al., 2018, managed meadow) in the fine root biomass after rewetting. In the latter study, the root response depended on the land use and was attributed to variable needs of water and nutrient uptake by fine roots, resulting from differences in the recovery of the dominant plant-microbial interactions. On the other side, in this study, the plant ^{13}C tracer uptake and allocation supports the hypothesis that carbon resources are preferentially invested into the regrowth of shoot biomass after rewetting (**Figure 3B**). Despite recovered ^{13}C tracer dynamics, the reduced shoot fructan pool indicates that, during the recovery phase, plants invested more carbon into structural carbohydrates or into respiration (e.g., for repair processes) than in storage. This investment was underpinned by the higher turnover of ^{13}C tracer in shoot starch, which suggests a faster utilization of recent assimilates from transitory

storage (Bahn et al., 2013) in plants recovering from drought. The reduced concentrations of root sucrose after rewetting could also be a result of the preferential use of newly assimilated carbon for shoot regrowth, decreasing the BCA during recovery (Zang et al., 2014). However, since only a marginal effect was observed on the average ^{13}C tracer incorporation in root sucrose and apparently a faster utilization of recent assimilates occurred in roots (**Supplementary Figures S6M–O**), most likely, the reduced sucrose concentrations were a result of increased root-rhizosphere carbon transfer (Hagedorn et al., 2016).

According to a shift in root functioning from resource preservation to nutrient acquisition, the uptake of fresh plant-derived carbon completely recovered for all microbial groups, and the carbon transfer to saprotrophic fungi even increased in the drought mesocosms (**Figure 3B**). These microorganisms were also found to rapidly take up recent plant-derived carbon in grasslands (de Deyn et al., 2011). In contrast to a previous study on the meadow (Karlowsky et al., 2018), we could not find significant excess uptake of ^{13}C tracer in bacteria. However, we cannot exclude that the use of sieved subsoil in this study led to altered microbial responses compared to the undisturbed topsoil in the previous study, as the initial microbial community and its functioning might have differed. Moreover, the rapid uptake of plant-derived carbon by saprotrophic fungi agrees with a recently introduced framework for carbon flow in the rhizosphere by Ballhausen and de Boer (2016), who proposed that a large fraction of the labile carbon from root exudation is primarily taken up by saprotrophic fungi prior to its consumption by fungus-feeding bacteria. As expected, AM fungi generally took up the largest fraction of plant-derived carbon in the soil microbial community (Drigo et al., 2010; Mellado-Vázquez et al., 2016; Karlowsky et al., 2018) but recovered slowly after rewetting the dried soil (de Vries et al., 2012; Meisner et al., 2013; Karlowsky et al., 2018). Interestingly, despite their lower abundance, AM fungi completely recovered their ^{13}C tracer uptake in drought treatments at the recovery labeling, suggesting that the efficiency of plant-mycorrhizal carbon flow increased at this time to support the recovery of the hyphal network.

The recovery of soil microbial growth after drought is typically preceded by a pulse of soil respiration directly after rewetting (Birch, 1958). However, those sources other than the released microbial osmolytes that contribute to the Birch effect are not well known, especially in planted soils (Canarini et al., 2017). Here, we found accumulations of carbon in the root sugar and soil EOC pools during drought, which quickly disappeared after rewetting. This strongly suggests that the release of these easy degradable carbon sources after the end of drought contributes to the acceleration of the soil microbial activity. Data not yet published on soil respiration from the ^{13}C pulse labeling experiment described by Karlowsky et al. (2018) indicate that carbon assimilated during drought contributes to the Birch effect, as ^{13}C applied to the monoliths during peak drought could be recovered in the soil respiration pulse after rewetting. Consequently, this means that the plant-derived carbon, which cannot be used by soil microorganisms during drought, is available for priming the microbial organic matter cycle in soil after rewetting. Such priming effects, e.g., observed after amending soil samples with fresh plant litter (Thiessen et al., 2013), are well-known to support plant growth by increasing nutrient mineralization from soil organic matter. An increase in nitrogen mineralization especially has been reported after rewetting dried soils (Borken and Matzner, 2009; Canarini and Dijkstra, 2015), and this increase probably contributed to the increased root and shoot nitrogen concentrations found at the recovery in this study. Additionally, the transport of preserved nitrogen from roots to shoots could have led to the significantly increased shoot nitrogen concentrations in drought treatments. As the leaf nitrogen concentration typically correlates with the photosynthetic activity (Wright et al., 2001; Milcu et al., 2014), the increased nitrogen uptake likely facilitated the higher assimilation rates needed for recovery (Ingrisch et al., 2018).

CONCLUSION

The results from this study confirm our first hypothesis that the frequently observed weakening of the link between plant photosynthesis and soil microbial carbon cycling during drought is due to reduced microbial uptake rather than to reduced root exudation. Our data from the ^{13}C pulse labeling experiments clearly show that recently assimilated plant carbon accumulates in the rhizosphere in the form of EOC during drought and that this accumulation is linked to reduced microbial uptake of plant-derived carbon. When the soil dries out, the limited diffusion leads to lower accessibility of root exudates for non-mycorrhizal fungi and bacteria. In addition, higher reductions of ^{13}C tracer allocation to growth-related fatty acid markers in comparison to the water-soluble MBC fraction, also in AM fungi, indicate adjustments in microbial metabolic activity; that is, the formation of osmolytes to prevent cell desiccation is favored over growth.

Our second hypothesis that drought leads to the accumulation of root sugars and EOC and that these easy degradable carbon sources are available for priming plant and soil microbial activity after rewetting, is only partially supported by the data. Indeed, we found that carbohydrates accumulated in roots and that the decreased microbial uptake was linked to increased EOC concentrations during drought. However, what causes the depletion of drought-accumulated carbon after rewetting remains unclear. Root sugars could either be used to support the regrowth of shoots or may be invested in plant-microbial interactions to gain more nutrients from soil organic matter decomposition. Drought-accumulated EOC that is not flushed away during the rewetting potentially further fuels the Birch effect, i.e., high microbial carbon and nitrogen mineralization shortly after rewetting. To determine how the preservation of belowground carbon pools during drought is related to microbial activity in the early phase of ecosystem recovery, future studies are needed to trace the flux of ^{13}C label applied at drought in soil after rewetting.

Ultimately, our results indicate that the link between plants and soil microorganisms plays a crucial role in the short-term response of carbon and nitrogen cycling to drought-rewetting events.

DATA ACCESSIBILITY

The datasets analyzed for this study can be found in the figshare repository: https://figshare.com/s/afd9c8f0fab5a572fdb3.

AUTHOR CONTRIBUTIONS

MB and GG conceived the ideas. SK, AA, JI, MB, and GG designed the methodology. SK, AA, JI, MA, and GG conducted the experiments and collected the data. SK, AA, and MA analyzed the data. SK and GG led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01593/full#supplementary-material

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Variations in Soil Bacterial Community Diversity and Structures Among Different Revegetation Types in the Baishilazi Nature Reserve

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We compared patterns of soil bacterial community diversity and structure in six secondary forests (JM, *Juglans mandshurica*; QM, *Quercus mongolica*; MB, mixed Broadleaf forest; BE, *Betula ermanii*; CB, conifer-broadleaf forest; PT, *Pinus tabuliformis*) and two plantation forests (LG, Larix gmelinii; PK, *Pinus koraiensis*) of the Baishilazi Nature Reserve, China, based on the 16S rRNA high-throughput Illumina sequencing data. The correlations between the bacterial community and soil environmental factors were also examined. The results showed that the broadleaf forests (JM, QM, MB) had higher levels of total C (TC), total N (TN), available N (AN), and available K (AK) compared to the coniferous forests (PT, LG, PK) and conifer-broadleaf forest (CB). Different revegetation pathways had different effects on the soil bacterial community diversity and structure. For the α-diversity, the highest Shannon index and Simpson index were found in JM. The Simpson index was significantly positively correlated with the available P (AP) ($P < 0.05$), and the Shannon index was significantly positively correlated with AK ($P < 0.05$). Compared with others, the increased ACE index and Chao1 index were observed in the CB and MB, and both of these α-diversity were significantly negative with AK ($P < 0.05$). The relative abundances of bacterial phyla and genera differed among different revegetation types. At the phylum level, the dominant phylum groups in all soils were Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Chloroflexi, Bacteroidetes, Gemmatimonadetes, and Planctomycetes. Significant differences in relative abundance of bacteria phyla were found for Acidobacteria, Actinobacteria, Chloroflexi, Gemmatimonadetes, and Proteobacteria. Correlation analysis showed that Soil pH, TC, TN, AP, and AK were the main abiotic factors structuring the bacterial communities. As revealed by the clear differentiation of bacterial communities and the clustering in the heatmap and in the PCA plots, broadleaf forests and coniferous forests harbored distinct bacterial communities, indicating a significant impact of the respective reforestation pathway on soil bacterial communities in the Baishilazi Nature Reserve.

Keywords: the Baishilazi Nature Reserve, secondary forests, plantation forest, soil bacterial community, soil characteristics

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INTRODUCTION

Forests have important ecosystem service functions; therefore, it is crucial to maintain the patterns, functions, and processes of the natural ecosystems. The restoration of the degraded forest ecosystem is a valid approach to solve a number of environmental problems and to increase plant biodiversity, consequently improving ecosystem service functions (Chazdon, 2008). Against this background, research on different forest restoration pathways is of great significance, especially in terms of plant community composition and diversity (Xie et al., 2013). Numerous studies have documented the effects of different revegetation pathways on soil physicochemical characteristics (Jin et al., 2016; Zhang et al., 2018), microbial biomass, and enzyme activities (Li et al., 2018), however, studies on the variations in microbial community composition are scarce (Kang et al., 2018), despite the crucial role of microorganisms in biogeochemical cycles (Smith et al., 2015).

Soil microorganisms, especially bacteria, which represent the most abundant group (Roesch et al., 2007), play central roles in ecosystems, including the carbon, nitrogen, and metals cycling as well as the biodegradation or stabilization of environmental contaminants (Green et al., 2008; He et al., 2011). As a consequence, shifts in the soil microbiome can directly affect soil ecosystem functions, especially carbon and nitrogen cycles (López-Lozano et al., 2013). In forest soils, the physicochemical properties of soil can influence the microbial community diversity, structure, and activity (Fierer and Jackson, 2006). Furthermore, vegetation types are regulatory factors that affect the soil physical and chemical properties (Curlevski et al., 2010; Liu et al., 2012; Oh et al., 2012). Tree species that determine leaf litter quality and quantity can significantly alter soil chemical properties, including soil pH, soil moisture, soil texture (Augusto et al., 2000), soil organic matter content (Frouz et al., 2009), soil nutrient availability (Menyailo et al., 2002), and the relative contents and chemical forms of macronutrients (Hagen-Thorn et al., 2004). This leads to quantitative and qualitative variations in soil carbon and nitrogen pools, combined with soil biological properties (Chen et al., 2004; Curlevski et al., 2010), which can influence the abundance of different bacterial groups (Scherer-Lorenzen and Potvin, 2007; Deyn et al., 2008; Orwin et al., 2010). The rates of microbial decomposition processes both in the litter and soil are substantially influenced by the dominant tree vegetation (Šnajdr et al., 2013). However, the extent to which vegetation type shifts the bacterial community diversity and structure, as well as the relationship between soil physical and chemical properties and the bacterial community are still remain poorly understood.

The Baishilazi Nature Reserve in China is located in the mountainous Region of the Eastern Liaoning Province, China. It was established in 1988 and belongs to the Changbai Mountain system. The original vegetation consisted of broadleaf *Pinus koraiensis* forests, which were severely damaged due to the over-exploitation in the past 100 years. At present, the vegetation mainly consists of natural secondary forests and conifer plantations, and this condition represents a unique opportunity to investigate the soil bacterial community under different reforestation pathways at the same climatic conditions. Numerous studies have investigated the changes in soil microbial biomass (Fan and Liu, 2014), and soil organic carbon contents (Qi, 2017) under different revegetation types, although the structure and diversity of the soil bacterial community have been studied scarcely. In this context, we applied pyrosequencing of the V3–V4 16S rRNA gene to explore both diversity and composition of the soil bacterial community in different revegetation types from eight sites in the Baishilazi Nature Reserve in Liaoning Province, China. Given the differences between conifer and broadleaf forests, we presumed that the soil bacterial community diversity and composition would also differ, although the sites have the same soil type and are subjected to the same climatic. We tested the following hypotheses: (1) different revegetation types impact soil characteristics; (2) the soil bacterial community diversity and structure are linked to soil characteristics; (3) the bacterial community structure of broadleaf forests differ from those of conifer-broadleaf forest and coniferous forests.

The overall goal of our study was to determine the effects of different reforestation pathways on soil bacterial communities. Understanding how plant species alter soil bacterial communities and their related course will conduce to extend our understanding of the biogeochemical elemental cycles that are affected by microbial communities in natural or anthropogenic forest ecosystems.

MATERIALS AND METHODS

Experiment Site

The field study was conducted at the Baishilazi Nature Reserve, in the mountainous region of the eastern Liaoning Province, China (40°50′00′′-40°57′12′′N, 124°44′07′′-124°57′30′′E). The total area of the Baishilazi Nature Reserve encompasses 7,407 hm^2 and belongs to the mountain range of the Changbai Mountain. This area is characterized by a continental monsoon climate, with warm wet summers, long cold winters, and strong diurnal temperature variation. The mean annual temperature is 6.4°C, with a mean annual precipitation of 1,158 mm. The characteristics of the eight stands are listed in **Table 1**; all forest stands were older than 40 years.

Soil Sampling

In July, 2017, we collected soil samples from *Juglans mandshurica* (JM), *Quercus mongolica* (QM), mixed broadleaf forest (MB), *Betula ermanii* (BE), conifer-broadleaf forest (CB), *Larix gmelinii* (LG), *Pinus koraiensis* (PK), and *Pinus tabuliformis* (PT) stands after removal of the litter layer. A total of 24 soil samples (three plots of 20 m × 20 m as three independent replicates) were taken from the A horizon of each stand with the use of a soil auger (8 cm in diameter, 10 cm deep). To ensure the representativeness of soil samples in each stands, the strip sampling method was used. The samples of 15–20 sampling plots within one stand were mixed together to obtain one

TABLE 1 | Sites information.

Samples	Main herb under the forest	Elevation (m)	Forest type
JM	*Acanthopanax senticosus*, *Padus racemose*, *Magnolia sieboldii*, *Pimpinella brachycarpa*, *Puccinellia tenuiflora*	901.8	Natural secondary forest
QM	*Acer mono*, *Cerasus tomentosa*, *Carpinus cordata*	842.3	Natural secondary forest
MB	*Smilacina japonica*, *Schisandra chinensis*, *Viola diamantiaca*	763.4	Natural secondary forest
BE	*Carpinus cordata*, *Acer mono*, *Maianthemum bifolium*, *Schisandra chinensis*	902.9	Natural secondary forest
CB	*Schisandra chinensis*, *Phryma leptostachya* L. subsp. *asiatica*	826.5	Natural secondary forest
PT	*Hemiptelea davidii*, *Leymus chinensis*	525.6	Natural secondary forest
LG	*Daemonorops margaritae*	552.7	Plantation forest
PK	*Daemonorops margaritae*, *Pteridium aquilinum*	552.7	Plantation forest

JM, Juglans mandshurica; QM, Quercus mongolica; MB, mixed broadleaf forest; BE, Betula ermanii; CB, Conifer-broadleaf forest; PT, Pinus tabuliformis; LG, Larix gmelinii; PK, Pinus koraiensis.

composite sample per stand. Identically, three real replicate samples stand and placed in cooled boxes. In the laboratory, the samples were sieved through a 2-mm mesh to remove roots and other debris and subsequently divided into two parts. One part was stored at −80°C for DNA extraction, while the other part was air-dried for soil physicochemical analyses. **Figure 1** shows the characteristics of the soils from all eight stands.

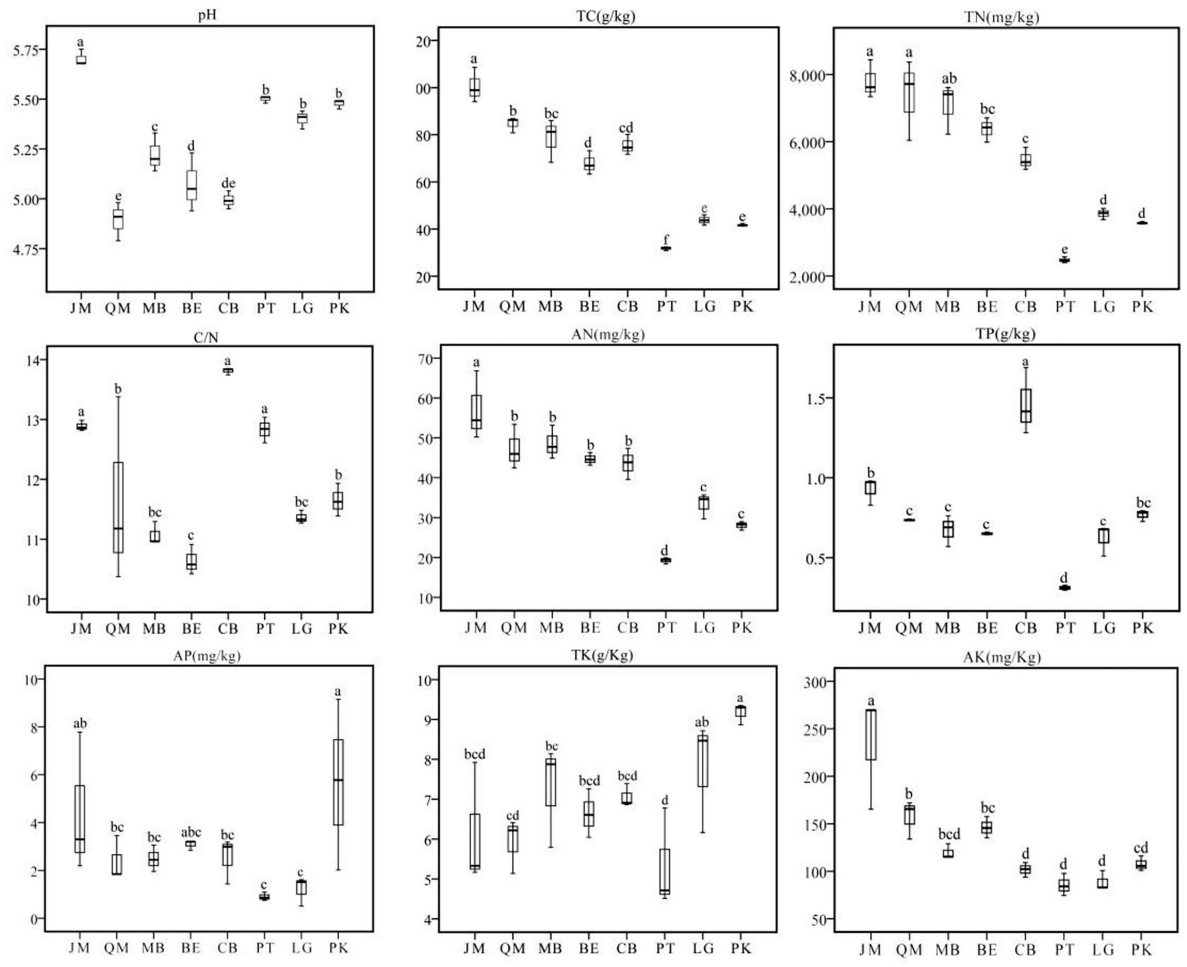

FIGURE 1 | Variations of soil physicochemical properties in JM, *Juglans mandshurica*; QM, *Quercus mongolica*; MB, mixed Broadleaf forest; BE, *Betula ermanii*; CB, Conifer-broadleaf forest; PT, *Pinus tabuliformis*; LG, *Larix gmelinii*; PK, *Pinus koraiensis*.

Soil DNA Extraction

Soil DNA was extracted from 0.5 g soil, using the Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, United States), according to the manufacturer's instructions. The NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) was used to measure the quantity and quality of the extracted DNAs.

16S rDNA Sequencing

A quantitative PCR amplification of the bacterial 16S rRNA genes V3–V4 region was performed, using the forward primer 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and the reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). Sample-specific 7-bp barcodes were incorporated into primers for multiplex sequencing. The PCR amplifications were performed in two steps. During the first step, each of three independent 25 μl mixtures per DNA sample contained 5 μl of Q5 reaction buffer (5×), 5 μl of Q5 High-Fidelity GC buffer (5×), 1 μl (10 μM) of each forward and reverse primer, 2 μl (2.5 mM) of dNTPs, 0.25 μl of Q5 High-Fidelity DNA polymerase (5 U/μl), 2 μl (40–50 ng) of DNA Template, and 8.75 μl of ddH2O. Cycling conditions were 98°C for 5 min (one cycle), 98°C for 15 s, 55°C for 30 s, 72°C for 30 s (25 cycles), followed by 72°C for 5 min. The PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). After the individual quantification step, amplicons were pooled at equal amounts, and pair-end 2 × 300 bp sequencing was performed using the Illumina MiSeq platform with the MiSeq Reagent Kit v3. We have uploaded all raw sequences to the NCBI Sequence Read Archive under submission number SUB4489099 and BioProject number PRJNA489351.

Statistical Analysis

Sequence data analyses were mainly performed using the software packages QIIME and R (v3.2.0). Operational taxonomic units (OTU)-level alpha diversity indices, such as the Chao1 richness estimator, the abundance-based Coverage Estimator (ACE) metric, the Shannon index, and the Simpson index, were calculated using the OTU table in QIIME. The shared and unique OTUs among samples were used to generate Venn diagrams with the R software package. The linear discriminant analysis (LDA) effect size (LEfSe) algorithm method was used to detect the potential biomarkers based on a normalized relative abundance matrix. The heatmap representation of the relative abundance of bacterial OTUs among samples was built using R. Principal components analysis (PCA) was also conducted based on the genus-level compositional profiles.

Analysis of variance (ANOVA) was performed using SPSS 19.0 software. Soil physicochemical characteristics as well as bacterial total diversity and abundance were compared using LSD tests. Pearson's correlation analysis was accustomed to estimate the correlations between soil characteristics and bacterial diversity indices. Canonical correspondence analysis (CCA), performed via Canoco 4.5, was used to evaluate the linkages between dominant bacterial groups related to soil environmental factors.

RESULTS

Changes in Soil Characteristics

We found significant differences among stands in terms of soil total Carbon (TC) and total Nitrogen (TN) (**Figure 1**). Interestingly, both TC and TN were highest in JM, with 100.53 and 7.80 g/kg, respectively; while in PT, we measured only 31.76 g/kg and 2.48 g/kg, respectively. Available N (AN) followed the order of JM > MB > QM > BE > CB > LG > PK > PT. In all sites, the soil C/N ratio was below 25:1, with CB showing the highest value. Soil pH value ranged from 4.89 to 5.70; under QM, pH was the lowest with 4.89, followed by CB, while JM had the highest soil pH value. Significant differences were found for available P (AP) and total P (TP), with the highest values under PK and CB (**Figure 1**).

Bacterial Community Richness and Diversity Indices of Different Revegetation Types

We obtained a total of 1,448,252 bacterial sequences, which remained after removing low quality sequences and chimeras (on average, 60,343 per sample). In total, 57,139 to 65,460 sequences per sample were obtained and classified into the domain level (**Table 2**); average sequence length was 330 bp. At the phylum, the remaining sequences were identified for 65,479 OTUs. A maximum of 2,845 OTUs were detected in CB; however, only 2,554 OTUs were obtained in the JM.

Venn diagrams were used to compare the bacterial communities under JM, QM, LG, CB, and PT, based on shared and unique OTUs among the samples. The number of shared OTUs among JM, QM, LG, CB, and PT was 1,187. A total of 392 unique OTUs were found in CB, while 344 unique OTUs were found in LG, 312 in PT, 199 in QM, and 407 in JM. In terms of shared OTUs, 290 were observed between LG and PT, and 502 between QM and JM. Under QM, the lowest number of unique OTUs was found (**Supplementary Figure S1**), while we detected most unique OTUs under JM.

The different soils showed high species richness, albeit with large variations. The highest species diversity (Shannon and Simpson) was found in JM, with 9.71 and 0.9950, respectively, which also showed the lowest values in ACE and Chao1 index, with 2635.51 and 2597.17, respectively (**Table 2**). The Shannon index value was lowest under the LG, with 9.10, followed by BE (9.20). However, the Simpson index varied considerably and was lowest under PT (0.9902), followed by BE (0.9906). Highest ACE index and Chao1 index value were observed for MB with 3,405.73 and 3,368.12, respectively, and for CB with 3,637.79 and 3,568.24, respectively, most likely because of the high amounts of leaf litter and roots in these sites. In addition, Person's rank correlation coefficients showed that the Simpson values were significantly positively correlated with the AP ($P < 0.05$). While, the Shannon value was significantly positively correlated with AK ($P < 0.05$). Values of ACE and

TABLE 2 | Soil bacterial diversity indexes of different samples.

Sample	No. of sequences	OTUs number (Phylum)	Shannon index	ACE index	Chao1 index	Simpson index
JM	57139 ± 4599b	2554 ± 151b	9.71 ± 0.03a	2635.51 ± 289.86c	2597.17 ± 223.89c	0.9950 ± 0.0002a
QM	59376 ± 5996ab	2710 ± 205ab	9.48 ± 0.07ab	2929.07 ± 553.72bc	2887.56 ± 487.75bc	0.9912 ± 0.0003bc
MB	61986 ± 4055ab	2816 ± 174a	9.32 ± 0.24bcd	3405.73 ± 285.75ab	3368.12 ± 395.94ab	0.9916 ± 0.0014bc
BE	62993 ± 4661ab	2738 ± 48ab	9.20 ± 0.07cd	3236.61 ± 320.61ab	3119.53 ± 410.62abc	0.9906 ± 0.0004bc
CB	65460 ± 1322a	2845 ± 21a	9.28 ± 0.08bcd	3637.79 ± 18.88a	3568.24 ± 149.81a	0.9920 ± 0.0007b
PT	57237 ± 4377b	2754 ± 153ab	9.37 ± 0.05bc	3168.73 ± 499.72abc	3118.16 ± 476.67abc	0.9902 ± 0.0002c
LG	59384 ± 4215ab	2627 ± 61ab	9.10 ± 0.09d	3220.67 ± 108.96ab	3082.82 ± 96.45abc	0.9919 ± 0.0005b
PK	59175 ± 1703ab	2782 ± 217ab	9.46 ± 0.31abc	3365.93 ± 271.01ab	3316.26 ± 318.03ab	0.9939 ± 0.0015a

Data are means ± standard error (n = 3). JM, Juglans mandshurica; QM, Quercus mongolica; MB, Mixed Broadleaf forest; BE, Betula ermanii; CB, Conifer-broadleaf forest; PT, Pinus tabuliformis; LG, Larix gmelinii; PK, Pinus koraiensis. Different letters in the same line (a, b) indicate a significant difference at P < 0.01.

TABLE 3 | Person's rank correlation coefficients between soil bacterial diversity indices and measured soil characteristics.

	pH	TC	TN	C/N	AN	TP	AP	TK	AK
Shannon	0.405	0.499	0.396	0.365	0.356	0.119	0.572	−0.244	**0.739***
ACE	−0.414	−0.411	−0.404	0.022	−0.362	0.298	−0.141	0.493	**−0.790***
Chao1	−0.406	−0.374	−0.377	0.069	−0.346	0.316	−0.100	0.475	**−0.764***
Simpson	0.576	0.361	0.243	0.255	0.360	0.409	**0.776***	0.419	0.561

**Correlation is significant at the 0.05 level (two-tailed). TC, Total Carbon; TN, Total Nitrogen; C/N, C-N ration; AN, Available Nitrogen; TP, Total Phosphorus; AP, Available Phosphorus; TK, Total Kalium; AK, Available Kalium.*

Chao1 were significantly negatively correlated with AK ($P < 0.05$) (**Table 3**).

The total number of sequences for each sample in the OTU abundance matrix was randomly sampled at different depths. The rarefaction curve was drawn with the number of sequences extracted at each depth and the corresponding OTU numbers. At a genetic distance of 3%, the rarefaction curve flattened with increasing numbers of measured sequences, indicating that the majority of the sample information was obtained (**Supplementary Figure S2**), adequately reflecting the microbial communities in the soil.

Bacterial Community Distribution and Composition in Soils

The relative abundances of bacterial phyla and genera differed among different reforestation pathways (**Table 4** and **Supplementary Figure S3**, **S4**). Across all samples, we detected 34 bacterial phyla and 1 archaeal phyla, of which 10 phyla had an average abundance greater than 1% (**Supplementary Figure S3**). The dominant phyla were Proteobacteria (39.98–46.77%), Acidobacteria (15.19–27.32%), Actinobacteria (7.03–16.61%), Verrucomicrobia (4.18–7.97%), Chloroflexi (3.03–7.36%), Bacteroidetes (1.82–4.64%), Gemmatimonadetes (1.93–3.15%), Planctomycetes (1.96–2.48%), Firmicutes (0.21–8.50%), and Nitrospiraea (0.35–3.33%) (**Table 4**), accounting for 97.12–99.07% of the bacterial sequences from each stands. In addition, Saccharibacteria, Cyanobacteria, Latescibacteria, Elusimicrobia, Armatimonadetes, Chlamydiae, and Parcubacteria were present in most soils, albeit at relatively low abundances (below 1%), and all of the other rarer phyla were identified. Among them, Proteobacteria were the absolute dominant species, followed by Acidobacteria and Actinobacteria (**Table 4** and **Supplementary Figure S3**). The relative abundance of Proteobacteria was highest in PT (46.77%) and lowest in PK (39.98%). The relative abundances of Verrucomicrobia and Planctomycetes in all samples did not significantly different. Actinobacteria were less abundant in coniferous forests [PK (8.26%), LG (7.02%), and PT (8.48%)] than in broadleaf forests [MB (16.40%), JM (15.88%), QM (14.82%), and BE (16.61%)] and conifer-broadleaf forest (14.93%). Firmicutes dominated in PK and were rare in the other stands. While JM had the highest relative abundances of Chloroflexi (7.36%), Gemmatimonadetes (3.15%), and Nitrospirae (3.33%), but the lowest abundances of Acidobacteria (15.19%) and Firmicutes (0.21%) (**Table 4** and **Supplementary Figure S3**).

At the genus level, average relative abundances below 1% were grouped into "others," and 11 groups were obtained. The relative abundances of several genera differed significantly between the different samples (**Supplementary Figure S4**). The soil was dominated by *Nitrobacter* (6.49%), followed by *Candidatus-Solibacte*r (3.72%), *Acidothermus* (2.86%), *Pseudolabrys* (2.43%), and *Bryobacter* (1.98%). Significant differences in relative abundance were not found for the genera *Variibacter* and *Haliangium* among samples. The relative abundances of *Nitrobacter*, *Candidatus-Solibacter*, *Acidothermus*, *Pseudolabrys*, *Bryobacter*, *Rhizomicrobium*, *H16*, and *Reyranella* showed significant differences between JM and QM. Similarly, the relative abundances of *Nitrobacter*, *Candidatus-Solibacter*, *Bryobacter*, and *H16* differed significantly among the JM, PT, and CB. The stand CB had the highest relative abundances of *Candidatus-Solibacter*, *Acidothermus*, *Bryobacter*, and *Rhizomicrobium*, but the lowest abundance of *H16*. PT had a significantly higher relative abundance of *Nitrobacter* compared to the other stands. Significant

TABLE 4 | Relative abundance of the most abundant bacterial phyla (>1%) present in the soil samples.

Samples	Proteobacteria (%)	Acidobacteria (%)	Actinobacteria (%)	Verrucomicrobia (%)	Chloroflexi (%)
JM	41.31 ± 0.59cd	15.19 ± 1.27e	15.87 ± 2.85a	6.94 ± 1.97a	7.36 ± 0.11a
QM	45.11 ± 1.19ab	19.33 ± 0.96cd	14.80 ± 2.94a	6.38 ± 1.80a	4.16 ± 0.12cd
MB	44.99 ± 0.31ab	19.01 ± 1.02cd	16.40 ± 3.17a	5.72 ± 4.92a	4.92 ± 1.58bcd
BE	44.08 ± 0.15ab	17.73 ± 1.31de	16.61 ± 4.72a	7.97 ± 4.13a	4.75 ± 0.85bcd
CB	44.12 ± 0.73ab	23.60 ± 0.68b	14.93 ± 1.62a	5.59 ± 0.57a	3.03 ± 0.35d
PT	46.77 ± 1.02a	21.52 ± 2.47bc	8.48 ± 2.16b	5.25 ± 1.46a	6.50 ± 0.53ab
LG	42.97 ± 0.82bc	27.32 ± 0.75a	7.03 ± 3.15b	7.22 ± 2.59a	5.39 ± 0.24bc
PK	39.98 ± 3.94d	19.13 ± 2.46cd	8.26 ± 2.48b	4.18 ± 1.27a	6.58 ± 2.48ab
Samples	**Bacteroidetes (%)**	**Gemmatimonadetes (%)**	**Planctomycetes (%)**	**Firmicutes (%)**	**Nitrospirae (%)**
JM	3.26 ± 0.42ab	3.15 ± 0.14a	1.97 ± 0.34a	0.21 ± 0.03b	3.33 ± 0.29a
QM	2.87 ± 0.36ab	2.83 ± 0.31ab	2.10 ± 0.42a	0.55 ± 0.09b	0.75 ± 0.46de
MB	1.82 ± 1.19b	2.41 ± 1.01abc	1.95 ± 0.71a	0.56 ± 0.13b	1.29 ± 0.53bcd
BE	1.61 ± 0.63b	2.19 ± 0.43bc	2.48 ± 0.03a	0.46 ± 0.15b	1.07 ± 0.22cd
CB	1.89 ± 0.26b	1.93 ± 0.09c	2.34 ± 0.26a	0.88 ± 0.04b	0.35 ± 0.07e
PT	2.58 ± 0.06b	3.12 ± 0.22a	2.29 ± 0.45a	0.52 ± 0.26b	0.94 ± 0.08d
LG	1.97 ± 0.15b	2.22 ± 0.39bc	2.17 ± 0.25a	0.55 ± 0.29b	1.53 ± 0.17bc
PK	4.64 ± 2.86a	2.17 ± 0.30bc	1.96 ± 0.40a	8.50 ± 7.83a	1.70 ± 0.46b

Values are means ± standard deviation (n = 3). JM, Juglans mandshurica; QM, Quercus mongolica; MB, Mixed Broadleaf forest; BE, Betula ermanii; CB, Conifer-broadleaf forest; PT, Pinus tabuliformis; LG, Larix gmelinii; PK, Pinus koraiensis. Different letters in the same line (a,b) indicate a significant difference at $P < 0.01$.

differences in relative abundance between LG and PK were found for the genera *Nitrobacter*, *Candidatus-Solibacter*, *Acidothermus*, *Bryobacter*, and *H16* (**Supplementary Figure S4**).

The LEfSe analysis was documented to determine the classified bacterial taxa with significant abundance differences among the different forest stands. As presented in **Figure 2**, 39 bacterial taxa were showed significantly different with LDA effect size scores were >4. At the phylum level, the biomarkers were affiliated with Proteobacteria, Acidobacteria, Actinobacteria, Chloroflexi, and Bacteroidetes.

We applied heatmap analysis to intuitively display the differences in relative abundances of the top 50 bacterial genera that appeared in all soil samples (**Figure 3**), which can reflect the differences in soil bacterial community structure between different vegetation types. The resulting heatmap could be divided into five clusters. Both relative abundance and distribution of soil bacteria in different stands were significantly different. In Cluster 1, higher relative abundances of 10 bacterial genera were found in JM, and significant differences were found for the other seven stands. The 19 bacterial genera with higher relative abundances in Cluster 2 were mainly found in QM, BE, and MB. In Cluster 3, the higher relative abundances of the six bacterial genera were mainly found in the PT and CB, and the three bacterial species with higher relative abundances in Cluster 4 were mainly found in PK and were rare in other stands. The three bacterial genera with higher relative abundances in Cluster 5 were mainly found in LG, CB, and MB. This indicates that the compositions and relative abundance of soil bacteria differed significantly between the stands. To further explore these differences, we applied principal components analysis (PCA) to extract the main components. The results of the PCA showed that soil bacterial community from the coniferous forest stand was separated from those of coniferous-broadleaf forest and the broadleaf forest, especially along PC2 (**Figure 4**).

Effect of Tree Species on Soil Properties and Microorganisms

Canonical correspondence analysis (CCA) was applied to explore the relative abundance of dominant bacteria phyla and genera as a function of soil variables (**Figure 5**). The first and second axes accounted for 91.2% and 80.5% of the variation, respectively, indicating that soil environmental factors significantly influence the bacterial community structure. At the phylum level (**Figure 5A**), AP (r = 0.6453) and TK (r = 0.7877) were significantly associated with axis1, which explained 72.4% of the variation. In contrast, soil pH (r = 0.8449) and AK (r = 0.6020) were significantly associated with axis 2. At the genus level (**Figure 5B**), pH (r = 0.8079) was associated with axis 1, accounting for 55.1% of the variation, while TC(r = 0.6839), TN (r = 0.7548), AN (r = 0.6798), and AK (r = 0.8993) were associated with axis 2. Based on this, soil pH, TC, TN, AN, TK, and AK significantly influenced the bacterial community structure.

We investigated the relationships between the relative abundances of different dominant bacterial phyla and genera and environmental variables (**Table 5**). In terms of soil chemical properties, AP was negatively correlated with the relative abundances of Proteobacteria, but positively correlated with Bacteroidetes abundance, while Acidobacterias' relative abundance was negatively correlated with AK ($P < 0.05$). The relative abundance of Actinobacteria was significantly positively correlated with TC, TN, and AN ($P < 0.01$), and Chloroflexi relative abundance was significantly positively correlated with pH ($P < 0.01$). The relative abundances of Firmicutes was positively correlated with TK ($P < 0.01$).

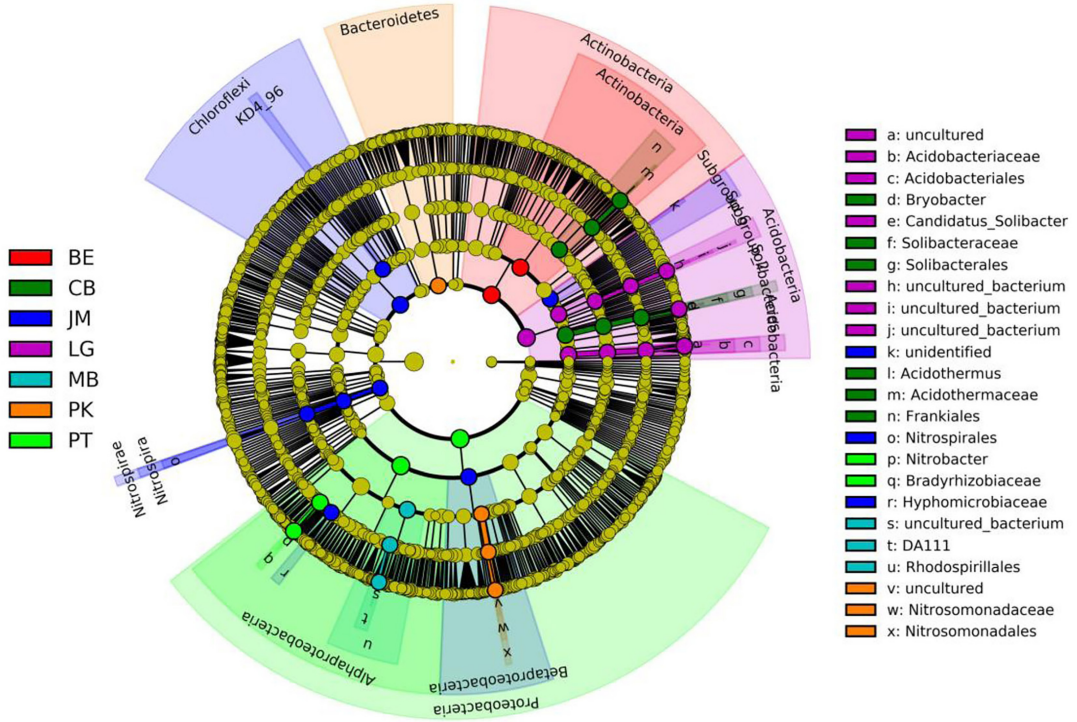

FIGURE 2 | Cladogram of soil bacterial in revegetation types via LEfSe method identifies the significantly different abundant taxa. Circle radiation from inner to outer of evolutionary branch figure represents the classification of the level from phylum doorgenus; every small circle represents the level of a classification in different classification level. The diameter of the circle is proportional to the relative abundance; the principle is that the species without significant differences uniformly color to yellow, and the other species are colored according to the highest abundance of the species. JM, *Juglans mandshurica*; QM, *Quercus mongolica*; MB, mixed broadleaf forest; BE, *Betula ermanii*; CB, Conifer-broadleaf forest; PT, *Pinus tabuliformis*; LG, *Larix gmelinii*.

At the genus level, *Acidothermus* was significantly negatively correlated with pH, while *Nitrobacter* was significantly negatively correlated with AP. The abundance of *Pseudolabrys* was positively correlated with TK, while *Candidatus-Solibacter*, *Bryobacter*, and *Rhizomicrobium* were significantly negatively correlated with AK.

DISCUSSION

Different Revegetation Types Contribute to Different Soil Physicochemical Characteristics

Reforestation pathways greatly affected the soil physicochemical characteristics through various processes, including changes in moisture and temperature, as well as the production of litter and root exudates. In our study, we observed that the broadleaf forests (JM, QM, MB) had higher levels of TC, TN, AN, and AK compared to the coniferous forests (PT, LG, PK) and the conifer-broadleaf forest (CB) (**Figure 1**), which is consistent with previous research suggesting that broadleaf-Korean pine mixed forest and secondary poplar-birch forest presented significantly higher OC and TN values than spruce fir and larch forest (Hui et al., 2014). The higher TC and TN values in the three broadleaf forests are most likely a result of constant organic matter inputs, rhizodeposition, and the release and recycling of nutrients (Finér et al., 2007; Wang and Cao, 2011). In complete contrast to the TC and TN pattern, the soil C/N ratios in coniferous forests (PT, LG, and PK) and the conifer-broadleaf forest were significantly higher than those in broadleaf forests (QM, BE, and MB). Previous studies have also reported that coniferous forests tend to be highly nitrogen deficient compared with broadleaf forests (Vitousek and Howarth, 1991; Mcgroddy et al., 2004). The soil C/N ratio directly reflects the soil's nitrogen mineralization capacity, and low soil C/N levels indicate a higher nitrogen mineralization rate, facilitating N uptake by microbes and plants. In turn, high C/N levels are beneficial for the fixation the of soil organic carbon. Litter C/N ratios of coniferous forests are generally higher than those of broadleaf forests, which may contribute to the higher C/N ratio in coniferous forest soils (Yang and Luo, 2011). Several studies have reported that higher C/N ratios and lower nutrient contents in coniferous forests compared with broadleaf forests (Barbier et al., 2008). In our study area, the Liaodong mountainous area, the soil was relatively acidic, with pH value ranging from 4.89 to 5.70, with the lowest levels under QM (pH = 4.89). These differences in pH may be due to different foliage properties and variations in litter quality (Haghdoost et al., 2011). Soil pH, nutrient contents, and numerous biogeochemical processes in forest ecosystems can be influenced by litter chemistry (Mueller et al., 2012; Fanin and

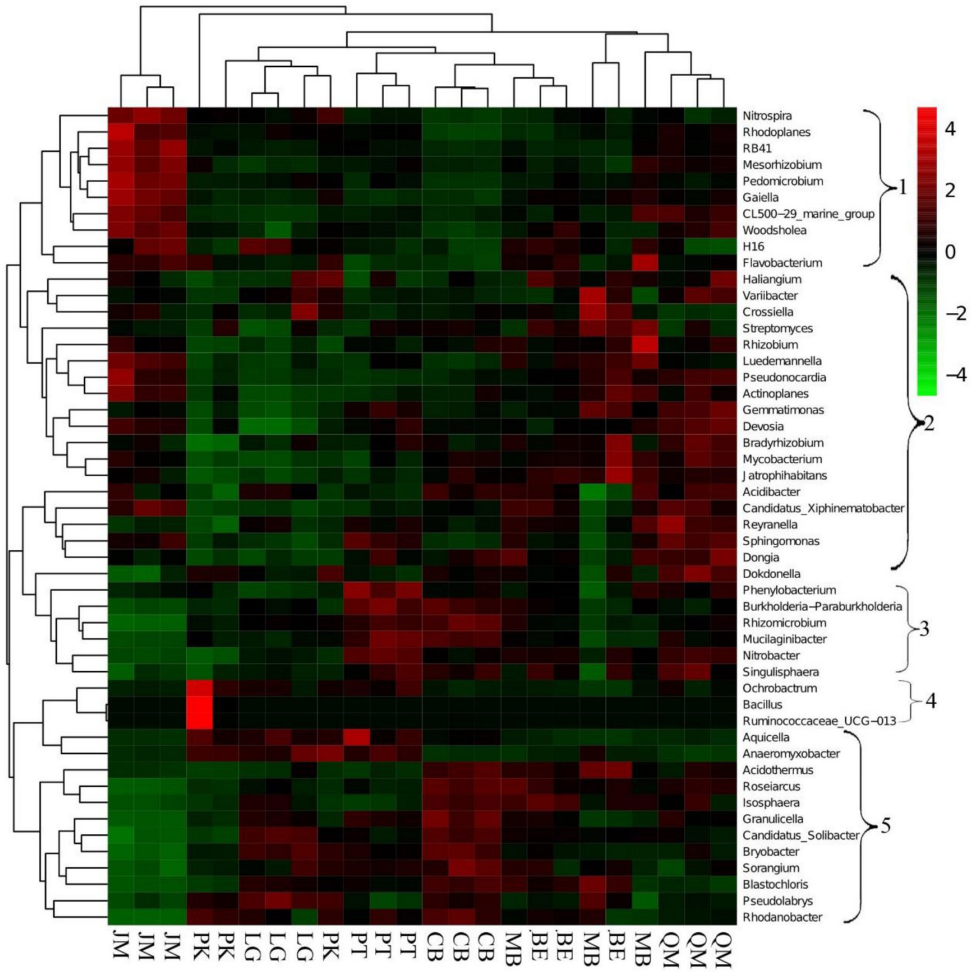

FIGURE 3 | Heatmap and hierarchical cluster analysis based on the relative abundances of the top 50 genera identified in the bacterial communities of the soils. The samples are grouped according to the similarity of each other, and the clustering results are arranged horizontally according to the clustering results. In the figure, red represents the genus with higher abundance in the corresponding sample, and green represents the genus with lower abundance. JM, *Juglans mandshurica*; QM, *Quercus mongolica*; MB, Mixed Broadleaf forest; BE, *Betula ermanii*; CB, Conifer-broadleaf forest; PT, *Pinus tabuliformis*; LG, *Larix gmelinii*; PK, *Pinus koraiensis*.

Bertrand, 2016). Compared to other broadleaf forests, litter leaf quality in QM was relatively low, with a low nitrogen content, a high C/N ratio, a higher lignin content, and higher lignin/N. Therefore, the decomposition rate of QM litter and the release rates of plant nutrients decreased gradually.

Response of Bacterial Community Diversity to Soil Physicochemical Characteristics in Different Reforestation Sites

Previous studies have demonstrated the crucial role of soil characteristics in altering the soil microbial communities during vegetation restoration (Thomson et al., 2015). Soil pH and nutrient contents, especially in terms of C, N, and P availability, are paramount factors (Ramirez et al., 2010; Tan et al., 2013) and significantly affect microbial abundance (Christianl et al., 2008; Paulinem and Davide, 2008; Zhong et al., 2010). When soil pH was below 6.5, the pH was supposed to be the primary factor which effected bacterial community diversity, structure and activity, and higher richness and diversity values were found at a pH close to neutral (Preem et al., 2012; Ramirez et al., 2012; Bergkemper et al., 2016). In contrast, Lauber et al. (2009) has proposed that there is a significantly negative relationship between soil pH and bacterial diversity. However, in my study, soil pH was not significantly correlated with any bacterial diversity index. However, we observed a relatively small pH rangs (4.89 to 5.70), making it different to find any correlation with bacterial diversity. Previous studies have suggested that bacterial alpha diversity is largely affected by SOC and TN (Siles and Margesin, 2016; Ren et al., 2017). However, in our study, TC and TN levels were not significantly correlated with bacterial diversity indexes, which was consistent with the observations obtained by Lin et al. (2014).

In most terrestrial ecosystems, the soil C/N ratio greatly influences soil microbial communities (Fierer and Jackson, 2006)

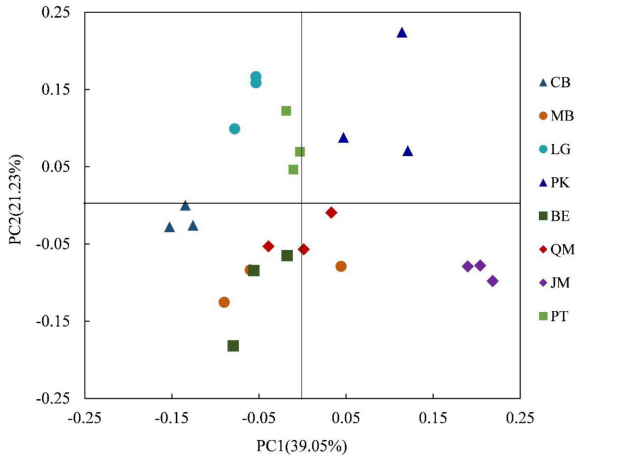

FIGURE 4 | Principal component analysis of the composition of bacterial communities in the soil of forests with different dominant trees. JM, *Juglans mandshurica*; QM, *Quercus mongolica*; MB, Mixed Broadleaf forest; BE, *Betula ermanii*; CB, Conifer-broadleaf forest; PT, *Pinus tabuliformis*; LG, *Larix gmelinii*; PK, *Pinus koraiensis*.

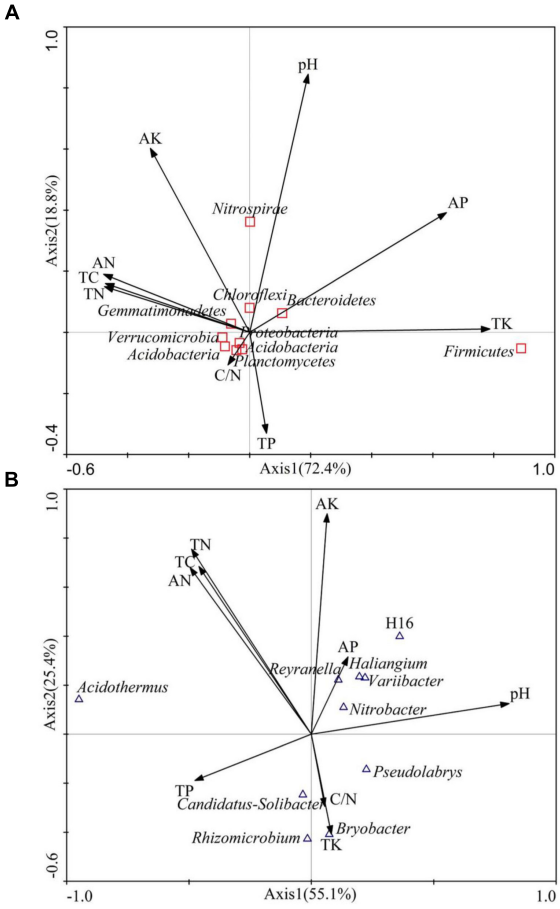

FIGURE 5 | Canonical correspondence analysis (CCA) on soil dominant bacteria phyla **(A)** and soil dominant bacteria genus **(B)** constrained by soil variables.

and is negatively correlated with the soil bacterial Shannon index (Zhou et al., 2017). However, in our study, C/N ratio showed no correlation with bacterial diversity indices, most likely because of the relatively small C/N range (10.64–13.81).

However, the Simpson index was significantly positively correlated with the AP, which is in accordance with a previous study suggesting that P increases bacterial diversity in pasture soil (Tan et al., 2013). Similarly, soil P is the second most significant driver of bacterial diversity in soil (Siciliano et al., 2014), although some authors have stated that bacterial diversity was not affected by P levels (Liu et al., 2018). In our study, the Shannon value was significantly positively correlated with AK ($P < 0.05$) (**Table 3**) and increased ACE index and Chao1 index values were observed in CB and MB, reflecting the large amounts of leaf litter and roots. Both of these α-diversity indicators were significantly negatively correlated with AK (**Table 3**), which leads us to infer that AP and AK availability may be the limiting factor affecting bacterial diversity in this area.

Response of the Bacterial Taxonomy to Different Revegetation Types

Similar to bacterial community diversity, the relative abundances of dominant bacterial phyla were influenced by different revegetation types. With regard to bacterial compositions, the different revegetation forest stands harbored distinct bacterial communities. In our study, Proteobacteria, Acidobacteria, and Actinobacteria were the most abundant soil bacterial phyla in the Baishilazi Nature Reserve, which is in agreement with a previous study in northern soils (Sun et al., 2014). In the light of bacterial classification, Proteobacteria, Acidobacteria, Actinobacteria, Firmicutes, Gemmatimonadetes, Nitrospirae, and Chloroflexi significantly differed between the different forest stands (**Table 4**). Acidobacteria was the dominant taxa in LG, while Actinobacteria was the dominant taxa in PK; Proteobacteria dominated the soil under PT. In constrast, Nitrospirae and Chloroflexi were dominant in JM. In contrast to our study, previous studies reported that coniferous forests were dominated by Proteobacteria and Gemmatimonadetes (Hui et al., 2014).

Proteobacteria was the most abundant phylum in our research, which is similar to the result of previous studies (Li et al., 2014a; Miyashita, 2015). However, according to Liu et al. (2013), Actinobacteria play a dominant role in the bacterial community of the mixed forest soil of the Dinghushan Mountain. Generally, members of the Proteobacteria and Acidobacteria are ubiquitous in almost all soil types (Zhang and Xu, 2008). In our study, despite different revegetation types, including broadleaf forests, coniferous forests, and conifer-broadleaf forest, the composition of bacterial groups was similar for all phyla detected. Previous studies have suggested that the Proteobacteria can be used as indicators of the prevalent nutrient status due to their different natures (Hartman et al., 2008). In our study, the relative abundance of Proteobacteria was highest in PT and largely affected by AP, but not by measured C, although a high

TABLE 5 | Person's rank correlations between the relative abundances of dominant bacteria groups and measured soil characteristics.

Bacteria group	pH	TC	TN	C/N	AN	TP	AP	TK	AK
Phylun	–	–	–	–	–	–	–	–	–
Proteobacteria	−0.456	−0.093	−0.047	0.044	−0.162	−0.316	**−0.837****	−0.706	−0.337
Acidobacteria	−0.169	−0.560	−0.594	0.121	−0.505	0.042	−0.629	0.227	**−0.772***
Actinobacteria	−0.445	**0.871****	**0.894****	−0.031	**0.860****	0.415	0.177	−0.355	0.614
Verrucomicrobia	−0.168	0.387	0.469	−0.351	0.504	−0.067	−0.271	−0.359	0.426
Chloroflexi	**0.932****	−0.215	−0.234	−0.026	−0.225	−0.509	0.357	0.025	0.322
Bacteroidetes	0.475	−0.150	−0.204	0.124	−0.232	−0.046	**0.716***	0.377	0.200
Gemmatimonadetes	0.452	0.184	0.144	0.210	0.055	−0.464	−0.129	−0.716*	0.498
Planctomycetes	−0.440	−0.235	−0.230	0.073	−0.217	0.039	−0.461	−0.346	−0.307
Firmicutes	0.235	−0.415	−0.409	−0.108	−0.414	0.029	0.701	**0.771***	−0.230
Nitrospirae	**0.796***	0.317	0.271	0.004	0.349	−0.078	0.520	0.075	0.702
Genus	–	–	–	–	–	–	–	–	–
Nitrobacter	−0.491	−0.212	−0.150	−0.015	−0.296	−0.406	**−0.754***	−0.700	−0.340
Candidatus-Solibacter	−0.550	−0.336	−0.321	−0.016	−0.256	0.210	−0.626	0.177	**−0.737***
Acidothermus	**−0.809***	0.406	0.461	−0.110	0.451	0.452	−0.215	−0.099	−0.090
Pseudolabrys	−0.183	−0.453	−0.435	−0.175	−0.325	0.223	−0.082	**0.738***	−0.626
Bryobacter	−0.415	−0.530	−0.573	0.199	−0.502	0.245	−0.500	0.264	**−0.835***
Variibacter	−0.423	0.349	0.495	−0.530	0.397	−0.199	−0.254	−0.126	0.205
Haliangium	−0.518	0.381	0.559	−0.666	0.465	−0.083	0.165	−0.069	0.400
Rhizomicrobium	−0.503	−0.476	−0.539	0.372	−0.541	0.192	−0.531	−0.053	**−0.791***
H16	0.578	0.262	0.295	−0.282	0.400	−0.179	0.077	0.056	0.459
Reyranella	−0.650	0.221	0.334	−0.256	0.172	−0.280	−0.607	−0.613	0.002

*TC, Total Carbon; TN, Total Nitrogen; C/N, C-N ration; AN, Available Nitrogen; TP, Total Phosphorus; AP, Available Phosphorus; TK, Total Kalium; AK, Available Kalium. **Correlation significant at 0.01 level (two-tailed); *Correlation significant at 0.05 level (two-tailed).*

abundance of Proteobacteria has previously been associated with a high C availability (Fazi et al., 2005; Fierer et al., 2007). This may be related to the composition of Proteobacteria in the different forest stands, and our results indicated that in the study region, soil TC had no significant impact on the bacterial community composition.

Recent studies have shown that Acidobacteria are generally oligotrophic (Kielak et al., 2009; López-Lozano et al., 2013), and Acidobacteria play a key role in biogeochemical nutrient cycling (Tringe et al., 2005). Apparently, the level of nitrogen input is negatively correlated with the relative abundance of Acidobacteria (Fierer et al., 2012), which is line with the results of our study. Acidobacteria belonged to the acidophilic bacteria, and Acidobacteria abundance is significantly higher under acidic conditions (Dion, 2008; Lauber et al., 2009; Bardhan et al., 2012). However, in our study, the relative abundance of the Acidobacteria was not correlated with soil pH (**Table 5**) but was significantly negatively correlated with AP. Soil properties, especially AP and AK, were significantly correlated with the bacterial, even at the very small geographical scale in our study, and this finding is in line with the previous research (Cai et al., 2018).

In our study, except for the Proteobacteria and Acidobacteria, the significant difference in the relative abundance of Actinobacteria was observed in coniferous forests (PK, LG, and PT) and was lower than the abundance in broadleaved forests (JM, QM, and BE). This finding is in agreement with a previous which reported that Actinobacteria are among the most important organic matter decomposers in soil (Kopecky et al., 2011). In our study, the differences in relative Actinobacteria abundance between the different reforestation pathways were related to changes in TC, TN, and AN (**Figure 5** and **Table 5**), which is in agreement with a previous study stating that Actinobacteria are significantly positively correlated with the soil organic matter accumulation, during secondary forest succession (Guo et al., 2018). In addition, PK showed a higher relative abundance of Firmicutes compared to the other forest stands, confirming the high resistance of these bacteria to unfavorable conditions (Hartmann et al., 2014). Other specific taxa, particularly Chloroflexi and Nitrospirae, were significantly correlated with the soil pH (**Table 5**).

The clear differentiation of bacterial communities and the clustering in the heatmap (**Figure 3**) and in PCA (**Figure 4**) plots, suggests that broadleaf forests and coniferous forests harbored different bacterial communities, indicating that the soil bacterial community composition largely depends on soil physicochemical characteristic. Soil physicochemical variables therefore play the decisive role in altering bacterial communities during vegetation restoration, which is consistent with previous studies (Kuramae et al., 2010; Li et al., 2014b).

CONCLUSION

Our results indicate that different revegetation types in the Baishilazi Nature Reserve have different impacts on the soil physicochemical characteristics and the bacterial communities.

Broadleaf forests had higher TC, TN, AN, and AK values when compared to coniferous forests. In the study area, AP and AK availability are limiting factors and significantly affect bacterial diversity. Broadleaf forests, conifer-broadleaf forests and coniferous forests harbored significantly different bacterial communities. Our study contributes to the understanding of the responses of bacterial communities to different reforestation pathways in mountainous regions.

AUTHOR CONTRIBUTIONS

All authors commented on the manuscript at all stages. JD and YY conceived and designed the study. JD and WZ contributed materials and analysis tools. JD, YY, WZ, and YZ contributed to the data analysis and paper preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02874/full#supplementary-material

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Effects of Afforestation Restoration on Soil Potential N_2O Emission and Denitrifying Bacteria After Farmland Abandonment in the Chinese Loess Plateau

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Denitrification is a critical component of soil nitrogen (N) cycling, including its role in the production and loss of nitrous oxide (N_2O) from the soil system. However, restoration effects on the contribution of denitrification to soil N_2O emissions, the abundance and diversity of denitrifying bacteria, and relationships among N_2O emissions, soil properties, and denitrifying bacterial community composition remains poorly known. This is particularly true for fragile semiarid ecosystems. In order to address this knowledge gap, we utilized 42-year chronosequence of *Robinia pseudoacacia* plantations in the Chinese hilly gullied Loess Plateau. Soil potential N_2O emission rates were measured using anaerobic incubation experiments. Quantitative polymerase chain reaction (Q-PCR) and Illumina MiSeq high-throughput sequencing were used to reveal the abundance and community composition of denitrifying bacteria. In this study, the afforestation practices following farmland abandonment had a strong negative effect on soil potential N_2O emission rates during the first 33 years. However, potential N_2O emission rates steadily increased in 42 years of restoration, leading to enhanced potential risk of greenhouse gas emissions. Furthermore, active afforestation increased the abundance of denitrifying functional genes, and enhanced microbial biomass. *Actinobacteria* and *Proteobacteria* were the dominant denitrifying bacterial phyla in the 0 to 33-years old sites, while the 42-years sites were dominated by *Planctomycetes* and *Actinobacteria*, implying that the restoration performed at these sites promoted soil microbial succession. Finally, correlation analyses revealed that soil organic carbon concentrations had the strongest relationship with potential N_2O emission rates, followed by the abundance of the *nosZ* functional gene, bulk density, and the abundance of *Bradyrhizobium* and *Variovorax* across restoration stages. Taken together, our data suggest above-ground restoration of plant communities results in microbial community succession, improved soil quality, and significantly altered N_2O emissions.

Keywords: denitrification, nitrous oxide, high-throughput sequencing, afforestation restoration, denitrifying bacteria, farmland abandonment, Loess Plateau

INTRODUCTION

Nitrogen management is one of the major environmental challenges for the 21st century (Sutton et al., 2011), and the mitigation of nitrous oxide (N_2O) emissions are of particular concern due to the strong role N_2O can play in climate change. In fact, the contribution of N_2O as a greenhouse gas affecting global temperatures is third only to CO_2 and CH_4, which have global warming potentials approximately 298 and 11.9 times larger than N_2O, respectively (Domeignoz-Horta et al., 2018). N_2O is also involved in the destruction of the stratospheric ozone layer (Crutzen, 1970) and has become the dominant ozone depleting substance (Ravishankara et al., 2009). With N_2O emissions on the rise [12–16 Tg N yr-1 from 2000 to 2050 (Bouwman et al., 2013; Hu et al., 2015)], studies that explore the factors that regulate N_2O fluxes for soil, ocean, estuaries, freshwater habitats, and wastewater treatment plants are increasing (Schreiber et al., 2012; Zhang Y. et al., 2016).

Soils constitute the largest source of N_2O emissions (Bremner and Blackmer, 1979; Hu et al., 2015), especially in agricultural and forested ecosystems (Kong et al., 2010; Smethurst, 2010; Levy-Booth et al., 2014). To enable more effective mitigation to counteract the steady increase in N_2O emissions, it is necessary to better understand the mechanisms that drive N_2O fluxes in different ecosystems (Hu et al., 2015). Numerous studies have focused on estimation and simulation of N_2O fluxes (Reay et al., 2012), assessing the impact of environmental factors on N_2O efflux (e.g., Bateman and Baggs, 2005; Chen et al., 2012; Németh et al., 2014), the central role of soil microbial communities in regulating nitrogen cycle processes and N_2O emissions (e.g., Baggs, 2011; Bouwman et al., 2013; Tatti et al., 2013; Chen et al., 2015) and microbial ecology that helps determine the production and consumption of N_2O (Barberan et al., 2012; Singh et al., 2010). Specifically, denitrification is one of the major microbial pathways fueling N_2O emissions from soil (Smith et al., 2003; Shaw et al., 2006; Levy-Booth et al., 2014). Denitrification consists of sequential reductions of soluble NO_3^- and NO_2^- to the nitrogen gases NO, N_2O, and N_2 via four enzymatic complexes (Philippot et al., 2011). For instance, the first step ($NO_3^- \rightarrow NO_2^-$) is catalyzed by nitrate reductase encoded by *napA* and *narG* genes; the second step ($NO_2^- \rightarrow NO$) is catalyzed by *nirK* and *nirS* genes encoding nitrite reductase; the third step leading to N_2O production ($NO \rightarrow N_2O$) is mediated by nitrous reductase, encoded by *qnorB* gene. And the last step, the reduction of N_2O to N_2 occurs through the *nosZ* gene encoding nitrous reductase, which is the only known sink for N_2O in the biosphere (Philippot et al., 2007). Denitrification is an enzymatically catalyzed process and can be strongly influenced by environmental factors, including soil conditions (Smith et al., 2003; Čuhel et al., 2010; Hu et al., 2014; Levy-Booth et al., 2014) and vegetation features (Zhang C. et al., 2016; Liu et al., 2018). For example, several studies have reported increasing N_2O emissions with decreasing soil pH (Bergaust et al., 2010; Čuhel et al., 2010) and oxygen concentrations (Chapuis-Lardy et al., 2007; Schreiber et al., 2012). Those effects have been attributed to a specific regulation of N_2O reductase activity (Richardson et al., 2009). Changes in soil pH and oxygen concentration can be influenced by several factors depending on vegetation type, land-use history, and time since active restoration occurred (Cheng and An, 2015; Kou et al., 2016; Nadal-Romero et al., 2016). This supports the hypothesis that vegetation, edaphic properties, and microbial community are closely linked over the course of ecosystem succession following disturbance (Lozano et al., 2014; Zhang C. et al., 2016; Barber et al., 2017). However, the rules that govern these interacting associations remain relatively poorly understood (Bernhard and Kelly, 2016), and few studies focus on the correlation between N_2O emission rates and the abundances, composition, and diversity of denitrifying bacterial community during vegetation management (e.g., afforestation) of abandoned farmland in semiarid regions over the long-term.

Due to the high degree of human activities that have occurred over the long-term, such as cultivation and grazing, the Chinese Loess Plateau had been one of the most eroded regions and one of the most vulnerable areas to desertification in China (Fu et al., 2011). To address this issue, a number of programs were initiated by the Chinese government in the 20st century, for example, "Grain for Green," which was the conversion of steep cultivated land to forest and grassland. In particular, afforestation was considered as a key restoration approach in China for the past few decades (Kou et al., 2016; Hu et al., 2017) and, due to its tolerance of a wide range of soil conditions (Liu et al., 2018), the nitrogen-fixing tree *Robinia pseudoacacia* has been widely planted as a pioneer afforestation species in the Chinese Loess Plateau. To date, the effects of this restoration on a vegetation competition, soil properties, and microbial communities during afforestation have been reported (Jiao et al., 2012; Cheng and An, 2015; Kou et al., 2016; Zhang C. et al., 2016; Liu et al., 2018). However, studies of N_2O emissions and the denitrifying microbial communities that drive these emissions during afforestation are still few, especially in arid and semiarid ecosystems.

In order to address this important knowledge gap, here we simultaneously assessed multiple aspects of plant and soil in a restoration chronosequence in the Chinese Loess Plateau. The goals of our study were to (1) assess the potential rates of N_2O efflux across reforested sites of different ages, (2) determine the abundance of denitrifying functional genes and discern the diversity and composition of total bacteria and denitrifying bacteria communities, and (3) explore the relationships among potential N_2O emission rates, soil properties, and denitrifying bacterial community across a range of restoration stages.

MATERIALS AND METHODS

Study Area

This study was conducted in the Zhifanggou watershed (36°43′21″–36°46′10″N, 109°14′26″–109°15′44″E), located in the hilly gullied region of the Chinese Loess Plateau. Climate is semiarid, characterized by a mean annual temperature of 8.8°C (with a mean minimum temperature in January of −6.2°C and a mean maximum temperature in August of 37.2°C). Mean annual precipitation is 504 mm and a mean annual evaporation is 1,000 mm (Kou et al., 2016; Zhang C. et al., 2016). The

soils are mainly Calcaric Cambisols, and the study area is distributed in the transition zone between forest and steppe. Species located in the area include *Sophora viciifolia*, *Periploca sepium*, *Rosa xanthina*, *Spiraea pubescens*, *Artemisia scoparia*, *Lespedeza davurica*, *Stipa bungeana*, *Artemisia giraldii*, *Artemisia gmelinii*, and *Bothriochloa ischaemun*.

Historical farming practices at the sites removed native species, resulting in severe soil erosion and land degradation (Fu et al., 2011). The primary approach to solve these problems has been vegetation restoration (conversion of steep cultivated land to forest and grassland) (Zheng, 2006). In this context, *R. pseudoacacia* was considered as a pioneer tree for afforestation and more than 70,000 ha of land was reforested with *R. pseudoacacia* from 1950 to 2005 in the Chinese Loess Plateau (Jiao et al., 2012; Kou et al., 2016). To investigate the restoration effects of *R. pseudoacacia* plantations on N_2O emission and denitrifier communities, the method of space for time substitution was used in our study. We selected *R. pseudoacacia* plantations of different ages (14, 26, 33, and 42-years), and farmland (0-year) for the control. All the *R. pseudoacacia* plantations had been planted with a layout of plantings that were 2 m × 2 m, resulting in similar initial tree densities. The ages of the *R. pseudoacacia* plantations were obtained from interviews with local farmers and compared with records registered with the An'sai Ecological Experimental Station of Soil and Water Conservation. Farmland agricultural practices included planting potatoes (*Solanum tuberosum*) and fertilizing with 600–900 kg ha^{-1} N urea and 400–600 kg ha^{-1} phosphorus pentoxide (P_2O_5) per year. *R. pseudoacacia* plantations and farmland control sites were selected with three replicates per restoration stage. Each of the three replicates was on a different hill slope. All sites were south-facing, and soil properties of the studied sites are presented in **Table 1** and **Supplementary Table S1**.

Vegetation Survey

A vegetation survey was conducted on our study sites in July 2016 (the peak of the growing season in the area). In each site, a 10 m × 10 m quadrant was established to estimate the canopy density and the vegetation cover, and five 1 m × 1 m quadrants were randomly selected to identify all plant species and record the number of individuals of each plant species. The canopy density and vegetation cover were estimated visually by three observers. The numbers of plant species were used to calculate the Pielou evenness index and Shannon diversity index, which were used to estimate the evenness and diversity, respectively (Kou et al., 2016). The number of species was used to estimate the richness (**Supplementary Tables S2**, **S3**).

Soil Sampling

Soil samples were collected in July 2016, when the soil moisture and temperature are conducive to microorganism activity (Bateman and Baggs, 2005). In each site, we sampled to a depth of 20 cm because most of the soil nutrients and microorganisms are in this upper soil layer (Kou et al., 2016). We then took three samples in each site, with each sample made up of six evenly distributed cores (5 cm in diameter and 20 cm deep). These soils were used to determine the organic carbon content, total nitrogen content, extractable ammonium and nitrate content, pH, potential N_2O emission rates, and the abundance and composition of soil denitrifying bacteria. The litter horizons were removed before soil sampling was performed. Soil samples were kept sealed and in a cooler with ice prior to analysis. After transportation to the laboratory, the soil samples were immediately sieved (< 2 mm), and visible plant roots, stones, and debris were removed. Soils were then separated into two subsamples: one subsample was immediately stored at −80°C for DNA analysis, and the other sample was air-dried at room temperature for chemo-physical analyses. Furthermore, another three evenly distributed cores (height = 5 cm, diameter = 5.05 cm) were sampled to determine soil bulk density in each site.

Soil Analysis

N_2O Emission Experiment

Laboratory incubations were used to determine potential N_2O emissions rates. The pathways of N_2O emission are strongly influenced by soil temperature and moisture (Bateman and Baggs, 2005). In our study area, the *in situ* soil temperature and moisture in the uppermost 20 cm were consistently measured from 23 June 2016 to 26 October 2016 using soil temperature-water monitors (L-99; Hangzhou Luge Science and Technology limited company, Hangzhou, China). The average temperature was 23.34 ± 1.80°C from 8:00 to 20:00 and 19.80 ± 0.31°C from 20:00 to 8:00 (**Supplementary Figure S1**). Therefore, to assess potential N_2O emission rates we set the temperatures to range from 20.1 to 25.4°C from 8:00 to 20:00 and from 18.8 to 21.7°C from 20:00 to 8:00, which was consistent with the measured data. The soil moisture was set at a water-filled pore space (WFPS) of 60%, the wet soil environment which is conducive to N_2O emission by denitrifiers (Bateman and Baggs, 2005; Li et al., 2016). Furthermore, the denitrification process occurs in anaerobic environments (Xiong et al., 2017) and thus an anaerobic environment was created using 150-ml

TABLE 1 | Soil physicochemical properties across different restoration n stages.

Sites	Bulk density (g cm^{-3})	pH	Organic C (g kg^{-1})	Total N (g kg^{-1})	Ammonium (mg kg^{-1})	Nitrate (mg kg^{-1})
0-year	1.22 ± 0.02a	8.28 ± 0.05c	3.84 ± 0.05e	0.44 ± 0.01e	35.04 ± 0.67a	12.77 ± 0.03a
14-year	1.15 ± 0.01b	8.38 ± 0.05b	5.72 ± 0.07d	0.61 ± 0.01c	4.24 ± 0.11c	13.01 ± 0.39a
26-year	1.13 ± 0.01b	8.30 ± 0.02c	12.93 ± 0.46b	1.04 ± 0.01a	4.56 ± 0.52c	14.96 ± 1.49a
33-year	1.06 ± 0.02c	8.54 ± 0.01a	15.17 ± 0.10a	0.47 ± 0.01d	4.82 ± 0.41c	5.31 ± 0.17c
42-year	0.99 ± 0.03d	8.39 ± 0.01b	10.59 ± 0.02c	0.72 ± 0.02b	6.67 ± 0.30b	6.90 ± 0.07b

Different letters within a row indicate significant differences ($P < 0.05$) among restoration stages based on a one-way ANOVA, followed by an LSD test.

Erlenmeyer flasks with gas-tight lids fitted with a gas sampling port and incubated an incubator (RXZ-380C; Ningbo Jiangnan Instrument Plant, Ningbo, China) (Bateman and Baggs, 2005). Soil (10 g, 7 repetitions, air-dried) was weighed into each flask and soil moisture amended to achieve the target WFPS of 60%. Soils were conditioned at 60% WFPS for 7 days to initiate microbial activity and to minimize changes in soil moisture in the incubator at the start of experiment. On day 8, the flasks were sealed, evacuated and filled with high argon gas 3 times (10 min each time) to keep an anaerobic environment. Samples were then incubated for 24 h and headspace samples (approximately 40 ml) were collected to assess for N_2O gas. The N_2O gas samples were analyzed using a gas chromatograph (Agilent 7890 gas chromatograph equipped with an ECD detector, Agilent, Santa Clara, CA, United States). The potential N_2O emission rates were calculated using the following formula (Lu et al., 2011):

$$V_{N_2O} = \frac{[\rho \times C \times (V_G + V \times \alpha) \times 273]}{[W \times (273 + T)]}$$

In this: V_{N_2O} shows the potential N_2O emission rates (mg kg^{-1} h^{-1}); ρ shows the density of N_2O–N in standard state, 1.25 kg m^{-3}; C shows the gas density of N_2O (m^3 m^{-3}); V_G shows the upper effective volume of the flask (m^3); V shows the liquid volume (m^3); α shows the Bunsen correction coefficient, 0.549 in 25°C (we used the value in our study); W shows the dry soil weight (g); T is the temperature at the time gases were measured (we used a mean value in of 22°C).

Quantitative Polymerase Chain Reaction (Q-PCR) and Illumina MiSeq High-Throughput Sequencing

The soil DNA was extracted from 0.5 to 1 g soil using a D5625-01 soil DNA kit (Omega Biotek, Winooski, VT, United States) according to the manufacturer's instructions. We extracted three soil DNA samples in each restoration stages for quantitative analysis, and then mixed the three DNA samples in one samples for Illumina MiSeq high-throughput sequencing analysis.

Quantitative analysis was conducted for fragments of the bacteria 16S rRNA and six denitrifying functional genes (i.e., *narG*, *napA*, *nirK*, *nirS*, *qnorB,* and *nosZ*). Q-PCR was performed in a CFX Real-Time PCR Detection System (Bio-Rad, Bio-Rad Laboratories Inc., Hercules, CA, United States) via a three-step thermal cycling procedure, with a 20 μL reaction mixture consisting of 10 μL of SYBR Green I PCR master mix (Applied Biosystems, Foster City, CA, United States), 1 μL of the DNA template (sample DNA or plasmid DNA for standard curves), 1 μL of forward primers, 1 μL of reverse primers, and 7 μL of sterile water (Millipore, Burlington, MA, United States). The protocol and parameter for each target gene are presented in **Supplementary Table S4**. The R-squared value for each standard curve exceeded 0.99, which indicated linear relationships across the ranges used in this study.

Successful PCR amplification was verified by 2% agarose gel electrophoresis. The triplicate amplicons were pooled and purified by gel extraction and quantified using a Quant-iT PicoGreen dsDNA Assay kit with a microplate reader (FLx800, BioTek Instruments, Inc., Winooski, VT, United States). The purified PCR amplicons were then mixed at equimolar ratios for sequencing analysis. Sequencing was conducted on the Illumina MiSeq platform using a TruSeq Nano DNA LT Library Prep Kit (Illumina Corporation, San Diego, CA, United States). Before sequencing, quality inspection was conducted on the Agilent Bioanalyzer using the Agilent High Sensitivity DNA Kit, and the sample was checked to have only a single peak. Then the sample was quantified using a Quant-iT PicoGreen dsDNA Assay Kit on a Promega QuantiFluor, and more than 2 nM was quantified. The sample was diluted and mixed with NaOH to denature the DNA into single strands for sequencing. Finally, the sample was analyzed using a MiSeq sequencer for paired-end sequencing of 2 × 300 bp using MiSeq Reagent Kit V3 (600 cycles).

Soil Physicochemical Analysis

The soil organic carbon and total nitrogen concentrations were determined using the oil bath-$K_2Cr_2O_7$ titration method and the Kjeldahl method, respectively. Extractable ammonium and nitrate concentrations were determined following extraction of fresh soil with 1 mol L^{-1} KCl and then using a colorimetric method on an Alpkem Autoanalyzer (AA3 Auto Analyzer 3; German SEAL; German). The soil pH was determined using an automatic titrator (PHSJ-4F pH meter, Shanghai Electric Instrument Science Instruments Ltd., Shanghai, China) in 1:2.5 soil: water suspensions. The soil bulk density was determined gravimetrically in the laboratory.

Statistical Analysis

To estimate the effects of *R. pseudoacacia* plantations on soil properties, potential N_2O emission rates and denitrifying bacterial community, we analyzed the differences in soil bulk density, pH, organic carbon content, extractable ammonium and nitrate content, potential N_2O emission rates, and the abundance of total bacteria and denitrifying functional genes (*napA*, *narG*, *nirK*, *nirS*, *qnorB*, and *nosZ*) with restoration age using general linear models. *Post hoc* comparisons with restoration stages were also performed using least significant difference (LSD) tests.

Principal Component Analysis (PCA) was used to analyze the similarity of total bacterial and denitrifying bacterial communities during restoration stages. The richness, evenness, and diversity of total bacterial communities were quantified by using Chao 1, Simpson and Shannon diversity indices, and the features of vegetation communities were quantified by using species richness, Pielou evenness and Shannon diversity indices. To quantify the correlations of potential N_2O emission rates and denitrifying functional genes, we used a normal distribution of the log of abundance of denitrifying functional genes. The correlations among soil properties, potential N_2O emission rates and denitrifying functional genes were quantified using Pearson correlation analyses before engaging in further analyses. Then, based on the results of Pearson correlation analyses, a multiple regression analysis was performed to determine the multiple linear regression equation relating potential N_2O emission rates and denitrifying functional genes. Likewise, the structural equation model (SEM) was applied to investigate the direct and indirect effects of the indices chosen by multiple regression analysis on potential N_2O emission rates ($P > 0.05$). Finally, PCA and Pearson correlation analyses were also used to determine the

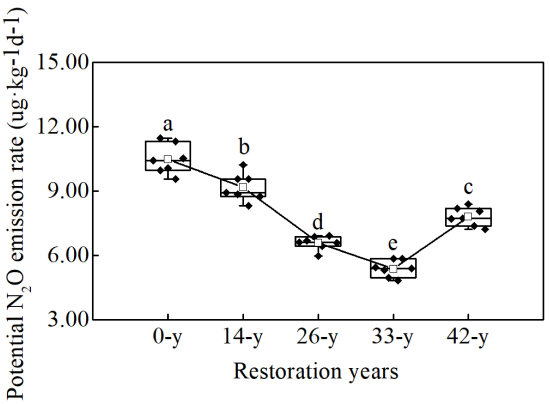

FIGURE 1 | Different letters indicate significant differences ($P < 0.05$) among afforestation restoration ages based on a general linear model followed by an LSD *post hoc* test. The box plot shows the following information from top to bottom: maximum, upper quartile, median, lower quartile, and minimum. The white square shows the average value and the black line shows the trend. The black rhombus shows potential N_2O emission rate data.

ordination of potential N_2O emission rates, soil properties and denitrifying bacterial community (the three major denitrifiers in genes level of each denitrifying functional genes).

All statistical analyses were performed using SPSS (IBM SPSS Statistics 20.0; International Business Machines Corporation, Armonk, NY, United States), and all graphs were made using Origin (OriginPro 2016; OriginLab Corporation, Northampton, MA, United States).

RESULTS

Potential N_2O Emission Rates on Denitrification After a Short Incubation

Potential N_2O emission rates varied significantly with restoration ages and ranged between 5.38 and 10.50 $\mu g\ kg^{-1}\ d^{-1}$ (**Figure 1**; $df = 4$; $F = 107.313$; $P < 0.000$). Rates were the highest at the 0-year sites, and decreased as the restoration ages increased from the 14-year to 33-year sites, and then increased between the 33-year and 42-year sites.

Abundance of Denitrifying Functional Genes

The absolute abundances of total bacteria and denitrifying functional genes, except *narG* genes, varied significantly with restoration stage (**Figure 2** and **Supplementary Table S5**). The absolute abundances of total bacteria ranged from 1.93×10^6 to 6.82×10^6 copies g^{-1}, and initially decreased compared with the 0-year sites and subsequently increased at the 14-year − 42-year sites. The *napA* and *narG* genes exhibited different trends. The absolute abundances of *napA* and *narG* genes varied $2.51 \times 10^4 - 8.11 \times 10^5$ copies g^{-1} and $5.35 \times 10^4 - 1.70 \times 10^5$ copies g^{-1}, respectively. Compared with *narG* gene, which showed no obvious trend across the sites, the *napA* gene initially decreased between 0-year and 14-year sites, and subsequently increased at the 14-year − 33-year sites, and then declined between the 33-year and 42-year sites. The absolute abundances of *nirK*, *qnorB* and *nosZ* genes appeared to be more abundant at the 42-year sites than at those of other sites and increased from the 0-year to 42-year sites. In contrast, the *nirS* gene abundances showed no obvious trend across sites. The *nirK*, *nirS*, *qnorB,* and *nosZ* genes varied $2.8 \times 10^3 - 7.59 \times 10^5$, $2.58 \times 10^3 - 9.77 \times 10^3$, $1.90 \times 10^4 - 9.61 \times 10^5$, and $1.94 \times 10^3 - 4.20 \times 10^5$ copies g^{-1}, respectively. Furthermore, the absolute abundances of *qnorB* were more than those of *nosZ* in all sites.

Diversity and Composition of Total Bacterial and Denitrifying Bacterial Communities

After quality trimming and chimera removal, the total bacterial and denitrifying bacterial sequences were assayed using Illumina MiSeq high-throughput sequencing (**Supplementary Table S6**). The variations of total bacteria and denitrifying bacteria composition with restoration stages were identified by PCA (**Supplementary Figures S2**, **S3**). The richness (shown by Chao 1 index) and diversity (shown by Shannon index) of the total bacterial communities revealed the similar trend, i.e., an initial increase followed by a decrease (except 42-year in richness), with the highest values at the 26-year sites (**Supplementary Table S7**). However, the evenness (shown by Simpson index) of the total bacterial communities showed no difference among restoration stages (**Supplementary Table S7**). Furthermore, the richness, evenness and diversity of denitrifying bacterial communities showed no obvious trends among restoration stages (**Supplementary Table S7**). The total bacterial community was dominated by *Actinobacteria* (23.1% on average), followed by *Proteobacteria* (21.7%), *Acidobacteria* (17.1%), *Planctomycetes* (12.8%), *Gemmatimonadetes* (7.2%), and *Chloroflexi* (7.2%) (with relative abundances of more than 5%; **Supplementary Table S8**). *Proteobacteria* was the major phylum in total bacterial communities at 0-year to 33-year sites. However, *Acidobacteria* and *Planctomycetes* were dominant at 42-year sites (**Supplementary Table S9**). Moreover, the denitrifying bacteria encoded by *napA*, *narG*, *nirS,* and *nosZ* genes were dominated by *Proteobacteria* in the phyla level, and the percentages of *Proteobacteria* in denitrifying bacteria communities were 47.8, 56.0, 5.8, and 89.8%, respectively. *Actinobacteria* and *Proteobacteria* were the major phyla of denitrifying bacteria encoded by *nirK* (16.8%, 15.7%) and *qnorB* (8.9%, 12.8%), but the denitrifying bacterial communities were still somewhat unclear.

Soil Properties, Abundance and Diversity of Denitrifying Bacterial Communities in Relation to the Potential N_2O Emission Rates

Some soil properties and soil microbial abundances were correlated with potential N_2O emission rates. Soil pH and organic carbon content were negatively related to potential N_2O emission rates (**Figure 3**; $P < 0.01$), while a positive relationship was found between extractable ammonium content and potential

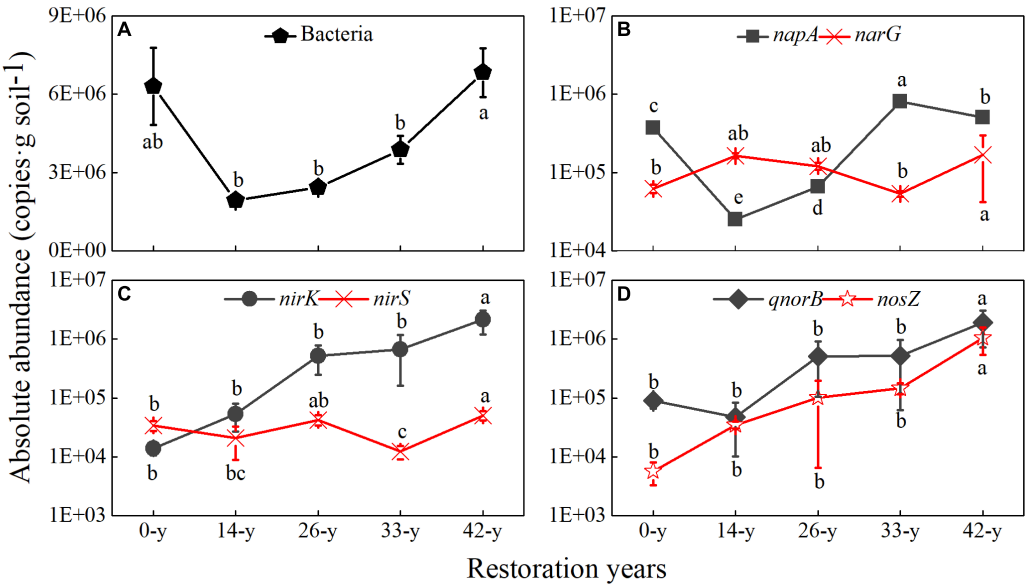

FIGURE 2 | The absolute abundances of total bacteria and denitrifying functional genes among afforestation restoration of abandoned farmland. **(A)** Bacteria; **(B)** *napA*, *narG*; **(C)** *nirK*, *nirS*; **(D)** *qnorB*, *nosZ*. The absolute abundances (copies g soil^{-1}) are shown on a log 10 scale (Y-axis). The standard deviations of the three replicates are indicated by error bars. Non-visible error bars indicate that the standard deviations are smaller than the marker size. Different letters indicate significant differences ($P < 0.05$) among restoration ages based on a general linear model followed by an LSD *post hoc* test.

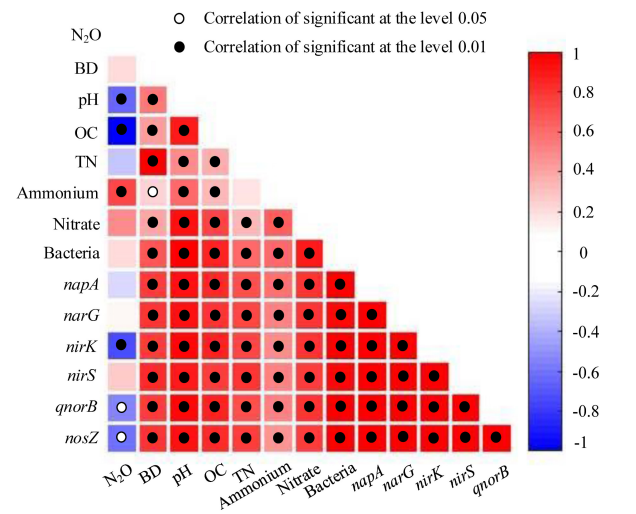

FIGURE 3 | The relationships between potential N_2O emission rates, denitrifying functional gene abundances, and soil properties among restoration stages. The strength of the linear relationship between two variables is described by the Pearson Correlation Coefficient and showed in different colors. *P*-values are given through black circles ($P < 0.01$) and unfilled circles ($P < 0.05$). N_2O = N_2O emission rates (μg kg^{-1} d^{-1}); BD = bulk density (g cm^{-1}); OC = organic carbon (g kg^{-1}); TN = total nitrogen (g kg^{-1}); extractable ammonium and nitrate (mg kg^{-1}); bacteria, *napA, narG, nirK, nirS, qnorB, and nosZ* show the abundances of denitrifying functional genes.

N_2O emission rates (**Figure 3**; $P < 0.01$). Furthermore, the abundances of denitrifying bacteria encoded by *nirK*, *qnorB*, and *nosZ* genes were negatively related to potential N_2O emission rates (**Figure 3**; $P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively). Multiple regression analysis was used to determine the relative contribution of denitrifying functional genes to the variation of potential N_2O emission rates. As a result, the ratios of *qnorB*/*nirK*, *nosZ*/*nirS*, (*nirK*+ *nirS*)/(*napA*+ *narG*), and (*napA*+ *narG*)/*bacteria* were the four major predictors, which could explain 53% of the variation in potential N_2O emission rates ($P = 0.018$). The ratios of *qnorB*/*nirK*, *nosZ*/*nirS*, and (*napA*+ *narG*)/*bacteria* were significantly explained 34, 51, and 35% of the variation in potential N_2O accumulation rates, respectively (**Figures 4A,B,D**). However, the ratios of (*nirK*+ *nirS*)/(*napA*+ *narG*) had no significant influence on potential N_2O emission rates (**Figure 4C**). Likewise, the *qnorB*/*nirK*, *nosZ*/*nirS*, and (*nirK*+ *nirS*)/(*napA*+ *narG*) ratios showed significant differences between the 0-year and 14-year − 42-year sites (**Supplementary Figures S4a–c**), and (*napA*+ *narG*)/*bacteria* ratios were significantly different between the sites with the lowest (0-year) and the highest (33-year) potential N_2O emission rates (**Supplementary Figure S4d**). Then, we used a SEM to examine the direct and indirect effects of the ratios of denitrifying functional genes on the potential emission rates of N_2O (**Figure 5**). The *qnorB*/*nirK*, *nosZ*/*nirS*, (*nirK*+ *nirS*)/(*napA*+ *narG*), and (*napA*+ *narG*)/*bacteria* ratios had direct different influences (β = 0.17,−0.25, −0.27, and −0.43) on the variation of the potential N_2O emission rates. Furthermore, the influences of (*nirK*+ *nirS*)/(*napA*+ *narG*) on the variation of the potential emission rates of N_2O were mediated through *qnorB*/*nirK* (β = −0.11) and *nosZ*/*nirS* (β = −0.12), and the (*napA*+ *narG*)/*bacteria* ratios affected the potential N_2O emission rates indirectly through *qnorB*/*nirK* (β = −0.04) and *nosZ*/*nirS* (β = −0.15). Overall, the SEM had a $P = 0.086$

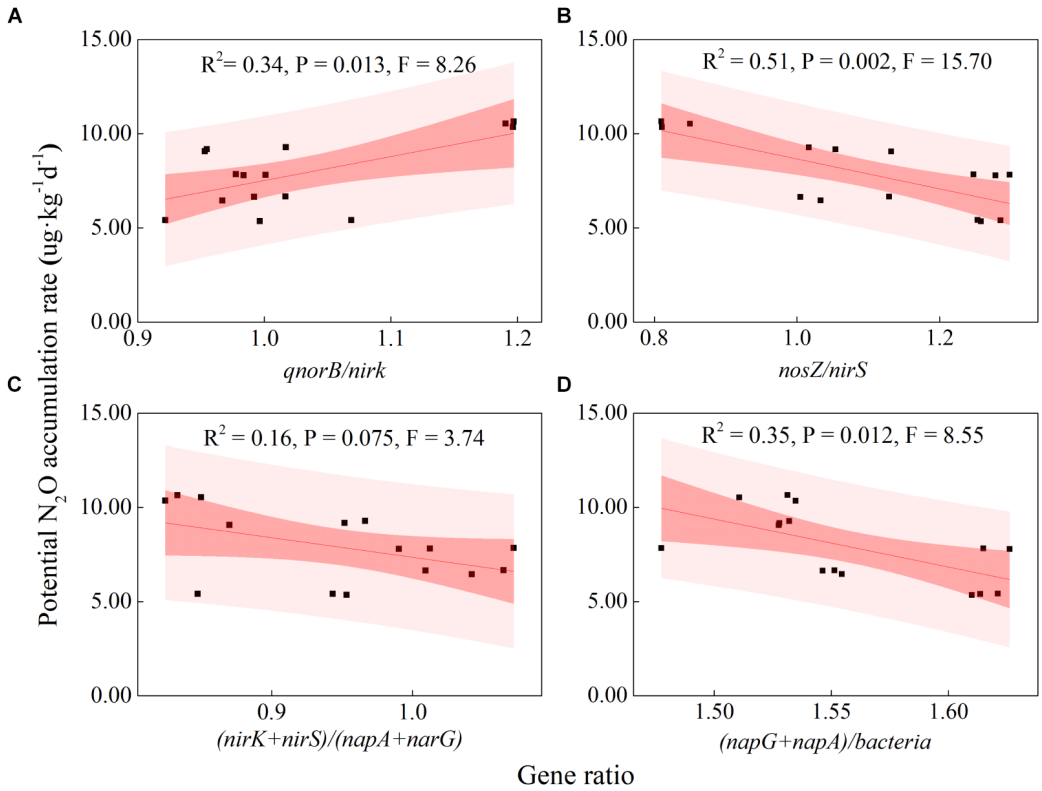

FIGURE 4 | Relationships between the potential N_2O emission rates and denitrifying functional gene ratios across restoration stages. **(A)** *qnorB/nirK* (NO transformation and N_2O production); **(B)** *nosZ/nirS* (NO to N_2 transformation); **(C)** (*nirK+nirS)/* (*napA+narG*) (nitrite consumption); **(D)** (*napA+narG)/bacteria* (nitrite production). The bacteria represent the abundance of the denitrifier bacterial 16S RNA gene. The black squares indicate the potential N_2O emission rates and the ratios of denitrifying functional genes. The red fitted lines are from ordinary least squares regression. The dark and shaded areas show the 95% confidence interval and the prediction band of the fit, respectively.

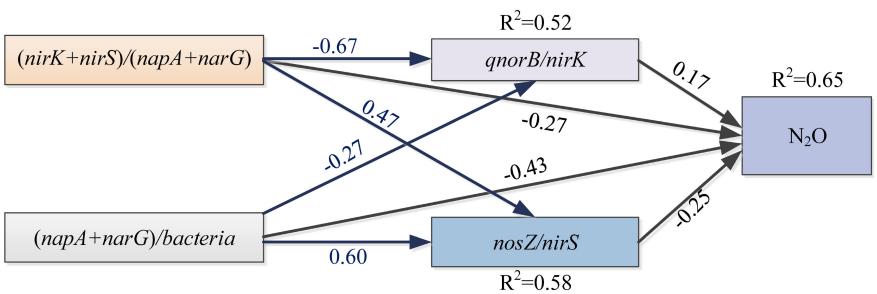

FIGURE 5 | The structural equation model (SEM) describing the direct and indirect contributions of gene fragments on the potential N_2O emission rates. Single-pointed arrows indicate causal paths. The black arrows represent the direct influences, and the blue arrows show indirect influences. R^2 values are shown for dependent variables. A non-significant *P*-value ($P = 0.086$) for the Chi-squared statistic indicate that there was no significant difference between the covariance pattern predicted by the SEM and that from the observed covariance, indicating a good fit of the data.

($P > 0.05$), which indicated that the SEM was applicable and explained 65% of the total variance influencing the potential N_2O emission rates.

Ordination of samples by PCA based on potential N_2O emission rates, soil properties, abundances and composition of denitrifying bacterial community showed a clear separation of restoration stages along the first axis, with the first two axes explaining 92.37% of the total variance (**Figure 6**). The ordination stressed the trends observed in **Supplementary Table S11**, showing a strong correlation of some soil properties, denitrifying bacterial community with potential N_2O emission rates. Of particular interest, we found positive correlations among extractable ammonium content, the abundance of *nosZ* and *napA* and potential N_2O emission rates ($P < 0.01$), while negative correlations among soil pH, organic carbon content, the abundances of *narG*, *nirS* and *qnorB*, *Bradyrhizobium* (*nirK*),

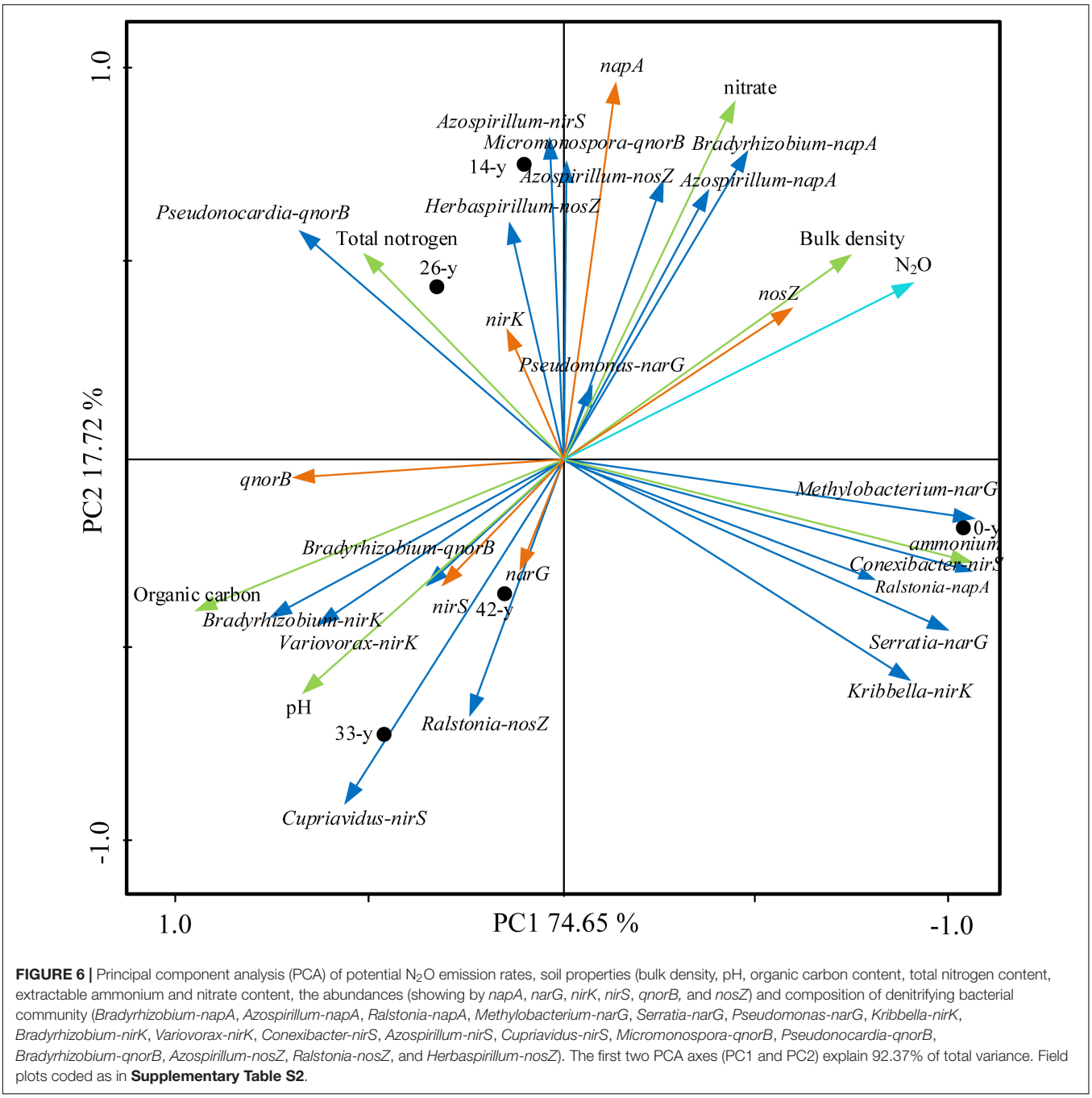

FIGURE 6 | Principal component analysis (PCA) of potential N_2O emission rates, soil properties (bulk density, pH, organic carbon content, total nitrogen content, extractable ammonium and nitrate content, the abundances (showing by *napA*, *narG*, *nirK*, *nirS*, *qnorB,* and *nosZ*) and composition of denitrifying bacterial community (*Bradyrhizobium-napA*, *Azospirillum-napA*, *Ralstonia-napA*, *Methylobacterium-narG*, *Serratia-narG*, *Pseudomonas-narG*, *Kribbella-nirK*, *Bradyrhizobium-nirK*, *Variovorax-nirK*, *Conexibacter-nirS*, *Azospirillum-nirS*, *Cupriavidus-nirS*, *Micromonospora-qnorB*, *Pseudonocardia-qnorB*, *Bradyrhizobium-qnorB*, *Azospirillum-nosZ*, *Ralstonia-nosZ*, and *Herbaspirillum-nosZ*). The first two PCA axes (PC1 and PC2) explain 92.37% of total variance. Field plots coded as in **Supplementary Table S2**.

Variovorax (*nirK*), *Bradyrhizobium* (*qnorB*), and potential N_2O emission rates were found ($P < 0.01$).

DISCUSSION

Potential Rates of N_2O Emission Across a Restoration Chronosequence

The potential N_2O emission rates through denitrification process varied significantly with restoration ages (**Figure 1**), suggesting that afforestation restoration does indeed alter N_2O emissions (Liu et al., 2018). Compared with the 14-year − 42-year sites in this study, the 0-year sites had by far the highest potential N_2O emission rates, indicating that farmland may have been the larger source of N_2O (Domeignoz-Horta et al., 2018). Unfortunately we cannot scale these potential rates to annual values; nevertheless, elevated rates in actively managed farmlands would not be a surprise. Agricultural practices, such as tillage and fertilization, can increase the soil organic carbon and extractable ammonium contents consequently alter N_2O emissions (Suleiman et al., 2013). Furthermore, afforestation restoration rapidly created a specific understory microhabitat due to canopy density, showing

a decreasing trend of soil potential N_2O emission (except at the 42-year sites; **Figure 1**). This supports the hypothesis that restoration in soils with a higher pH, such as those common in arid environments, may reduce N_2O emission (Gundersen et al., 2012). Šimek et al. (2002) also reported that $N_2O/(N_2O + N_2)$ ratios decreased with increasing soil pH values, consistent with our finding that soil potential N_2O emission rates decreased and pH increased at 14-year − 42-year sites (**Figure 1** and **Table 1**). The 42-year sites had a higher potential N_2O emission rates than did the 33-year sites (**Figure 1**), following a decrease in soil pH and organic carbon content, which translated into a decline in ecological function in the oldest sites. This phenomenon was consistent with the finding by Kou et al. (2016), who found that the soil nutrients and the density and cover of *R. pseudoacacia* plantations decreased after 30 years of afforestation in the Chinese Loess Plateau. Understory vegetation communities can dramatically alter soil microbial process through root exudation and litter inputs (Singh et al., 2004). In this study, we found that the potential fluxes of N_2O were negatively correlated with trends in understory vegetation features, such as richness, evenness and diversity (**Supplementary Table S3**), and that soil properties, such as soil pH and organic carbon content (**Table 1**), were also negatively correlated with potential N_2O emission rates, perhaps through their interactions with changes in understory vegetation (Čuhel et al., 2010; Levy-Booth et al., 2014). Therefore, N_2O emission can be influenced by understory vegetation, soil pH and organic carbon content over the course afforestation and the role of denitrifying bacteria may also be critical.

The Abundance Dynamic of Denitrifying Functional Genes During Afforestation Restoration

The absolute abundances of total bacteria and the denitrifying functional genes of *nirK*, *qnorB,* and *nosZ* increased across age since restoration plantings (**Figure 2**), suggesting that afforestation restoration can increase microbial biomass (Lozano et al., 2014; Barber et al., 2017). However, the absolute abundances of total bacteria and the *qnorB* gene in the 0-year sites were higher than in the 14-year sites (**Figure 2**), which was inconsistent with a previous study showing that frequent human intervention can reduce the abundance of bacteria (Lin et al., 2012). Added nutrient resources in farmlands may support a more plentiful microbial community (Zhang C. et al., 2016), an idea which was supported by the Shannon diversity of the total bacterial community (**Supplementary Table S5**). Organic carbon content was positively correlated with the abundance of *napA* and *narG* genes (Bru et al., 2011). In this study, the absolute abundances of *napA* and *narG* genes showed different changes with restoration stages (**Figure 2B**), which may be explained by the special roles of *napA* and *narG* genes (Chèneby et al., 2009). The *napA* genes had a similar change with soil pH and organic carbon content (**Figure 2B** and **Table 1**), suggesting that the *napA* gene is more sensitive than *narG* gene on the variations in soil nutrients, which is contrary to the finding by Chèneby et al. (2009). The *narG* gene showed no obvious change with restoration age, inconsistent with the finding that the *narG* gene is easily promoted by increases in soil nutrients (Tang et al., 2016). This discrepancy might be due to differences in the environmental conditions in the two studies. The Chinese Loess Plateau is located in a semiarid region, whereas the *nirS* gene is likely more adapted to waterlogged soils than the *nirK* gene (Azziz et al., 2017). The *nirS* genes also showed no obvious changes in absolute abundance, while the *nirK* gene increased among restoration ages (**Figure 2C**). Likewise, the *qnorB* genes had higher abundances than *nosZ* genes in all sites (**Figure 2D**), which offered a molecular-level explanation for the viewpoint that soil acts as a source for N_2O, especially in farmlands and forests (Bremner and Blackmer, 1979; Kong et al., 2010; Levy-Booth et al., 2014; Hu et al., 2015). Briefly, denitrifying functional genes have different roles in denitrification and have close relationships with each other (**Figure 3**), which can change the products of denitrification, especially N_2O emission (Hu et al., 2015).

Variation in Denitrifying Bacterial Community Over the Course of Afforestation Restoration

PCA of the compositions and structures of the total bacterial and denitrifying bacterial communities identified large shifts in the distributions of microbial communities during restoration stages (**Supplementary Figures S2**, **S3**), which was consistent with the results of Lozano et al. (2014) and Zhang C. et al. (2016), who found obvious variations during the succession of bacterial communities in abandoned farmland. *Actinobacteria* and *Proteobacteria* were the most two abundant phyla of the total bacterial communities regardless of the restoration stages (except 42-year sites; **Supplementary Table S8**). Soil nutrients play an important role in the total bacterial community composition for *Actinobacteria* can be widely distributed in both terrestrial and aquatic ecosystems (Ventura et al., 2007) and *Proteobacteria* is partial to nutrient-rich soils (Liu et al., 2018). In this study, the total bacterial communities transitioned from *Actinobacteria*-dominant (0-year) to *Proteobacteria*-dominant (14 and 26-year) and then returned to *Actinobacteria*-dominant (33-year) (**Supplementary Table S9**), consistent with the findings by Desnues et al. (2007) and Liu et al. (2018). Taken together, these results indicate that total bacterial community composition was more influenced by soil total nitrogen and extractable nitrate contents. Furthermore, we found that the highest richness, evenness, and diversity of the understory vegetation community were in the 33-year sites, while the highest richness and diversity of total bacterial community were in the 26-year sites (**Supplementary Tables S3**, **S7**), suggesting an incongruous process which understory vegetation succession followed soil bacteria succession. These patterns are inconsistent with Lozano et al. (2014), who found that microbial succession followed plant succession in natural recovery processes. The mechanism underlying this difference is unclear, and the effects of artificial restoration process, especially for *R. pseudoacacia* plantations, on soil ecosystem may aid in understanding. Specifically, the total bacterial community composition in 42-y sites was dominated by *Planctomycetes*

(22.3%), followed by *Actinobacteria* (19.4%), *Acidobacteria* (18.7%), and *Proteobacteria* (14.4%) (**Supplementary Table S9**), indicating a unique distribution of total bacterial community composition was found in our study. The mechanism underlying this control is unclear, but vegetation community in the herbaceous layer and the limitation by soil nutrients are likely playing a role.

Furthermore, *Actinobacteria* and *Proteobacteria* were the major phyla of denitrifying bacteria encoded by *nirK* and *qnorB* genes, which indicated that N_2O products can occur in most environments (Liu et al., 2018) Although other soil processes controlled by microbes, such as nitrification, can also contribute to N_2O efflux, denitrification is often a dominant pathway. *Proteobacteria* was the major phylum of denitrifying bacteria encoded by *napA*, *narG*, *nirS,* and *nosZ* regardless of restoration stages (**Supplementary Table S3**), suggesting that the dynamics of N_2O emission is influenced by the changes in soil nutrients (Azziz et al., 2017; Tatti et al., 2017), which is in agreement with the relationship analysis indicating that denitrifying functional genes and soil properties were closely related (**Figure 3**). However, the richness, evenness, and diversity of denitrifying bacterial community showed no obvious trends across the chronosequence (**Supplementary Table S7**). The mechanism underlying this is unclear.

Relationships Among Potential N_2O Emission Rates, Soil Properties and Denitrifying Bacteria

Taken together, the correlation analyses and the PCA (**Figure 6**) suggested that the variation in potential N_2O emission rates were partially attributable to changes in the soil properties and in the abundance and composition of denitrifying bacteria. In addition, the restoration occurring from afforestation studied here changed the canopy density and altered the understory community (Liu et al., 2018). Such changes to the vegetation can affect soil properties through changes to root exudation and litterfall (Singh et al., 2004).

Response of Potential N_2O Emission Rates to Soil Properties

Afforestation via planting trees can rapidly create an understory microhabitat from increases canopy density, which can, in turn, alter soil properties and N_2O fluxes (Liu et al., 2018). In our study, we found that soil extractable ammonium content, pH, organic carbon content, and bulk density all had close correlations with potential N_2O emission rates (**Figures 3**, **6**). Specifically, we found a strong positive correlation between extractable ammonium content and potential N_2O emission rates (**Figure 3**). Our study area was farmlands that were fertilized and agriculturally managed, especially with fertilizer addition, which can supply ample ammonium for denitrification, and N_2O emission (Hu et al., 2015). Furthermore, soil pH had a negative correlation with potential N_2O emission rates (**Figures 3**, **6** and **Supplementary Table S11**), consistent with the findings that higher soil pH could reduce N_2O efflux (Wrage et al., 2001; Hu et al., 2015). Our results indicated that the denitrifying bacterial community encoded by *nosZ* genes preferred low pH in soils, which is seen in the negative relationships between soil pH and the Shannon diversity of denitrifying bacterial community encoded by *nosZ* genes (**Figure 6**). Soil organic carbon serves as the energy supply in heterotrophic microbial pathways, yet soil carbon content was negatively correlated with potential N_2O emission rates (**Figures 3**, **6** and **Supplementary Table S11**). These patterns may be explained by the denitrifying bacterial community in the phyla level. We found that denitrifying bacterial community encoded by *qnorB* genes, which can produce N_2O, belongs to *Actinobacteria* and *Proteobacteria*, leading to a large number of N_2O emission in soils (Liu et al., 2018). However, the *nosZ* genes, acting as the only microbial pathway for N_2O consumption, were made up more by *Proteobacteria*, meaning that eutrophic environments can had lower N_2O emissions (Hu et al., 2015). Soil bulk density, which can be related to soil aeration, was positively correlation with potential N_2O emission rates (**Figure 6**), suggesting that soil compaction could represent a significant control over N_2O emission rates and losses to the atmosphere (Hu et al., 2015). Therefore, fertilizer addition and soil compaction (i.e., higher soil bulk density) may increase N_2O emissions, whereas higher soil pH and organic carbon content can reduce N_2O emissions.

Response of Potential N_2O Emission Rates to Denitrifying Functional Genes

The multiple regression analysis, SEM and PCA showed that total bacteria and denitrifying functional genes were crucial in mediating potential N_2O emission rates (**Supplementary Table S10** and **Figure 5**), which could reflect and integrate a part of the fluctuations in bio-ecological processes (Hu et al., 2015). More specifically, the *qnorB*/*nirK* denotes the level of NO transformation and N_2O production as the *qnorB* genes are directly involved in N_2O production and NO consumption, while *nirK* genes perform NO production. The significant positive correlation between *qnorB*/*nirK* and potential N_2O emission rates indicated the importance of the reaction substrate supply in N_2O emissions. The *nirK* and *nosZ* genes are directly involved in NO production and N_2 production processes, and *nosZ*/*nirS* ratio reflects the level of NO to N_2 transformation. The *nosZ*/*nirS* had a negative correlation with potential N_2O emission rates and because N_2 production is the only pathway for N_2O consumption, these data suggested that *nosZ* genes play an important role in controlling N_2O emissions for this system. The *napA* and *narG* genes are involved in nitrite consumption level in denitrification process. The (*napA*+ *narG*)/*bacteria* and (*nirK*+ *nirS*)/(*napA*+ *narG*) were negatively associated with potential N_2O emission rates, likely because they reflect nitrite transformation in the denitrification process. (*napA*+ *narG*)/*bacteria* reflects the nitrite production level and (*nirK*+ *nirS*)/(*napA*+ *narG*) was directly involved in nitrite consumption. This is in agreement with the finding of Levy-Booth et al. (2014), who found that nitrite reduction had a major influence on N_2O emission rates. Furthermore, the indirect influence of denitrifying functional genes on potential N_2O emission rates reflects the close links among different microbial

processes of denitrification. The ratios of (*nirK*+ *nirS*)/(*napA*+ *narG*) and (*napA*+ *narG*)/*bacteria* had indirect influences on potential N_2O emission rates through *qnorB*/*nirK*, suggesting that nitrite transformation showed an indirect effect on potential N_2O emission rates by influencing the N_2O production. We also found that the ratios of (*nirK*+ *nirS*)/(*napA*+ *narG*) and (*napA*+ *narG*)/*bacteria* had indirect influences on potential N_2O emission rates through *nosZ*/*nirS*, suggesting that nitrite transformation showed an indirect effect on N_2O emission rates by influencing the N_2O consumption. Notably, we also found that the abundances of denitrifying bacteria encoded by *nosZ* were positively correlated with potential N_2O emission rates, while *qnorB* was negatively correlated with potential N_2O emission rates. The *qnorB*/*nosZ* ratios were positively correlated with potential N_2O emission rates (r = 0.41), indicating that denitrifying functional genes have close relationships with each other (**Figure 3**) and N_2O emissions can be regulated by the abundance of denitrifying functional genes (Hu et al., 2015).

Therefore, nitrite transformation is a key step in denitrification process and N_2O emission (Domeignoz-Horta et al., 2018). However, the relationships between denitrifying functional genes and potential N_2O emission rates were not sufficient to fully explain patterns in N_2O emissions, and the underlying mechanisms and the response of denitrifying bacterial community on N_2O emission rates is worthy of further study.

Response of Potential N_2O Emission Rates to Denitrifying Bacterial Community

N_2O emission is driven by soil microorganisms, especially denitrifiers in anaerobic environments (Hu et al., 2015). In our study, we found *Bradyrhizobium* (*nirK*) and *Variovorax* (*nirK*) were negatively correlated with potential N_2O emission rates ($P < 0.01$) (**Figure 6** and **Supplementary Table S11**), suggesting that the denitrifying functional genes encoded by *nirK*, which produce NO, were crucial to N_2O emission. These data point to nitrite transformation as a key step in determining overall N_2O emission rates, at least under the conditions simulated here (found in section "Response of Potential N_2O Emission Rates to Denitrifying Functional Genes"). Specifically, a negative correlation between *Bradyrhizobium* (*qnorB*) and potential N_2O emission rates was found, inconsistent with the finding that denitrifiers encoded by *qnorB* can produce N_2O. In our study, soil organic carbon content had the strongest relationship with potential N_2O emission rates, followed by the abundance of *nosZ*, soil bulk density, and *Bradyrhizobium* and *Variovorax* abundance, consistent with the findings by Levy-Booth et al. (2014), Lozano et al. (2014), Zhang C. et al. (2016), and Domeignoz-Horta et al. (2018), which indicated that the changes of soil properties can alter the microorganism composition and then influence the products of soil microbial pathway. Taken together, our study and others emphasize the importance of denitrifying bacterial communities in responding to environmental changes and modulating denitrification process (Bernhard and Kelly, 2016).

CONCLUSION

Afforestation restoration rapidly created a special understory microhabitat that acted as driving force to alter N_2O emission and the diversities and compositions of vegetation and soil denitrifying bacteria communities. Compared with farmlands, afforestation restoration can increase soil nutrients (soil organic carbon content) and improve soil physical characteristics (soil bulk density), followed by increasing the abundances of denitrifying functional genes and altering the structure of denitrifying bacterial communities (*Actinobacteria*-dominant to *Proteobacteria*-dominant). These changes can interact to alter the dynamics of potential N_2O emission rates. Specifically, correlation analysis indicated that NO transformation was the key step in determining potential N_2O emissions in our study. However, some denitrifying bacteria were remaining unknown, and these organisms could be targeted for future soil nitrogen-focused studies in semiarid ecosystems.

AUTHOR CONTRIBUTIONS

ND made the text, figures, and tables (including the **Supplementary Material**). HW, SH, and JJ contributed to experimental design method, manuscript frame, and manuscript modification.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00262/full#supplementary-material

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Belowground Consequences of Intracontinental Range-Expanding Plants and Related Natives in Novel Environments

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Introduced exotic plant species that originate from other continents are known to alter soil microbial community composition and nutrient cycling. Plant species that expand range to higher latitudes and altitudes as a consequence of current climate warming might as well affect the composition and functioning of native soil communities in their new range. However, the functional consequences of plant origin have been poorly studied in the case of plant range shifts. Here, we determined rhizosphere bacterial communities of four intracontinental range-expanding plant species in comparison with their four congeneric natives grown in soils collected from underneath those plant species in the field and in soils that are novel to them. We show that, when controlling for both species relatedness and soil characteristics, range-expanding plant species in higher latitude ecosystems will influence soil bacterial community composition and nutrient cycling in a manner similar to congeneric related native species. Our results highlight the importance to include phylogenetically controlled comparisons to disentangle the effect of origin from the effect of contrasting plant traits in the context of exotic plant species.

Keywords: ecological novelty, habitat novelty, phylogenetic distance, plant range expansion, rhizosphere community assembly

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INTRODUCTION

Current climate change is reshaping natural communities by enabling species range expansions to higher altitudes and latitudes (Parmesan and Yohe, 2003; Chen et al., 2011; Pauli et al., 2012). Whereas patterns for plants and animals have been relatively well explored, consequences of these range shifts for cryptic species assemblages, such as soil biota, are poorly known (Van Nuland et al., 2017). During range expansion, specific interactions between plants and their co-evolved soil organisms will become disrupted when they have different dispersal capacities (Berg et al., 2010). In the new habitat, range-expanding plant species may benefit from the absence of specialized pathogens (Engelkes et al., 2008; Van Grunsven et al., 2010b; Morriën et al., 2013; Dostálek et al., 2016). Such enemy release has been documented for exotic plant species that have been introduced from other continents (Mitchell and Power, 2003; Reinhart et al., 2010), and have been proposed to contribute to increased performance of exotics over co-occurring natives

(Keane and Crawley, 2002; Blumenthal et al., 2009). However, the soil community contains not only pathogens, but also numerous other symbionts and saprophytic microbes that are involved in a variety of ecosystem processes, such as decomposition and nutrient cycling. A key question that is still not well addressed for range-expanding plant species is how multifunctional soil communities, also including saprophytic microorganisms, may respond to novel host plants with which they lack a co-evolutionary history (Van der Putten, 2012; Evans et al., 2016).

In the rhizosphere, bacterial community composition is determined by plant species and soil characteristics (Kowalchuk et al., 2002; Berg and Smalla, 2009). Saprophytic soil microbes are indirectly affected by plants through the quality and quantity of plant litter and root exudates (De Deyn et al., 2008; Eilers et al., 2010). Novel plant species that have different root exudation patterns or tissue chemistry compared to natives (Macel et al., 2014) will select a specific assemblage of belowground microorganisms (Lankau et al., 2009; Lankau, 2011). Depending on the novel plant characteristics, soil communities may shift in their composition and functions when an exotic plant species invade (Kourtev et al., 2002a; Wolfe and Klironomos, 2005; Vilà et al., 2011; Gibbons et al., 2017; Harkes et al., 2017; Mamet et al., 2017). However, many studies on belowground functional consequences of exotic invaders are based on comparing species with different traits and life history strategies. Therefore, phylogenetically controlled comparisons, which exert higher control for factors known to influence soil community composition and functioning, are important to elucidate the effects of species origin (Agrawal et al., 2005; Funk and Vitousek, 2007).

Across-species comparison has shown that the invasive potential of exotic species may result from distant relatedness to native plant species, rather than from an effect of geographical origin *per se* (Strauss et al., 2006). Therefore, identifying functional consequences of ecological novelty of exotic plant species may be more accurate when comparing exotic species with related natives. Several experimental studies have singled out effects of ecological novelty (i.e., plant geographical origin in this case) by comparing exotic plant species with congeneric natives, demonstrating that even when controlling for species relatedness exotics can differ from natives (Agrawal et al., 2005; Funk and Vitousek, 2007; Funk and Throop, 2010; Meisner et al., 2013). Here, we aim at understanding how plant species that expand their range to higher latitudes within continents will impact the composition and functioning of the soil bacterial community in the new range as a result of their ecological novelty. We determined the impact of novel range-expanding plant species on native ecosystems in comparison with congeneric natives according to a phylogenetically controlled experimental set up (Engelkes et al., 2008; Meisner et al., 2011, 2012).

In the present study, we compared rhizosphere bacterial communities of range-expanding plant species and their related natives in two different contexts; grown in soils from their own field populations and in soils where both are ecologically novel. By using these two different context, we investigated whether potential differences in rhizosphere bacterial community composition between range-expanders and native plant species are the result of selection effects by plants or a response to existing soil heterogeneity. Because differences in rhizosphere bacterial communities may depend on plant ontogeny, we repeated our assessments in a time series, so that we could control for differences that may result from plant development. Our first hypothesis was that rhizosphere community composition of range-expanding plants differs from related natives when plants grow in their "own" field soils. Our second hypothesis was that plant origin-specific differences in bacterial rhizosphere communities increase over time when plants are grown in "novel" soils, as that would reveal plant selection effects on soil communities determined by their geographical origin. We assessed functional consequences of differences in soil community composition by measuring catabolic response profiles and soil enzymatic activities. We tested our hypotheses using a controlled greenhouse experiment with four pairs of range-expanding plant species and congeneric natives. Each plant species was grown in "own" and "novel" soils. We determined bacterial community composition and community-level functioning in the rhizosphere of all plants after four, eight and twelve weeks of plant growth.

MATERIALS AND METHODS

Plant Species Selection and Seed Origin

We used four pairs of range-expanding and congeneric native plant species and all eight species co-occur in riverine habitat of the Netherlands (**Supplementary Table S1**). This river-accompanying ecosystem is connected to Central Europe through the Rhine river, and to South-East Europe through the Rhine-Danube canal. In Central and South-East Europe, >800 km away from the Netherlands, the range-expanding and congeneric native plant species are all native. The plant species were selected based on the same criteria used in previous studies (Engelkes et al., 2008; Meisner et al., 2011). Briefly, we selected range-expanding plant species that are present in the Netherlands and co-occur in the same ecosystem with an abundant native plant species of the same genus. The range-expanding plant species were first recorded in the Netherlands during the second half of the 20th century with the exception of *Geranium*, which was first recorded in 19th century (Dutch flora is very well tracked by many volunteer florists) and show an increasing trend in abundance in the Netherlands over the last decades (NDFF, 2018). Because of their co-occurrence in the same riverine habitat type and their close phylogenetic (intra-genus) relationship, plant species belonging to the same pair differ in their geographical origin (i.e., range-expander vs. native), but are otherwise expected to be similar in genetic background and general ecology.

Seeds of *Rorippa* species, native *Geranium molle*, and range-expanders *Centaurea stoebe* and *Tragopogon dubius* were collected from the field in the Netherlands. Seeds of native *Centaurea jacea, Tragopogon pratensis* and the range-expander *Geranium pyrenaicum* were purchased from an external supplier that collects and propagates seeds from wild plant populations (Cruydt Hoeck, Nijeberkoop, Netherlands). All seeds were surface sterilized (3 min, 10% bleach solution) and germinated

on glass beads under controlled conditions (16 h of daylight at 20°C and 8 h of darkness at 10°C). *Rorippa* seeds were not surface sterilized due to their small size and were germinated in gamma-sterilized soil (minimally 25 KGray, Syngenta BV, Ede, Netherlands).

Soil Collection

During August–October 2015, we collected soils from five independent plant populations of each plant species in order to act as soil inocula in our experiment (**Supplementary Table S1**). All soils were sampled from field populations in the new range. Therefore, initial differences in soil community composition could not have been the result from environmental differences between the original and new range. A total of 40 inoculum soils were collected and kept separate as five experimental replicates throughout the experiment. Even though the plant species of interest occurred in mixed plant communities, soils were collected from underneath individuals of the species of interest in each of the five populations. Soil samples were collected from locations that were at least 60 m apart from each other (**Supplementary Table S1**). Field soils were sieved using a 4 mm mesh size to remove coarse elements and stored at 4°C in the dark until the experiment started. A subsample of each soil was stored at −80°C for further molecular analyses of the soil microbial community. A second subsample was oven dried at 40°C for 5 days in order to determine moisture content and soil C:N ratio using an elemental analyzer (Flash EA 1112, Thermo Fisher Scientific Inc., Waltham, MA, United States). Soil available phosphate (P-Olsen) was extracted in a 0.5 M $NaHCO_3$ solution and quantified using an autoanalyzer (QuAAtro Autoanalyzer, SEAL Analytical Ltd., Southampton, United Kingdom). Finally, we extracted available N (nitrate and ammonia) from field moist soils by shaking a 10 g dry weight equivalent in a 50 ml of 1 M KCl solution for 2 h. We measured soil pH in the KCl extracts and determined the concentration of mineral nitrogen (NH_4^+ and NO_3^-–NO_2^-) using an autoanalyzer (QuAAtro Autoanalyzer, SEAL Analytical Ltd., Southampton, United Kingdom).

In our experiment, we inoculated a sterilized background soil with living (non-sterilized) field soil. This method is commonly used and allows studying plant responses to soil biota while controlling for potential differences in abiotic properties of the soils (Engelkes et al., 2008). Background soil was collected from a riparian area near Beneden-Leeuwen, Netherlands (N51°53.952, E05°33.670). Background soil was sieved using a 1 cm mesh size, homogenized and gamma-sterilized (minimally 25 KGray, Syngenta BV, Ede, Netherlands).

Experimental Setup

We inoculated the sterilized background soil with 10% of live field soil (dry weight basis). We grew each plant species in soils inoculated with two different types of soil inocula: an inoculum from field sites where the plant species was present in the field ("own" soils), and an inoculum that is novel to the plant species ("novel" soils) (**Figure 1**). Novel soils for each congeneric plant pair were created by mixing the soils of all non-congeneric species using equal amounts on a dry weight basis. For both "own" and "novel" soils there were five independent replicates (**Figure 1**). Therefore, the "novel" soil mixes also originated from habitats within the riverine ecosystem where plant species could occur, but had not been previously conditioned by the plant species grown in the experiment. The novel soil of each replicate was split into two halves, one for growing the range expander and the other for growing the congeneric native.

Pots of 1.1-L were filled with the equivalent of 850 g of dry weight of soil. We adjusted soil moisture to 60% of the soil water holding capacity and kept it constant during the experiment by watering two times per week to re-set weight. Pots with soil only were pre-incubated in the greenhouse for 4 days in order to adjust to the water content and allow the inoculated soil microbial communities to establish in the sterilized soil. Afterwards, one seedling of each plant species was planted in the pots. During the first week, we replaced the seedlings that failed to establish. Greenhouse conditions represented an average growth season in the new range and were controlled to 16 h day length with day temperature of 21°C, night temperature of 16°C and average air humidity of 60%. Artificial light was supplied when required [High pressure Sodium (Son-T, 600 W Philips GP)].

We destructively sampled rhizosphere soil at 3 different time points during plant development and measured plant biomass after 4, 8, and 12 weeks since the start of the experiment. Pots were organized in a randomized block design in the greenhouse with 5 replicate blocks. In total, 240 pots were set up (8 plant species × 2 soil treatments ("own" and "novel") × 3 time points × 5 replicates).

Soil and Plant Biomass Sampling

At each sampling time, we destructively harvested 80 pots (8 plant species × 2 soil treatments ("own" and "novel") × 5 replicates). First, we removed the whole plant and soil from the pot. Then, the top soil in the pots and the soil attached loosely to plant roots was separated and discarded. Finally, roots were shaken vigorously and the soil that detached last from the roots was considered the "rhizosphere soil." We filled an Eppendorf tube with that rhizosphere soil and stored it at −80°C for further molecular analyses of the bacterial community composition. We used the remaining rhizosphere soil to analyze soil community functioning. Plant roots and shoots were separated, roots were washed and above and belowground plant biomass was measured after oven drying to constant weight at 70°C for 48 h (data not shown).

DNA Extraction and Community-Level Sequencing Analyses

We extracted DNA from all soil samples and subsequently amplified the 16S rRNA gene to determine bacterial community composition. Eppendorf tubes containing rhizosphere soil were freeze-dried prior to DNA extraction (FreeZone 12, Labconco, Kansas City, MO, United States). DNA was extracted from 0.25 g of soil using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, United States) following the manufacturer's instructions. We then amplified DNA using duplicate PCR reactions with bar-coded primers. Bacterial

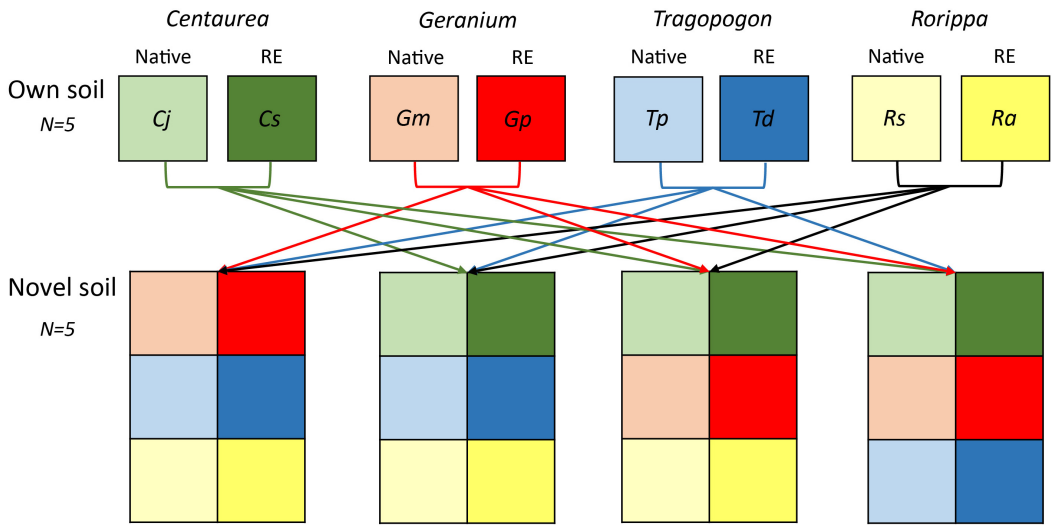

FIGURE 1 | Scheme of soil inocula treatments used in the experiment. Each plant species was grown in sterile soils inoculated with live soil originating from underneath plant individuals in the field (own soils). Furthermore, each species of each congeneric pair was grown in sterile soils inoculated with a mixture of live soils origination from underneath the non-congeneric species in the field (novel soils). A total of 5 independent soil live inocula replicates were collected from the field and kept separate throughout the experiment.

community composition was determined by targeting 16S rRNA gene using 515F/806R primers (Caporaso et al., 2012). PCR products were purified using the Agencourt AMPure XP magnetic bead system (Beckman Coulter Life Sciences, Indianapolis, IN, United States) with a volume of PCR product to beads of 1 to 0.7. Purified PCR products were analyzed in a Fragment Analyzer using a Standard Sensitivity NGS Fragment Analysis kit (1–6000 bp) and following manufacturer's instructions (Advanced Analytical Technologies GmbH, Heidelberg, Germany). Finally, bacterial PCR amplicons were sequenced using Illumina MiSeq platform.

The 16S rRNA amplicon reads, MiSeq paired-end reads, were merged when reads had a minimum overlap of 150 bp and at least a PHRED score of 25 using the RDP extension of PANDASeq (Masella et al., 2012) named Assembler (Cole et al., 2014). Primer sequences were removed using Flexbar version 2.5 (Dodt et al., 2012). Sequences were clustered to OTU with VSEARCH version 1.0.10 (Rognes et al., 2016), using the UPARSE strategy by dereplication, sorting by abundance with removing singletons and clustering using the UCLUST smallmem algorithm (Edgar, 2010). Chimeric sequences were detected using the UCHIME algorithm. All reads were mapped to OTUs and an OTU Table was created and converted to BIOM-Format 1.3.1 (McDonald et al., 2012). Taxonomic information for each OTU obtained using the RDP Classifier version 2.10 (Cole et al., 2014). All steps where implemented in a workflow made with Snakemake (Köster and Rahmann, 2012). Samples with a total sequence number lower than 1000 reads and singleton OTUs (e.g., OTU which is only found once in one sample) were removed from further analyses. The sequencing analyses of the 16S rRNA region of all soils yielded an average of 2.761 OTUs per sample (±944 SD), with a total of 5.452.733 reads. OTUs belonged to 25 different phyla (including bacteria and archaea), 82 classes, 136 orders, 274 families and 630 genera.

Catabolic Response Profile of the Soil Community

We used a catabolic response profile method as described in Fierer et al. (2012) in order to assess how soil communities differ in their ability to mineralize different organic carbon compounds. For each pot, we measured the CO_2 production response of the soil communities after the addition of 8 organic substrates of varying complexity (i.e., glucose, sucrose, glycine, oxalic acid, citric acid, yeast, lignin and cellulose). Organic carbon solutions were made before the soil sampling started and adjusted to a pH of 6.0. These analyses were carried out immediately after sampling of rhizosphere soil. Briefly, the equivalent of 4 g of dry soil was weighed into 9 different 50 ml centrifuge tubes. Then, each tube received 8 ml of one of the organic carbon substrate solutions. Additionally, one of the tubes received water as a control. Tubes containing soils and substrates were incubated for 1 h uncapped in a horizontal shaker (20°C). Centrifuge tubes were then closed tightly with a modified lid equipped with a rubber septum and a rubber O-ring in order to ensure air tightness. We then flushed the headspace air in the tubes with CO_2-free air for 2 min at 1 bar (Westfalen Gassen Nederland BV, Deventer, Netherlands). We incubated the tubes at constant temperature of 20°C in the dark using a climate-controlled chamber (Economic Lux chamber, Snijders Labs, Tilburg, Netherlands).

After incubation, we collected 6.2 ml of headspace air from each tube using a syringe and stored it into pre-evacuated 5.9 ml Exetainer vial (Labco Ltd., Buckinghamshire, United Kingdom). Samples were collected after 4 h of incubation for the water

control and the labile substrates (glucose, sucrose, glycine, oxalic acid, citric acid and yeast) and after 24 h for more recalcitrant substrates (lignin, cellulose). The concentration of CO_2 in the gas vials (over pressure of 1 bar) was measured by injecting 250 µl of each sample in a Trace Ultra GC gas chromatograph equipped with a flame ionization detector with methanizer (mFID) (Interscience BV, Breda, Netherlands) and a TriplusRSH auto-sampler (Interscience BV, Breda, Netherlands), and a Rt-QBOND (30 m, 0.32 mm ID) capillary column (Restek, Bellefonte, PA, United States). We used helium 5.0 as a carrier gas, a sample split ratio of 1:20 and set oven temperature at 50°C with a flow of 5 ml. We used a calibration curve of known concentrations of CO_2 ranging from 0 to 4600 ppm of CO_2 prepared out of a reference gas (2.38% CO2 in synthetic air, Westfalen AG, Munster, Germany) to determine the amount of CO_2 in our samples. Chromeleon 7.2 Data System Software (Thermo Fisher Scientific, Waltham, MA, United States) was used to automatize the measurements and process data. Respiration profiles were determined for each sample by calculating the relative respiration response from each of the incubations (in the control and the 8 different substrate additions) with respect to the total amount of respiration measured for that sample.

Extracellular Hydrolytic Enzyme Activity

Remaining rhizosphere soil was kept at –20°C for further analyses of extracellular enzyme activity in the soil. We measured soil enzyme activity using high-throughput fluorometric measurements, where a gain of fluorescence over the incubation time represents the amount of enzymatic activity (Baldrian, 2009). We determined the potential activity of 3 enzymes in soils involved in different pathways of carbon and nutrient cycling: glucosidase, phosphatase and aminopeptidase. Enzyme activity was measured in the 80 soil samples of the last time point (12 weeks). Briefly, 1 g of fresh soil was weighed into a clean glass jar before adding 50 ml of sodium acetate buffer (2.5 mM, pH 5.5). Vials were then capped tightly and shaken in a horizontal shaker for 10 min at 330 rpm in order to obtain the soil homogenate. Fluorogenic substrates 4-methylumbellyferyl-β-D-glucopyranoside (MUFG), 4-methylumbellyferyl-phosphate (MUFP) and L-alanine-7-amido-4-methylcoumarin (AMCA) were purchased (Sigma-Aldrich Chemie NV, Zwijndrecht, Netherlands). We dissolved all substrates in DMSO at concentration of 2.5 mM for AMCA and 2.75 mM for MUFG and MUFP. A 40ul of substrate solution was mixed with 250 µl of soil homogenate in each well of a black 96-well plate. Three technical replicates were included per soil sample and enzyme activity. We calibrated concentrations of enzyme activity product by a dilution curve made from a stable form of the fluorogenic compounds (1.0 mM methylumbellyferol (MUF) and 1.0 mM 7-aminomethyl-4-coumarin (AMC) (Sigma-Aldrich Chemie NV, Zwijndrecht, Netherlands). Fluorescence was measured at time 0 h and after 2 h of incubation at 40°C. We used a 96-well plate reader with an excitation and emission wavelengths of 360 and 460 nm, respectively (Synergy HT, BioTek Instruments, Winooski, VT, United States). We compared the measured fluorescence in our samples, after subtraction of the blank, with standard curves of MUF and AMC to calculate the amount of enzymatic product formed over the incubation time. A unit of enzyme activity is defined as the amount of enzyme reaction product (µmol) per gram of dry soil and hour.

Statistical Analyses

Abiotic properties of field inocula soils were analyzed with 2-way ANOVA in R (R Core Team, 2017). We tested the effect of plant genera and plant origin on each of the soil abiotic parameters. We considered plant genus as a fixed factor and not random since we selected the genera available after accounting for our selection criteria (Engelkes et al., 2008; Meisner et al., 2011). We tested the effect of plant origin within each plant genus for each of the soil parameters using *post hoc* comparisons of least square means with Tukey adjustment. Data was transformed prior analyses to meet assumptions of normality using Box-Cox power transformation for linear models in R (R Core Team, 2017).

Canoco 5 software was used to conduct multivariate statistics on bacterial community composition of field soils used as inoculum, and on compositional and functional (CRP) data of rhizosphere bacterial communities (Ter Braak and Šmilauer, 2012). Relative abundances of bacterial OTUs in soil communities and on soil respiration responses were log transformed prior the analyses. We performed Principal Coordinate Analyses (PCoA) of the dissimilarity matrix based on Bray–Curtis distances to visualize differences in bacterial community composition and soil functioning between our treatments. For the inoculum soils, soil abiotic properties measured (pH, C:N ratio, nitrate, ammonia and plant-available phosphate) were projected as (supplementary variables) in the PCoA ordination. Furthermore, we statistically tested the effect of the soil parameters measured and plant species on bacterial community composition of the inoculum soils using PERMANOVA (9999 permutations) (Oksanen et al., 2018). For the experimental soils, we tested the effect of plant genera, plant origin, soil and time point on bacterial community composition and community functioning using PERMANOVA (9999 permutations) (Oksanen et al., 2018). As explained earlier in the Methods section, during the experiment, each plant pair formed by a native and range-expanding plant species was grown in novel soils created by mixing the soils from the replicate sites of the non-congeneric plant species. Thereby, the novel soils were different from one plant pair to another and, for this reason data analyses were performed for each plant pair separately. Permutation tests were performed within each plant pair and we tested the effect of individual and interaction effects of plant origin, soil inocula and time of soil conditioning by the plants. For the same reason, we also conducted the analyses in "novel" and "own" soils per plant pair separately. The analyses within "novel" and "own" soils allowed us to zoom in on the plant-driven variation in rhizosphere communities and their functions in the case of novel soils, and examine differences in more detail in the case of own soils. To statistically test the significance of plant origin, soil inocula and time of harvest effects on community composition and functioning, we performed PERMANOVA analyses (9999 permutations) using the "adonis" function in the "vegan" package in R (Oksanen et al., 2018). We performed pairwise comparisons using the "pairwiseAdonis" function (Martinez Arbizu, 2017). In all cases,

block was included as a covariate in the analyses. We also performed multivariate dispersion analyses (999 permutations) to test for homogeneity of dispersion between the different plant origin, soil and time point groups and, thereby, validate the PERMANOVA tests (Anderson, 2006). Homogeneity of dispersion was measured using the "betadisper" function in the "vegan" package (Oksanen et al., 2018).

Bacterial OTU richness and Shannon's diversity indices of bacterial communities (H') and evenness were computed for each sample using Canoco 5 (Ter Braak and Šmilauer, 2012). These community parameters were determined in inocula soils and analyzed in the same way as the abiotic soil properties described above. For the experimental soils, we analyzed community parameters with linear mixed models using "lmerTest" package (Kuznetsova et al., 2017). We modeled community parameters for each of the three time points separately with plant genus, plant origin and soil as fixed effect factors and block as random factor. All analyses were conducted in R (R Core Team, 2017). OTU richness was log transformed prior to analyses. Diversity and evenness data were transformed prior to analyses to meet assumptions of normality using Box-Cox transformation for linear models in R.

Additionally, the effects of plant origin and soil inocula on soil enzyme activity were tested using linear mixed models with plant genus, plant origin (native or range expander) and soil inocula ("own" or "novel") as fixed effect factors, and block as a random factor. Enzyme activity rates were log transformed prior to analyses to meet assumptions of normality. All analyses were conducted in R (R Core Team, 2017).

RESULTS

Bacterial Community in Field Soils (Inocula Soils)

The variation of bacterial community composition represented by the first two axes of the PCoA was 39% of the total variation (**Supplementary Figure S1**). Soil abiotics and plant species identity explained 40 and 21% of the variation in soil bacterial communities, respectively, as tested with PERMANOVA

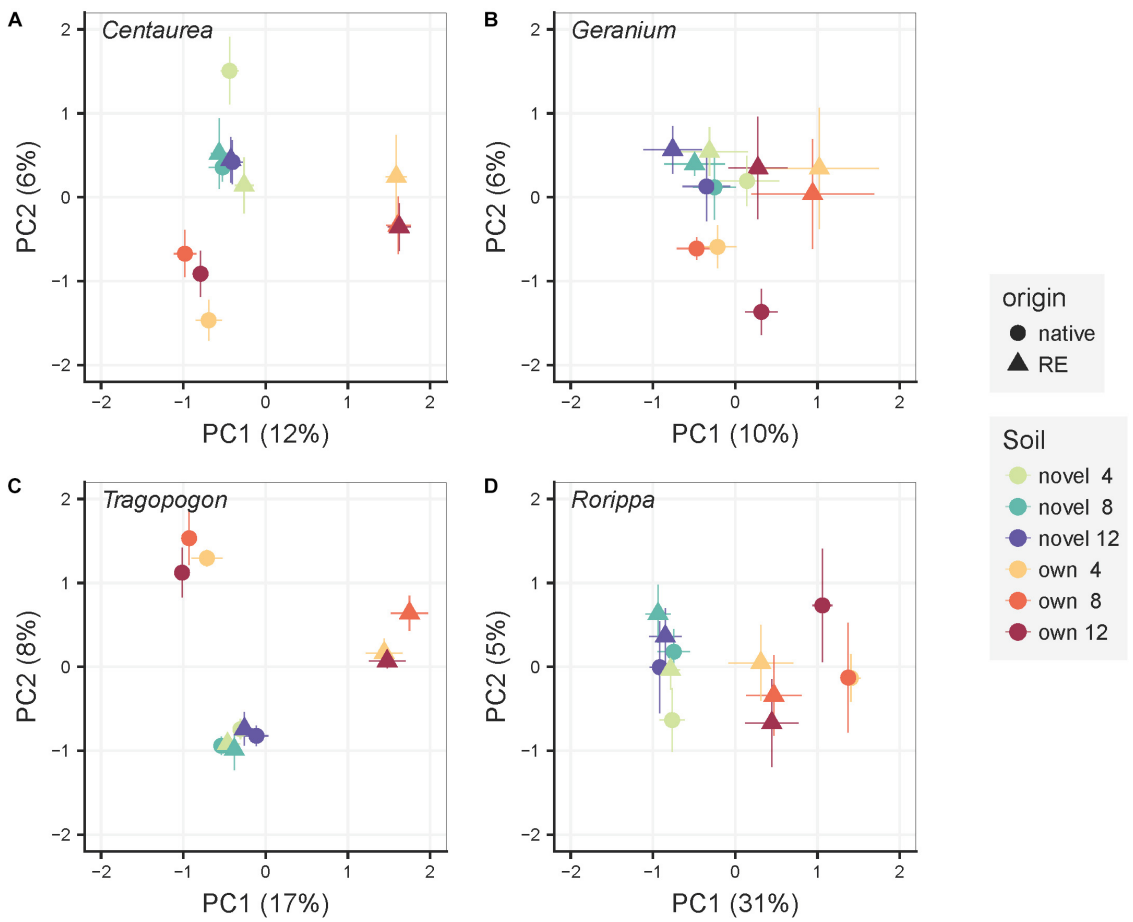

FIGURE 2 | Principal Coordinate Analyses of the rhizosphere bacterial community composition for each pair of a range-expander and its congeneric native plant species (**A**: *Centaurea*, **B**: *Geranium*, **C**: *Tragopogon*, **D**: *Rorippa*) grown in soils from their own field locations (own) and soils that are novel to both of them (novel). The symbols are means ± SE ($N = 5$). Within each pair, circles represent the native plant species and triangles the range-expander. Colors indicate soil treatment ("own" and "novel") and time of harvest (4, 8, and 12 weeks) as noted in the legend.

(**Supplementary Table S2**). Among soil abiotic properties measured, soil pH explained the largest amount of variation in soil bacterial communities (**Supplementary Table S2**). Bacterial communities in field soils of native and range-expanding *Geranium* species were more similar to each other than for the other plant pairs. However, soil bacterial communities associated with the natives *C. jacea* and *T. pratensis* were more similar to each other than to their related range-expanders *C. stoebe* and *T. dubius* (**Supplementary Figure S1**). Soil bacterial communities of *Rorippa* species were most different from the rest and were associated to soils with higher soil pH and C:N ratio compared to the other plant species (**Supplementary Figures S1**, **S2** and **Supplementary Table S3**). Overall, bacterial community richness, diversity and evenness was affected by plant genus, yet no significant effect was found for plant origin (**Supplementary Table S3** and **Supplementary Figure S2**). Soil nitrate availability was significantly higher in soils of native plant species than in soils of range expanders. In the case of ammonia, soils of native species had lower ammonia than soils of range-expanders, with the exception of the *Tragopogon* species (**Supplementary Table S3** and **Supplementary Figure S2**).

Bacterial Community Composition in the Rhizosphere of the Experimental Plants

The variation of bacterial community composition represented by the first two axes of the PCoA accounted for 18, 16, 25, and 36% of the total variation in *Centaurea*, *Geranium*, *Tragopogon* and *Rorippa* species, respectively (**Figure 2**). In the overall PERMANOVA test, the effect of plant origin on rhizosphere bacterial community was interacting with plant genera and soil (**Supplementary Table S4**). Furthermore, there was a main effect of time of sampling. Bacterial communities after 4 weeks of plant growth significantly differ from those samples at week eight and twelve of the experiment (p = 0.002 and p = 0.001, respectively), while there were no differences between the latter time points as indicated by pairwise testing. When considering plant pairs separately, soil inocula ("novel" or "own") was the most important factor explaining variation in bacterial community composition in all plant pairs with the exception of the *Tragopogon* pair, where soil inocula and plant origin explained the same amount of variation (**Table 1**). The interaction of plant origin and soil inoculum explained bacterial community in the rhizosphere in all plant pairs except in *Geranium* (**Table 1**). Overall, bacterial communities were separated between native and range expanders when they were grown in their "own" field soils, but did not differ when grown in "novel" soils. Multivariate dispersion analyses indicated that dispersion within groups was homogeneous between range-expanders and natives and between time points for each of the four plant pairs. However, "own" soils had significantly higher dispersion than "novel" soils in *Geranium*, *Tragopogon* and *Rorippa* plant pairs (F = 8.8, p = 0.004, F = 58.2, p = 0.001 and F = 28.8, p = 0.001, respectively). This indicated that community composition was most similar between samples of "novel" soils, while there was most variation between samples in "own" soils. As a result of the soil mixing scheme (**Figure 1**), own soils for each plant pair consisted of ten different soils originating from individual locations in the field, causing a higher multivariate variation, while novel soils consisted of five different soil mixes from locations of the non-congeneric species in the field.

Bacterial Communities Within "Novel" and "Own" Soils

To test the effect of plant origin more accurately we performed the same analyses within each soil inocula treatment, which allows to disentangle the effect of plant origin from the effect of pre-existing differences in soil bacterial communities. We then observed that, in "novel" soils, plant origin no longer

TABLE 1 | PERMANOVA tests on Bray–Curtis dissimilarity matrix (9999 permutations).

		Centaurea		*Geranium*		*Tragopogon*		*Rorippa*	
	Factor	R^2	**Signif.**	R^2	**Signif.**	R^2	**Signif.**	R^2	**Signif.**
Bacterial community	Plant origin (P)	**0.057**	***	**0.036**	**	**0.082**	***	**0.030**	*
	Soil inocula (S)	**0.067**	***	**0.049**	***	**0.082**	***	**0.199**	***
	Time (T)	**0.050**	**	**0.054**	**	**0.047**	*	0.040	ns
	P × S	**0.055**	***	0.023	ns	**0.081**	***	**0.026**	*
	P × T	0.020	ns	0.024	ns	0.022	ns	0.018	ns
	S × T	0.022	ns	0.021	ns	0.021	ns	0.023	ns
	P × S × T	0.019	ns	0.019	ns	0.023	ns	0.016	ns
Community functioning	Plant origin (P)	0.003	ns	0.001	ns	0.001	ns	0.000	ns
	Soil inocula (S)	0.031	ns	0.028	ns	0.028	ns	0.028	ns
	Time (T)	0.009	ns	0.005	ns	0.009	ns	0.010	ns
	P × S	0.002	ns	0.001	ns	0.000	ns	0.000	ns
	P × T	0.001	ns	0.000	ns	0.000	ns	0.001	ns
	S × T	0.000	ns	–0.001	ns	–0.001	ns	0.000	ns
	P × S × T	0.001	ns	0.000	ns	0.000	ns	0.001	ns

*Significance levels: ns $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Values in bold indicate $p < 0.05$.*

explained variation in bacterial community composition (**Supplementary Figure S3** and **Supplementary Table S5**). Instead, time of harvest appeared to explain around 10% of the total variation in *Geranium* and *Tragopogon* plant pairs (**Supplementary Table S5**). In "own" soils, i.e., originating from field populations of each species, plant origin significantly explained bacterial community composition at the end of the growth experiment (**Supplementary Figure S4** and **Supplementary Table S5**). In both "novel" and "own" soils, the variation in community composition of the samples belonging to range-expanders and natives and to the different time points showed homogeneous multivariate dispersion, indicating that the variation in community composition was equal between the groups. There was only the exception of the *Geranium* plant pair grown in "own" soil, where soils of range-expanders had higher variation in community composition than soils of natives ($F = 5.8$, $p = 0.02$).

Bacterial Community Richness, Diversity and Evenness

Soil treatments ("own" and "novel") significantly differed in their bacterial richness and diversity at all sampling times (**Supplementary Table S6**; Fixed factors, Soil). Novel soils, which were mixes of all soil samples collected from field sites with non-congeneric plant species, had significantly higher richness and diversity of bacterial OTUs than own soils, which originated from individual locations where the tested plant species were present in the field. Bacterial communities in own soils of *Rorippa* species were most different from the other plant pairs (**Supplementary Figure S2** and **Supplementary Table S6**) and also from their novel soil, which is represented by a significant soil and plant genera interaction. Overall, richness, diversity and evenness of rhizosphere bacterial communities were not significantly affected by plant origin itself (**Supplementary Table S6**).

Community-Level Functional Analyses

Catabolic Response Profile

We used PCoA ordination to assess differences in the functional responses of soil communities to the various added organic substrates (catabolic response profile). The first and second axis represented 75, 68, 57, and 55% of the variation in catabolic response profiles of *Centaurea*, *Geranium*, *Tragopogon* and *Rorippa* soil communities, respectively (**Figure 3**). In the overall PERMANOVA test, there was only a significant effect of time point on explaining variation in soil community functioning (**Supplementary Table S4**). However, this effect of time point was weak and pairwise *post hoc* testing resulted non-significant. Even though time seems to drive dissimilarity in community functioning in the ordination PCoA plots of each plant pair (**Figure 3**), none of the experimental treatments (plant origin, soil inocula, time of harvest) explained differences in community level functioning (**Table 1**). Similarly, when functioning of the "novel" or "own" soils were examined separately, neither plant origin nor time of harvest explained the variation in community level functioning (**Supplementary Figures S5**, **S6** and **Supplementary Table S4**). Overall, compositional differences in bacterial communities were not consistently linked to shifts in catabolic response profiles in our experiment. Multivariate dispersion analyses for community functioning showed that variation among functional profiles was not significantly different between groups of samples with the same plant origin, soil inoculum type and across time points.

Extracellular Enzyme Activity

To study soil functions related to nutrient cycling, the activity of three extracellular enzymes was measured in the rhizosphere soil collected after 12 weeks of plant growth (**Table 2**). There were no main effects of plant origin, indicating that both range-expanders and related native species were associated with the same levels of enzyme activity in their rhizosphere soil. Plant genus marginally affected glucosidase enzyme activity in the soil ($F_{3,60} = 2.91$, $p = 0.04$); however, *post hoc* testing using Tukey HSD did not yield significant differences between any specific group. Soil treatment ("own" and "novel") significantly affected phosphatase activity ($F_{1,60} = 11.00$, $p = 0.001$), with higher levels of phosphatase activity in "own" than in "novel" soils.

DISCUSSION

It is generally assumed that exotic plant species may alter composition and functioning of soil microbial communities (Ehrenfeld et al., 2001; Allison et al., 2006; Ehrenfeld, 2010). However, in most case studies where exotics are compared with natives, the exotics that replace the natives not only differ in origin, but also in traits or life histories, whereas pre-invasion site conditions cannot easily be controlled for (Kourtev et al., 2002a; Wolfe and Klironomos, 2005; Vilà et al., 2011). Here, we compare how intra-continental range expanding plant species and congeneric natives influence bacterial community composition and functioning in their rhizosphere, while minimizing genetic differences between range expanders and natives, and controlling for ecological novelty. We paired plant species that expand range most likely as a result of climate warming with species from the same genus that are native in the expansion range (Engelkes et al., 2008; van Grunsven et al., 2010a; Meisner et al., 2011; Morriën et al., 2013). All plant species were grown in soils collected from established field populations, as well as in soils from sites where neither the range expander nor the native currently occurred. In support of our first hypothesis, rhizosphere bacterial communities differ between range-expanding and native plant species when plants are grown in soils collected from their own field populations. Interestingly, when both species were grown in novel soils, there were no compositional differences in rhizosphere bacterial communities. Therefore, and opposite to our second hypothesis, we argue that in the present comparison plant origin *per se* has little effect on rhizosphere bacterial community composition.

Many field studies on plant invasions and soil communities have shown that exotic plant species have distinct soil communities compared to native plants growing in adjacent areas (Kourtev et al., 2002a,b; Scharfy et al., 2009; Collins et al., 2016; Stefanowicz et al., 2016; Gibbons et al., 2017). Consistent with these results, in our experiment we also observe that the

composition of the rhizosphere bacterial community differed by plant origin in all four plant pairs when plants were grown in their own field soils (**Figure 2** and **Supplementary Figure S4**). These results suggest that plant origin indeed influences bacterial community composition even in a phylogenetically constrained comparison between range expanders and natives. However, in the present study, as well as in most field studies it is difficult to exclude the possibility that invaded sites were already different from adjacent sites prior arrival of the exotic species. If that is the case, the bacterial assembly in the rhizosphere may simply reflect differences of initial bulk soil communities (de Ridder-Duine et al., 2005), or a combination of site and origin differences and, overall, the capacity to disentangle the effect of ecological novelty from other co-varying effects is limited. We tried to rule out such confounding factors as much as possible by growing range-expanding and native plant species also in the same "novel" soil.

In contrast to our second hypothesis, the geographical origin of the plant species (range-expanding or native) did not affect rhizosphere bacterial community composition differently when both range-expanding and congeneric native plant species were grown in the same "novel" soils (**Figure 2** and **Supplementary Figure S3**). Our results diverge from previous controlled experiments, which concluded that plant origin may play a role in influencing soil community composition in both intercontinental exotic plant invasion (Kourtev et al., 2003) and plant range-expansion (Morriën et al., 2013). In contrast with many intercontinental exotic plant invasion studies, we used phylogenetically controlled comparisons to assess the effect of plant species origin, while intending to minimize their ecological differences. Consequently, plant species in each plant pair were not expected to differ strongly in e.g., life history and plant functional type, which have been suggested as important

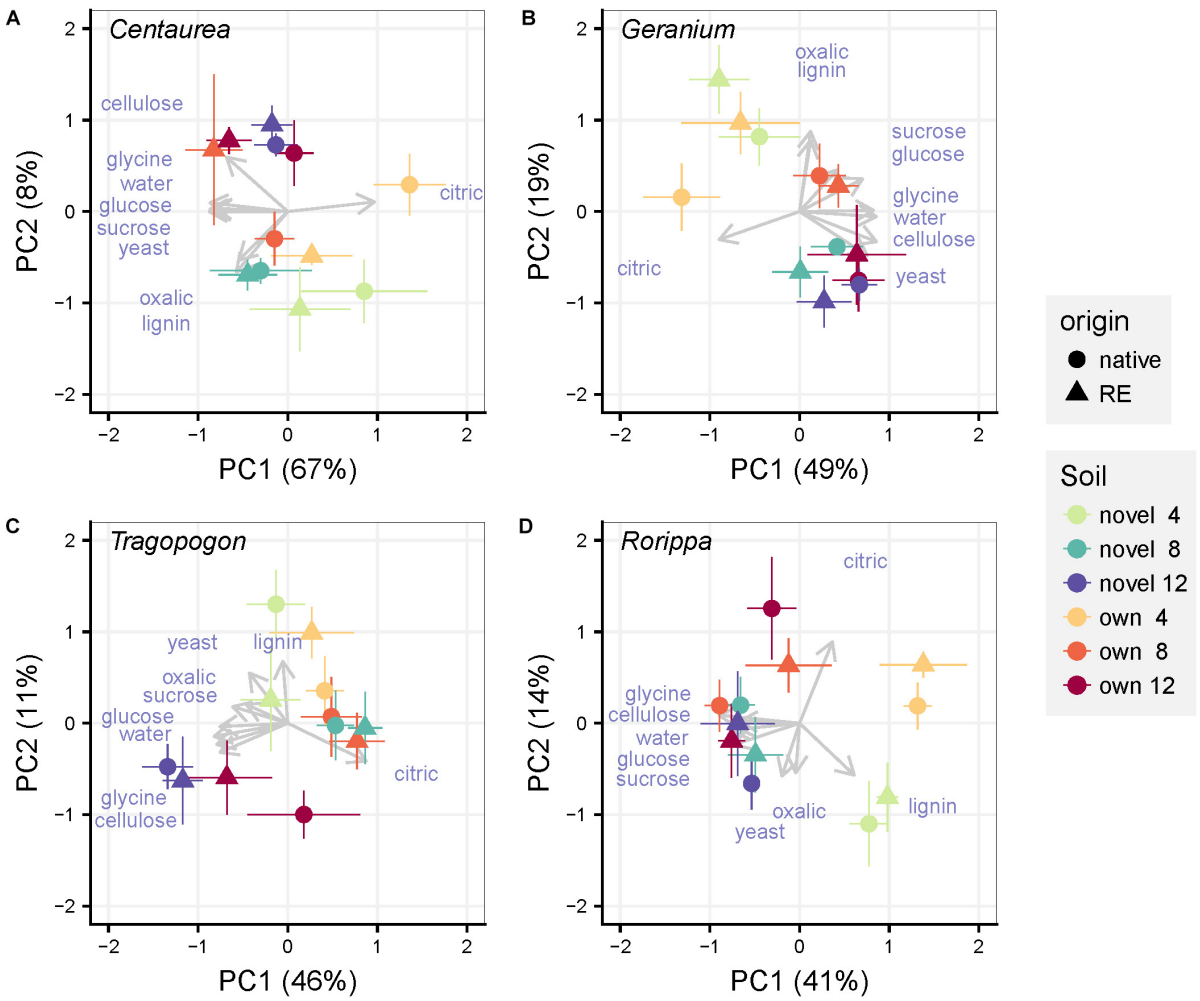

FIGURE 3 | Principal Coordinate Analyses of the catabolic response profiles for each pair of a range-expander and its congeneric native plant species (**A**: *Centaurea*, **B**: *Geranium*, **C**: *Tragopogon*, **D**: *Rorippa*) grown in soils from their own field locations (own) and soils that are novel to both of them (novel). The symbols are means ± SE ($N = 5$). Within each pair, circles represent the native plant species and triangles the range-expander. Arrows representing each substrate are displayed over the ordination plot as supplementary variables. Colors indicate soil ("own" and "novel") and time of harvest (4, 8, and 12 weeks) as noted in the legend.

TABLE 2 | Hydrolytic enzyme activity in the rhizosphere soil after 12 weeks of plant growth. Values are means ± SE ($N = 5$).

Plant genus	Plant species	Plant origin	Soil inocula	Glucosidase activity (μmol * kg^{-1} * h^{-1})	Phosphatase activity (μmol * kg^{-1} * h^{-1})	Aminopeptidase activity (μmol * kg^{-1} * h^{-1})
Centaurea	*C. jacea*	Native	own	10.18 ± 1.89	10.81 ± 0.34	0.77 ± 0.16
			novel	8.78± 2.25	9.73 ± 1.53	0.68 ± 0.12
	C. stoebe	Range-expander	own	10.43 ± 1.41	10.60 ± 1.49	1.27 ± 0.19
			novel	9.98 ± 1.67	8.77 ± 1.22	0.58 ± 0.06
Geranium	*G. molle*	Native	own	15.73 ± 3.16	21.14 ± 5.83	1.32 ± 0.25
			novel	12.50 ± 0.68	15.90 ± 9.98	1.47 ± 0.25
	G. pyrenaicum	Range-expander	own	18.32 ± 4.36	22.79 ± 5.68	1.38 ± 0.13
			novel	10.50 ± 2.39	9.18 ± 2.15	1.56 ± 0.46
Tragopogon	*T. pratensis*	Native	own	13.12 ± 1.24	13.36 ± 2.02	0.91 ± 0.25
			novel	14.45 ± 1.79	12.63 ± 0.63	1.59 ± 0.57
	T. dubius	Range-expander	own	10.51 ± 1.24	17.23 ± 2.52	0.95 ± 0.09
			novel	11.66 ± 1.83	12.23 ± 1.68	0.90 ± 0.17
Rorippa	*R. sylvestris*	Native	own	11.54 ± 1.48	13.59 ± 1.71	1.14 ± 0.53
			novel	9.89 ± 2.25	9.58 ± 1.92	0.96 ± 0.21
	R. austriaca	Range-expander	own	12.82 ± 2.02	17.35 ± 3.80	1.41 ± 0.43
			novel	13.75 ± 1.61	11.40 ± 3.55	3.07 ± 1.54
Fixed factors		Plant genus	*F*-value	2.915	2.664	2.566
			p-value	**0.041**	0.056	ns
		Plant origin	*F*-value	0.035	0.068	0.524
			p-value	ns	ns	ns
		Soil inocula	*F*-value	2.108	11.008	0.227
			p-value	ns	**0.001**	ns

Effects of plant genus, plant origin and soil inocula ("own" and "novel") on enzyme activity were analyzed using ANOVA with block as random factor in the mixed linear models. Significance levels of 2-way and 3-way factor interactions resulted all non-significant and are not included in this table. Values in bold indicate $p < 0.05$.

biotic predictors of soil community and soil functional shifts (Scharfy et al., 2011; de Vries et al., 2012; Legay et al., 2014; Lee et al., 2017).

The relatively short running time of our experiment (3 months) will not have allowed divergence of the bacterial communities between plants from different origins. However, the running time of our experiment is not shorter than that of most plant-soil feedback experiments where plants produced different soil microbial community structure and composition (Morriën et al., 2013; Heinen et al., 2017). In addition, *Geranium* and *Rorippa* species started to senesce within the 12 weeks of experiment, so that the length of the growth period was natural. Furthermore, the value of this 12 weeks experiment is that it reveals the microbiome response to plant identity (structural and chemical plant traits) without much influence of evolutionary dynamics between plants and soil microbial communities (Lankau, 2012). Furthermore, our main interest was to assess shifts in saprophytic microbes in the rhizosphere community and thereby, we analyzed the composition of the whole bacterial community rather than looking at the abundance of specific microbial groups, such as potential plant pathogens (Morriën et al., 2013). Seed surface sterilization might have also influenced bacterial community composition in the rhizosphere compared to when using unsterilized seeds. However, it allowed to disentangle the selection effects of plants on soil bacteria in the rhizosphere from the differences that may be caused by the pre-existing community in the seed surface. Furthermore, in a recent study it has been shown that seed-borne endophytic oomycetes of these range-expanding plant species and congeneric natives could not be found in the rhizosphere (Geisen et al., 2017) suggesting a limited effect of endophytic microbial community to the composition of the rhizosphere.

In spite of the substantial differences in bacterial community composition in the rhizosphere of plants growing in soil collected from field populations (**Figure 2**), we observed limited differences in community functioning (**Figure 3** and **Table 2**). Previous experiments manipulating the composition of microbial communities have shown high levels of functional redundancy in microbial communities (Franklin and Mills, 2006; Wertz et al., 2006). Plant-induced changes in microbial-mediated soil processes may be the result of comparing phylogenetically distant plant species (Ehrenfeld et al., 2001) or major plant community shifts (Carney and Matson, 2005). They may also be derived from studies focusing on longer time scales (Collins et al., 2016) or on specific soil processes such as nitrogen cycling (Hawkes et al., 2005). Nevertheless, in spite of the possibility that plant selection effects may have influenced soil fungal communities (Dassen et al., 2017; Hannula et al., 2017), it is obvious that those changes, if occurring in our study, have not influenced soil microbial functioning either. Although the results of our experiment are consistent across the four plant pairs examined, we stress that future studies should test for consistency of our results by repeating such manipulative experiments under similar and contrasting environmental conditions.

We conclude that intracontinental plant range expansions may lead to populations of novel plant species that have different bacterial communities than congeneric natives, but that this is not necessarily due to their different geographical origin. When range expanding and native plant species from the same genus pair were grown for 3 months in novel soils, bacterial rhizosphere communities of the range expander and the congeneric native were indistinguishable. Interestingly, the differences in bacterial community composition when plants were grown in their own soils did not result in altered ecosystem processes as is demonstrated by the respiration of different organic substrates. Therefore, our results demonstrate that plant origin *per se* does not necessarily have a major impact on bacterial community composition and soil microbial functioning when keeping all other aspects the same. This does not exclude the possibility that range expanders may influence community composition and ecosystem functioning when they are exposed to the soils for longer time periods, or in other ways, such as by responding differently to extreme weather events (Meisner et al., 2013), natural enemies (Engelkes et al., 2008; Van Grunsven et al., 2010b; Dostálek et al., 2016), and other conditions that may typify their novel environments.

DATA AVAILABILITY

All sequence data has been uploaded to the European Nucleotide Archive (ENA) under the accession number PRJEB26590.

AUTHOR CONTRIBUTIONS

MM, GFV, and WP designed the study and wrote the manuscript with input from all authors. MM, GFV, and CW performed the experiments and collected the data. MM and LBS analyzed the data with input from GFV and WP.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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Plant Stage, Not Drought Stress, Determines the Effect of Cultivars on Bacterial Community Diversity in the Rhizosphere of Broomcorn Millet (*Panicum miliaceum* L.)

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Broomcorn millet (*Panicum miliaceum* L.) is one of the oldest domesticated crops and has been grown in arid and semiarid areas in China since 10,000 cal. BP. However, limited information is available about how bacterial communities within the rhizosphere of different broomcorn millet cultivars respond to drought stress. Here, we characterized the changes in the rhizobacterial assemblages of two broomcorn millet cultivars, namely, *P. miliaceum* cv. *HeQu Red* (HQR) and *P. miliaceum YanLi 10* (YL10), from the jointing stage to the grain filling stage after they were exposed to a short-term drought stress treatment at the seedling stage. Drought significantly inhibited the growth of both cultivars, but the effect on YL10 was higher than that on HQR, indicating that the drought tolerance of HQR was greater than that of YL10. *Proteobacteria* (33.8%), *Actinobacteria* (21.0%), *Acidobacteria* (10.7%), *Bacteroidetes* (8.2%), *Chloroflexi* (6.3%), *Gemmatimonadetes* (5.9%), *Firmicutes* (3.5%), *Verrucomicrobia* (2.9%), and *Planctomycetes* (2.7%) were the core bacterial components of broomcorn millet rhizosphere as suggested by 16S rDNA sequencing results. The diversity and composition of bacterial rhizosphere communities substantially varied at different developmental stages of broomcorn millet. As the plants matured, the richness and evenness of the rhizobacterial community significantly decreased. Principal coordinate analysis showed that the structure of the bacterial rhizosphere community changed notably only at the flowering stage between the two cultivars, suggesting a stage-dependent effect. Although drought stress had no significant effect on the diversity and structure of the bacterial rhizosphere community between the two cultivars, differential responses to drought was found in Actinobacteria and *Acinetobacter*, *Lysobacter*, *Streptomyces*, and *Cellvibrio*. The relative abundance of Actinobacteria and *Lysobacter*, *Streptomyces*, and *Cellvibrio* in the YL10 rhizosphere was stimulated by the

drought treatment compared with that in the HQR rhizosphere, whereas the opposite effect was found in *Acinetobacter*. Our results suggested that the effects of cultivars on bacterial rhizosphere communities were highly dependent on plant developmental stage, reflecting the genetic variations in the two broomcorn millet cultivars.

Keywords: broomcorn millet, cultivar, bacterial community, rhizosphere, drought stress, plant age

INTRODUCTION

The rhizosphere is a specific microenvironment in terrestrial ecosystems, and it permits a sophisticated exchange between numerous organisms and their environment. Diverse bacteria, fungi, and viruses live in the rhizosphere of plants, and many of these microorganisms facilitate various processes, including nutrient uptake, hormone production, and disease and pest prevention (de Vrieze, 2015). Given the vital roles of these organisms to plant growth and health, certain microbes, such as nitrogen-fixing bacteria and arbuscular mycorrhizal fungi, have been found to help plants successfully live under stressful conditions (Sánchez-Blanco et al., 2004) and can be used to increase crop yield in agro-ecosystems with minimal use of chemical fertilizers and pesticides (Glick, 2014). Therefore, understanding the mechanisms of plant–microorganism interactions within the rhizosphere can help us tackle challenges associated with the sustainability and productivity of agro-ecosystems.

For annual and perennial plant species, plant stage is an important factor that drives shifts in microbial rhizosphere community composition under field and greenhouse conditions (Houlden et al., 2008; Micallef et al., 2009; Na et al., 2017a). As plants develop, variations in the physiological status of plants affect the growth of distinct microbial groups within the rhizosphere mediated by variation in the composition of plant root exudates and depositions (Mougel et al., 2006). The diversity and quantity of carbon flow to and from the roots into the rhizosphere change with plant development (Swinnen et al., 1994; Duineveld et al., 2001). These results have suggested that plants can initiate and actively recruit their rhizosphere microbiome to meet their requirements at a given developmental stage. Peiffer et al. (2013) found a small but significant proportion of heritable variation in bacterial rhizosphere diversity among 27 modern maize inbreds under field conditions. Similar results have also been found in the rhizosphere of different genotypes of *Arabidopsis thaliana* under controlled conditions (Bulgarelli et al., 2012; Lundberg et al., 2012). Given that the genotype of a plant corresponds to its phenotype, the different responses of rhizosphere microbiota may reflect the differences in physiological conditions among genotypes or cultivars at a specific developmental stage. Thus, analyzing interactions between microbiota and their host plants may be an alternative way to identify plant alleles that control plant performance (Peiffer et al., 2013).

Shifts in plant–bacterium interactions in the rhizosphere have been observed when plants are subjected to drought stress, and research on how this interaction contributes to plant drought tolerance has been widely performed (Dimkpa et al., 2009; Xu et al., 2018). As a response to water deficit, osmotolerant rhizobacteria increase the synthesis of osmolytes, such as glycine betaine, and enhance the drought tolerance of host plants (Dimkpa et al., 2009). These osmotolerant bacteria can also stimulate root growth and reconstruct the root architecture of host plants by producing indole-3-acetic-acid (Yuwono et al., 2005). Improved root growth enhances the efficiency of water acquisition and nutrient uptake of host plants under drought stress. However, most studies on the plant–rhizobacterium interactions under drought stress have focused on various species, such as rice (Yuwono et al., 2005), maize (Casanovas et al., 2002), tomato (Mayak et al., 2004), and common bean (German et al., 2000), which do not have notable drought resistance. The response of the bacterial rhizosphere community of plant species with inherently low water requirements to water deficit has yet to be further investigated.

Broomcorn millet (*P. miliaceum* L.), also known as common, hog or proso millet, is one of the oldest cultivated and domesticated crops. In 10,000 cal. BP, this crop was cultivated in China (Hunt et al., 2008, 2014); since then, it has been considered a staple cereal in Northern China (Hunt et al., 2011). It has several unique characteristics among cereal species in terms of its ecology, geography, and cultivation history (Hunt et al., 2011). Among cereal crops, broomcorn millet has the lowest water requirement and shortest growth period (Baltensperger, 2002). It also has a low nutrient requirement (Rajput et al., 2016) and can be cultivated in marginal agricultural lands, where the cultivation of other cereals dose not succeed, because it can tolerate drought and high-temperature stresses and is well adapted to saline–alkaline soils (Hunt et al., 2011; Wang et al., 2016). Currently, this important cereal is cultivated in large areas in arid and semi-arid steppe regions in Northern China, Russia, Central Asia, and North America (Zohary and Hopf, 2000).

Although previous studies investigated the agronomic trait diversity (Wang et al., 2016), genetic diversity (Hunt et al., 2011; Liu et al., 2016; Rajput et al., 2016), and stress tolerance mechanisms (Karyudi and Fletcher, 2002; Dai et al., 2011; Zhang et al., 2012) of broomcorn millet, a comprehensive understanding of its diversity and the structure of its rhizosphere bacterial assemblages is lacking. Plant–microbe interactions in the rhizosphere are genetically controlled (Peiffer et al., 2013). Hence, broomcorn millet provides an ideal system to reveal the mechanisms of heritable plant–microorganism interactions and the role of bacterial rhizosphere populations in drought tolerance. For these purposes, we characterized the rhizosphere bacterial community across two cultivars of broomcorn millet under drought stress by monitoring their different developmental stages via pyrosequencing 16S rRNA gene amplicons. On the basis of previous studies suggesting that bacterial rhizosphere

community diversity was driven by the developmental stage of host plants and our preliminary experiments demonstrating that the two broomcorn millet cultivars differed in drought tolerance, we hypothesized that (1) the effect of cultivar on the bacterial community composition depended on the developmental stage of the plant; (2) drought profoundly affected rhizobacterial community structure; and (3) the responsive pattern of the relative abundance of individual bacterial populations to drought depended on the cultivar of broomcorn millet. Knowledge about the temporal shift of plant-associated microbial communities would help enhance our understanding of the differences in the drought tolerance of broomcorn millet cultivars and the interactions between this oldest drought-tolerant crop and its associated microbiota.

MATERIALS AND METHODS

Experimental Design

Pot experiments, which had a completely randomized block design with three replicates, were conducted with two broomcorn millet cultivars, namely *P. miliaceum* L. cv. *HeQu Red* (HQR) and *YanLi 10* (YL10), at the Hequ Research Station of the Shanxi Academy of Agricultural Sciences (39°08′20.78″N, 110°14′18.74″E) at the eastern fringe of the Loess Plateau, Shanxi Province, China. The soil samples (sandy loam) were collected from a local agricultural field (soil auger, 5 cm in diameter and 20 cm in length), which had not been used previously to grow broomcorn millet. The field soils were air dried and sieved to 2 mm to remove rocks and plant debris.

Broomcorn millet plants were planted in pots (height; 35 cm; diameter: 30 cm) containing 10 kg of the sieved dry soil. All of the experiments were conducted in a greenhouse to avoid rainfall. Thirty surface-sterilized seeds bleached for 5 min and rinsed at least three times with sterile water were planted in each pot on June 11, 2015, and 16 seedlings were kept after germination. After 55 days of growth, the soil water content of half of the pots decreased to 15% water holding capacity without watering (about 4 days) and maintained at 15% for another 15 days by weighing the pots every day (drought stress), whereas the remaining pots were maintained at 55% of water holding capacity (control). The experiment consisted of 36 samples (3 replicates × 2 cultivars × 2 treatments × 3 stages). Two pots were randomly selected from each of the replicates, and all of the plant roots were bulked as a single sample. Therefore, 72 pots were used in this study. The experimental procedure is shown in detail in **Supplementary Figure S1**.

Sampling

The rhizosphere soil was first sampled after 15 days of drought stress (i.e., the jointing stage). The water holding capacity of the remainder pots was returned to 55%. Additional samples were harvested 10 (flowering stage) and 20 (grain filling stage) days after the first 15 days in the control and drought stress treatments. Destructive sampling was performed 15, 25, and 35 days after the drought treatment, and all of the plants (total plants = 32) in two random pots were pooled and considered as one replicate (total replicates = 3). After the loosely adhered soil was removed by shaking, the root samples of the harvest plants were transferred into a 5 ml sterilized tube and stored in ice immediately. The roots with adhered soil were washed with 5 ml of 0.9% sterile NaCl solution to collect the rhizosphere soil. The resulting solution was centrifuged at 12,000 rpm and at 4°C for 10 min, and deposition was defined as the rhizosphere soil sample. These rhizosphere soils were then stored at –20°C until further analysis.

After the last sampling was conducted, the shoots of all of the plants were harvested on September 30, 2015, and used for quantifying the agronomic traits of plant height, number of internodes, culm diameter, and panicle length. Plants were dried to constant weight at 60°C before the dry weights of panicles and grains were determined.

Total Genomic DNA Extraction and Amplicon Generation

Total genomic DNA was extracted from the rhizosphere soil samples by using a PowerSoil DNA isolation kit (MoBio, United States) in according with the manufacturer's instructions. DNA integrity and purity were monitored on 1% agarose gels. Partial 16S rDNA-based high-throughput sequencing was performed to detect the bacterial diversity and composition of each sample according to Caporaso et al. (2010). The V4 region of the 16S rRNA gene was amplified with the specific primer 515F and 806R with a 6 bp error-correcting barcode unique to each sample. PCR was conducted in 30 μl reactions with 15 μl of Phusion High-Fidelity PCR Master Mix (New England Biolabs, United Kingdom), approximately 10 ng of template DNA, and 0.2 μM forward and reverse primers. Thermal cycling involved initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 60 s, and a final round of elongation at 72°C for 5 min. The PCR products were detected on 2% agarose gel, and the samples with bright main strips between 200 and 300 bp were chosen and purified for further analyses.

Library Preparation and Sequencing

Amplicon libraries were generated using a NEB Next Ultra™ DNA Library Prep kit for Illumina (NEB, United States) in accordance with the manufacturer's recommendations, and index codes were added. Library quality was assessed using a Qubit 2.0 fluorometer (Thermo Fisher Scientific) and an Agilent Bioanalyzer 2100 system. Subsequently, high-throughput sequencing was performed on an Illumina MiSeq 2500 platform in Novogene (Beijing, China), and 250 bp/300 bp reads were generated. The paired end reads were merged using FLASH (Magoč and Salzberg, 2011) and then assigned to each sample based on the unique barcodes. Sequence analysis was performed by the UPARSE software package by using UPARSE–OTU and UPARSE–OTU ref algorithms. UPARSE pipeline[1] was utilized to cluster the sequences into operational taxonomic units (OTUs) at a minimum pair-wise identity of 97%. We chose representative sequences for each OTU and used the RDP

[1]http://drive5.com/uparse/

Classifier (Wang et al., 2007) to annotate taxonomic information for each representative sequence. The sequencing of bacterial 16S rRNA gene amplifications from all of the rhizosphere samples resulted in a total of 641,244 high-quality reads. The results of quality control confirmed that these data could be used for further analysis (**Supplementary Table S1**). Then, bacterial community alpha diversity indices, such as the Shannon index, OTU number (the number of total OTU observed), and beta diversity based on weighted/unweighted UniFrac distance, were calculated with QIIME (Caporaso et al., 2010). All of the tags in the Core Set (GreenGene data base) were aligned using PyNAST (Version 1.2) to analyze the phylogenetic relationship of the OTUs at the phylum level. The unweighted pair group method with arithmetic mean (UPGMA) clustering was calculated with QIIME.

Statistical Analysis

One-way ANOVA followed by Tukey's *post hoc* comparisons was conducted to detect whether the paired data among different treatments were significantly different (n = 3). Before analysis was performed, non-normal data were log transformed to achieve Gaussian distribution as checked via a Shapiro–Wilk test. Multi-factor ANOVA was performed by using SPSS version 22.0 (IBM Corp., Armonk, NY, United States) to examine the individual and interactive effects of drought stress and cultivar on the growth of broomcorn millet and the effects of drought stress, cultivar, and developmental stage of broomcorn millet on the α diversity index of rhizosphere bacterial community. Principal coordinate analysis (PCoA) based on the weighted UniFrac distance was performed with vegan package in *R* (v 3.4.3) to test the differences between cultivars, drought stress, and developmental stage on bacterial community composition. Variance partitioning was simultaneously carried out to determine the explanation rates of cultivar, drought, and plant stage on community variance across the samples. Variance partitioning was further conducted on the basis of the data collected only at the stage to detect the explanation rate of cultivar on the variation in bacterial community at the flowering stage. Prior to PCoA and variance partitioning analysis, relative abundance data were Hellinger transformed.

A heat map of the 35 most-abundant bacterial phyla was plotted using *R* to systematically analyze the variation patterns in relative abundance among different developmental stages. The relative abundance of the dominant bacterial rhizosphere phyla (i.e., 10 most dominant) and genera (i.e., 50 most dominant) under drought stress was normalized by dividing the relative abundances of the bacteria under control condition to test whether the individual bacterial rhizosphere population of the two cultivars responded differently to drought stress. Two-factor ANOVA was then performed to examine the individual and interactive effects of the cultivar and developmental stage of broomcorn millet on the variations in the ratio. This multiple testing was an efficient method for screening bacterial populations, which differed in their responses to drought between cultivars, although introducing increased type I errors was possible (Finner and Roters, 2002). Bacterial responsive pattern, which was significantly affected by cultivar, was further calculated using the following equation: (relative abundance under drought/relative abundance under control) -1 and compared using a Tukey *post hoc* test at each developmental stage.

RESULTS

Effects of Drought Stress on the Growth of Broomcorn Millet

The phenotype of the two cultivars grown in the control treatment without drought stress significantly differed (**Table 1** and **Supplementary Table S2**). For example, HQR had more internodes, wider diameter culms, and shorter panicles than YL10 (**Supplementary Table S2**). However, drought stress had a marked effect on plant phenotype (**Supplementary Table S2**). The drought-exposed plants were shorter than the control plants. The panicle of the former was also shorter than that of the latter. Furthermore, the dry weight of panicles and grains of the former was lower than that of the latter, but the number of internodes and culm diameter of the drought-exposed plants were higher than those of the control plants (**Table 1** and **Supplementary Table S2**). Two-way ANOVA revealed that only the length of panicle was significantly affected by the interactive effect of drought stress and cultivar (**Table 1**). The effect of drought stress was greater on YL10, which had shorter panicles with lower dry mass than HQR (**Supplementary Figure S2**). Drought stress stimulated significantly the diameter of the culm of HQR compared with that of the control, whereas the diameter of the culm of YL10 was not (**Supplementary Table S2**).

Variation in the α Diversity and Composition of Bacterial Rhizosphere Community

Across treatments, the number of OTUs varied from 1515.3 ± 52.5 to 3004.7 ± 249.9 (mean ± SD), and the Shannon index ranged from 7.1 ± 1.3 to 9.3 ± 0.4. The effects of plant developmental stage, cultivar, and drought stress on the diversity of the bacterial rhizosphere community varied (**Table 2**). Developmental stage had a significantly negative effect on the number of OTUs and Shannon index. However, no significant effect of cultivar, drought stress, or their interaction on either metric was observed (**Table 2**).

TABLE 1 | Results of two-way ANOVA on the effects of cultivar and drought stress on agronomic traits of broomcorn millet as dependent variables (n = 3).

	Cultivar		Drought stress		Cultivar × Drought stress	
	F	*p* value	*F*	*p* value	*F*	*p* value
Plant height (cm)	0.104	0.755	22.435	0.001	0.739	0.415
Number of internode	20.167	0.002	0.167	0.694	1.500	0.256
Culm diameter (cm)	74.064	<0.001	23.170	0.001	0.191	0.673
Panicle length (cm)	4.009	0.080	51.136	<0.001	21.827	0.002
Dry weight of panicle (g)	5.516	0.047	45.991	<0.001	0.298	0.600
Grain weight per plant (g)	1.353	0.278	42.148	<0.001	0.023	0.883

TABLE 2 | Results of three-way ANOVA on the effects of cultivar, drought stress, and developmental stage on the richness and evenness of the bacterial community within the rhizosphere of broomcorn millet (n = 3).

Factor	OTU number		Shannon index	
	F	*p* value	*F*	*p* value
Cultivar	1.235	0.277	2.854	0.104
Developmental stage	30.647	<0.001	3.969	0.032
Drought	0.203	0.657	0.021	0.887
Cultivar × Drought	2.243	0.147	1.244	0.276
Cultivar × Developmental stage	0.050	0.951	0.126	0.882
Developmental stage × Drought	1.588	0.225	1.103	0.348
Cultivar × Developmental stage × Drought	2.410	0.111	1.358	0.276

Similarly, the rhizosphere bacterial community showed variation in the PCoA community structure as a function of plant developmental stage (**Figure 1A**). At the development stages, the result of variance partitioning demonstrated that 12.3% of variance could be explained by the developmental stage (adjusted R^2 = 12.3%, F = 5.6, p = 0.004), whereas the effect of drought (adjusted R^2 = 0.0%, F = 0.6, p = 0.682) or cultivar (adjusted R^2 = 0.8%, F = 1.3, p = 0.232) was not significant. At the flowering stage, the rhizobacterial assemblages of HQR and YL10 were obviously different from each other (**Figure 1A**). Cultivar explained a small but significant fraction of the total variation in the bacterial rhizosphere community composition at the specific stage (adjusted R^2 = 8.0%, F = 1.9, p = 0.036). UPGMA analysis showed the same trends as that of PCoA (**Figure 1B**). Rhizobacterial communities from HQR and YL10 rhizospheres at the jointing and grain filling stages were clustered into one clade. The bacterial community structures of these samples were more similar to one another compared with that of the samples collected at the flowering stage (**Figure 1B**).

Variation in Bacterial Abundance

We recorded a total of 923 core OTUs in all of the samples, which were composed of Proteobacteria (33.8%), Actinobacteria (21.0%), Acidobacteria (10.7%), Bacteroidetes (8.2%), Chloroflexi (6.3%), Gemmatimonadetes (5.9%), Firmicutes (3.5%), Verrucomicrobia (2.9%), and Planctomycetes (2.7%, **Supplementary Figure S3**). Across all samples, these nine bacterial phyla, together with TM7, were the 10 dominant phyla detected in this study, which represented 93.6–96.3% of the

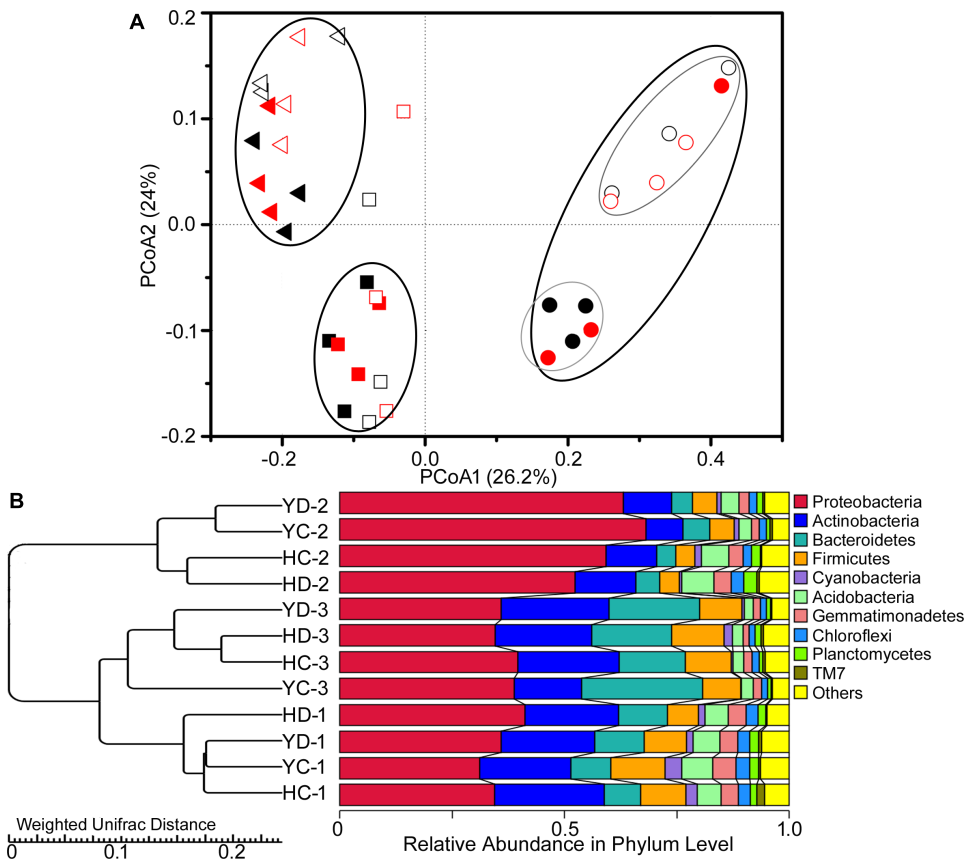

FIGURE 1 | Variations in the composition of the bacterial rhizosphere community of HQR and YL10 at distinct developmental stages and under drought stress. **(A)** Principal coordination analysis (PCoA) based on the weighted UniFrac distances. Square, jointing stage; circular, flowering stage; triangle, grain filling stage. Black, control; red, drought treatment. The solid square, circular, and triangle represent HQR, whereas the open ones represent YL10. **(B)** UPGMA clustering using Weighted UniFrac Distances. H = HQR; Y = YL10; C = control, D = drought stress; 1 = jointing stage; 2 = flowering stage; 3 = grain filling stage.

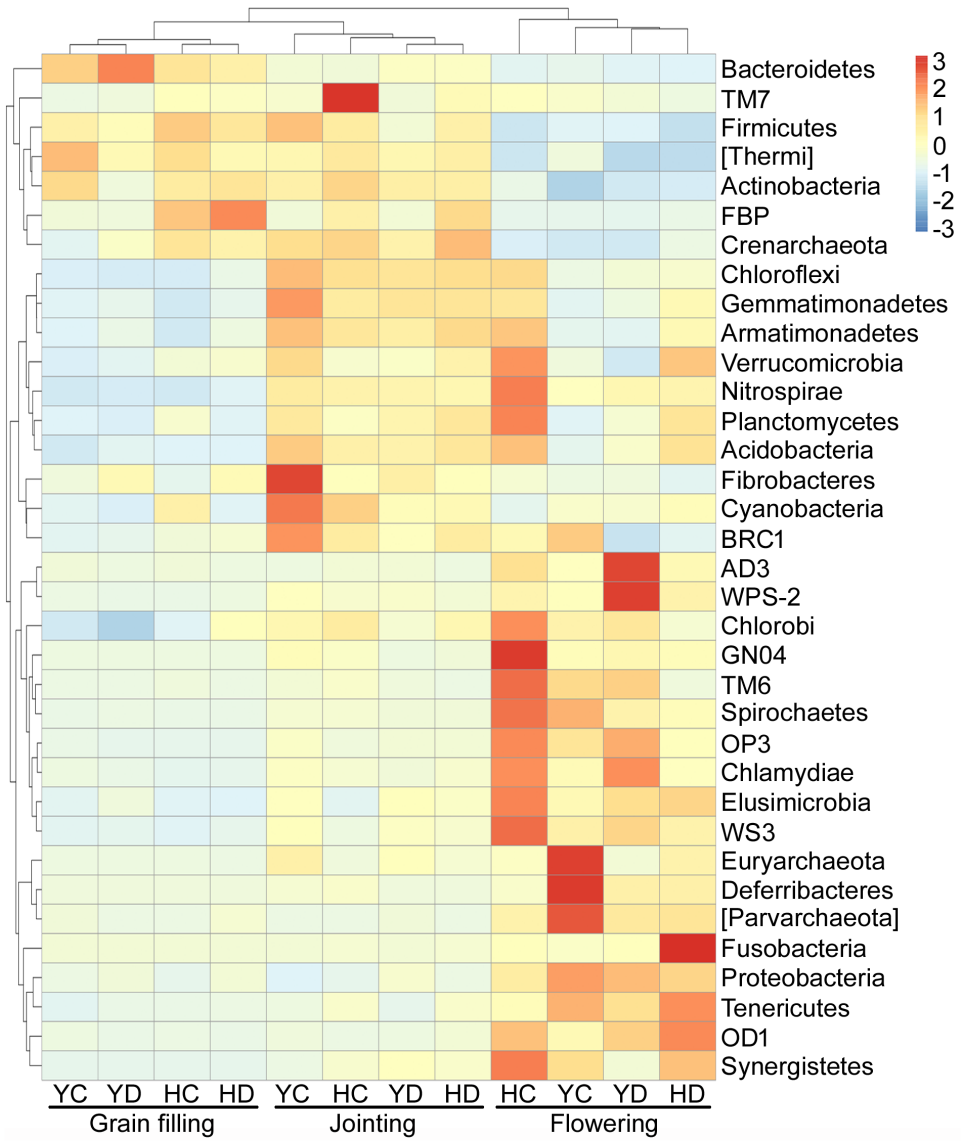

FIGURE 2 | Distribution heat map of bacterial phyla arranged in terms of the developmental stage of broomcorn millet. H = HQR; Y = YL10; C = control, D = drought stress.

assigned sequences (**Supplementary Table S3**). The relative abundances of these bacterial phyla in each rhizosphere varied with plant developmental stage except Cyanobacteria and TM7, which were consistently present at 1.31 and 0.55%, respectively (**Supplementary Table S3**).

The variations in the relative abundances of bacterial phyla were further analyzed because the developmental stage of broomcorn millet drove the changes in the community composition of rhizosphere bacteria (**Figure 1**), and three distinct groups of bacteria with respect to developmental stage were found (**Figure 2**). The relative abundances of Proteobacteria, AD3, WPS-2, Chlorobi, GN04, TM6, Spirochaetes, OP3, Chlamydiae, Elusimicrobia, WS3, Euryarchaeota, Deferribacteres, Parvarchaeota, Fusobacteria, Tenericutes, OD1, and Synergistetes were enriched at the flowering stage relative to the jointing and grain filling stages (**Figure 2**). Actinobacteria, Bacteroidetes, Firmicutes, and Thermi were enriched in the jointing and grain filling stages (**Figure 2**). The third group, including Acidobacteria, Chloroflexi, Gemmatimonadetes, Planctomycetes, Cyanobacteria, Verrucomicrobia, Nitrospirae, Armatimonadetes, Fibrobacteres, and BRC1, were specifically enriched at the jointing stage and then decreased with plant development (**Figure 2**).

A significant effect of cultivar on the response of Actinobacteria to drought stress was detected through two-way ANOVA (**Supplementary Table S4**). Actinobacteria mainly accumulated in the rhizosphere of YL10, whereas an opposite pattern was found in the rhizosphere of HQR under drought

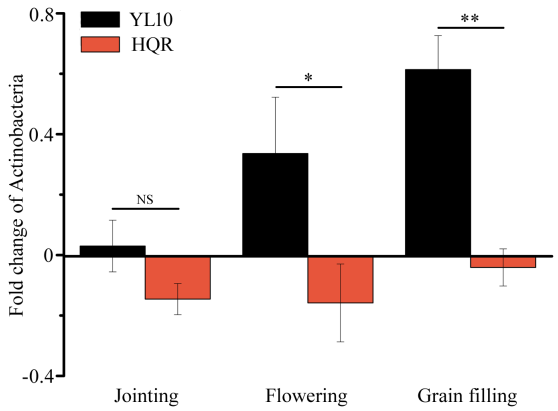

FIGURE 3 | Effect of drought stress on the relative abundance of Actinobacteria in the rhizosphere of broomcorn millet. The fold change of Actinobacteria was calculated using the following equation: (relative abundance under drought/relative abundance under control) −1. Error bars are standard error over three independent replicates (n = 3). NS indicates no significant difference between HQR and YL10; * $p < 0.05$; ** $p < 0.01$.

stress (**Figure 3**). Five of the 50 most dominant bacterial genera were affected by the cultivar and/or developmental stage of broomcorn millet (**Supplementary Table S5**). In these genera, developmental stage significantly affected the responses of *Adhaeribacter* and *Acinetobacter* to drought stress, whereas cultivar influenced the responses of *Lysobacter*, *Cellvibrio*, *Streptomyces*, and *Acinetobacter* (**Supplementary Table S5**). The ratio of drought-exposed *Acinetobacter* to the control was also significantly affected by the interactive effect of developmental stage and cultivar (**Supplementary Table S5**). The relative abundance of *Acinetobacter* was notably upregulated in the rhizosphere of HQR under drought stress but was downregulated in the rhizosphere of YL10 (**Figure 4A**). By contrast, the relative abundances of *Lysobacter*, *Streptomyces*, and *Cellvibrio* in the rhizosphere of YL10 were mainly stimulated by drought stress compared with that of HQR (**Figures 4B–D**).

DISCUSSION

Plant Stage Is the Major Factor Driving the Succession of Bacterial Rhizosphere Community of Broomcorn Millet

The effects of plant developmental stage on the diversity, dynamics, and composition of bacterial rhizosphere communities have been comprehensively elucidated. For example, the growth of a seedling to a mature plant significantly affects the microbial community structure in the rhizosphere of various plants,

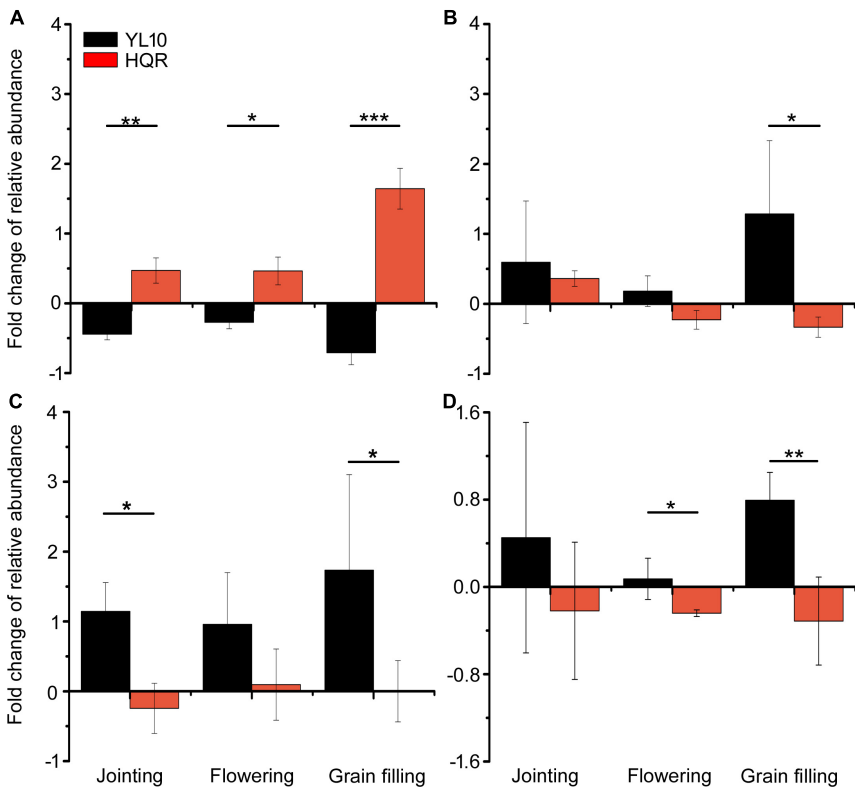

FIGURE 4 | Effects of drought stress on the relative abundances of *Acinetobacter* **(A)**, *Lysobacter* **(B)**, *Streptomyces* **(C)**, and *Cellvibrio* **(D)** in the rhizosphere of broomcorn millet. The fold change of each bacterial genera was calculated using the following equation: (relative abundance under drought/relative abundance under control) −1. Error bars are standard error of three independent replicates (n = 3). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

including *Arabidopsis* (Micallef et al., 2009; Chaparro et al., 2014), maize (Baudoin et al., 2003; Haichar et al., 2008), sweet potato (Marques et al., 2014), pea, wheat, sugar beet (Houlden et al., 2008), and *Caragana liouana* (Na et al., 2017a). In our study, similar effects were observed, and significant changes in the rhizosphere bacterial community structure and α diversity were found as the millet matured from the seedling to the grain filling stage (**Figure 1** and **Table 2**).

Changes in bacterial communities may be explained by the outputs of the plants themselves. Root exudate quantity, quality, and exudation rate vary with plant development and rapidly affect the growth of specific microorganisms in the rhizosphere, especially fast-growing microbes, and can cause structural divergences (Aulakh et al., 2001; Baudoin et al., 2003; Chaparro et al., 2014). As such, the effects of plant developmental stages on microbial community diversity and composition may be mediated by the varying root exudation patterns (Dunfield and Germida, 2003; Chaparro et al., 2014).

As broomcorn millet matured, the richness and evenness of the bacterial community in the rhizosphere gradually decreased. These results could be attributed to the change in the relative allocation of photosynthesis products between above and belowground parts of the plants (Brady and Weil, 1999; Brimecombe et al., 2001). After the flowering stage, the photosynthates are mainly used to maintain seed development and less are allocated to root exudates (Brady and Weil, 1999; Lucas García et al., 2010). This variation in root exudates may then disrupt the recruitment of bacteria and weaken the dominance of copiotrophic populations in the rhizosphere (**Figure 2**).

The changes in root exudation patterns might drive specific rhizosphere bacterial assemblages based on the developmental stage of the millet. The unique bacterial rhizosphere assemblage of each stage might represent the specific physiological needs or requirements of broomcorn millet at a particular stage because root exudates and physiological status varied with plant development (Brady and Weil, 1999; Brimecombe et al., 2001), driving the colonization of particular functional microbial populations in the rhizosphere (Wasson et al., 2006). For example, the enrichment of some less dominant bacteria at the flowering stage relative to the jointing and grain filling stages indicated a specific variation in the diversity and quantity of root depositions during plant flowering (**Figure 3**). Plants can secrete high levels of low-molecular-weight organic acids to the rhizosphere to acquire more nutrients during flowering than fruiting (Aulakh et al., 2001; Lucas García et al., 2010). Aulakh et al. (2001) also suggested that the highest exudation rates are detected at the flowering stage among the stages of the life cycle of rice.

Stage-Dependent Effects of Cultivar on the Development of Bacterial Rhizosphere Community Composition

Comparisons between plant species and cultivars have demonstrated the differences in microbial rhizosphere communities when community structure and function are considered at specific times (Haichar et al., 2008; Peiffer et al., 2013; Marques et al., 2014). In the present study, the PCoA results showed obvious differences in the structure of the bacterial rhizosphere community between HQR and YL10 at the flowering stage only (**Figure 1A**). This result demonstrated that the effects of cultivars on the rhizobacterial community composition of broomcorn millet were plant stage dependent. Previous studies confirmed that root exudation pattern (i.e., in both quantity and quality) among species is genetically controlled (Rengel, 2002). As such, this unique cultivar effect of broomcorn millet on its rhizosphere bacterial community composition might precisely reflect the genetic difference in HQR and YL10. From this perspective, the most obvious divergence in physiological status during the life cycle of HQR and YL10 might occur at the flowering stage, following the results of the PCoA.

However, we have yet to determine why the variation in community structure occurs only at the flowering stage of the two cultivars. The flowering stage is the transition phase from vegetative growth to reproductive growth in herbaceous plants. After the flowering stage, photosynthesis products are mainly allocated to and accumulated in the seeds (Aulakh et al., 2001). Our results showed that the plant height and culm diameter of HQR were larger than those of YL10, whereas the panicle length of HQR was significant shorter than that of YL10; nevertheless, they had the same grain weight per plant (**Supplementary Table S2**). These results indicated that YL10 allocated more carbon to increase grain yield relative to that of HQR after flowering. As such, the differences in photosynthate redistribution between the two broomcorn millet cultivars at the flowering stage could partly explain the divergence of bacterial rhizosphere community composition. Another reason might be the differences in the concentration and composition of the root exudates of the two cultivars (Aulakh et al., 2001; Lucas García et al., 2010).

Bacterial Rhizosphere Community of Broomcorn Millet Is Robust Under Drought Stress

The results of a commonly used drought-stress method (Earl, 2003) showed that neither the α diversity nor the composition of the bacterial rhizosphere community of the two broomcorn millet cultivars changed in response to drought stress (**Figure 1** and **Table 2**). These results were inconsistent with our hypothesis 2, as well as the results of previous studies, suggesting that soil moisture is an important factor that affects the composition of soil microbial communities (Griffiths et al., 2011; Na et al., 2017b). Three reasons might explain this paradox. First, 15 days of drought treatment might be too short to affect the rhizosphere bacterial community structure of broomcorn millet. Although the growth of broomcorn millet was significantly inhibited under drought stress (**Supplementary Table S2**), the rhizosphere bacterial communities did not respond to the lack of water. Second, broomcorn millet only requires a small amount of water for growth and development (Baltensperger, 2002; Lágler et al., 2005; Jana and Jan, 2006; Gulati et al., 2016). As such,

a short period of drought stress at the seedling stage might not affect the interactions between the root of broomcorn millet and the associated soil microbes in the rhizosphere. Third, the diversity and structure of rhizobacterial community detected under control and drought stress conditions might represent the normal status of broomcorn millet grown in arid and semi-arid areas. Thus, the bacterial rhizosphere community of broomcorn millet as a whole was robust under drought. The drought tolerance of broomcorn millet and its local microbiota might be indicative of co-evolutionary mechanisms that have occurred over time.

Cultivar-Dependent Responses of Specific Rhizosphere Bacterial Populations to Drought Stress

Xu et al. (2018) demonstrated that drought stress stimulates the abundance and activity of monoderm bacteria, such as Actinobacteria and Firmicutes, in the rhizosphere of *Sorghum bicolor* via the shifts in root metabolism. Consistent with this finding, our results confirmed that a proportion of Actinobacteria accumulated in the rhizosphere of YL10 under drought stress (**Figure 3**). However, the response pattern of Actinobacteria differed between HQR and YL10 (**Figure 3**), which inferred that the recruiting mechanism of these bacterial species depended on the cultivar or genotype of host plants. Xu et al. (2018) suggested that this difference may be associated with the altered metabolism of roots under drought. Further metabolic and genetic experimentations would help verify this hypothesis.

Actinobacteria species have great economic importance to humans because of their functions in agricultural and forest soil ecosystems, such as organic matter decomposition (Steger et al., 2007), nitrogen fixation (Gtari et al., 2007), antibiotic production (Jiang et al., 2017), and plant defense induction (Conn et al., 2008). *Streptomyces* and *Lysobacter* are widely studied bacterial genera in the production of a wide range of antibiotic and bioactive secondary metabolites that may be useful against phytopathogens (Haruo et al., 2003; Hashizume et al., 2004). *Acinetobacter* spp. exhibit plant growth-promoting properties, including phosphate solubilization, nitrogen fixation, and auxin production (Gulati et al., 2009; Sachdev et al., 2010). Consistent with the functions of these bacteria, our results indicated that HQR might maintain its growth performance under drought stress by recruiting plant growth-promoting bacteria, whereas YL10 might recruit antibiotic-producing bacterial species. HQR strengthened its ability to acquire nutrients, whereas YL10 enhanced its health status in the rhizosphere under drought stress. Although this observation was not confirmed by our study, our results suggested that the two cultivars of broomcorn millet might have evolved distinct response mechanisms to drought stress through interactions with specific bacterial populations. The influence of plant–microbe interactions in the rhizosphere on the drought tolerance of broomcorn millet, as well as the underlying genetic mechanism controlling these processes, should be further examined.

Given that cultivar and drought stress had no significant effect on the α diversity and composition of bacterial rhizosphere community (**Figure 1** and **Table 2**), considering the functions of specific microbes in the rhizosphere might be more important than comparing the bacterial rhizosphere community as a whole to understand the drought resistance mechanism of broomcorn millet. Further metabolomic, metagenomic, and metatranscriptomic analyses are needed to assess the function of microbial associations to understand the co-evolution mechanisms between this oldest drought-tolerant crop and its local microbiota.

CONCLUSION

With Illumina MiSeq technology, our study revealed that (1) plant stage was the major driver of the α diversity and structure of bacterial rhizosphere communities of two broomcorn millet cultivars; (2) the effect of broomcorn millet cultivar on the bacterial community composition was stage dependent; and (3) the bacterial rhizosphere community of both cultivars was robust to drought stress. Overall, these results might contribute to our understanding of the co-evolutionary mechanisms of drought-tolerant broomcorn millet and its associated microbiome.

AUTHOR CONTRIBUTIONS

XN and XC designed the research. XC, SL, JW, HW, and LC carried out the pot experiments. XN, CM, SM, and PX performed DNA extraction and detection. XN analyzed the data and drafted the manuscript. XC and ZQ supervised the study and participated in its coordination. All authors read and approved the final manuscript.

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Variable Influences of Water Availability and Rhizobacteria on the Growth of *Schizachyrium scoparium* (Little Bluestem) at Different Ages

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There is significant interest in understanding the role of plant growth-promoting rhizobacteria (PGPR) in alleviating different types of plant stress. *Schizachyrium scoparium* (little bluestem) is a moderately drought tolerant, perennial bunchgrass native to North America. The goal of this experiment was to evaluate whether the addition of a bacterial root isolate in the *Pseudomonas* genus promoted the growth of *S. scoparium* with changes in water availability. *Pseudomonas* are common rhizobacteria and have been shown to improve plant growth. It was hypothesized that plants inoculated with the PGPR strain would have greater growth and health, and would be less affected by shifts in water availability. *Pseudomonas* strains were isolated from the roots of native *S. scoparium* plants. After germination, *S. scoparium* seedlings were subjected to four treatment groups: low water; high water; low water with PGPR; and high water with PGPR. The experiment was run three times with plants at different starting ages; 14-, 28-, and 70-day-old plants. The effects of the water and PGPR treatments were variable between the experimental trials. There were no significant effects of the water treatments on plant growth in Trial 1 (14-day-old plants) or Trial 2 (28-day-old plants), however, there was a significant negative effect of the high watering treatment on the shoot length and biomass in Trial 3. High water availability was significantly associated with greater plant health in Trial 1, but appeared to reduce plant health in Trials 2 and 3. The PGPR treatment appeared to promote root growth and biomass in Trial 2, and was associated with greater plant health in all three trials, especially when paired with the low water treatment. Results from a permutational MANOVA indicate that plant growth was significantly different between the trials due to differences in the starting age of the plants and the duration of the experiments. Thus, methodological choices, such as plant life history stage and experiment duration, may affect the response of plants to PGPR in the rhizosphere. This research provides an insight into the interactions between PGPR and water availability on the growth and health of native plants.

Keywords: PGPR, native grassland species, environmental stress, water limitation, rhizosphere manipulation, rhizosphere microbes

INTRODUCTION

Interactions with both the abiotic and biotic components of a plant's environment significantly impact its growth and reproduction (Lambers et al., 2008; Suzuki et al., 2014). These include abiotic resources, such as water, temperature, light, and nutrients, as well as other organisms, including competitors, herbivores, pathogens, and beneficial microorganisms. Root-associated microorganisms, also known as rhizosphere microorganisms, form complex, and often beneficial, interactions with plants. They can promote plant growth by shifting biotic conditions in soil, primarily through decreasing infection by microbial pathogens (Cook et al., 1995; El-Sayed et al., 2014). Rhizobacteria may also alter abiotic conditions in the soil environment, for example, through increasing nutrient availability (Richardson et al., 2009; Pii et al., 2015). Likewise, rhizobacterial growth and activity is strongly affected by soil environmental conditions (Schimel et al., 2007; Berg and Smalla, 2009; Philippot et al., 2013; terHorst et al., 2014; Xiao et al., 2017), and plants appear to have a strong selective pressure on the bacteria that colonize the rhizosphere through the production of root exudates (Berg and Smalla, 2009; Ling et al., 2011; Patel et al., 2015; Guyonnet et al., 2018). The complex interactions between plants, rhizobacteria, and the abiotic environment are critical to understanding the many factors affecting plant growth.

Plants have a suite of physiological and morphological mechanisms to cope with abiotic (e.g., drought, flood, high salt) and biotic (e.g., herbivory, attack from pathogens) stress (Lambers et al., 2008). For example, either increasing or decreasing water-use efficiency (the ratio of photosynthesis to stomatal conductance) can improve plant fitness under drought, depending on the timing of drought onset (Heschel et al., 2002; Heschel and Riginos, 2005). Production of secondary metabolites and many proteins can function as defense against herbivores and pathogens (Walling, 2000; Wittstock and Gershenzon, 2002). Morphological examples include structural defense against herbivory, such as spines and pubscence (Hanley et al., 2007), and specialized tissues to promote gas exchange between the soil, root, and shoot in flooded soil, an important trait for flood tolerance (Mommer et al., 2006; Colmer and Voesenek, 2009). Recent research has shown that interactions with beneficial rhizobacteria can also confer resistance to various abiotic stressors such as drought (Kang et al., 2014; Naylor and Coleman-Derr, 2018).

Plant responses to drought influence the function of the root microbiome (Antoun, 2013; Badri et al., 2013). Microbes utilize amino acids, plant hormones, and organic compounds directly released from the plant roots (Henry et al., 2007; Antoun, 2013; Canarini et al., 2016; Calvo et al., 2017). Often the root exudates secreted by plants shift when a plant is under stress (Patel et al., 2015; Naylor and Coleman-Derr, 2018). Stress, for example, caused by high levels of solar radiation, nutrient deficiency, or drought, stimulates the production of 1-aminocyclopropane-1-carboxylate (ACC), a precursor for the plant stress hormone ethylene (Lynch and Brown, 1997; Yang et al., 2009). Some strains of rhizobacteria are able to degrade ACC using the enzyme ACC deaminase, which minimizes the plant's stress response (Glick, 2004; Zahir et al., 2008; Timmusk et al., 2011; Saikia et al., 2018). Many different plant-derived nutrients promote rhizobacteria growth and activity, which drives a feedback in which bacteria solubilize essential plant nutrients, such as phosphorus and potassium, which in turn drives overall plant productivity (Micallef et al., 2009; Naylor and Coleman-Derr, 2018).

Plant growth-promoting rhizobacteria (PGPR) are usually free-living rhizosphere bacteria that comprise a stable part of the rhizosphere microbial community (Mayak et al., 2004; Grönemeyer et al., 2012; Antoun, 2013; Timmusk et al., 2014). PGPR form close associations with plants which lead to increases in overall plant growth through the production of growth-stimulating phytohormones and metabolites that promote plant growth (Mayak et al., 2004; Belimov et al., 2009; Dimkpa et al., 2009; Timmusk et al., 2014). In addition, PGPR outcompete and suppress pathogens (Cook et al., 1995; Pierson and Pierson, 1996; Whipps, 2001). PGPR also help plants via the synthesis of signaling molecules that trigger protective responses within the plant (Cho et al., 2008; Marasco et al., 2012), which affects plants' susceptibility to stress. In addition, while undergoing stress plants may excrete higher levels of organic acids, which increases the recruitment of PGPR (Patel et al., 2015). The root architecture of plants, under normal and stress conditions, may also play an important role in recruiting PGPR to colonize root surfaces (Saleem et al., 2018). The dynamics of how rhizobacteria affect plants' response to stress is highly dependent on environmental factors.

Soil conditions exert a strong influence on the way in which rhizobacteria shape plants' response to certain stressors. In soils with high levels of contaminants, PGPR decrease the negative effects of toxins on plant growth by degrading the contaminants, and by affecting the expression of plant genes encoding for stress responses (Gurska et al., 2015; Zhang et al., 2017). Soil organic matter concentrations also affect the degree to which PGPR stimulate plant growth. Results from recent studies indicate that soil organic matter and PGPR inoculation have a synergistic effect on plant growth, especially for experiments involving organic matter additions and amendments in degraded soils (Çakmakçi et al., 2006; Mengual et al., 2014). Plants and rhizobacteria, as well as their interactions, are strongly influenced by soil water availability. For example, Lau and Lennon (2011) observed that plant acclimation to drought stress was promoted by shifts in rhizosphere microbial community structure. PGPR promote plant acclimation to water limitation by degrading ACC, thereby dampening plants' stress response pathways (Saleem et al., 2007; Zahir et al., 2008; Belimov et al., 2009; Saikia et al., 2018). In addition, Sandhya et al. (2009) isolated multiple *Pseudomonas* strains that produced exopolysaccharides which promoted the growth of seedlings in response to drought stress (Sandhya et al., 2009). For older plants, inoculation with PGPR may indirectly alleviate drought stress by stimulating root growth and increasing root surface area, thereby increasing water uptake by plants (Marasco et al., 2013; Wang et al., 2014).

Bacteria in the *Pseudomonas* genus are one of the most dominant and well characterized PGPR in literature, and their role in improving stress tolerance amongst a variety of plant host species has been well-documented (Podile and Kishore, 2006; de Bruijin et al., 2007; Sandhya et al., 2010; Beneduzi et al., 2012;

Gera Hol et al., 2013; Vurukonda et al., 2016; and others). *Pseudomonas* is one of the most dominant PGPR genera found natively in soils (Cook et al., 1995; El-Sayed et al., 2014; Wang et al., 2014). *Pseudomonas* are able to colonize the roots of several different plant species, and their root associations with plants enhance root length, shoot length, and biomass under a range of environmental conditions (Timmusk et al., 2014; Oteino et al., 2015; Lally et al., 2017). *Pseudomonas* strains assist plants undergoing stress by producing antibiotic compounds and inducing plant immune defenses, which defend the plant from pathogen attachment and invasion (Cook et al., 1995; Pierson and Pierson, 1996; Chen et al., 2000). Experimental evidence has documented the effectiveness of PGPR in the *Pseudomonas* genus in promoting plant growth in response to water stress (Çakmakçi et al., 2006; Zahir et al., 2008; Sandhya et al., 2009; Gurska et al., 2015; Saikia et al., 2018). *Pseudomonas* strains, such as *P. putida*, likely modulate plant physiology and plant tissue stoichiometry during drought through the production of signaling molecules that shift hormone and antioxidant production (Kang et al., 2014). Thus, research investigating the interactions between PGPR and plants has focused on different aspects of *Pseudomonas* effects on plant growth under a range of conditions. However, a majority of this research has evaluated PGPR effects on crop plants, and there is relatively little known about PGPR effects on wild species, especially grasses.

The goal of this research was to evaluate the individual and synergistic effects of a native *Pseudomonas* rhizobacterium and water availability on the growth of the perennial bunchgrass *Schizachyrium scoparium* (Michx.) Nash (little bluestem). *S. scoparium* is native to grasslands throughout North America and occurs in many different soils and ecosystems across a wide precipitation gradient (Steinberg, 2002; Tober and Jensen, 2013; PRISM Climate Group, Oregon State University, 2015; USDA-NRCS, 2019). Furthermore, *S. scoparium* experiences periodic water limitation in its native range and is moderately tolerant to drought conditions (Mueller and Weaver, 1942; Maricle and Adler, 2011; Tober and Jensen, 2013; Maricle et al., 2015). However, it has historically succumbed to prolonged extreme drought (Weaver et al., 1935; Weaver and Albertson, 1939; Weaver and Albertson, 1943). *S. scoparium* accesses most of its water in relatively shallow soil layers (∼5–50 cm, Eggemeyer et al., 2009; Mueller et al., 2013), and its response to water limitation is to resist the negative effects of drought through physiological adjustments (Hake et al., 1984; Knapp, 1984; Maricle and Adler, 2011; Maricle et al., 2015). Due to its adaptability to various soil conditions, *S. scoparium* serves as an important forage species for livestock and wildlife, and can be used in erosion control (USDA-NRCS, 2002). In addition, the response of *S. scoparium* and other native grass species to a range of environmental conditions, especially with respect to water availability, is especially critical in the face of global change. Climate projections for Central Texas, where this study took place, forecast an increase in extreme drought frequency over the coming decades (Chen et al., 2012; Deng et al., 2018). Thus, it is beneficial to understand the extent of *S. scoparium*'s tolerance to shifts in water availability, as well as the potential for native PGPR strains to affect the response of *S. scoparium* to changing environmental conditions.

This research was designed to test the hypothesis that the addition of a single PGPR *Pseudomonas* strain will cause a significant positive effect on the growth of *S. scoparium* across a range of water availability, and in plants of different ages. Specifically, we predicted that the addition of a PGPR strain at regular intervals will result in improved observable plant health, longer root and shoot lengths, and greater plant biomass compared to plants that did not receive the bacterial addition. In addition, we predicted that the effect of PGPR addition would vary with water availability. Importantly, this study also evaluated the repeatability of these effects across three different trials of a full-factorial greenhouse experiment, while varying the starting age of plants among trials. Results from this research show that supplementing the rhizosphere microbiome with a native PGPR can increase plant growth. However, we observed a high degree of variability in the response of *S. scoparium* to PGPR addition between the trials. Our results illustrate the importance of methodological choices, such as the starting age of plants and experiment duration, in the responses of plants to PGPR addition. Furthermore, most research on plant-microbe interactions has been performed on semi to highly domesticated agricultural plants (Cook et al., 1995; Chen et al., 2000; Belimov et al., 2009; Marasco et al., 2013), which are often ecologically distinct compared to many wild species (Milla et al., 2015). Therefore, studying plant–microbe interactions in a wild species represents an important contribution to understanding how rhizobacteria affect plant growth and mediate response to stress in natural ecosystems.

MATERIALS AND METHODS

Overview of Experimental Design

In order to test the effect of a native *Pseudomonas* rhizobacteria on *S. scoparium* growth under both non-stress and stress conditions, we used a two-way full factorial experimental design with water availability and the addition of a *Pseudomonas* culture as factors. The four treatment groups were: (1) well-watered plants with the addition of uncultured, sterile *Pseudomonas* broth (W); (2) plants watered at a reduced, or drought, level with the addition of uncultured, sterile *Pseudomonas* broth (D); (3) well-watered plants with the addition of a *Pseudomonas* culture grown in *Pseudomonas* broth (BW); and (4) watering at a reduced level and the addition of a *Pseudomonas* culture grown in *Pseudomonas* broth (BD). Three trials were conducted, with sixty plants for each trial and 15 individuals per treatment.

PGPR Isolation, Characterization, and Growth

Wild samples of *S. scoparium* were collected from Blunn Creek Nature Preserve in Austin, TX, United States (30°13′57.59″ N; Longitude: 97°44′52.20″ W) in order to isolate native PGPR *Pseudomonas* strains from its roots. For the first experimental trial *S. scoparium* samples were collected on May 31, 2016. To

isolate the strain used in the second and third experimental trials *S. scoparium* samples were collected on February 26, 2017.

To isolate *Pseudomonas* strains, surrounding bulk soil was removed from the roots. Then the roots were placed in 500 mL of sterilized *Pseudomonas* broth containing 20 g/L enzymatic digest of soybean or tryptone, 1.4 g/L magnesium chloride, 10 g/L potassium sulfate, and 0.025 g/L Irgasan, for 1 h. The roots were then removed from the broth, and the inoculated broth was incubated shaking at 30°C for 24 h. After 24 h, an aliquot of the bacterial culture was transferred to a *Pseudomonas* agar plate using the streak plate method, and was incubated at 30°C for 24 h. A single *Pseudomonas* isolate from this agar plate was transferred to a fresh *Pseudomonas* agar plate in order to obtain a pure culture. The isolates were visualized using the gram staining method and 100× bright field microscopy. In addition, the identity of the isolates as *Pseudomonas* was confirmed using microscopy and the Gen III BIOLOG system (Hayward, CA, United States[1]). The isolates were then used for plant inoculation during the experiment (see section "Experimental Treatments: Water Availability and PGPR"). *Pseudomonas* strain A was isolated using these methods in May 2016. In January 2017 it was not possible to revive this isolate from a glycerol stock stored at −20°C. As a result, *Pseudomonas* strain B was isolated using a different *S. scoparium* collected from the same area of Blunn Creek Nature Preserve in February 2017. Strain A was used for Trial 1, in which the experiment started with 14-day-old plants (**Table 1**). Strain B was used in Trials 2 and 3, which started with 28- and 70-day-old plants, respectively (**Table 1**). Following pure culture isolation, strains were preserved as a glycerol stock by adding 500 μl of bacterial culture with 500 μl of 50% sterilized glycerol, and stored at 20°C.

Following the isolation of strain A, a growth curve was performed to characterize the rate at which the bacterium would achieve its maximum cell density. The optical density (OD) was measured at 600 nm using a spectrophotometer every hour for 10 h. Strain A approached its maximum OD, 0.489 ± 0.005, after growing in *Pseudomonas* broth for 9 h at 30°C. Similar methods were used to characterize the growth of strain B. Strain B grew more slowly, and thus the OD was measured at 600 nm for 24 h. After 24 h strain B grew to its maximum OD of 0.389 ± 0.005. The times at which these isolates reached their maximum ODs were used in the design of the bacterial addition treatments (see section "Experimental Treatments: Water Availability and PGPR").

Source of Plant Material for Greenhouse Experiments

Schizachyrium scoparium seeds (Central Texas mix) were purchased from Native American Seed (Junction, TX, United States[2]). Seeds were stored in an air-conditioned laboratory at room temperature until they were germinated for the experimental trials.

Experimental Treatments: Water Availability and PGPR

Drought and bacterial inoculations were conducted to understand how drought and the addition of a *Pseudomonas* isolate affect the growth of *S. scoparium*. There were two different methods in which the treatments were allocated. Method one was used for Trial 1 (14-day-old plants at the start of the experiment) and Trial 3 (70-day-old plants). Method two was used for Trial 2 (28-day-old plants). For each trial, 60 seedlings were transplanted into 1 gallon pots with a soil ratio of 3:1 potting soil and sand. The seedlings were haphazardly chosen, initial root length and shoot length were measured, and placed into the pots labeled according to treatment.

Treatment Method One

The bacteria treatment (B) included 10 mL of a *Pseudomonas* culture grown in *Pseudomonas* broth diluted with 10 mL of DI water (20 mL total volume). Plants that did not receive the bacterial addition treatment received sterile *Pseudomonas* broth with DI water (20 mL) that had not been cultured to act as a control. Plants that were well-watered (HW) were watered with 100 mL of water every other day. The low water treatment plants (LW) did not receive any water in addition to the 20 mL of sterile *Pseudomonas* culture or broth diluted with DI water every other day. **Table 1** includes a summary of the amount of total liquid received by plants in each experimental trial.

The mean annual precipitation range of *S. scoparium* is 10–60 inches of rain per year, according to Tober and Jensen (2013). The mean annual range for the Central Texas *S. scoparium* mix used in this study is 20–40 inches (see text footnote 2). Thus, the

[1]www.biolog.com

[2]http://www.seedsource.com

TABLE 1 | A summary of the methodological differences between the trials.

Trial	Starting age	Experiment duration	Bacterial strain	Amount of liquid added per addition	Total amount of liquid received during the experiment	Equivalent inches of rain/year
1	14 days	8 days	A	HW: 120 LW: 20	HW: 480 mL LW: 80 mL	HW: 49 in LW: 8 in
2	28 days	25 days	B	HW: 25 LW: 20	HW: 300 mL LW: 240 mL	HW: 10 in LW: 8 in
3	70 days	25 days	B	HW: 120 LW: 20	HW: 1560 mL LW: 260 mL	HW: 49 in LW: 8 in

HW indicates the High Water treatment while LW is an abbreviation for the Low Water treatment.

high water treatment, which was approximately equivalent to 49 inches of rain per year (**Table 1**), is near the upper quadrant of the mean annual precipitation tolerance of *S. scoparium* overall, and above the range for Central Texas. By contrast, the low water treatment was roughly equivalent to plants receiving eight inches of rain per year, which is slightly below the minimum mean annual precipitation for *S. scoparium* overall, but well below the range for Central Texas *S. scoparium*.

Treatment Method Two

Plants receiving the bacterial addition treatment (B) received 10 mL of *Pseudomonas* culture grown in *Pseudomonas* broth every other day. The plants that did not receive the bacterial treatment received 10 mL of sterile *Pseudomonas* broth every other day. Plants that were well-watered (HW) received 15 mL of water every other day, whereas plants receiving the low water treatment (LW) received 10 mL of water every other day. The high water treatment for this trial was equivalent to approximately 10 inches of rain per year, while the low water treatment was equivalent to 8 inches of rain per year (**Table 1**). Thus, the high and low water treatments were just above and below the minimum mean annual precipitation for *S. scoparium* overall, and well below the minimum for Central Texas *S. scoparium*.

Trials

In Trial 1 *S. scoparium* seeds were germinated on May 18, 2016 on a sand bed and were misted daily. Sprouting occurred 2 weeks after the seeds were placed on a sandbed. Plants were transplanted into pots on June 11, 2016, and were misted daily until June 14, 2016 when the treatments began. Initial root length was recorded at transplanting. At the start of the experimental treatments the plants in Trial 1 were 14 days old. The treatments were applied every other day during the experiment, and continued until June 22, 2016. The total duration of treatment for this trial was 8 days (**Table 1**). The final shoot and root lengths, plant health, and biomass were measured on June 22, 2016. The final root lengths were recorded by measuring the length of the longest root in millimeters. Final plant health was recorded as the presence/absence of leaf rolling and discoloration, with the presence of these leaf conditions indicating reduced plant health. The types of leaf discoloration observed included yellowing and browning. In order to measure total biomass, the shoots of each plant were separated from roots by cutting the plant at the shoot base, and the roots were carefully harvested from the soil. Then, the tissues were dried in an oven at 80°C for 48 h, and weighed on an analytical balance in milligrams.

For Trial 2 *S. scoparium* seedlings were germinated on May 1, 2017 on a sand bed while misting daily. Seedlings were transplanted into pots May 22, 2017. Treatment method two and strain B were used for this trial (**Table 1**). The plants used for Trial 2 were 28 days old at the start of the treatments. The experimental treatments were applied to the plants starting on May 29, 2017, and occurred every other day until June 21, 2017. Dead and alive leaf count were recorded on June 7, June 14, and again at the end of the experiment on June 22, 2017. Final root and shoot length, total shoot length measurements, and final leaf counts were recorded on June 22, 2017. Similar to Trial 1, plant biomass was measured by drying and weighing the entire plant tissue (shoots and roots).

In Trial 3 *S. scoparium* seedlings were germinated on February 1, 2017 on a sand bed while misted daily. Seedlings were transplanted into pots between March 22, 2017 and April 3, 2017. Treatment method one and strain B were used for this trial (**Table 1**). The plants used for Trial 3 were 70 days old at the start of the treatments. The treatments began on April 12, 2017 and occurred every other day until May 6, 2017, with a treatment duration of 24 days. The number of leaves alive and dead were recorded May 3, 2017 and again at the end of the experiment on May 10, 2017. Final root length and shoot length was also measured on May 10, 2017. Similar to Trial 2, plant biomass was measured by drying and weighing the entire plant tissue.

Statistical Analysis

In order to test the hypothesis that the effect of inoculation with a PGPR strain would interact with water availability to influence plant growth we compared final root length, final shoot length, and biomass among the four treatments. A two-way ANOVA was performed using R (R Core Team, 2017) separately for each trial to analyze the statistical significance of differences in the following parameters between the treatment groups: (1) final shoot length; (2) final root length; and (3) final total biomass. Also, for each trial, one-way ANOVA tests were paired with Tukey's Honest Significant Difference (HSD) Tests in order to identify significant pairwise differences between the treatment groups.

To determine whether the variation in plant parameters between the three trials was due to methodological differences, a hierarchical clustering analysis was conducted on root length, shoot length, and biomass data using R (R Core Team, 2017). Then the cutree function was employed on the dendrogram generated from the hierarchical cluster analysis to determine if the plant samples were clustering based on starting age, duration of treatment, total water volume added during the experiment, and/or bacterial strain. We then performed a permutational multivariate analysis of variance (MANOVA) using the adonis function in the vegan package of R project (Oksanen, 2013) to determine if treatment had a significant effect on plant growth overall, using final root length, final shoot length, and final plant biomass as inputs to the model. In addition, another permutational MANOVA was performed using the adonis function to evaluate whether methodological differences between the trials explained a statistically significant proportion of the variability in the observed plant growth parameters across all three trials. This second MANOVA was written such that the permutations were constrained to within the treatment groups using strata as an argument in the model (Oksanen, 2013).

To determine if PGPR addition and water availability affected plant health, we tested whether leaf condition (rolling and discoloration in Trial 1, proportion of leaves alive in Trials 2 and 3) varied significantly among the treatments. For Trial 1, the format of the data was a $2 \times 2 \times 2$ contingency table: bacteria addition (yes/no) $\times$ water treatment (high/low) $\times$ leaf

condition (rolling yes/no or discoloration yes/no). We used log-linear modeling in R (loglm in the MASS package; R Core Team, 2017) to test whether there was a significant association among the number of plants with rolled or discolored leaves, bacteria treatment, and water treatment. The step function was used to select the best fitting model based on the Akaike's Information Criterion (AIC). Rolling and discoloration were analyzed separately.

For Trials 2 and 3, we evaluated the effects of PGPR addition and water availability on plant health (proportion of senesced leaves over time) using a repeated measures ANOVA in R (R Core Team, 2017). In addition, we evaluated the individual effects and interaction between PGPR addition and water availability on plant health at individual time points using a two-way ANOVA in R (R Core Team, 2017).

RESULTS

Shoot Length Differences Between Treatments and Experimental Trials

Results from two-way ANOVA tests indicate that watering treatment did not have a significant effect on shoot growth in Trials 1 and 2 (**Figure 1**). However, the watering treatment did have a significant effect on the final shoot lengths of plants in Trial 3 ($p \leq 0.05$). In this trial, plants that received the low water treatment (LW) had larger shoot lengths than plants in the high water treatment (HW; **Figure 1**). Also, the plants that received both the low water and PGPR treatments (LWB) had larger shoot lengths than well-watered plants receiving additional bacteria (HWB; **Figure 1**). The PGPR treatment did not have a significant difference on final shoot length in any of the experimental trials.

Root Length Differences Between Treatments and Experimental Trials

The water treatments did not have a significant effect on the final root lengths of plants in any of the experimental trials (**Figure 2**). However, results from a two-way ANOVA identified that the PGPR treatment had a statistically significant effect on root growth in Trial 2 (**Figure 2**; $p \leq 0.01$). Based on a Tukey's HSD test, plants in Trial 2 that received the PGPR addition (LWB, HWB) had significantly greater increases in root length compared to plants that did not receive the bacterial addition (LW, HW; $p \leq 0.01$). There were no significant effects of the PGPR treatment on the root lengths for Trials 1 and 3.

Biomass Between Treatments and Experimental Trials

Results from a two-way ANOVA indicate that the watering treatment had a statistically significant effect on the final biomass of plants in Trial 3, where plants were 70 days old at the start of the experiment (**Figure 3**; $p = 0.005$). According to results from a Tukey's HSD test, the plants in the low water treatment, with or without PGPR addition, had significantly higher mean biomass than plants in the high water treatment group (**Figure 3**; $p \leq 0.05$). By contrast, the watering treatment did not have a significant effect on final plant biomass in Trials 1 or 2, where plants were much younger (14 and 28 days at the start of the experiment, respectively) at the start of the experiment.

In Trial 2, the bacterial treatment had a statistically significant effect on the final biomass of plants (two-way ANOVA; $p = 0.05$). In this trial, plants that received the PGPR addition had significantly higher biomass than plants that did not receive the additional bacteria, regardless of the watering treatment (Tukey's HSD: $p = 0.05$). Based on the results from two-way ANOVA tests, there were no other statistically significant effects of the bacterial treatment on final plant biomass in Trials 1 or 3.

Comprehensive Influences on Plant Growth Across Experimental Trials

The water and PGPR treatments had a statistically significant effect on plant growth overall across the trials, as identified by a MANOVA (adonis function: $F = 7.6$, $R^2 = 0.12$, $p \leq 0.001$). However, plants in the three trials responded very differently to the treatments. A hierarchical clustering analysis showed that the plants (based on final root length, final shoot length, and plant biomass data) form three clusters, but the plants did not cluster based on experimental treatment. Instead, the plants clustered based on experimental trial. Results from a MANOVA analysis suggest that the experimental trial (Trials 1, 2, or 3), and the experiment duration (8 or 25 days; **Table 1**) had statistically significant effects on overall plant growth ($F = 119.8$, $R^2 = 0.5$, $p \leq 0.001$). The addition of the other methodological variables, such as the bacterial strain (A or B), the total amount of water received during the experiment, and/or the amount of water received per week during the experiment, did not increase the R^2 of the model output. The primary difference between each of the three experimental trials was the starting age of the plants (**Table 1**).

Treatment Effects on Plant Health

To determine whether PGPR addition and water availability impacted the health of the *S. scoparium* seedlings in Trial 1, we used log-linear modeling to test for an association between leaf condition (rolling or discoloration), bacteria treatment, and water treatment. PGPR addition and high water availability were both positively associated with reduced incidence of leaf rolling and leaf discoloration, indicating these treatments improved seedling health (**Figure 4**). In the mosaic plots in **Figure 4**, this is represented as the size of the boxes for rolled vs. not rolled (**Figure 4A**) or discolored vs. not discolored (**Figure 4B**) leaves for each combination of bacteria and water treatment. The treatment group with no PGPR addition and low water availability had the greatest proportion of plants with rolled or discolored leaves, while the group with PGPR addition and high water availability had the lowest proportions (**Figure 4**). Groups with either PGPR addition or high water had intermediate proportions (**Figure 4**). For both leaf rolling and discoloration, the model that best fit the data included two-way interactions between leaf condition and water treatment and between leaf condition and bacteria treatment, but no three-way interaction between leaf condition,

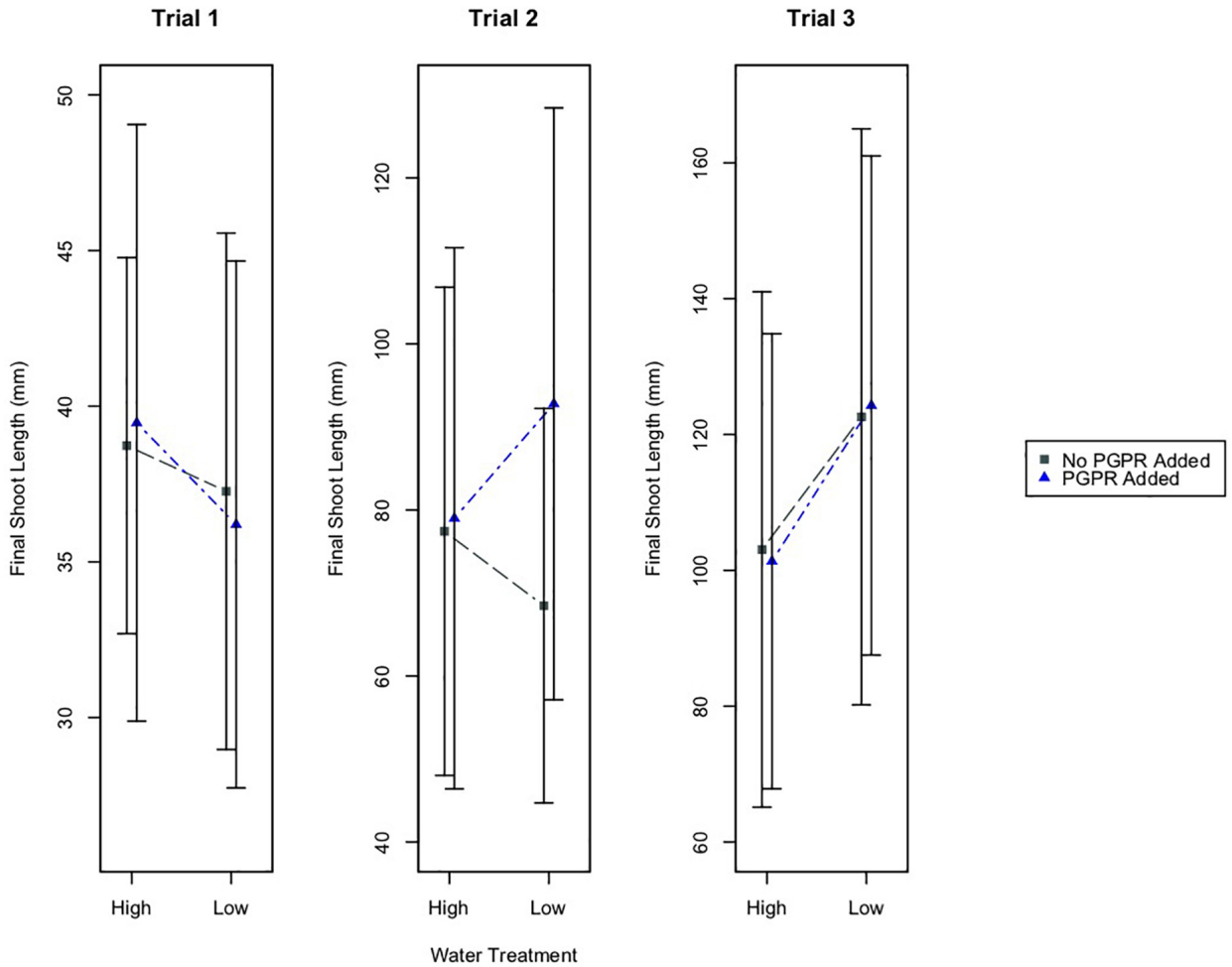

FIGURE 1 | Mean final shoot length of plants, plus/minus one standard deviation, for the different water treatments (High and Low Water), and the PGPR treatments (No PGPR Added, PGPR Added). There was a statistically significant difference in the final shoot length for plants in the high water versus low water treatment in Trial 3 ($p \leq 0.05$).

bacteria treatment, and water treatment (leaf rolling best model: AIC = 12.40, LR X^2 = 0.400, df = 2, p = 0.819; leaf discoloration best model: AIC = 14.59, LR X^2 = 2.589, df = 2, p = 0.24). In contrast, models without interactions between leaf condition and bacteria or water treatment were poorer fits to the data (leaf rolling simple model: AIC = 17.38, LR X^2 = 9.38, df = 4, p = 0.052; leaf discoloration simple model: AIC = 18.12, LR X^2 = 10.12, df = 4, p = 0.038). These results indicate that water availability and *Pseudomonas* addition each had significant additive effects on leaf condition, but there were no interactive effects of water availability and PGPR on leaf condition.

To evaluate the effects of the experimental trials on the proportion of senesced leaves (plant health metric) over time in Trials 2 and 3, we performed repeated measures ANOVA tests. Results from this analysis indicated that in Trial 2 the experimental treatments significantly affected the number of senesced leaves (**Figure 5**; F = 4.7, p = 0.003), and the number of senesced leaves significantly changed over time (**Figure 5**; F = 89.5, $p \leq 10^{-12}$). Specifically, in Trial 2 the water treatment significantly affected the proportion of leaves that senesced over the course of the experiment (F = 0.2, p = 0.003), and there was a significant interaction between the water and PGPR treatments on leaf senescence over time (F = 0.1, p = 0.04). In Trial 3 there was also a significant difference in leaf senescence between the experimental treatments (F = 3.5, p = 0.02), and over time (F = 60.0, $p \leq 10^{-13}$), as well as a significant interaction between the treatments and time (F = 2.74, p = 0.05; **Figure 6**). Leaf senescence was significantly different between the watering treatments over the course of the experiment in Trial 3 (F = 6.0, $p \leq 0.03$), whereas there was only a significant effect of the PGPR treatment on leaf senescence at the end of the experiment (Day 28; F = 5.4, p = 0.02). In both Trials 2 and 3, the greatest average amount of leaf senescence was observed for plants in the high water treatment that did not receive additional PGPR, whereas the lowest average amount of senescence was recorded for plants in the low water treatment that received the PGPR addition (**Figures 5**, **6**).

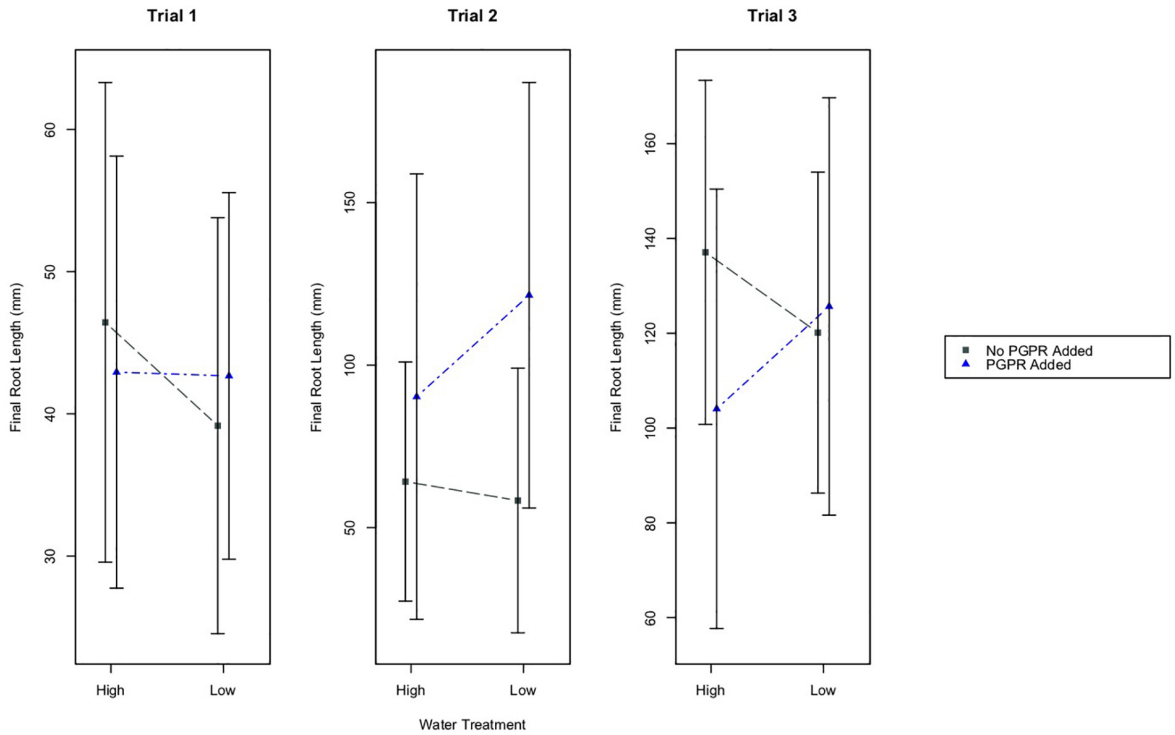

FIGURE 2 | Mean final root length of plants, plus/minus one standard deviation, for the different water treatments (High and Low Water), and the PGPR treatments (No PGPR Added, PGPR Added). There was a statistically significant difference in the final root length for plants that received the PGPR treatment versus those that did not receive the addition of PGPR in Trial 2 ($p \leq 0.01$).

DISCUSSION

Recently, there has been a considerable research focus on understanding how abiotic stress influences plant growth, as well as plant–microbial interactions, in cultivated plants, especially high-value domesticated crop species (Ström et al., 2002; Mayak et al., 2004; Zahir et al., 2008; Sandhya et al., 2010; Timmusk et al., 2011; Grönemeyer et al., 2012; Kang et al., 2014; Nadeem et al., 2014; Timmusk et al., 2014; Vurukonda et al., 2016). However, it is likely that wild plants respond differently to environmental pressures, such as water limitation, than cultivated varieties due to inherent differences in adaptive traits and selective pressures (El-Sayed et al., 2014; Milla et al., 2015; Eida et al., 2018). In addition, evidence suggests that wild plants have closer associations with their rhizobacterial partners, and that rhizobacteria have a greater effect on plant growth in native plant varieties compared to domesticated varieties (Pérez-Jaramillo et al., 2018).

The primary goal of this study was to increase our understanding of the effects of PGPR on the growth of *S. scoparium*, a wild bunchgrass species native to North America. We also evaluated whether the addition of a *Pseudomonas* PGPR influences the growth and health of *S. scoparium* in response to environmental stress, specifically differences in water availability, and whether these effects would be repeatable across different experimental trials. Since the starting age of the plants in each trial was different (14, 28, and 70 days old), we were also able to compare plant responses to PGPR and water availability across life history stages. Overall, the experimental treatments significantly affected plant growth (MANOVA: $F = 7.6$, $R^2 = 0.12$, $p \leq 0.001$). However, we observed significant differences between the experimental trials in terms of plant growth responses to PGPR addition and water availability, likely due to differences in the starting age of the plants and experiment duration (MANOVA: $F = 119.8$, $R^2 = 0.5$, $p \leq 0.001$). While the treatments had no significant effects on the growth of plants in Trial 1, there were significant effects of the treatments on plant growth in Trial 2 (starting age of 28 days) and Trial 3 (starting age of 70 days), however, plants responded differently to the treatments between these two trials. Across the trials, the water treatments and PGPR addition significantly affected plant health. Our results provide insight into how factors such as plant age and methodological choices affect the responses of a native plant species to PGPR and water availability.

Variable Responses of *S. scoparium* to Water Availability at Different Ages

Water limitation is likely a common form of environmental stress for *S. scoparium* since it is adapted to seasonally dry habitats across North America (Tober and Jensen, 2013; PRISM Climate Group, Oregon State University, 2015; USDA-NRCS, 2019). We observed variation in how *S. scoparium* responded to differences in water availability between the experimental trials. For plants

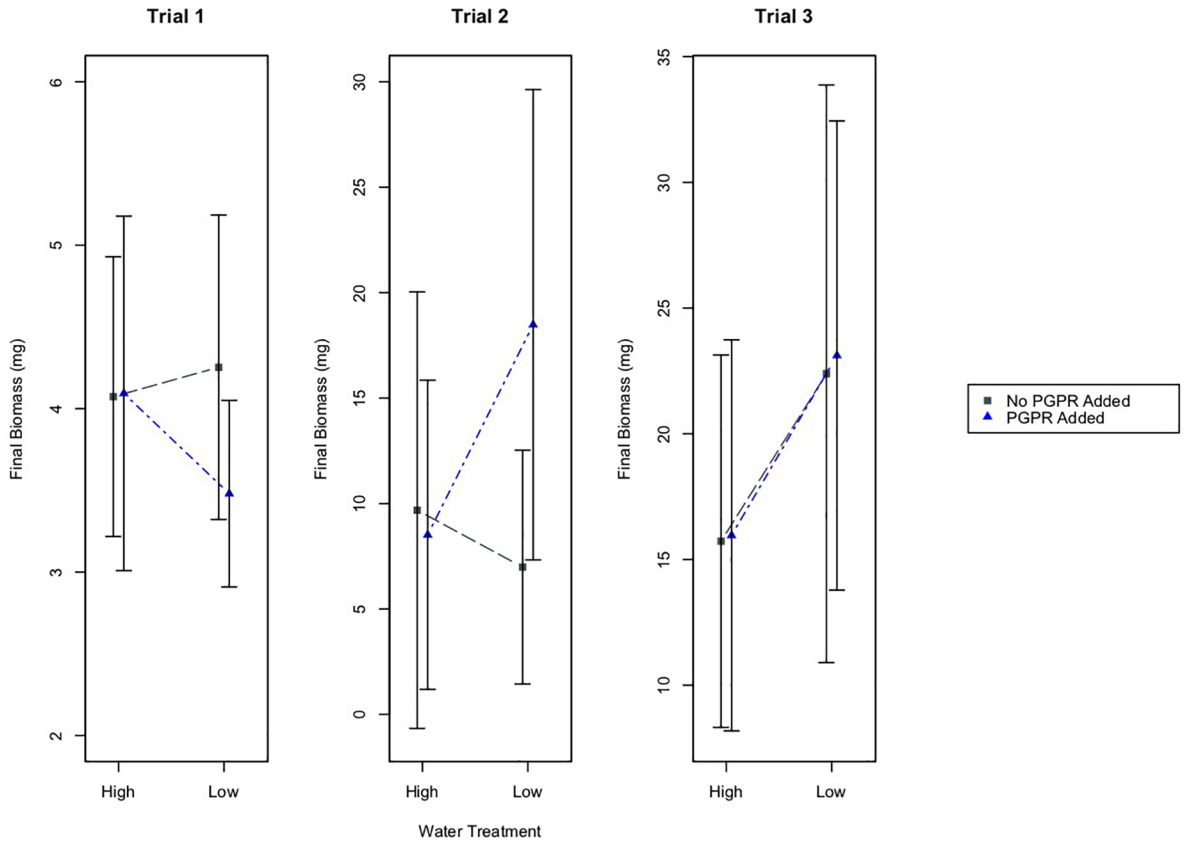

FIGURE 3 | Mean final biomass of plants, plus/minus one standard deviation, for the different water treatments (High and Low Water), and the PGPR treatments (No PGPR Added, PGPR Added). In Trial 2 there was a statistically significant difference in the final biomass for plants that received the PGPR treatment versus those that did not receive the addition of PGPR ($p = 0.05$). In Trial 3 there was a statistically significant difference in the final biomass for plants in the high water versus low water treatment ($p \leq 0.05$).

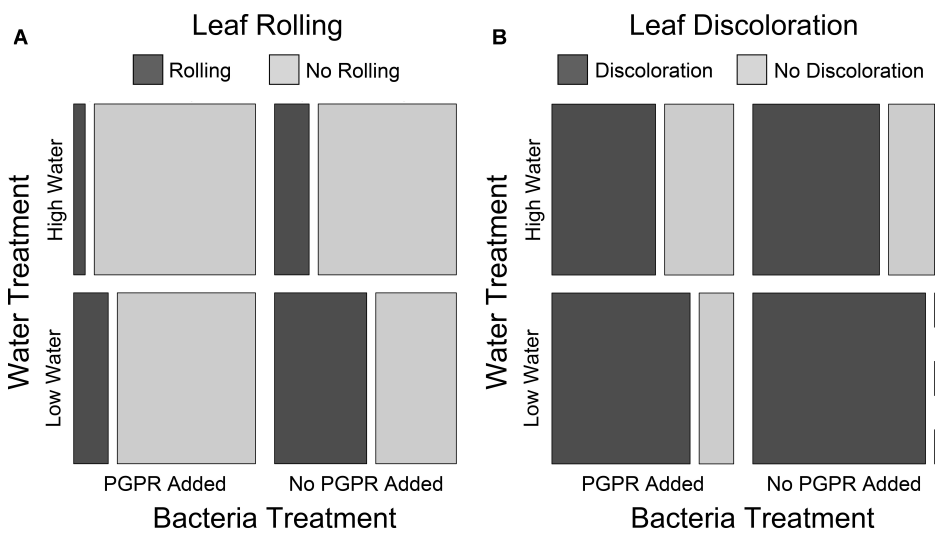

FIGURE 4 | The number of Trial 1 seedlings with or without rolled **(A)** or discolored **(B)** leaves for each bacteria + water treatment combination. $N = 15$ plants for each bacteria + treatment combination; total $N = 60$ plants. Leaf condition observations were made at the end of the trial. Bacteria treatment and water treatment were both significantly associated with leaf rolling and discoloration (see main text for statistical details).

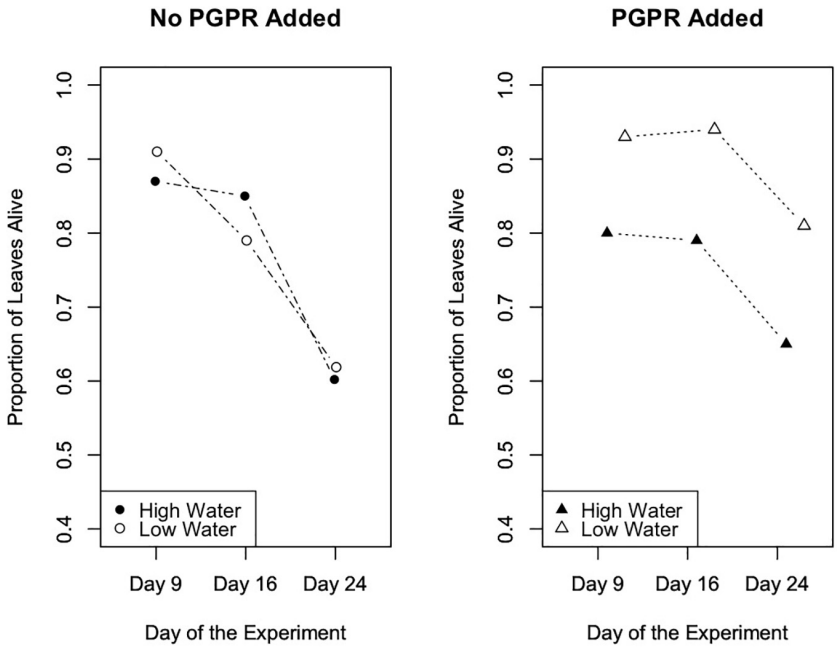

FIGURE 5 | Leaf senescence, as measured by the proportion of leaves that remained alive over the course of the experiment, in Trial 2. The experimental treatments, especially the water treatments, significantly affected the number of senesced leaves ($p = 0.003$). Also, the number of senesced leaves significantly changed over time ($p \leq 10^{-12}$).

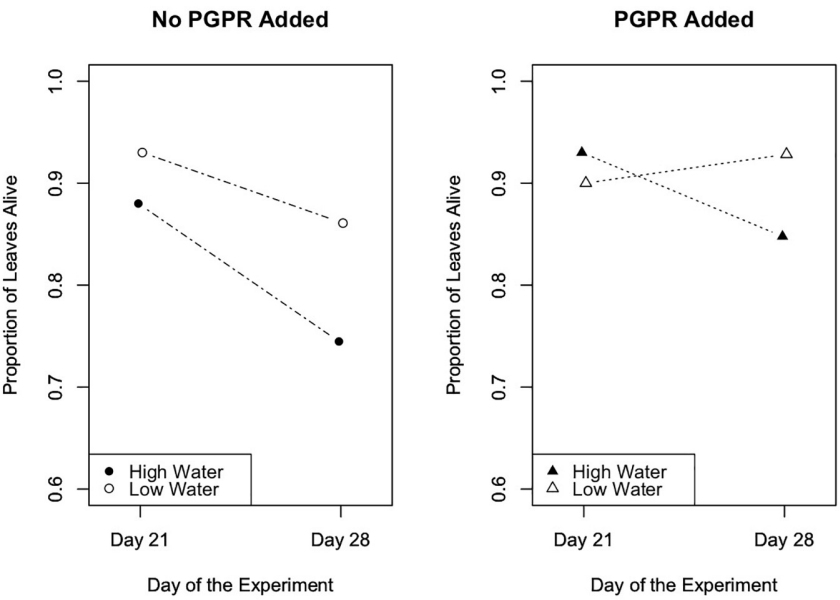

FIGURE 6 | Leaf senescence, as measured by the proportion of leaves that remained alive over the course of the experiment, in Trial 3. There was a significant difference in leaf senescence between the experimental treatments ($p = 0.02$), and over time ($F = 60.0$, $p \leq 10^{-13}$). The watering treatments had a significant effect on leaf senescence throughout the experiment ($p \leq 0.03$), while the PGPR treatment significantly affected leaf senescence at the end of the experiment (Day 28; $p = 0.02$).

that were 14 days old at the start of the experiment (Trial 1), high water availability appeared to improve plant health, indicated by a lower incidence of leaf rolling and discoloration. Leaf rolling is a common stress response in grasses (Kadioglu et al., 2012) and *S. scoparium* is known to roll its leaves during drought (Knapp, 1984). In addition, the yellowing and browning observed was indicative of stressed and dying tissue. In contrast, for plants that were 70 days old at the start of the trial (Trial

3), the same high water treatment appeared to reduce plant health, indicated by more leaf death/senescence. High water also had a negative effect on growth in Trial 3; plants in the high water treatment had significantly shorter shoot lengths and lower biomass. These observations indicate that the low water treatment imposed drought stress on the young seedlings in Trial 1, but that high water was more stressful than low water for the older plants in Trial 3.

Differences in root system development between new seedlings and older more established plants, as well as differences between the local climate where these seeds were from and the water treatments applied, may explain these contrasting responses. According to the seed supplier, the *S. scoparium* Central Texas mix is adapted to oak woods and prairies with annual rainfalls of 20–40" (see text footnote 2). In this experiment, the low water treatment in all three trials was equivalent to 8" of rain per year, which is well below the Central Texas range. High water in Trials 1 and 3 (49" of rain per year) was above the range for the Central Texas mix. Resource availability is generally critical for plant seedling establishment (Lambers et al., 2008), and the stress response of *S. scoparium* seedlings in low water from Trial 1 is consistent with their native climate range. In addition, while *S. scoparium* seedlings are moderately tolerant to drought compared to other prairie species (Mueller and Weaver, 1942), water limitation can reduce their growth (LaGory et al., 1982). Older plants with more established root systems may be less sensitive to water limitation, but we would still expect the low water treatment in Trial 3 to be stressful. Furthermore, previous work on *S. scoparium* has observed reductions in shoot production under water limited and drought conditions (LaGory et al., 1982; Knapp, 1984; Maricle et al., 2015). Why then, would the high water treatment be more stressful for older plants? One explanation is that for older plants with more developed root systems, water levels much higher than the native precipitation range could sometimes be more detrimental than periodic water limitation. Field experiments in natural and transplanted *S. scoparium* populations indicate there may be an optimum level of soil moisture, above which there is no benefit (Knapp, 1984) and potentially even detrimental effects on growth (Rozum, 2014). Collectively, this is consistent with *S. scoparium* being tolerant of moderate, but not extreme drought, and not adapted to highly mesic environments.

Some of *S. scoparium*'s responses (or lack thereof) to water availability were more difficult to understand. First, despite effects on seedling health in Trial 1, we did not observe reduced growth of seedlings in low water. However, it is possible that the trial duration (8 days) was too short to see significant differences in biomass accumulation. Second, given that both water treatments in Trial 2 were below the precipitation range for Central Texas *S. scoparium*, it is unclear why the plants in the high water treatment (which presumably would be less stressful), had more leaf death/senescence than those in low water. In addition, there was no main effect of water availability on growth in Trial 2, despite a trial duration of 28 days. This lack of treatment effect could reflect the relatively small difference in water volume between treatments in Trial 2. Given that both treatments were well below the native precipitation range, it is likely that neither was optimal for *S. scoparium* growth.

The results from this experiment indicate that low and high water availability can impose stress on Central Texas *S. scoparium*, with effects on both plant health and growth. *S. scoparium*'s native range spans a wide precipitation gradient, including regions that experience periodic drought (Tober and Jensen, 2013). Importantly, *S. scoparium* generally does not access water in very deep soil layers (Eggemeyer et al., 2009; Mueller et al., 2013), and thus responds to water limitation through physiological adjustments to resist the negative effects of drought (Hake et al., 1984; Knapp, 1984; Maricle and Adler, 2011; Maricle et al., 2015). Its physiological response to excess water is less well studied. Collectively, this indicates that there is an opportunity for interactions between *S. scoparium* and rhizobacteria to mediate response to water stress.

Positive Effects of PGPR on *S. scoparium* Growth and Health

Previous research has documented the plant growth promotion of rhizosphere *Pseudomonas* strains for a variety of plants growing under typical and stressed conditions (Sandhya et al., 2010; Timmusk et al., 2014; Oteino et al., 2015; Lally et al., 2017; Taketani et al., 2017; and others). In our study, surprisingly, we did not observe a consistent effect of the PGPR addition on plant growth across the three experimental trials. There was a significant positive effect of PGPR addition on root growth and biomass for plants that were 28 days old at the start of the experiment (Trial 2), but we did not observe any plant growth promotion in the other experimental trials. In fact, root growth was significantly lower for plants that received the PGPR addition for the oldest plants (70 day starting age; Trial 3). In addition, we observed that PGPR addition did not significantly affect shoot growth in any of the experimental trials under high or low water conditions. Previous research found that inoculation with PGPR strains in the *Pseudomonas* genus promoted root growth more than shoot growth (Sandhya et al., 2010). PGPR inoculum may have a greater effect on root growth than shoot growth when the interactions between PGPR strains and the plant are more localized and do not translate into systemic physiological changes within the plant. These localized interactions may be especially important under stress conditions.

Under stress, root growth is suppressed when plants produce compounds such as ethylene, and its precursor ACC, whereas many PGPR strains can reverse stress-induced growth reduction through the degradation of ACC (Yang et al., 2009). When plants experience drought, the positive effects of PGPR on root growth and biomass accumulation are especially pronounced (Jaleel et al., 2007; Timmusk et al., 2014). Root development, such as the growth of root hairs and lateral roots, may be maintained despite water limitation because PGPR create water-resistant matrices, thus allowing the root system to stay hydrated (Sandhya et al., 2009; Timmusk et al., 2014; Timmusk et al., 2015). In fact, environmental stress, such as drought, may necessitate more coordinated functions between the plants and rhizosphere microorganisms (Zang et al., 2014; Panke-Buisse et al., 2015; Naylor and Coleman-Derr, 2018). Thus, interactions between host plants and PGPR affect plants' allocation of

resources toward shoot and root growth under a range of environmental conditions.

Plants host a diversity of microbes within their native rhizosphere community and it is likely that colonization by multiple PGPR strains provides additional benefit, in terms of plant growth. Previous research has documented greater boosts in plant growth in response to inoculation with multiple PGPR strains (Mathivanan et al., 2014; Nadeem et al., 2014). In our study, we did not use sterilized soil and thus, the *Pseudomonas* isolate added during the PGPR treatments was competing for access to the plant roots. This may explain why we did not observe a more widespread effect of the PGPR addition on plant growth across our experimental trials. There is some evidence that there is a reduction in the influence of PGPR inoculation on plant growth when the native soil microbiome is intact due to competitive interactions (Çakmakçi et al., 2006; Al-Khaliel, 2010). This is possibly due to the fact that plants can be specific in the rhizobacterial partners they recruit (Vacheron et al., 2013), and other bacterial strains may be preferentially selected over the PGPR strain(s) added under experimental conditions. Also, the majority of PGPR research has been conducted with cultivated crop species, and it is possible that wild plants may interact differently with PGPR. In fact, the application of multiple PGPR strains in high concentrations had no effect on the growth of several rare wild plant species in the field, however, soil type strongly influenced plant growth (Michaelis and Diekmann, 2018). Thus, the degree to which PGPR inoculation may promote plant growth is likely dependent on plant adaptations to environmental conditions, as well as the competitive interactions between added PGPR strains and the rhizosphere microbial community.

While in our study PGPR addition seemed to have variable effects on plant growth, we did observe a measurable effect of PGPR on plant health. In Trial 1, plants that received the PGPR and high water treatments had the least amount of discoloration and rolling, while in Trials 2 and 3 leaf senescence was lowest for plants that received both the PGPR and low water treatments. By contrast, in these Trials 2 and 3 the greatest leaf senescence was measured for plants receiving only the high water treatment. The majority of similar studies focus solely on the effects of PGPR on shoot, root, or biomass yield, although a few studies have characterized PGPR effects on leaf senescence. PGPR inoculation was associated with a decline in leaf and flower senescence, as well as a delay in fruit ripening in crop species, under normal and drought conditions (Saleem et al., 2007; Ghanbari Zarmehri et al., 2013). In switchgrass and *Arabidopsis thaliana*, earlier leaf senescence was observed in plants inoculated with PGPR, however, this was attributed to an expedition of plant development in general, as opposed to a direct effect of PGPR on plant health (Poupin et al., 2013; Wang et al., 2015, 2016). Leaf senescence is often attributed to the production of ethylene and ACC in response to stress. Thus it is possible that these improvements in plant health, and declines in leaf senescence, are due to PGPR strains degrading ACC through ACC deaminase. These previous research studies have focused on cultivated and model species, and our results suggest that PGPR may confer similar plant health benefits for wild, uncultivated plant species.

Age-Dependent Effects of PGPR on *S. scoparium* Growth

Plant age may play an important role in coordinating interactions between plants and PGPR. Results from our research suggest that younger plants may respond to water availability and the addition of PGPR differently than older plants. Low water availability may have a more detrimental effect on early seedlings (Trial 1) compared to older plants that are more established and are in a later stage of development. There is some evidence that older plants display greater resistance to stress (Hong and Hwang, 1998). The growth of the oldest plants in our study (Trial 3) also seemed to be relatively unaffected by PGPR inoculation as compared to intermediate-aged plants (Trial 2), while the growth of the youngest plants also did not appear to be affected by the addition of PGPR. In Trial 1, it is likely that the short experimental duration (**Table 1**) did not provide sufficient time to observe an effect of the PGPR on growth. In addition, the root microbiome membership is often more dynamic during earlier stages of development (Micallef et al., 2009; Wagner et al., 2016), which suggests the heightened importance of competitive interactions for the establishment of PGPR on the roots of young plants.

As plants age there are shifts in the secretion of root exudates (Marschner et al., 2004; Jones et al., 2009; Chaparro et al., 2014), which is an important mechanism through which plants recruit and maintain associations with rhizobacteria (Berendsen et al., 2012). Microbial species richness in the root microbiome may decrease over the plant's lifetime (Wagner et al., 2016), and rhizosphere microbial communities often become more specialized and distinct as plants age (Roesti et al., 2006; Micallef et al., 2009). Thus, it is likely that the timing of PGPR inoculation is critical to their effect on plant growth. In our study it is possible that the oldest plants (Trial 3) had already established associations with other microbes within the rhizosphere, and the added PGPR strain was at a competitive disadvantage. The intermediate-aged plants in Trial 2 may have had a less developed microbiome at the start of the experiment, and the experiment was also long enough for a PGPR association to affect growth. In addition, the stress associated with the low water treatments could have primed the plants for PGPR association. Plants secrete different root exudates in response to environmental pressures, and these exudates select for microorganisms with functions that increase plant survival under stress (Marschner et al., 2004; Zang et al., 2014; Panke-Buisse et al., 2015; Wagner et al., 2016). The life history stage at which a plant experiences low water availability may exert strong control over the synergy between the plant and its rhizosphere microorganisms.

CONCLUSION

Our study demonstrates the complex factors that influence the effects of PGPR inoculation on plant growth and plant health. Plant age, experiment duration, and water availability strongly influenced the response of *S. scoparium* to PGPR. Our results demonstrate that plant health parameters, such as leaf discoloration, rolling, and senescence, are helpful for

understanding the effects of PGPR in alleviating stress. This research was distinctive in that it studied the effects of PGPR inoculation on plant growth in a wild plant species, which potentially responds differently to PGPR associations than highly domesticated crop species. Understanding the ways in which native plants respond to PGPR under a range of environmental conditions provides insight into the factors affecting the dynamics of plant–microbe interactions in an era of global change.

AUTHOR CONTRIBUTIONS

TB, RV, and AK conceived the idea for the study. TB, RV, and AK designed the experiment for Trial 1. RV and AK conducted the plant germination, the experimental set-up and treatment applications, and the final plant measurements for Trial 1. TB and RV conducted the bacterial isolation for all of the experimental trials, and designed and conducted the experiments for Trials 2 and 3. RV maintained and grew the bacterial cultures for all three experimental trials. TB, RV, and AK conducted data analysis and created figures for the manuscript. TB and RV created tables for the manuscript. TB and RV wrote the first three drafts of the manuscript; AK wrote sections of the manuscript. TB, RV, and AK revised, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00860/full#supplementary-material

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Alder Distribution and Expansion Across a Tundra Hillslope: Implications for Local N Cycling

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Increases in the availability of nitrogen (N) may have consequences for plant growth and nutrient cycling in N-limited tundra plant communities. We investigated the impact alder (*Alnus viridis* spp. *fruticosa*), an N-fixing deciduous shrub, has on tundra N cycling at a hillslope located on Alaska's Seward Peninsula. We quantified N fixation using $^{15}N_2$ incubations within two distinct alder communities at this site: alder shrublands located on well-drained, rocky outcroppings in the uplands and alder savannas located in water tracks along the moist toeslope of the hill. Annual N fixation rates in alder shrublands were 1.95 ± 0.68 g N m^{-2} $year^{-1}$, leading to elevated N levels in adjacent soils and plants. Alder savannas had lower N fixation rates (0.53 ± 0.19 g N m^{-2} $year^{-1}$), perhaps due to low phosphorus availability and poor drainage in these highly organic soil profiles underlain by permafrost. In addition to supporting higher rates of N fixation, tall-statured alder shrublands had different foliar traits than relatively short-statured alder in savannas, providing an opportunity to link N fixation to remotely-sensed variables. We were able to generate a map of the alder shrubland distribution at this site using a multi-sensor fusion approach. The change in alder shrubland distribution through time was also determined from historic aerial and satellite imagery. Analysis of historic imagery showed that the area of alder shrublands at this site has increased by 40% from 1956 to 2014. We estimate this increase in alder shrublands was associated with a 22% increase in N fixation. Our results suggest that expansion of alder shrublands has the potential to substantially alter N cycling, increase plant productivity, and redistribute C storage in upland tundra regions. An improved understanding of the consequences of N fixation within N-limited tundra plant communities will therefore be crucial for predicting the biogeochemistry of these warming ecosystems.

Keywords: *Alnus* (alder), arctic, nitrogen cycling, nitrogen fixation, shrub encroachment, tundra, tundra greening, nutrient limitation

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INTRODUCTION

Since the late 20th century, tundra regions have been greening in response to changing climate and an accelerated disturbance regime (Jia et al., 2003; Goetz et al., 2005; Lara et al., 2018). An important component of greening in the low Arctic has been the expansion of deciduous shrubs into previously graminoid-dominated tundra communities (Sturm et al., 2001; Tape et al., 2006; Myers-Smith et al., 2011).

Deciduous shrubs store a greater amount of carbon (C) in live plant biomass and are associated with increased magnitude, quality, and turnover of litter nitrogen (N; DeMarco et al., 2014). Transitioning plant communities therefore have important implications for C and N cycling within tundra ecosystems that have historically been nutrient-limited (Chapin and Shaver, 1985; Hobbie, 1992; Mack et al., 2004).

As tundra plant communities in low Arctic regions shift toward greater dominance by deciduous shrubs, the trajectory of these transitioning ecosystems will likely be underpinned by keystone species like alder (Hollingsworth et al., 2010; Nossov et al., 2010). Alder broadly refers to the genus *Alnus*, which comprises approximately 25 deciduous tree and shrub species found in temperate, boreal and arctic regions of the northern hemisphere that introduce biologically available N to an ecosystem via symbiotic N fixation. *Frankia* bacteria housed within alder root nodules fix N in exchange for plant C. This exchange allows alder to colonize recently-disturbed areas with low levels of N availability such as fire scars, floodplains, deglaciated surfaces, and cryoturbated mineral soils (Crocker and Major, 1955; Uliassi and Ruess, 2002; Mitchell and Ruess, 2009; Frost et al., 2013). In addition to being an early successional species, alder is also one of the shrubs contributing to the high latitude greening trend that has been observed since the 1980's (Goetz et al., 2005; Bhatt et al., 2010; Myers-Smith et al., 2011; Epstein et al., 2012). Ground-based observations of shrub expansion are spatially and temporally limited compared to the satellite record, but these direct observations provide important insight into the dynamics and species behind regional greening trends observed in the satellite record (Epstein et al., 2012). Shrub expansion into Alaskan tundra has been attributed in part to alder (*Alnus viridis* ssp. *crispa* (Aiton); Sturm et al., 2001; Tape et al., 2006) and recent decades have also seen elevated levels of alder recruitment at the northern boreal tree line in NW Canada (*Alnus viridis* ssp. *fruticosa* (Rupr.); Lantz et al., 2010). Warmer summer temperatures in the tundra regions of the Brooks Range and on the North Slope of Alaska have led to alder expansion along well-drained, rocky hillslopes (*A. viridis* ssp. *fruticosa*; Tape et al., 2012). Alder's role in shrub expansion is likely greatest in Alaska and Western Canada (Myers-Smith et al., 2011), but analyses of regional satellite data generally do not distinguish alder from other arctic shrub species. The impact of symbiotic N fixation by arctic alder has therefore been difficult to assess at a landscape scale. Separating alder from other deciduous shrubs in regional analysis is valuable because alder at lower latitudes has been associated with high N inputs, low nutrient use efficiency and a relatively open, or "leaky," N cycle (*Alnus rubra* (Bong.) in Binkley et al., 1992; *Alnus incana* ssp. *tenuifolia* (Nutt) in Clein and Schimel, 1995). In the Alps, alder expansion into N-poor grasslands has been shown to increase local soil N availability as well as the N content of neighboring plants (*Alnus viridis* (Chaix); Bühlmann et al., 2016). This caused not only a shift of N from soils into plant biomass, but also a loss of N via leaching into downslope catchments (*A. viridis*; Bühlmann et al., 2014; Bühlmann et al., 2016). The tundra biome is poor in nutrients, with N availability limiting both plant productivity and decomposition by soil microbes (Chapin and Shaver, 1985; Shaver and Chapin, 1980; Hobbie, 1992; Mack et al., 2004). Nitrogen introduced to tundra ecosystems by alders therefore has the potential to exert an important influence on the terrestrial and aquatic biogeochemistry of these rapidly warming systems.

Alder N fixation in boreal and alpine systems are relatively well-characterized (*A. incana ssp*. tenuifolia and *A. viridis ssp*. *fruticosa*; Anderson et al., 2009; Mitchell and Ruess, 2009; Hollingsworth et al., 2010; Nossov et al., 2011; Ruess et al., 2013), but we know little about tundra-dwelling alders and their impact on N cycling within the tundra biome. A quantitative investigation of N fixation by alder growing in tundra has not yet been performed despite the novelty of a N-fixing shrub expanding its range in this nutrient-poor biome. The arctic research community also lacks a way to distinguish alders from other deciduous shrubs in remotely sensed data products, something that would be invaluable for identifying areas alders may expand in the future and quantifying their impact on landscape-level N cycling. We address these knowledge gaps by examining the above- and belowground traits of *A. viridis ssp. fruticosa* growing in two distinct plant communities at a hillslope tundra site on Alaska's Seward Peninsula. Our goal is to answer the following questions:

Q1) Does alder significantly impact local N cycling and N fixation rates in this tundra ecosystem?
Q2) What are the sources of variation in above- and belowground traits of alder, and can observed variation be used to quantify symbiotic N fixation at the landscape scale?
Q3) Is alder cover expanding at this site and if so, what are the implications for the N cycle?

We hypothesize that alder N cycling, above- and belowground traits, and range expansion will vary according to community and landscape position. Alder shrubland communities located near the crest of the hill contain alder that are tall in stature and grow in high densities. Alder savanna communities located along the toeslope of the hill contain short alder that are dispersed throughout a community of mixed shrubs and graminoids. Since large, dense stands of alder likely support more symbiotic bacteria, we hypothesize that alder in upland shrublands will fix more N, have distinct above and belowground traits, and be capable of greater expansion in response to regional warming than their counterparts in lowland savannas.

MATERIALS AND METHODS

Study Site

The Kougarok Hillslope site (65°09'50.1"N, 164°49'34.2"W) is in the interior of the Seward Peninsula, 103 km from the town of Nome. The historic ATLAS site (65°25'46.3"N, 164°38'48.0"W; L.D. Hinzman et al., 2003) is 32 km northeast of the Kougarok Hillslope and has a mean annual air temperature of -4.02°C, growing season precipitation of 131.6 mm, and a mean snowpack depth of 82 cm (2000-2017 data, Busey unpublished data; Hinzman et al., 2003). This region of the Seward Peninsula was likely last glaciated during the early Pleistocene (Kaufman and

Hopkins, 1986; Kaufman and Manley, 2004) and permafrost here is discontinuous (Hinzman et al., 2003; Schuur and Mack, 2018). Warming temperatures have impacted the Seward Peninsula in the past half century. Glacial extent in the Kigluaik Mountains to the south of the Kougarok Hillslope has been decreasing since the 1980's, and these glaciers are expected to disappear by 2035 (Calkin et al., 1998). Remote-sensing studies suggest shrub cover is increasing across the Seward Peninsula (Silapaswan et al., 2001; Stow et al., 2004), and the white spruce forest near the village of Council is expanding into tundra regions (Lloyd et al., 2002).

The Kougarok Hillslope site encompasses an exposed, rocky outcropping surrounded by steep, well-drained slopes that transition to a lowland wetland tundra. The hillslope site spans a roughly 100-m change in elevation and a variety of tundra plant communities are present across the varying topography. *A. viridis* ssp. *fruticosa* (herein alder) grows in two of the communities present at this site: alder shrublands and alder savannas. Alder shrublands are found along the well-drained slopes below the crest of the hill and are interspersed with patches of dwarf shrub lichen tundra. Within alder shrubland communities, alder shrubs grow to height of at least 2 m and form dense, closed canopies (**Figure 1A**). Alder shrubland communities at Kougarok are similar to the upland community described in Frost et al. (2014). Soil profiles under alder shrublands at Kougarok Hillslope are quite rocky and are underlain by bedrock. Alder savannas, on the other hand, are found along the Kougarok Hillslope's gently-sloping toeslope and are intermingled with willow-birch tundra and tussock tundra communities. Alder savannas consist of short-statured (1 m), evenly spaced alder that grow amongst other deciduous shrubs and tussock tundra along poorly-developed water tracks (**Figure 1B**) and lowland areas. Alder savanna communities at Kougarok are akin to those described in Frost et al. (2013) and the regular spacing of alder shrubs could potentially be attributed to nutrient competition, local hydrology, and/or cryogenic disturbances (Chapin et al., 1989). Within the alder savanna, alder makes up about 15% cover and the understory is characterized by sedges [*Eriophorum vaginatum* (L.) and *Carex bigelowii* (Torr. ex Schwein)], dwarf erect shrubs [*Betula nana* ssp. *exilis* (Sukaczev), *Rhododendron tomentosum* (Harmaja), *Vaccinium uliginosum* (L.)], lichens (*Cladonia* spp., *Cetraria* spp.) and moss [*Sphagnum* spp., *Hylocomium splendens* (Hedw.)]. Alder savanna soil profiles are underlain by permafrost and the active layer depth is approximately 40 cm. The variation in topography and presence of the same alder species in two distinct plant communities at this site makes this an ideal location for assessing the impact of alder on tundra N cycling.

Environmental Conditions

We characterized soil pH, organic horizon depth, soil temperature at 2 cm depth and soil moisture at 8 cm depth within these two alder communities. Surface soil moisture and temperature sensors were installed in July 2016 (2 per community; H21-002, S-TMB-M002,S-MBD-005, Onset Computer Corporation, Bourne, MA). Data from July 2016 through July 2018 were summarized for summer (June-August) versus winter (September-May) seasons as well as on an annual basis. In 2016, we collected four replicate soil cores from alder shrubland and alder savanna communities. The depth of organic horizon was recorded in the field, and pH of surface soils was measured in the laboratory. In early September of 2017, we measured soil profile depth at five replicate patches of alder shrubland and alder savanna using a thaw probe. Environmental data and detailed methods are publicly available (see https://doi.org/10.5440/1346195 and https://doi.org/10.5440/1346200).

Soil Nutrient Availability and Depth

Soil inorganic N and phosphorus (P) availability were measured using anion- and cation-binding resins deployed at 30 locations across the Kougarok Hillslope. There are a total of six plant communities present at Kougarok Hillslope, with alder shrublands and alder savannas representing just two of the six. In 2016, resin plots were established across the six plant communities (n = 5

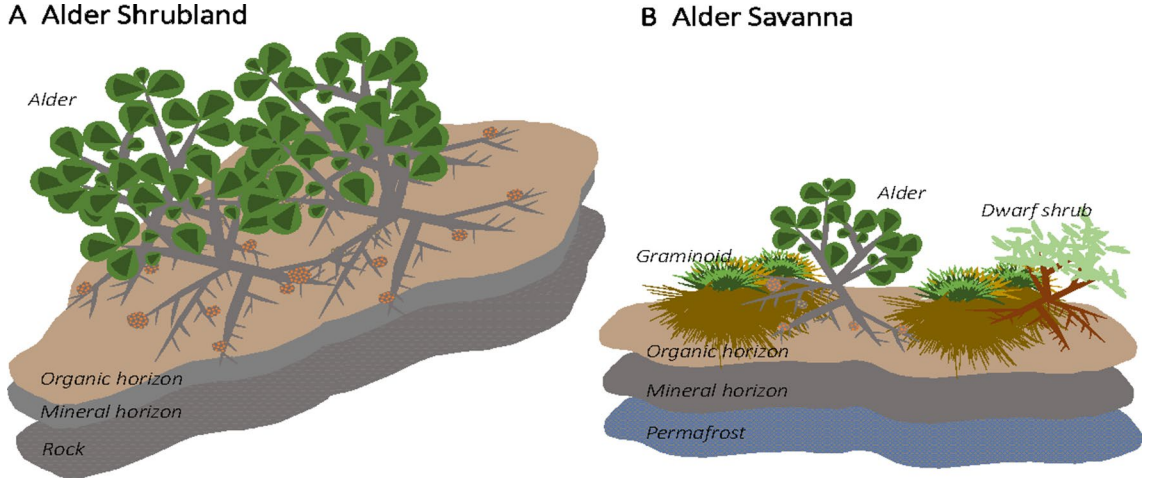

FIGURE 1 | Alder (*Alnus viridis* ssp. *fruticosa*) is found in two distinct communities at the Kougarok Hillslope site. Short-statured, dispersed alder grow in lowland tundra communities ("alder savanna") while tall alder grows in dense shrublands along the rocky ridge of the hillslope ("alder shrubland")

replicates per community) and a 10-cm deep PVC resin-access tube was installed (WECSA, LLC, Saint Ignatius, MT, USA). Resins were deployed for the summer and winter seasons from July 2016 through September 2017. In the lab, resins were rinsed with distilled water, air-dried, and serially extracted three times with 20 ml 2 M KCl solution. The three batches of extract were combined, filtered, and frozen prior to analysis of NH_4, NO_3, and PO_4 on a Lachat autoanalyzer (Lachat QuikChem 8500, Hach Company, Loveland, CO). Concentrations were blank-corrected and standardized to μg N or P extracted per unit resin surface area. Resin-N was calculated as the sum of extracted NH_4-N and NO_3-N. Resin data and detailed methods are publicly available (see https://doi.org/10.5440/1346201).

Aboveground Plant Traits

We measured a suite of aboveground traits on alder growing in alder shrubland and alder savanna communities. We recorded the height individual alder shrubs and measured the basal diameter of all ramets associated with the shrub at the soil surface. To determine whether ramets were part of the same alder individual, ramets were shaken and shallow, coarse roots were traced. For each alder individual we collected 10 sun and 10 shade leaves. For the purposes of this study, we defined shade leaves as those present in the lower canopy where there was the potential for shading by upper canopy leaves, neighboring shrubs, or tussock mounds. Specific leaf area (SLA, cm^2 leaf area g^{-1} dry leaf) was determined from leaf punches of known diameter that were counted, pooled, dried, and weighed prior to being ground for elemental and isotopic analysis. Leaf %C was measured on an elemental analyzer (Costech) while leaf %N and $\delta^{15}N$ were measured using isotope ratio mass spectrometry (IRMS, Integra CN, SerCon, Crewe, UK). Leaf %P was measured by Kjeldahl digest and resulting solutions were run on a Lachat QuikChem 8500 analyzer (Lachat Instruments, Loveland, CO, USA). SLA and leaf %N and leaf %P were used to calculate N_{area} (g N m^{-2}) and P_{area} (g P m^{-2}). In September 2017, senesced alder litter was collected from alder shrublands by brushing a hand along branches. Litter was not collected from alder savanna communities. SLA of litter assumed a 7% loss of dry mass during senescence and resorption efficiency (%) during senescence was calculated as the change in N_{area} and P_{area} (Norby et al., 2000; Uliassi and Ruess, 2002).

Leaf samples from non-alder species were collected in July 2016 at 12 resin plots. Plots were chosen so that all six plant communities at Kougarok Hillslope were represented (n = 2 replicates per community). Foliar chemistry and SLA were measured as described above. The leaf trait data associated with alder and non-alder samples are publicly available (https://doi.org/10.5440/1346199).

Alder Cover Using Remotely-Sensed Data

Remote-sensing datasets from multiple sensor platforms including EO-1 Hyperion, SPOT-5, Landsat and the USGS IfSAR-based digital elevation model (**Supplementary Table 1**) were compiled using a multi-sensor fusion approach similar to that employed by Langford et al. (2019). The spectral properties of alder shrubland were particularly distinct within data from the EO-1 Hyperion hyperspectral instrument (**Supplementary Figure 1**). All remote-sensing datasets were processed at 5-m spatial resolution for analysis. A k-means, clustering based, unsupervised classification was then performed on the 211-dimensional remote sensing dataset. This resulted in classification of the entire Kougarok Hillslope into 50 clusters with similar spectral properties that were compared to ground-based observations of alder shrubland at Kougarok Hillslope (https://doi.org/10.5440/1465967). Two of the clusters were found to capture the spectral characteristics of alder shrublands and were subsequently used to generate a spatial map of alder shrubland distribution. The unsupervised classification-based (UCB) alder shrubland map was validated further by comparison with GPS coordinates of alder shrublands recorded during field sampling (red Xs in **Figure 4A**).

A time series of four cloud-free, high-resolution aerial and satellite images was analyzed using established photo-interpretation methods to determine whether alder shrubland distribution has changed in the last half-century (Lara et al., 2018; Frost et al., 2014). Geospatial image products included: 1) a 1956 panchromatic aerial photograph from USGS, 2) a 1985 color-infrared aerial photograph from the Alaska High Altitude Aerial Photography, 3) a 2006 panchromatic OrbView-3 satellite image, and 4) a 2014 multispectral Worldview-2 satellite image. All images were acquired during mid- to late summer and orthorectified, georeferenced (mean RMS error: 2.5 m), and resampled to 1 m resolution. Alder shrubland patches were digitized manually in each image at a map scale of 1:2,500 in ArcMap GIS (ArcMap 10.6; ESRI, Redlands, CA, USA). This time-series was only able to characterize change in alder shrublands; alder within alder savanna communities do not form homogenous canopies larger than 1 m in diameter and were therefore below the detection limit of the image products.

Nodule Biomass

Nodule biomass per unit ground area was determined by extensive collection of shallow soil cores during June and July of 2017, similar to methodologies described in Ruess et al. (2013) and Uliassi and Ruess (2002). Cores were taken to a depth of 15 cm or until rock, large-diameter coarse roots, or standing water were encountered. All cores were collected with a power drill connected to a 7.3-cm diameter circular hole-saw. Measuring nodule biomass in these two plant communities required different sampling techniques due to the fact that alder in shrubland communities formed a closed canopy while alder in savanna communities grew interspersed with sedges and a variety of other low-statured evergreen and deciduous shrub species.

In five distinct stands of alder shrubland, replicate 2.5 × 2.5 m square plots were established and a grid containing 64 cells was demarcated. One soil core was collected per grid cell unless soil surface was inaccessible due to the presence of by rock or alder stem. We recorded which grid cells had individual alders rooted in them. Nodule biomass plots in the alder shrubland community contained between 4 and 8 individual alder shrubs. This sampling protocol resulted in destructive sampling of 3.9%

of the total plot area and involved the collection of 288 soil cores across the five plots.

In alder savanna communities, alder shrubs were evenly dispersed with approximately one shrub per 4.1 m^2 (unpublished data). A sampling method like the one utilized in the alder shrublands would therefore oversample the space between alder shrubs and under sample the areas closest to the shrub where coarse root biomass is highest. In order to take the spatial structure of the alder savanna community into account, we decided to sample nodule biomass within 10 circular plots, each with an the alder at the center. An inner circle within the plot spanned the canopy of the alder, while an outer circle extended this radius by 50 cm. Each plot contained only one alder shrub, located at the "bullseye" of the circles. On average, nodule plots in the alder savanna were 3.75 m^2 (±0.47). The concentric circles were divided into eight equal slices and we collected approximately twice as many cores from the inner circle as from the outer circle. This sampling protocol resulted in destructive sampling of 3.4% of the total plot area and involved the collection of 299 soil cores across the 10 plots. Following collection, all soil cores were frozen and shipped to Oak Ridge National Laboratory (ORNL) for processing.

In the laboratory, thawed cores were examined for 10 min and live nodules were collected. Live nodules were differentiated from dead nodules based on their lighter color.and structural integrity (Ruess et al., 2009). Dead nodules were dark, spongy, and easily pulled apart. The core was sorted for additional 5 min intervals until an interval passed without discovery of live nodules. Live nodules were then rinsed, dried, and weighed. No discernable spatial patterns were observed; nodule biomass per soil core did not increase with proximity to alder stems in alder shrublands or in alder savannas. There was similarly no significant relationship between shrub basal area (average cm^2 per shrub or shrub cm^2 per m^2) and nodule biomass within the data from either community. This result differed from that of Ruess et al. (2009) who used shrub basal area to scale nodule biomass measurements within stands of boreal alders. Given absence of a spatial pattern in nodule biomass observations and absence of community-specific relationships between basal area and nodule biomass, we decided to calculate average nodule biomass values for the two communities directly from the nodule biomass plots rather than scaling based on correlation with a covariate. Within alder shrublands, nodule biomass per m^2 ground area was averaged across all soil cores collected in the square gridded plots (n = 5 plots). In alder savannas, nodule biomass was calculated as the area-weighted average of values from inner- and outer circles (n = 10 plots). Since alder savanna nodule biomass plots were similar in size to the overall density of shrubs in the alder savanna community, we feel that this approach adequately takes into account the spacing of alders within the alder savanna community. Nodule biomass data and detailed methods are publicly available (see https://doi.org/10.5440/1493669).

N Fixation Within Nodules

Rates of N fixation within alder root nodules were determined in the field by incubating excised nodules with ^{15}N-labeled N_2 gas, as described in Anderson et al. (2004). Rates of N fixation were determined for 21 alder shrubs growing in shrublands and 12 alder shrubs growing in alder savannas in July of 2017 and 2018. Briefly, ~2.5 g fresh weight of nodules was collected and placed immediately in a 60-ml syringe with 10 ml of 98 atm% $^{15}N_2$ gas. A subsample of headspace gas was collected. After 10 min, the incubation was halted by flash-freezing the nodules in liquid N. Another ~2.5 g of fresh nodule biomass was collected from the same alder individual to determine the initial (natural abundance) ^{15}N signature of the incubated nodules. Nodule ^{15}N and %N were determined by isotope ratio mass spectrometry (IRMS, Integra CN, SerCon, Crewe, UK) at ORNL. Headspace gas samples were sent to UC Davis Stable Isotope Facility for determination of atm% $^{15}N_2$ ($^{15}N_{headspace}$). Atom percent excess of the nodule (APE_{nodule}) was calculated as the difference between atm% ^{15}N of incubated and non-incubated nodules. The rate of N fixation (Nfix) in µmole N g^{-1} nodule h^{-1} was calculated as:

$$Nfix = \frac{(APE_{\text{nodule}} \times N_{\text{nodule}})}{incubation\ time \times {}^{15}N_{headspace}}$$

where N_{nodule} was the µmole N present per g dry nodule. Rates of N fixation within alder nodules and detailed methods are publicly available (see https://doi.org/10.5440/1493688).

Scaling N Fixation Inputs

Peak growing season N fixation was calculated as the product of nodule biomass (g nodule m^{-2} ground) and N fixation (µmole N g $nodule^{-1}$ h^{-1}). Maximal daily N fixation measurements (µmole N m^{-2} ground day^{-1}) were then scaled to the entire growing season using a step function that assumes daily rates are half-maximal from 20 to 31 May, maximal from June 1 to August 15, half-maximal for the last 2 weeks of August, and quarter-maximal for the first 2 weeks of September (Uliassi and Ruess, 2002; Anderson et al., 2004; Ruess et al., 2009; Ruess et al., 2013). This assumes that daily N fixation at peak growing season is stable throughout the day, as has been observed in *A. incana* ssp. *tenuifolia* (Huss-Danell et al., 1992).

Statistical Analyses

All statistical analyses were performed in R (R Core Team, 2018). Differences in environmental observations and aboveground alder traits of alder shrubland and alder savanna communities were compared using t-tests ($p < 0.05$). The effect of deployment date and plant community on resin-available resin-N and resin-P were initially determined with a two-way ANOVA. Plant community provided little explanatory power for the variation in resin-N observed, so we explored the relationship between resin-N availability and the distance to the nearest alder shrubland using the spatial data analysis packages *ggmap* (Kahle and Wickham, 2013), *sp* (Pebesma and Bivand, 2005), *raster* (Hijmans, 2019), *rgeos* (Bivand and Rundel, 2018) and *rgdal* (Bivand et al., 2018). The minimum 2-dimensional distance

between each resin-N measurement and the nearest edge of the alder shrubland classification region in the modern, UCB map was determined and then the elevational difference between points was calculated. For resin-N measurements taken within the mapped extent of alder shrubland, the distance and elevation difference measures were both considered zero. The relationship between resin-N availability and the distance to alder shrubland (d) was fit to a negative exponential model using generalized non-linear least squares in the *nlme* package.

$$TIN = Ae^{-bd}$$

This two-term model fit was compared to models in which A and b were fitted to summer and winter deployments separately (A_{winter}, b_{winter}, & A_{summer}, b_{summer}) as well as a model fit to a common extinction coefficient (b) and deployment-specific intercepts. Nested models were compared based on a likelihood ratio test, and non-nested models were compared by AIC. The 95% confidence intervals for the final model fit were determined by bootstrapping (1,000 iterations).

A multiple linear regression model was used to examine the relationship between foliar chemistry of non-alder species and winter resin-N. Alder data were excluded from this analysis because the goal was to determine whether alder N inputs were impacting nearby species %N in addition to impacting resin-N. Leaf %N and $\delta^{15}N$ of non-alder species were averaged for the plant functional types (PFTs) present in each plot. The relationship between nodule biomass and sun leaf N:P was analyzed with a similar simple multiple linear regression model.

The covariance of alder traits was also explored using Principal Components Analysis (PCA). PCAs were performed on the covariance matrix of aboveground traits (height, sun leaf SLA, sun leaf %N, sun leaf $\delta^{15}N$, sun leaf %P) and either nodule biomass or rates of N fixation. Following PCA analysis, the significance effect of traits on principal components was determined by bootstrapping eigenvectors ($p < 0.05$).

RESULTS

Environmental Conditions

Soils within alder shrubland communities had much thinner organic horizons than alder savannas (**Table 1**). These two plant communities both had slightly acidic surface soils, and shallow soil temperatures were similar when compared on an annual and a seasonal basis. During the winter months (September through May), surface soils in alder shrublands were wetter than surface soils of alder savanna communities, potentially due to the accumulation of drifting snow around the tall alder shrubs that forms a deeper snowpack than at the toeslope. The wetter winter season in alder shrublands drove a significant difference in annual surface soil moisture content between the two communities. In deeper portions of the soil profile, however, it is likely that alder savannas had a greater moisture content; soils at 15-cm depth in inter-tussock areas of alder savannas were often saturated (*personal observation*). Indeed, soil moisture measured on 15 cm deep bulk soil in July 2017 was significantly greater alder savannas than alder shrublands (51.1% versus 30.4%, p = 0.01; McCaully et al, in preparation).

TABLE 1 | Annual average surface soil temperature and soil volumetric water content within plant communities containing alder.

	Soil Properties		
	Surface soil pH	**Depth of organic horizon (cm)**	**Soil Profile Depth (cm)**
Alder Shrubland	4.89 ± 0.09	**7.13 ± 1.48 ***	**26.86 ± 2.55 ***
Alder Savanna	4.69 ± 0.14	**21.25 ± 2.69 ***	**38.96 ± 1.78 ***
		Soil Temperature (C°)	
	Annual	**Summer**	**Winter**
Alder Shrubland	1.24 (-13.89, 14.96)	8.80 (2.34, 14.96)	-0.36 (-13.89, 11.95)
Alder Savanna	0.73 (-14.16, 20.48)	8.89 (0.47, 20.48)	-1.00 (-14.16, 15.44)
		Soil VWC (m^3/m^3)	
	Annual	**Summer**	**Winter**
Alder Shrubland	**0.26 (0.11, 0.41) ***	0.30 (0.21, 0.37)	**0.25 (0.11, 0.45) ***
Alder Savanna	**0.19 (0.00, 0.48) ***	0.30 (0.17, 0.41)	**0.16 (0, 0.49) ***

Data is from summer 2016 through summer 2018 and summer was defined as June, July & August. Averages are followed by minimum and maximum values in parentheses or ± standard error. Asterisk (*) and bold indicate statistically significant differences between communities ($p < 0.05$).

Alder Above- and Belowground Traits Vary Across Communities

Alder growing in different plant communities exhibited contrasting above- and belowground traits. Alder in shrublands were taller and had a greater basal area (**Table 2**). Shade leaves in alder shrublands also had a greater SLA, higher %N, and lower N_{area} than their counterparts growing in alder savannas (**Table 3**). Leaves throughout the canopy of alder growing in shrublands had higher %P and P_{area}, a lower N:P, and a more depleted $\delta^{15}N$

TABLE 2 | Structural traits of alder growing in two plant communities.

Community	Height (cm)	Basal Area Per Shrub (cm^2 stem)	Basal Area (cm^2/ m^2 ground)	Nodule Biomass (g/ m^2 ground)	Nodule Biomass (g/ cm^2 stem)
Alder Shrubland	**267.89 ± 8.13 ***	**391.94 ± 69.62 ***	**64.86 ± 16.65 ***	**18.54 ± 6.14 ***	**0.47 ± 0.25**
Alder Savanna	**111.55 ± 7.56 ***	**32.18 ± 7.38 ***	**11.39 ± 2.42 ***	**3.64 ± 1.21 ***	**0.44 ± 0.15**

Averages are given ± standard error. Asterisk (*) and bold indicate statistically significant differences between communities ($p < 0.05$).

TABLE 3 | Leaf traits of alder growing in two plant communities. Averages are given ± standard error.

Leaf Type	Community	SLA (cm^2/ g)	$\delta^{15}N$	C:N	%N	N_{area} (g N/ m^2 leaf)	N:P	%P	Parea (g P/ m^2)
Sun	Alder Shrubland	120.55 ± 7.09	**-1.74 ± 0.11 ***	21.01 ± 1.73	2.33 ± 0.06	2.03 ± 0.08	**14.40 ± 0.36 ***	**0.16 ± 0.01 ***	**0.14 ± 0.01 ***
	Alder Savanna	116.56 ± 6.29	**-1.36 ± 0.09 ***	19.25 ± 0.88	2.25 ± 0.12	1.92 ± 0.09	**20.43 ± 1.12 ***	**0.11 ± 0.01 ***	**0.10 ± 0.01 ***
Shade	Alder Shrubland	**160.6 ± 7.1 ***	**-1.76 ± 0.15 ***	21.70 ± 1.76	**2.29 ± 0.05 ***	**1.49 ± 0.07 ***	**14.67 ± 0.40 ***	**0.16 ± 0.01 ***	**0.10 ± 0.01 ***
	Alder Savanna	**120.42 ± 4.9 ***	**-1.22 ± 0.08 ***	23.08 ± 0.85	**1.98 ± 0.08 ***	**1.71 ± 0.10 ***	**23.52 ± 2.65 ***	**0.09 ± 0.01 ***	**0.07 ± 0.00 ***
Litter	Alder Shrubland	151.16	-2.26 ± 0.18	32.61 ± 0.50	1.56 ± 0.02	1.03 ± 0.01	24.46 ± 2.76	0.07 ± 0.01	0.05 ± 0.01

Asterisk (*) and bold indicate statistically significant differences between communities ($p < 0.05$).

signature. The alder communities did not differ in leaf C:N or sun leaf %N, N_{area}, or SLA. Alder litter collected within alder shrublands had a relatively high %N and N_{area}, indicating a low N resorption efficiency (41.2%). In contrast, P resorption efficiency in alder shrublands was 62.4%.

Nodule biomass per m^2 ground was greater in alder shrublands than in alder savannas (**Table 2**, **Figure 2**, p = 0.04). When the nodule biomass was normalized to stem basal area, there was no significant difference between alder shrublands and alder savanna (**Table 2**). PCA of aboveground alder traits collected alongside nodule biomass showed a distinct separation of community traits along PC1, which explained 54.0% of the observed variation (**Figure 2B**, **Supplementary Table 2**). PC1 was positively correlated sun leaf $\delta^{15}N$ and negatively correlated

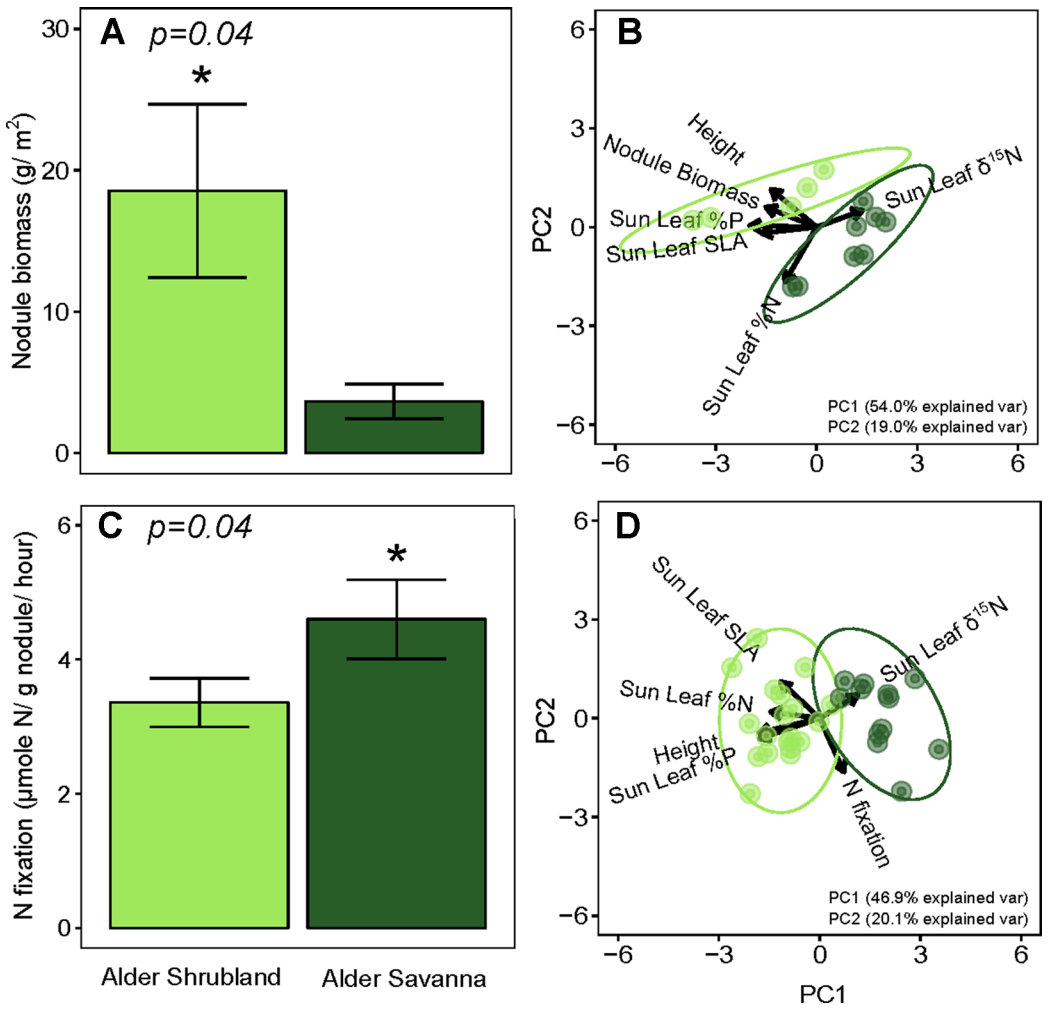

FIGURE 2 | Below- versus aboveground traits of alder growing in shrublands and savannas. Alder nodule biomass **(A)** and its relationship to aboveground alder traits **(B)**, rates of N fixation **(C)** and its relationship to aboveground alder traits **(D)**. Asterisks (*) indicate statistically significant differences between communities ($p < 0.05$).

with nodule biomass, height, sun leaf SLA, sun leaf %N, and sun leaf %P.

N fixation rates within nodules were lower for alder growing in shrublands than alder growing in alder savannas (P = 0.04, **Figure 2C**). Similar to the pattern seen with nodule biomass, PCA of aboveground traits and N fixation exhibited a strong separation of communities (**Figure 2D**, **Supplementary Table 3**). PC1 explained 46.9% of variation in the dataset and was positively correlated with sun leaf $\delta^{15}N$ and negatively correlated with N fixation rate, height, sun leaf SLA, sun leaf %N, and sun leaf %P.

The overall rate of N inputs via N fixation was primarily driven by variation in nodule biomass. Fixation rates were higher in nodules from alder savannas, but the higher nodule biomass within alder shrublands more than compensated for their lower nodule fixation rate. Alder shrublands had nodule biomass that was roughly four times that of alder savannas, but N fixation per unit nodule biomass in alder savannas was only 37% higher than rates in observed in shrublands. The greater nodule biomass within alder shrublands was associated with lower leaf N:P and there was a significant negative correlation between sun leaf N:P and nodule biomass in this community (**Figure 3**). Alder in savannas, however, had higher sun leaf N:P and no relationship between leaf N:P and nodule biomass.

Peak Season and Annual Rates of N Fixation

Alder shrublands had an average peak season N fixation rate of 62 μmole N m^{-2} h^{-1} (±22 SE) while alder savannas had an averaged 17 μmole N m^{-2} h^{-1} (±6 SE). There was a wide range in N fixation rates per unit ground area within both communities, predominately driven by nodule biomass variation. When these peak season N fixation rates were integrated to yearly fluxes, annual N fixation by alder shrublands was over 3.5 times thats of alder savannas (**Table 4**).

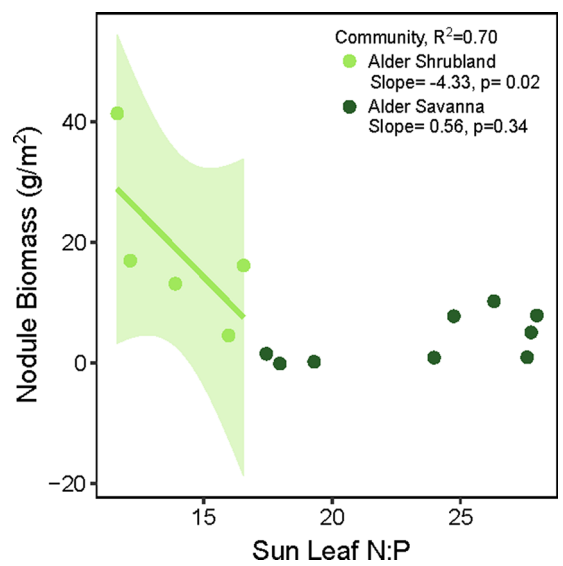

FIGURE 3 | Alder nodule biomass in relation to sun leaf N:P (g:g basis). In alder shrubland nodule biomass plots, there were 4-8 alder individuals so the N:P ratio on the y axis is an average value. The colored line and shaded 95% confidence intervals denote significant relationship between nodule biomass and sun leaf N:P within the alder shrubland community.

Alder's Impact on Local N Cycling

Deployment period (winter vs summer) and plant community both had a significant impact on total inorganic nitrogen extracted from resin capsules (Resin-N, **Supplementary Figure 2A**, P = 0.003 and P = 0.013 respectively). Overall, resin-N in winter was greater than resin-N in summer, and alder shrubland communities tended to have the highest levels of resin-N. Within the each of the six communities at Kougarok Hillslope, however, there was a high degree of variation in resin-N replicates. P availability followed a similar pattern but with greater variation and only the deployment term was significant in the model (**Supplementary Figure 2B**, p = 0.019). The extreme variation in nutrient availability within the six plant communities led us to hypothesize that high resin-N was associated with proximity to alder shrublands rather than a location's community classification. Comparing the modern UCB alder shrubland map (**Figure 4A**) with locations of resins confirmed that resins near alder shrublands were indeed higher in resin-N (**Figure 4B**). The negative exponential model was best fit with a deployment-specific intercept term (A) and a common extinction coefficient (b = 0.22, 95% CI from 0.09 to 0.36). The intercept term for resins deployed in winter, A_{winter} was higher (33.84, 95% CI from 19.20 to 48.49) than that for resins deployed in the summer (A_{summer}, 1.73, 95% CI from -3.71 to 7.17).

When leaf %N of non-alder species was examined, overall leaf %N increased with resin-N (**Figure 5A**, overall slope = 0.002, p = 0.004). Overall, this pattern was weak, however, and was driven primarily by the few locations where resin-N was high. Within PFTs, this trend was significant for both deciduous (slope = 0.005, $p < 0.001$) and evergreen shrubs (slope = 0.002, $p < 0.002$), but the leaf %N of forbs and graminoids was not significantly correlated with resin-N. The $\delta^{15}N$ signature of non-alder leaves tended to be heavier when leaves had higher leaf %N (**Figure 5B**, overall slope = 6.4, $p < 0.001$). Within PFTs, however, this slope was only significant for evergreen shrubs (slope = 4.32, $p < 0.001$).

Extent of Alder Shrublands Through Time

Time-series image analysis showed that alder shrublands at Kougarok Hillslope have expanded their range by 40% since 1956 (**Figure 6**, **Table 5**). This analysis does not include alder in savannas, which could not be differentiated from other deciduous shrubs given their low density and short stature in these areas. We found alder shrubland cover increased continually from 7.4 ha in 1956 to 10.4 ha in 2014. Rates peaked between 1956 and 1985 and alder shrubland expansion appeared to slow in the 2000's (inset in **Figure 6**). The average rate of alder shrubland expansion was 513 m^2 $year^{-1}$ and most of this occurred by infilling gaps between patches of existing alder shrublands.

TABLE 4 | Annual N fixation within alder shrublands and alder savannas. Rates from this tundra ecosystem are at the lower end of the range reported for alder N fixation in boreal regions.

Study	Species	Biome/Habitat	Methods	Annual N fixation (g N/ m²/ yr)
This study	*A. viridis spp. fruticosa*	Tundra (Alder shrubland community)	$_{15}N_2$ incubation of nodules Soil core collection for nodule biomass	1.95 ± 0.68
This study	*A. viridis spp. fruticosa*	Tundra (Alder savanna community)	$_{15}N_2$ incubation of nodules Soil core collection for nodule biomass	0.53 ± 0.19
Mitchell and Ruess (2009)	*A. viridis spp. fruticosa*	Boreal (post-fire succession)	ARA incubation of nodules Soil core collection for nodule biomass	0.25 - 0.66
Uliassi and Ruess (2002)	*Alnus incana spp. tenuifolia*	Boreal (floodplain, unfertilized)	ARA incubation of nodules Soil core collection for nodule biomass	3.9 - 8.8
Ruess et al. (2009)	*Alnus incana spp. tenuifolia*	Boreal (alder stands impacted by canker)	$_{15}N_2$ incubation of nodules Soil core collection for nodule biomass	2.2 - 10.7
Ruess et al. (2013)	*Alnus incana spp. tenuifolia*	Boreal (floodplain, unfertilized)	$_{15}N_2$ incubation of nodules Soil core collection for nodule biomass	2.6 - 3.8
Crocker and Major (1955)	*Alnus viridis spp. crispa*	Boreal (recently deglaciated area)	Annual N increment in soil 40 year period	6.2
Van Cleve et al. (1971)	*Alnus incana spp. tenuifolia*	Boreal (floodplain, unfertilized)	Annual N increment in soil 20 year period	15.6

DISCUSSION

We quantified N fixation by alder across the Kougarok Hillslope to better understand the role alder may play in warming tundra ecosystems. We hypothesized that the tall, dense alder in shrublands would have a greater impact on local N cycling than the short, dispersed alder in savanna communities and our findings support this hypothesis. Annual rates of N fixation within alder shrublands were over three times as high as N fixation in alder savannas, and N-limited tundra plant species growing in communities adjacent to alder shrublands had greater access to N. The observed expansion of alder shrublands therefore has important implications for N cycling within tundra ecosystems, and our data provide intriguing evidence that we may be able to predict the biogeochemical consequences of alder expansion across the tundra based upon aboveground alder traits.

Alder Shrublands Have an "Open" N-Cycle That Impacts Neighboring Plants and Soils

Context for the N fixation rates presented in this study can be provided by comparison with the N required for annual net primary productivity (N_{req}). We estimate the N_{req} of the entire alder shrubland community to be 13 g N m^{-2} $year^{-1}$ and the N_{req} of the entire alder savanna community to be 11 g N m^{-2} $year^{-1}$ based on data from biomass harvests of understory species,

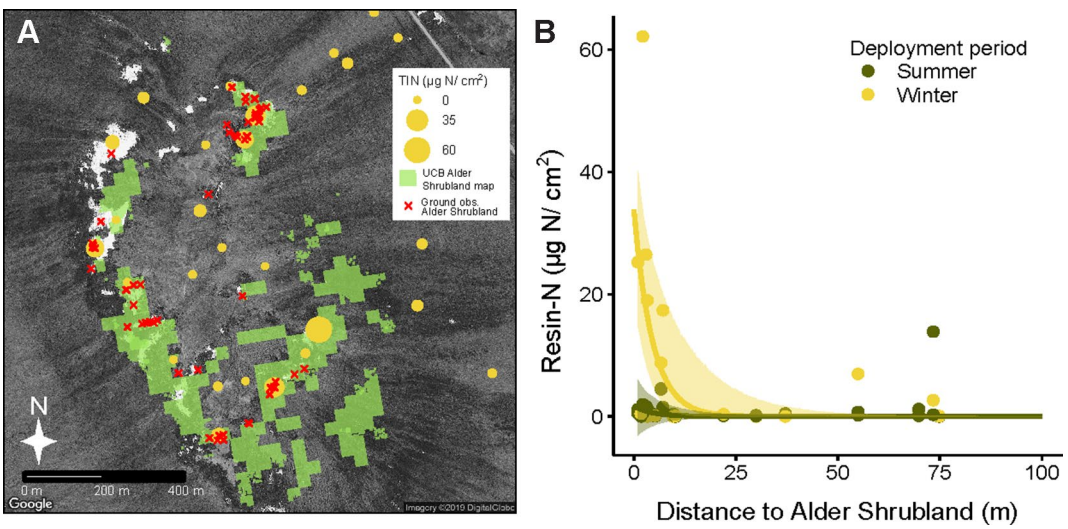

FIGURE 4 | Inorganic N availability (Resin-N) at Kougarok Hillslope increases with proximity to alder shrublands. Light green areas in panel **A** represent an unsupervised classification-based (UCB) alder shrubland map derived from multisensor data. The accuracy of the alder shrubland map was verified with ground-based observations (red x's). Resin-N was measured with ion-exchange resins deployed across the hillslope site and was found to decrease with increasing distance from alder shrublands (panel **B**). Shaded regions in panel B represent 95% confidence intervals fit to negative exponential model with separate intercepts for summer and winter seasons. Several near-zero resin-N values from 100 m to 380 m distance are not pictured in panel B but were included in model fitting.

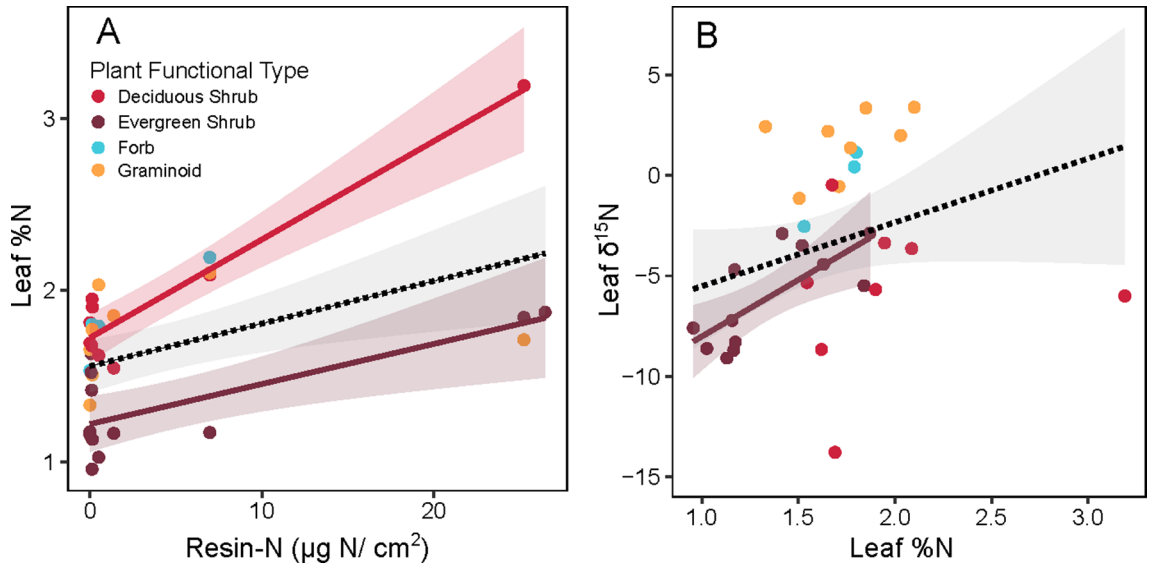

FIGURE 5 | Relationship between inorganic N availability (Resin-N) and leaf chemistry of non-alder plant species at Kougarok Hillslope. Panel **A** shows Resin-N versus leaf %N while panel **B** shows Leaf %N versus Leaf %15N. Colored lines and shaded 95% confidence intervals denote significant slope of a given plant functional type ($p < 0.05$). Dashed lines and grey 95% confidence intervals represents a significant slope fit across all PFTs ($p < 0.05$).

shrub allometries, and gapfilled tissue %N and rhizome pools based on existing arctic literature (Salmon et al., in preparation). Arctic plant communities lacking alder have N_{req} ranging from 0.42-4.40 g N m^{-2} $year^{-1}$ (Shaver and Chapin, 1991). The annual N fixation rates reported in this study (0.53-1.95 g N m^{-2} $year^{-1}$) are at the lower end of the range of reported values for alder growing in boreal regions though reported rates vary widely between habitat types as well as methodologies (**Table 4**). The lower rates of N inputs by tundra versus boreal alders could be the result of a colder climate, lower phosphorus availability, or a shorter growing season. However, when we consider these N inputs by tundra alders next to the N_{req} of neighboring tundra communities, their magnitude is striking. Within alder shrubland and alder savanna communities, N fixation supplies 16% and 5% of N_{req} respectively. Typically, plants with a greater reliance on symbiotic N fixation are associated with leaf $\delta^{15}N$ near zero (Shearer and Kohl, 1986), but we found alder leaf $\delta^{15}N$ in shrubland communities was more negative than alder leaf $\delta^{15}N$ in savanna communities (**Table 3**). This does not discredit the higher proportion of N_{req} met by N fixation in the alder shrubland community we calculate, however. Increased N contribution from ectomycorrhizal symbionts or greater reliance on organic N forms in alder shrublands could similarly explain the depleted $\delta^{15}N$ signature of alder shrublands (Hobbie and Hobbie, 2006; Yano et al., 2009).

The high proportion of N_{req} met by N fixation in alder shrublands in conjunction with the observed increased resin-N

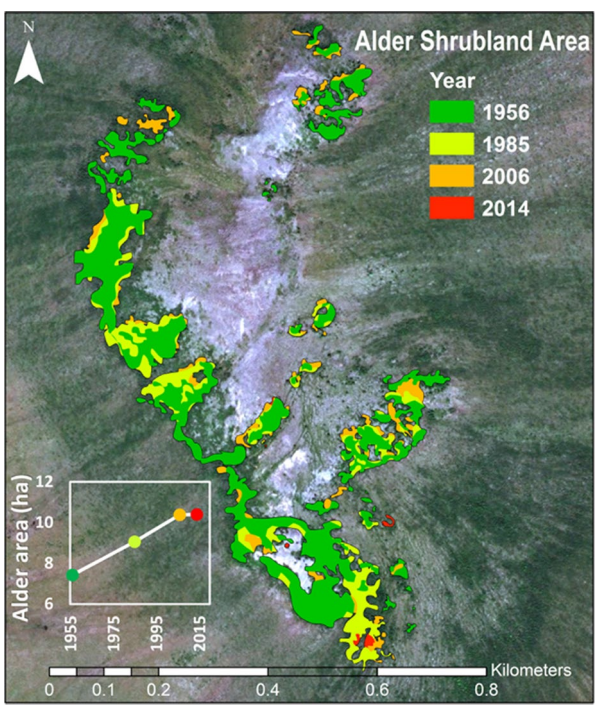

FIGURE 6 | Expansion of alder shrubland at the Kougarok Hillslope (1956-2014). The photo-interpreted area alder shrublands through time are overlaid on the 2014 WorldView2 image of the site.

TABLE 5 | Alder shrubland expansion at Kougarok Hillslope since 1956.

Year	Cover by Alder Shrublands
1956	74,231 m_2
1985	90,621 m_2
2006	103,812 m_2
2014	103,967 m_2
1956-2014	29735 m_2 increase
Rate of change	513 m_2/ year
Percent change (1956-2014)	40%

in soils located near alder shrublands soils suggest that alder shrublands do not have a very tightly constrained N cycle. Alder shrublands may fail to retain N due to their high rates of litter N deposition and their location in well-drained uplands where they are presumably susceptible to downhill losses of N in the form of litter and leachate. We found that alder leaf litter from shrublands was high in N (1.56% N by weight). N resorption efficiency was still higher than reported for boreal alders (41.2% versus 13.9%; Uliassi and Ruess, 2002). The N resorption efficiency of tundra alders we observed, however, was lower than the 65% figure cited for non-fixing tundra species (Jonasson and Shaver, 1999). By applying allometric equations relating basal area to aboveground biomass and assuming leaves makes up roughly 80% of new growth, we can calculate the annual flux of N in alder leaf litter to be around 3.8 g N m^{-2} (Berner et al., 2015; personal communication L. Berner). The downslope movement of alder litter and leachate is therefore a likely mechanism behind the elevated levels of resin-N we observed within a 20 m radius of alder shrublands. Resin-N was greatest when resins were deployed through the winter season, potentially due to winter decomposition of alder litter (Kielland et al., 2006; Buckeridge et al., 2010), moisture limitation of summer N mineralization, or competition between resins and roots for N during the growing season. Transport of N from alder shrublands to surrounding soils and water bodies could therefore have important implications for biogeochemical cycling across arctic landscapes. Related work at the Kougarok hillslope suggests that nitrate from these alder shrublands is transported downslope during rainfall events (McCaully et al., in preparation,). Future research on the hydrological transport of alder-sourced N across arctic landscapes is needed and we believe that an expanded version of our UCB alder shrubland map could prove integral to these efforts.

The positive relationship between resin-N and leaf %N of deciduous and evergreen shrubs suggests that the hotspots in resin-N associated alder shrublands (**Figure 4B**) explained some of the observed variation in shrub leaf %N. This suggesting that evergreen and deciduous shrubs may have access to N "leaked" from alder shrublands. With the data at hand it is not possible to directly attribute this increased leaf %N to increased reliance on alder-fixed N (see Binkley et al., 1985). Leaf $\delta^{15}N$ signatures tended to less depleted in when leaf %N was high. This could indicate an increased dependence on recently-fixed N since $\delta^{15}N$ signatures close to zero are associated with N fixation (Shearer and Kohl, 1986). However, alder shrubland leaf $\delta^{15}N$ was around -1.75 (**Table 3**), so this source is unlikely to explain the high degree of depletion we observed in evergreen and deciduous shrubs (around -5, **Figure 5B**). An altered balance between fractionating processes in the N cycle may explain the low values observed in **Figure 5B**. At the Kougarok Hillslope, graminoid and forb $\delta^{15}N$ signatures were near or greater than zero as is common for non-mycorrhizal plants. These PFTs are known to access N from deeper mineral soils (Hewitt et al., 2019) which may buffer them from the isotopic signature of alder-derived N as well as explain why %N of these PTFs was not correlated with inorganic N availability of surface soils.

Alder Shrublands Are Expanding

The observed expansion of alder shrublands at Kougarok Hillslope has important implications for N inputs at this site. If the observed rate of N fixation by alder shrublands is applied to annual area of alder shrubland from 1956-2014, we see that the 40% increase in alder shrubland area was associated with a 22% increase in N fixation. Such an increase in available N has the potential to reshape plant productivity, alter plant community composition, and accelerate microbial decomposition in downslope, N-limited communities (Shaver et al., 2001; Mack et al., 2004). Nitrogen saturation also has the potential to increase N leaching and impact downstream ecosystems (Hiltbrunner et al., 2014). Expansion of alder shrubland communities at Kougarok Hillslope was associated with colonization of rocky, well-drained outcroppings near the crest of the hill and did not extend down into the tundra lowlands. This heterogeneous pattern of expansion parallels observations made farther north in Alaska (Tape et al., 2012). The 513 m^2 $year^{-1}$ of alder expansion reported here, therefore, could be used along with topographical maps and classification of suitable alder shrubland terrain to estimate future rates of alder expansion in Alaska and Northwest Canada. Care should be taken not to apply this rate to the entire arctic biome.

Sources of Variation in Alder N Fixation Rates

We posit that the variation in annual N fixation by alder shrubland and alder savanna communities was driven by a combination of P limitation and soil moisture dynamics. Nodule biomass exerted stronger control on annual N fixation inputs than the rates of fixation within individual nodules. The relationship between leaf N:P and nodule biomass suggests P availability varies across communities and higher sun leaf N:P was associated with lower nodule biomass. Alder savanna sun leaves generally had N:P over 20, a threshold often used to signify P limitation (Güsewell, 2004; Koerselman and Meuleman, 1996). Fertilization studies in tussock tundra plant communities have shown them to be jointly limited by N and P availability (Chapin and Shaver, 1985) and the regular spacing of alder growing in graminoid dominated tundra has been attributed to intra-specific competition for nutrients (Chapin et al., 1989). Furthermore, P availability was found to limit alder nodule production in a primary succession within a boreal floodplain (Uliassi and Ruess, 2002). The low availability of soil P and the higher foliar N:P in alder savannas, the propensity for graminoid-dominated tundra to be jointly N and P limited, and the fact that alder access to P limits alder nodule biomass in boreal ecosystems together suggest our observed differences in nodule biomass at Kougarok Hillslope are driven in part by P limitation within alder savanna communities.

Differences in soil moisture between the two plant communities may also be a key factor driving the observed variation in N fixation by alder shrubland and savanna communities. Though the surface soil moisture within the two plant communities was similar, the overall soil profiles differed greatly in their drainage potential and alder savannas had higher soil moisture deeper in

the soil profile. The shallow, sloping soils within alder shrublands were underlain by bedrock and would likely drain well during the growing season while the deep, flat soils in alder savannas would tend to retain moisture were are often saturated beneath the soil surface. The wet conditions in alder savannas were likely also a result of the underlying permafrost in these soil profiles: this impermeable layer would force the water table to remain perched near the soil surface. Though root nodules create an anaerobic zone for N fixation by *Frankia* bacteria, alder roots themselves do not appear to thrive in saturated soils (Tape et al., 2012). If the rooting zone of alder in savanna communities is restricted to the dry, organic horizon, alder access to nutrients in mineral horizons could be reduced. This could limit alder biomass production as well as slow the C supply to N-fixing symbionts.

Scaling Up Alder N Fixation

The strong association between alder basal area and rates of N fixation we observed across alder shrubland and alder savanna communities suggests that aboveground biomass could be a reliable scaler for estimating rates of N fixation by alder. Nodule biomass of alder shrublands was greater than in alder savannas when nodule biomass was expressed per unit ground area but nodule biomass of the two communities was similar when expressed per unit alder stem basal area (**Table 3**). The range of nodule biomass per m^2 ground was similar to that reported for the same species in boreal regions (Mitchell and Ruess, 2009) and N fixation rates within nodules were on the lower end of the range reported for boreal *A. incana* ssp. *tenuifolia* and *A. viridis* ssp. *crispa* measured using the same $^{15}N_2$ method (Anderson et al., 2004; Ruess et al., 2013). Work by Rhoades et al (2001) and Uliassi and Ruess (2002) in the boreal zone found similar positive correlations between N indices (resin-N, N fixation) and alder aboveground biomass (height, LAI). These lines of evidence suggest the relationship between alder aboveground biomass and inputs of N via symbiotic fixation may be consistent across boreal and arctic ecosystems and therefore may be an integral part of quantifying landscape level fluxes of N in high latitudes. These findings substantiate the assumptions in Epstein et al. (2000) and Menge et al. (2009), both of which model N fixation of alders by scaling this flux to plant biomass. Scaling N fixation rates from this study to other tundra landscapes, however, should be undertaken with caution. There are known sources of variation not considered in this study: nitrogen fixation by boreal alder is impacted by choice of *Frankia* bacterial partner (Anderson et al., 2009; Ruess et al., 2013), fungal infection (Ruess et al., 2009; Nossov et al., 2011), and stand age (Mitchell and Ruess, 2009). Further research is needed to quantify the impact these factors have on tundra alders.

Implications for Modeling Warming Arctic Ecosystems

Here we have shown that alder can be an important source of N to surrounding tundra plant communities and that N-fixation rates vary the community and landscape position the alder occupies. The area of tall alder shrublands has increased over past decades in upland areas and may increase further as tundra regions continue to warm. This expansion has important consequences for tundra N cycling, especially in areas downslope of alder shrublands. Our data both inform the mechanistic representation of N-fixation in models and provide observations to evaluate model projections.

Though alder has long been present in tundra ecosystems, few large-scale, high-latitude models include an N-fixing PFT. Epstein and colleagues (2000) incorporated *Alnus* species in ArcVeg, but other arctic models do not (ie, Rupp et al., 2000; Euskirchen et al., 2009). Climate-scale earth system models not simulate N-fixing species in high latitudes. The data reported in this study provide useful constraints for initiating implementation of a N-fixing shrub PFT in large-scale models. Our results suggest that N fixation by alder could be scaled to the landscape level using aboveground alder traits (e.g. height and biomass) that can be derived from remote-sensing products. The observed seasonal variation in resin-N may also help constrain alternative hypotheses for the processes driving plant nutrient uptake, and the seasonal timing of uptake (Riley et al., 2018). Capturing the variation in N fixation observed in this study may require community specific- parameterization of a N-fixing PFT or dynamic trait distributions that respond to environmental cues (Wullschleger et al., 2014). Parameterization of an N-fixing PFT would likely help capture changing vegetation dynamics of arctic ecosystems and could lead to more robust predictions of C and N feedbacks at high latitudes.

DATA AVAILABILITY STATEMENT

All data are publicly available. DOIs and hyperlinks are provided in the methods section.

AUTHOR CONTRIBUTIONS

VS led data collection in field and laboratory and analyzed data with significant assistance from CI. AB classified vegetation communities and plant species at the site, established plots, and performed field work. JK performed remote sensing neural network analysis and helped with field measurements. ML analyzed historic images from the site and helped with field measurements. PT and SW helped with field measurements and gave feedback on early drafts of the analysis. VS and CI wrote the first draft of the manuscript and all authors contributed to the final draft.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01099/full#supplementary-material

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Fine-Root Turnover, Litterfall, and Soil Microbial Community of Three Mixed Coniferous–Deciduous Forests Dominated by Korean Pine (*Pinus koraiensis*) Along a Latitudinal Gradient

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Carbon dynamics in forests and in particular in soils are of primary importance in the context of climate change. A better understanding of the drivers controlling carbon storage is needed to improve climate mitigation strategies. Carbon storage is the result of a balance between inputs and outputs. Carbon inputs in the soil come from plant residues and root exudates, which are further transformed by microorganisms and stored in the long term. Here, we measured litter and fine-root production in three mixed forests dominated by *Pinus koraiensis* along a latitudinal gradient and performed a litterbag experiment to better understand the driving factors of decomposition. We found that over the three sites litter production was controlled by climatic factors, soil properties, and forest stand characteristics, whereas decay rates were mainly controlled by microbial community structure and soil stoichiometry. For fine roots, production differed among sites, and higher production was consistently observed in the top soil layers compared to deep soil, although the root distribution along the soil profile differed among sites. Fine-root decay rates were mainly controlled by fine-root stoichiometric characteristics. This article emphasizes the complexity of fine-root dynamics even for a single species. Environmental drivers impact on both production and decay, and we suggest performing manipulative field experiments to better identify the mechanisms involved in soil carbon cycling.

Keywords: fine roots, litter decomposition, soil organic carbon, Korean pine, latitudinal gradient

INTRODUCTION

The interest in soil organic carbon (SOC) has increased in recent decades (Feller and Bernoux, 2008). Soils contain the largest stock of organic carbon, exceeding the amount of carbon stored in the atmosphere and in plant biomass (Jobbágy and Jackson, 2000; Scharlemann et al., 2014). As a consequence, even a small change in soil carbon storage may significantly affect the atmospheric concentrations of greenhouse gases and therefore the climate (Rustad

et al., 2000). Thus, SOC is considered of major importance in the global carbon cycle (Schmidt et al., 2011), and a better understanding of the factors controlling SOC dynamics is urgently needed to mitigate climate change (Luo et al., 2016; Dignac et al., 2017).

The SOC stocks are controlled by balance between the outputs (i.e., heterotrophic respiration and dissolved organic carbon lateral transport and erosion) and the inputs from primary production and sometimes deposition of eroded materials (Guo et al., 2005). The OC inputs into the soils can originate from aboveground litter entering the soil or come from root exudates (Rasse et al., 2005). Root production depends on local conditions, such as soil water content (SWC) and nutrient availability, plant species and ecological interactions with other species (Gill and Jackson, 2000; Finér et al., 2011; Keel et al., 2012). However, inputs into the soil depend also on root mortality, which is controlled by plant phenology and external drivers such as herbivory, fire, or environmental conditions (Riley et al., 2010). Finally, to be stored in the long term, dead plant material must be incorporated into the microbial biomass (Kallenbach et al., 2016). Microbial uptake and biotransformation are controlled by the structure of the microbial community as well as environmental drivers (Kaiser et al., 2014). Disentangling the effects of plant characteristics (e.g., litter quality) from the effects of environmental conditions (e.g., soil characteristics) is very complex. Therefore, major efforts are still needed toward a better understanding of root dynamics and in particular of the controlling factors of dead root decomposition. However, fine-root and litter turnover, decomposition of fine root and litter, and characteristics of soil microbial biomass have been rarely reported simultaneously in past studies. So, it might be important to find out its underlying mechanism.

In this study, we focused on factors driving the dynamics of root and litterfall at three different mixed forest sites in Northeast China dominated by *Pinus koraiensis* Sieb. et Zucc. (Korean pine) along a latitudinal gradient. These three sites have different vegetation composition and soil characteristics. Our major goals were to determine the seasonal dynamics of fine-root biomass and litterfall and fine-root and litter turnover rate and to understand the major drivers. We assumed that (1) fine-root and leaf litter decomposition rates were related to soil microbial properties in the Korean pine forests from different sites, but (2) those relationships regulating the fine-root and litter dynamics vary along latitudinal and gradients.

MATERIALS AND METHODS

Site Description

Three mixed coniferous–deciduous forests dominated by the Korean pine along a latitudinal gradient in Northeast China were assessed in this study: (1) Kuandian (40°91′N, 124°79′E, 930 m a.s.l.), (2) Dongsheng (44°41′N, 128°12′E, 804 m a.s.l.), (3) Shengshan (49°48′N, 126°78′E, 504 m a.s.l.). This region has a temperate continental climate with long cold winters but warm summers. The mean annual temperature and mean annual precipitation in Kuandian, Dongsheng, and Shengshan are 4.92°C and 1,082 mm, 0°C and 717 mm, and −1.54°C and 561 mm, respectively. Soils at the three sites are classified as Eutric cambisols (FAO-WRB (ISSS-ISRIC-FAO-UNESCO), 1998).

Three 20 × 20-m plots of mixed coniferous–deciduous forests dominated by Korean pine at each site were selected for this study. In Kuandian, the forest was dominated by *P. koraiensis* Sieb. et Zucc.(Korean pine), admixed with *Abies nephrolepis* (Trautv.) Maxim., *Padus maackii* (Rupr.) Kom., *Betula costata* Trautv., *Acer pseudosieboldianum* (Pax) Komarov, *Tilia amurensis* Rupr., *Quercus mongolica* Fisch. ex Ledeb., *Ulmus laciniata* (Trautv.) Mayr. In Dongsheng, the forest was dominated by Korean pine, admixed *Abies holophylla* Maxim., *Acer mono* Maxim., *Betula platyphylla* Suk., *Quercus mongolica* Fisch. ex Ledeb., and *T. amurensis* Rupr. Finally, in Shengshan, the forest was dominated by *P. koraiensis* Sieb. et Zucc., admixed with *T. amurensis* Rupr., *U. laciniata* (Trautv.) Mayr., and *A. mono* Maxim. Tree density, stand basal area, and Korean pine basal area of the three forests are shown in **Table 1**.

Soil Properties

Soil samples (0–20 cm) for measuring gravimetric SWC were collected from five randomly placed cores (3-cm diameter) in each plot in May, June, July, August, September, and October 2015, and SWC was measured by oven-drying samples at 105°C for 24 h. Soil bulk density was determined by weighing oven-dried samples of known volume by using a cutting ring (volume 100 cm3, inner diameter 5 cm). Soil samples (0–20 cm) for measuring physical and chemical properties were collected from five randomly placed cores (3-cm diameter) in each plot in July 2015. The five replicates in each plot were pooled and mixed to get one composite sample. Composite soil samples were air dried, passed through a 2-mm sieve, and manually cleaned of any visible plant tissues for laboratory analysis. Soil organic carbon content and soil total nitrogen content were determined by an element analyzer (Vario EL III), and soil

TABLE 1 | The community and soil properties of three mixed coniferous–deciduous forests dominated by Korean pine and located in Kuandian, Dongsheng, and Shengshan. Values represent mean ± standard error (n = 3). Different letters within a soil layer are significantly different ($P < 0.05$) according to the Duncan *post hoc* test.

	Stand density (stem ha^{-1})	Stand basal area (m^2 ha^{-1})	Korean pine basal area (m^2 ha^{-1})	Soil water content (%)	Soil bulk density (g cm^{-3})	pH	Soil organic carbon (g kg^{-1})	Soil total nitrogen (g kg^{-1})	Soil total phosphorus (g kg^{-1})	C:N	C:P	N:P
Kuandian	867 ± 203a	35.5 ± 0.5b	12.2 ± 2.4b	69.07 ± 1.77a	1.08 ± 0.01a	4.87 ± 0.13a	66.4 ± 3.2ab	4.8 ± 0.1a	0.75 ± 0.04a	13.8 ± 0.3b	88.6 ± 2.0b	6.5 ± 0.2a
Dongsheng	878 ± 121a	65.6 ± 10.7a	20.6 ± 5.0a	49.6 ± 2.71b	1.1 ± 0.02a	4.97 ± 0.09a	74.1 ± 3.4a	5.1 ± 0.1a	0.79 ± 0.03a	14.7 ± 0.4b	93.8 ± 2.1b	6.4 ± 0.1a
Shengshan	692 ± 224a	28.6 ± 2.4b	21.8 ± 0.9a	35.8 ± 1.63c	1.07 ± 0.01a	4.90 ± 0.06a	57.8 ± 3.0b	3.1 ± 0.1b	0.53 ± 0.02b	18.6 ± 0.5a	109.7 ± 2.6a	5.9 ± 0.1a

total phosphorous content (Moty et al., 2016) was analyzed by using the molybdenum blue colorimetric method with a UV/visible spectrophotometer after H_2SO_4-H_2O_2 digestion (UV-2550; Shimadzu, Kyoto, Japan).

Fine-Root Biomass, Production, and Turnover Rate

The core sampling method (Roberts, 1976) was used to measure fine-root (< 2 mm) biomass in each plot down to 40-cm soil depth in May, July, and September 2015. At each sampling date, soil samples (0–10, 10–20, and 20–40 cm) were collected from five randomly located cores (8 cm in diameter and 10 cm in length) at each plot. Fine roots were manually removed from the soil samples and washed. These separated fine roots were further classified into live and dead fine roots by the following distinct criteria. Live fine roots were characterized as roots with a varying degree of brownish tissues, often well branched, with light and turgid root tips, and with white to slightly brown and elastic stele (Vogt and Persson, 1990; Persson and Stadenberg, 2010; Yang et al., 2010). Dry biomass was determined after oven drying at 65°C for 2 days.

A modified in-growth core technique (Lund et al., 1970) was used to determine fine-root (< 2 mm) production. Five holes were created in each plot and were refilled with native soil without any root biomass, and their boundaries were marked with sticks in October 2014. These in-growth cores were harvested at the end of October 2015. Soil samples from different depths (0–10, 10–20, and 20–40 cm) for each in-growth core were labeled, and fine-root biomass was subsequently estimated using exactly the same procedures as described above. Total fine-root production was estimated as the sum of live and dead roots present in the in-growth core at the end of October 2015.

Fine-root turnover rate is defined here as the ratio of the total amount of fine root produced over the mean standing biomass of fine roots (Aber et al., 1985). Mean fine-root biomass was estimated as the average of live root biomass in May, July, and September 2015.

Forest Floor Mass, Litter Fall, and Litter Turnover

Forest floor mass was collected in October 2014 and every month from May to October 2015 by placing a 0.5 × 0.5-m quadrant at five randomly located places in each plot. Collected forest floor mass was further sorted into woody and nonwoody fractions. Litterfall samples were trapped and collected using five randomly located 0.5 × 0.5-m rectangular baskets in each plot. Samples were frequently collected from October 2014 to October 2015 and sorted into woody and nonwoody fractions. Dry litter mass was determined after oven drying at 65°C for 2 to 3 days.

The turnover rate of litter was calculated as the ratio of the annual litter production over the mean annual floor mass according to (Rochow, 1974). Mean annual floor mass was estimated as the average of floor mass between October 2014 and October 2015.

Soil Microbial Properties

Five 0- to 20-cm soil core samples (3-cm diameter) per plot were randomly collected for determination of soil microbial properties in July 2015. Each sample was labeled and then stored at 2°C in a cooler before transporting to the laboratory. In the laboratory, the fresh samples were passed through a 2-mm sieve and manually cleaned of any visible plant tissues. Soil microbial biomass carbon (SMBC) and nitrogen (SMBN) were measured by the chloroform fumigation–extraction method (Vance et al., 1987). Briefly, after adjusting to approximately 60% of water-holding capacity, the fresh soil samples were incubated for 1 week in the dark at 25°C. Twenty grams (dry weight equivalent) of fumigated and nonfumigated soil samples were extracted with 0.5 M K_2SO_4. Extracts were filtered through 0.45-μm filters and frozen at −20°C before analysis of extractable carbon by dichromate digestion as described by Lovell et al. (1995). SMBC and SMBN were calculated as the difference in extractable organic C and inorganic N contents between the fumigated and the nonfumigated samples using conversion factors (kec and ken) of 0.38 and 0.45 (Lovell et al., 1995), respectively.

The soil microbial community was characterized using phospholipid fatty acids (PLFAs) analysis as described by Bossio and Scow (1998). The separation and identification of extracted PLFAs were carried out according to the standard protocol of the Sherlock Microbial Identification System $V_{4.5}$ (MIDI) and a Gas Chromatograph (Agilent 6850, USA). The abundance of individual fatty acids was determined as relative nanomoles per gram of dry soil, and standard nomenclature was used. The fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω7c, 16:1ω5c, i17:0, a17:0, 17:0cy, and 19:0cy were chosen to represent the PLFAs of the bacterial group, and fungi were considered to be represented by the PLFAs 18:2ω6c and 18:1ω9c (Frostegård and Bååth, 1996; Bossio and Scow, 1998). All of the PLFAs mentioned above were used to calculate the total PLFAs of soil microbial community. The ratio of fungal to bacterial PLFAs was also included in the data analysis. This ratio has often been used as an indicator of changes in the soil microbial community structure (Bardgett et al., 1999).

Decomposition of Leaf Litter and Fine Roots

Decomposition rates of leaf litter and fine root were determined using the nylon bag (or litterbag) method (Arunachalam et al., 1996). Recently fallen leaves and fine roots from the 0- to 10-cm mineral soil layer were collected from each plot.

Each nylon bag had a dimension of 15 × 15 cm and a mesh of 1 mm. In each plot, 25 nylon bags containing 3 g of air-dried leaves or fine roots were placed on the forest floor and at the10-cm soil depth, respectively, in October 2014. These nylon bags were sampled after 0, 218, 263, 307, and 365 days in Kuandian; 0, 220, 264, 308, and 365 days in Dongsheng; and 0, 221, 266, 309, and 365 days in Shengshan, respectively. Five nylon bags were collected at each sampling date. Mass of leaf litter and fine roots in each nylon bag was determined after oven drying at 65°C for 2 to 3 days. In addition, we also determined total C, N, and P of leaf litter and fine-root material (**Table 2**). Initial OC, total N, and P content of each litter category were measured using the same methods as for soil.

TABLE 2 | The chemical contents of leaf and fine-root litter of mixed coniferous–deciduous forests dominated by Korean pine in Kuandian, Dongsheng, and Shengshan. Values represent mean ± standard error (n = 3). Different letters within a soil layer are significantly different ($P < 0.05$) according to the Duncan *post hoc* test.

		Kuandian	Dongsheng	Shengshan
Leaf litter	C	504.7 ± 4.2b	513.2 ± 7.3b	533.5 ± 3.3a
	N	14.9 ± 0.1a	13.7 ± 0.3b	12.5 ± 0.2c
	P	1.1 ± 0.01a	1.1 ± 0.02a	0.98 ± 0.02b
	C:N	34.0 ± 0.2b	37.5 ± 0.6c	42.7 ± 0.6a
	N:P	13.0 ± 0.12a	12.7 ± 0.02a	12.7 ± 0.11a
	C:P	441.5 ± 2.1b	475.5 ± 8.2c	544.9 ± 11.5a
Fine-root litter	C	472.4 ± 3.1b	482.3 ± 3.6b	497.9 ± 2.1a
	N	14.2 ± 0.4a	12.2 ± 0.2b	11.2 ± 0.4b
	P	1.2 ± 0.03a	1.2 ± 0.01a	1.0 ± 0.02b
	C:N	33.2 ± 0.8b	39.6 ± 0.5c	44.7 ± 1.5a
	N:P	11.6 ± 0.1a	10.6 ± 0.2b	10.8 ± 0.5ab
	C:P	386.5 ± 7.6b	419.5 ± 3.0c	480.6 ± 7.9a

Statistical Analysis

Data management and statistical analyses were performed using SPSS software (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) was used to test for significant differences of community and soil properties, initial chemical content of leaf litter and fine root, and the annual decay constant (k). Two-way ANOVA was used to test the effects of sampling position and soil layer on fine-root production, and two-way ANOVA was used to test the effects of sampling position and sampling date on litter fall and forest floor mass. Three-way ANOVA was used to test the effects of sampling position, soil layer, and sampling time and all their interactions on fine-root biomass. Multiple comparisons were also performed to permit separation of effect means using Duncan *post hoc* test at a significance level of $P < 0.05$. A stepwise regression was used to measure the relationships of the annual decay constant (k) of leaf litter or fine-root litter to soil microbial community and soil properties, initial chemical content of leaf litter and fine root, fine-root biomass, production and turnover rate, litter fall and forest floor mass, and soil microbial properties. This stepwise regression was performed on the entire dataset without distinction per site. Annual decay constant was calculated using Equation 1 (Olson, 1963):

$$Y_t = Y_0 \times e^{kt} \quad (1)$$

with t being the time in years; Y_0, the weight of litter at the beginning of the experiment; Y_t, the weight of remaining litter at certain time (t); and k, the decay constant.

RESULTS

Forest Floor Mass and Litter Fall

Total litter fall was site dependent (**Table 3**, $P < 0.001$), with the highest litter production in Donsheng, followed by Kuandian and Shengshan (**Figure 1**). For all sites, litter production varied seasonally (**Table 3**, $P < 0.001$), and a significant interaction

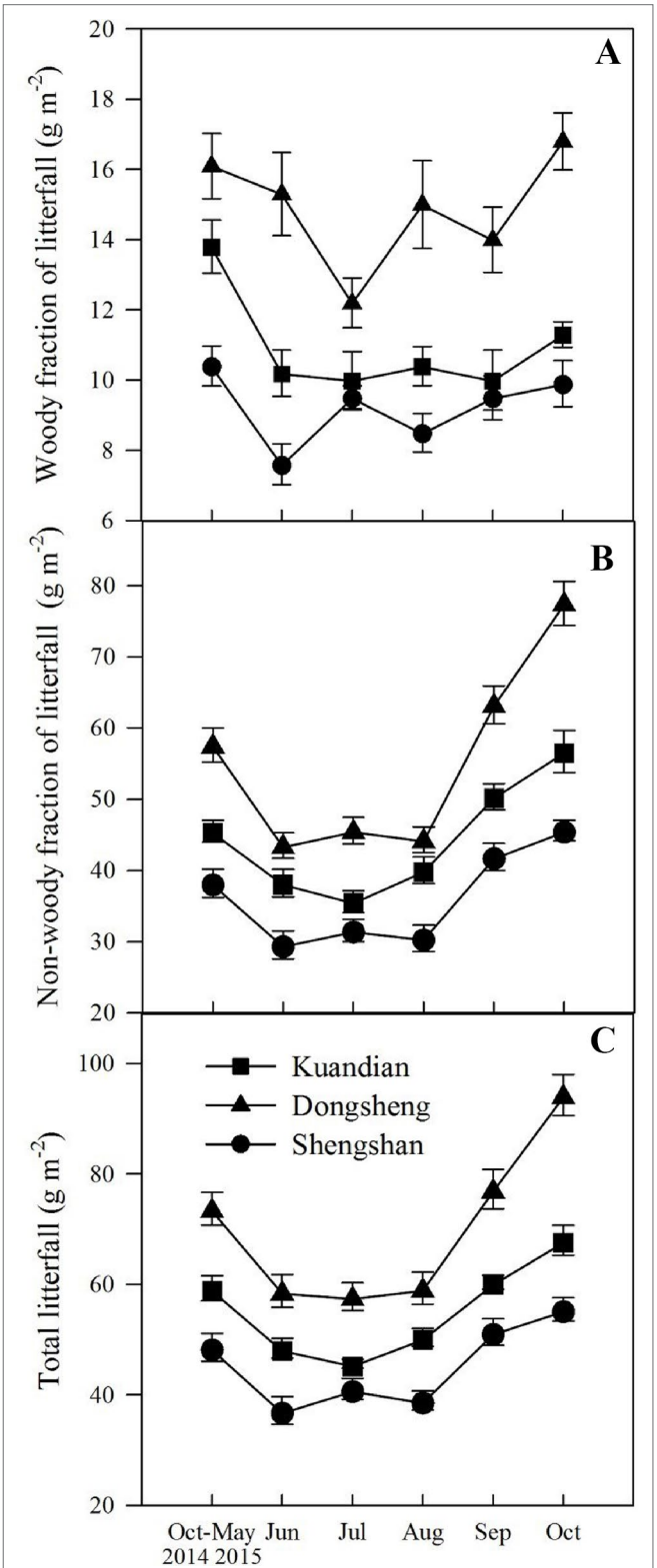

FIGURE 1 | Seasonal changes in woody **(A)** and nonwoody **(B)** fractions of litterfall and total litterfall **(C)** of three mixed coniferous–deciduous forests dominated by Korean pine and located in Kuandian, Dongsheng, and Shengshan from October 2014 to October 2015. Vertical bars indicate standard errors of means (n = 3).

TABLE 3 | Results (*F* values) of three-way ANOVA on the effects of site, soil layer (SL), sampling time (ST), and all their interactions on fine-root biomass and production, woody and nonwoody fraction of litter fall and total litter fall, and woody fraction and nonwoody fraction of forest floor mass and total forest floor mass.

Sources	Fine-root biomass	Fine-root production	Woody fraction of litter fall	Nonwoody fraction of litter fall	Total litter fall	Woody fraction of forest floor mass	Nonwoody fraction of forest floor mass	Total forest floor mass
Site	174.30***	127.01***	86***	130.38***	154.06***	29.67***	913.49***	466.81***
ST	12.79***		6.23***	62.06***	50.02***	0.76	7.05***	4.39**
SL	174.30***	505.28***						
Site * ST	0.35		1.88	3.622**	2.94**	0.10	0.34	0.15
ST * SL	3.46*							
Site * SL	38.47***	16.26***						
Site * ST * SL	0.11							

, **, and * represent significance at P<0.05, P<0.01, and P<0.001.*

between site and time was observed (**Table 3**, $P<0.001$). In particular, the peak in litter fall in September was higher for Donsheng than for the other sites. The woody fraction of the litter fall followed the same seasonal pattern as total litter fall, with a site and a time effect, but no interaction between both (**Table 3**). For the nonwoody fraction, a significant interaction between site and time was also detected.

Total floor mass showed a similar pattern to that of litterfall, except that no interaction between site and time was observed (**Table 3**). The total floor mass was higher for Donsheng followed by Kuandian and Shengshan (**Figure 2**), and following the litter fall peak, also total floor litter mass was higher in September and October. For the woody fraction, only a site effect was detected (**Table 3**, $P<0.001$), and no significantly temporal variation was observed. For the nonwoody fraction, a site effect together with a time effect was detected (for both **Table 3**, $P<0.001$).

Fine-Root Biomass, Production, and Turnover Rate

Fine-root biomass production was site dependent (**Table 3**, $P<0.001$), with higher production at Donsheng, followed by Kuandian and Shengshan (**Figure 3**). All sites presented higher fine-root production in top soil compared to deeper soil layers (**Figure 3 Table 3**). This vertical pattern was site dependent, as suggested by the significant interaction between site and soil layers presented in **Table 3**. Nevertheless, this effect was not quantitatively important (**Figure 3**). Fine-root biomass followed a similar pattern (**Figure 4**), with higher fine-root biomass in the top soil layers than in the deep soil layers. We also observed a site effect with higher biomass for Donsheng followed by Kuandian and Shengshan. Fine-root biomass also varied with time (**Table 3**, $P<0.001$), with higher biomass in July compared to the other periods. For fine-root biomass, the significant interaction between time and soil layers (**Table 3**, $P<0.05$) suggests that the increase observed in July was mainly due to an increase in the top layers (**Figure 4**). Finally, the significant interaction between site and soil layers (**Table 3**, $P<0.001$) suggests that the vertical root biomass distribution was site dependent. Indeed, the relative proportion of fine-root biomass was highest in the top layer; this vertical difference was larger at Shengshan than at the other sites.

Soil Microbial Properties

Soil microbial biomass C was similar in Donsheng and Kuandian, but lower in Shengshan (**Figure 5**). For soil microbial N, a higher stock was observed in Donsheng, followed by Kuandian and Shengshan. Nevertheless, the microbial C:N ratio was similar at all sites, being close to 4. For all sites, the microbial community was dominated by bacteria, as suggested by the PLFA analysis presented in **Figure 6**. Indeed, the ratio of fungi to bacteria (F:B ratio) was low: between 0.0298 ± 0.0011 for Kuandian and 0.0471 ± 0.0035 for Shengshan. Some differences among sites were observed. In particular, in agreement with the other proxy for microbial biomass, soil microbial C, also the total PLFA was significantly lower at Shengshan compared to the other sites. Also, fungal biomass was higher at this site, explaining its significantly higher F:B ratio.

Leaf Litter and Fine-Root Decomposition

After 1 year in the field, the least litter mass remained in the leaf litter and root litter bags in Kundian, followed by Dongshen and Shengshan (**Figure 7**). Nevertheless, the temporal pattern of decomposition was quite similar among the sites, with limited decomposition during the cold winter and then a rapid decrease in remaining mass between March and October. When estimating the turnover rates (**Table 4** and **Figure 8**), we observed a statistically significantly higher turnover rate for Kundian, followed by Dongshen and Shengshan, for both leaf litter and fine roots. The stepwise regression indicated that for all sites fine-root turnover rates were controlled by their C:N and C:P ratio, as well as by the SWC and the soil C:N ratio (**Table 5**). No microbial-related variables were significantly correlated to the turnover rate. For the leaf litter, turnover rate was controlled by its C:N ratio, but also by the soil C:N ratio and the F:B ratio. In addition, Korean pine basal area and tree density also partially controlled the leaf litter decay constant (**Table 5**).

DISCUSSION

Forest ecosystems, as a large and persistent carbon sink, play a more and more important role in the global carbon cycle under the context of climate change (Pan et al., 2011). Major carbon inputs into soils are fine root and leave litter, and it is necessary to analyse how they vary. Aboveground litterfall is the key ecosystem

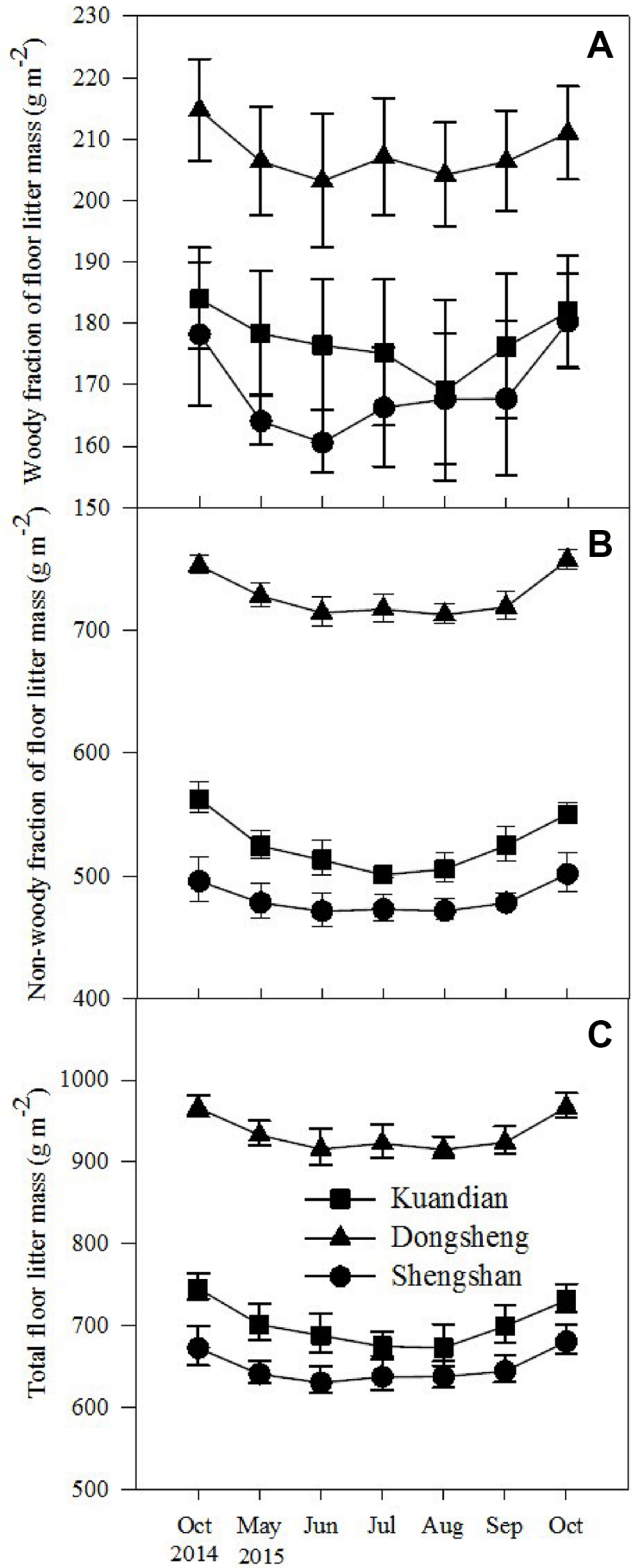

FIGURE 2 | Seasonal changes in woody fraction **(A)** and nonwoody fraction **(B)** of forest floor mass and total forest floor mass **(C)** of three mixed coniferous–deciduous forests from October 2014 to October 2015. Vertical bars indicate standard errors of means (n = 3).

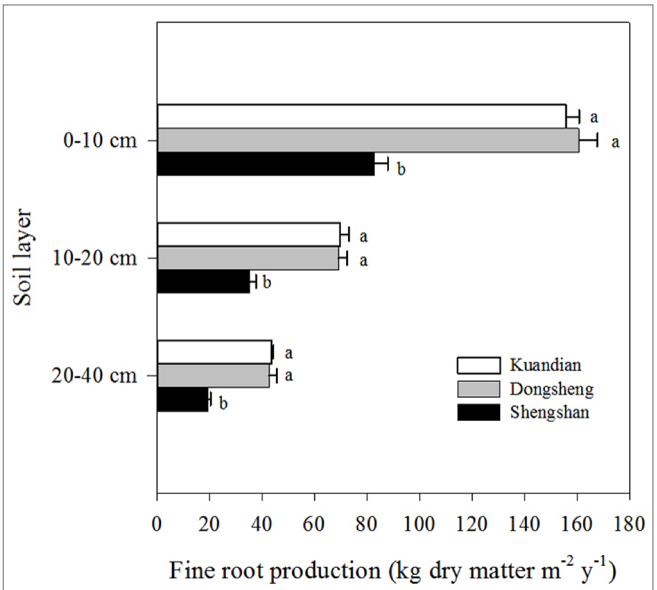

FIGURE 3 | Vertical distribution of fine-root production of three mixed coniferous–deciduous forests. Horizontal bars indicate standard errors of means (n = 3). Different letters within a soil layer indicate significant difference ($P < 0.05$) according to the Duncan *post hoc* test.

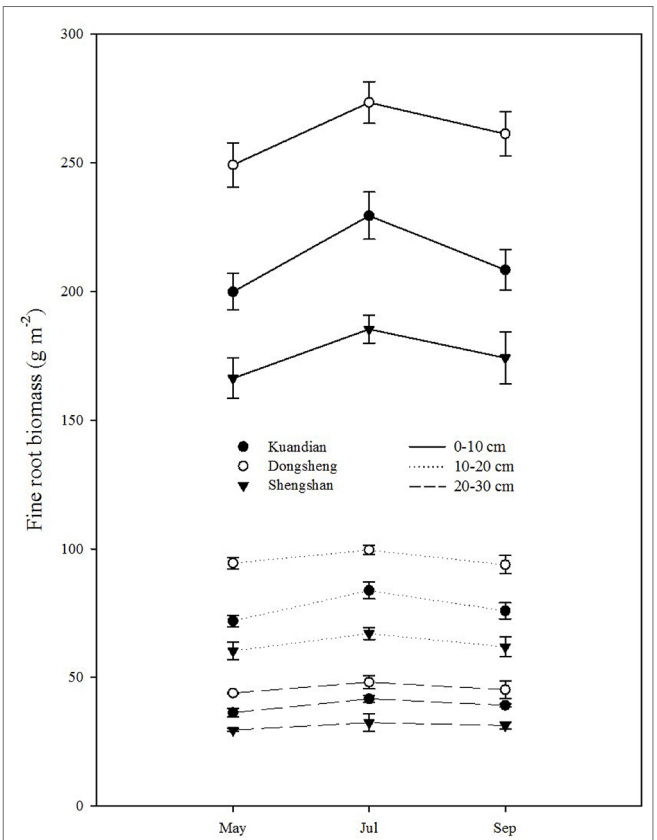

FIGURE 4 | Vertical distribution of fine-root biomass of three mixed coniferous–deciduous forests in May, July, and September 2015. Vertical bars indicate standard errors of means (n = 3).

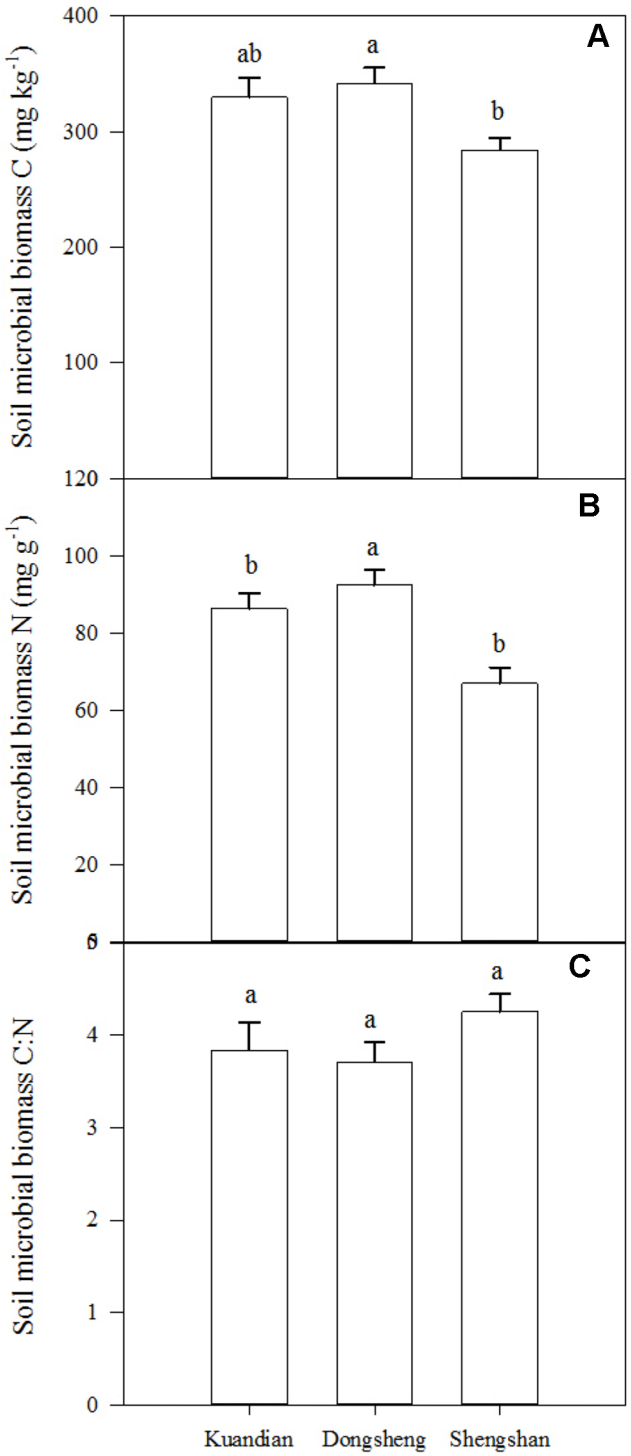

FIGURE 5 | Soil microbial biomass C **(A)**, N **(B)**, and C: N **(C)** ratio of three mixed coniferous–deciduous forests in the 0- to 20-cm-depth increment. Vertical bars indicate standard errors of means (n = 3). Different letters indicate significant difference ($P < 0.05$) according to the Duncan *post hoc* test.

process for both nutrient and energy transfer from plant canopies to the soils. We observed that, for all study sites, the forest floor litter mass in fall was higher than in summer, as the litter of mixed deciduous–coniferous forests is mainly produced during fall when physiological activity is reduced (Richardson et al., 2010). The three sites had different litter and root production rates, inducing different litter stocks and fine-root biomass. This might be due to climatic factors, which are known to be major drivers of plant productivity (Beer et al., 2010), since the least productive site, Shengshan, was also the coldest and the driest. Nevertheless, it is difficult to clearly disentangle the effect since the sites also differed by their stand basal areas and their soil characteristics such as total SOC, STN, C:N, and C:P ratios. Nevertheless, litter mass is the result of a balance between litter inputs and decomposition, which we measured using the litter-bag method.

For leaf litter decomposition, the mass remaining after 1 year was in line with previous studies (Prescott et al., 2000; Parton et al., 2007). The main drivers of decomposition are related to climate and litter chemistry. We observed the fastest litter turnover rate at Kundian, which was also the site with the highest mean annual air temperature. Temperature indeed enhances microbial metabolic activity potentially leading to accelerated litter decomposition rate (Schindlbacher et al., 2011). C:N and C:P ratios of litter are generally higher than those of the microbial decomposers (Xu et al., 2013). Therefore, decomposers need to access exogenous nutrients to decompose the litter (Hobbie and Vitousek, 2000; Norris et al., 2013), explaining the significant effect of soil stoichiometry on litter decay rates. Moreover, other forest stand characteristics also appeared to be of major importance in our case (**Table 3**). Tree species composition effects on SOC stocks were already reported in a large-scale meta-analysis study (Vesterdal et al., 2013). This effect might be explained by an impact of the tree community and litter production on some soil characteristics driving the decomposition, such as moisture or temperature as observed by Villalobos-Vega et al. (2011) in neotropical savanna. Trees can also influence decomposition through the priming effect of their fresh litter inputs (Prévost-Bouré et al., 2010). Finally, decomposition rate is also partially controlled by soil microbial characteristics, in particular the microbial biomass and its F:B ratio (Salamanca et al., 2003). In our study, the slowest litter turnover rates were observed at Shengshan, where we also measured the lowest microbial biomass and the highest F:B ratio. Our F:B ratio values are in line with previous observations (Bååth and Anderson, 2003), and it is known that fungi play a major role in the first stages of litter decomposition (Voříšková and Baldrian, 2013). Therefore, the slowest decomposition and turnover rates observed at Shengshan are probably due to the lowest microbial biomass, although the highest F:B ratio was observed at Shengshan. Additionally, the F:B ratio in the three Korean pine forests decreased along the latitudinal gradient, reflecting that there is a shift in the function and composition of microbial communities among the three Korean pine forests along this latitudinal gradient that might be due to changes in the environmental conditions (e.g., temperature or moisture) or to changes in the stoichiometry of the litter.

Fine roots take an important part on the biogeochemical cycle of carbon in forest ecosystems, and up to 67% of the annual net primary production in forest ecosystem can be allocated to fine

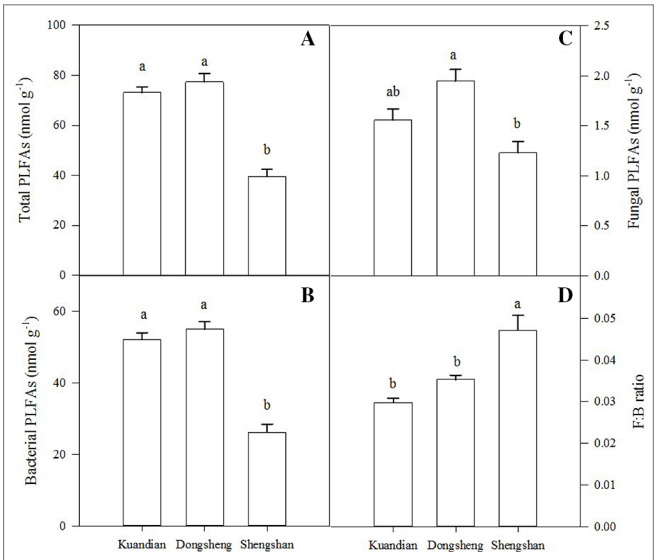

FIGURE 6 | Total PLFAs **(A)**, bacterial PLFAs **(B)**, fungal PLFAs **(C)**, and F:B ratio **(D)** of mixed coniferous–deciduous forests dominated by Korean pine in Kuandian, Dongsheng, and Shengshan. Vertical bars indicate standard errors of means (n = 3). Different letters within a soil layer are significantly different ($P < 0.05$) according to the Duncan *post hoc* test.

roots (Jackson et al., 1997; Janssens et al., 2002). Fine roots combine a short life span (< 1–9 years) (Matamala et al., 2003) and high decomposition rate (Silver and Miya, 2001). In our study, fine-root biomass profiles were in line with expectations (Riley et al., 2010), whereas their production was low compared to other studies (Finér et al., 2011). Measuring root productivity is difficult, and results are method dependent. Indeed, in-growth methods similar to what was applied in this study generally deliver lower productivity estimates than other methods (Finér et al., 2011). We speculate that this explains the rather low root productivity estimates in our study. Higher fine-root biomass was observed in Dongshen, compared to the other sites. Because the fine-root production rate was similar between Dongshen and Kuandian, the higher fine-root biomass at Dongshen was therefore probably due to the reduced mortality at that site, likely because of different physical conditions and nutrient availability. It is known that rapid fine-root turnover requires more nutrient and energy cost, such that at nutrient-limited sites root life span is typically longer, resulting in reduced turnover rates and lower efficiency of resource uptake (Schoettle et al., 1994).

Decay rates of fine roots were in line with previous observations (Gill and Jackson, 2000). In our study, decay rates depended primarily on the fine-root characteristics, and not on soil characteristics, with the exception of SWC and C:N ratio. The importance of C:N and C:P ratio of the decomposed material has been already underlined as a major driver of decomposition (Zhou et al., 2008). Interestingly, no microbial-related variables significantly affected the decomposition of fine roots in our study. This is in accordance with the work of (Wei et al. (2015), who observed that heterotrophic respiration was not controlled by microbial characteristics in subtropical forests.

In conclusion, in all study sites, the forest fine-root biomass and floor litter mass in fall were higher than in summer.

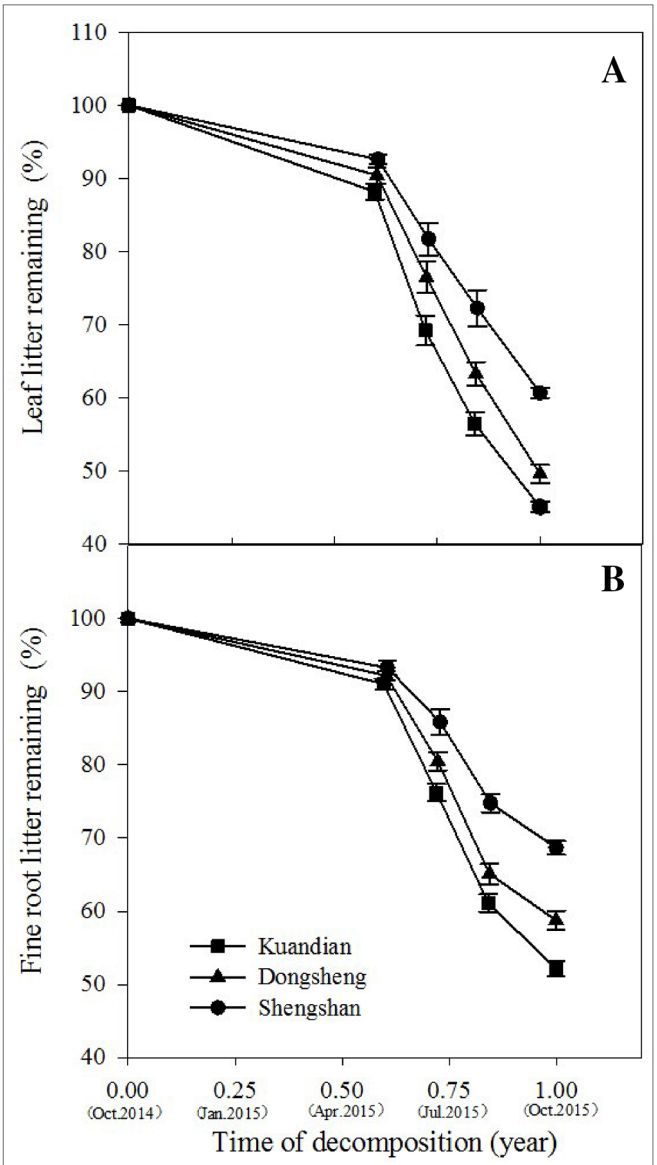

FIGURE 7 | Leaf litter mass **(A)** and fine-root litter mass **(B)** remaining (% of initial) of three mixed coniferous–deciduous forests during 1-year period. Vertical bars indicate standard errors of means (n = 3).

TABLE 4 | Mean annual decay constant (*k*) of leaf and fine-root litter of mixed coniferous–deciduous forests dominated by Korean pine in Kuandian, Dongsheng, and Shengshan. R^2, multiple coefficient of determination, and significance level of the exponential model ($P < 0.05$; $P < 0.01$) are also given. Values represent mean ± standard error (n = 3). Different letters within a soil layer are significantly different ($P < 0.05$) according to the Duncan *post hoc* test.

		k	R^2
Leaf litter	Kuandian	0.751(0.147)a	0.783**
	Dongsheng	0.636(0.165)b	0.738**
	Shengshan	0.449(0.152)c	0.740**
Fine-root litter	Kuandian	0.619(0.061)a	0.873**
	Dongsheng	0.514(0.076)c	0.750**
	Shengshan	0.355(0.067)b	0.762**

** *represents significant at P < 0.01.*

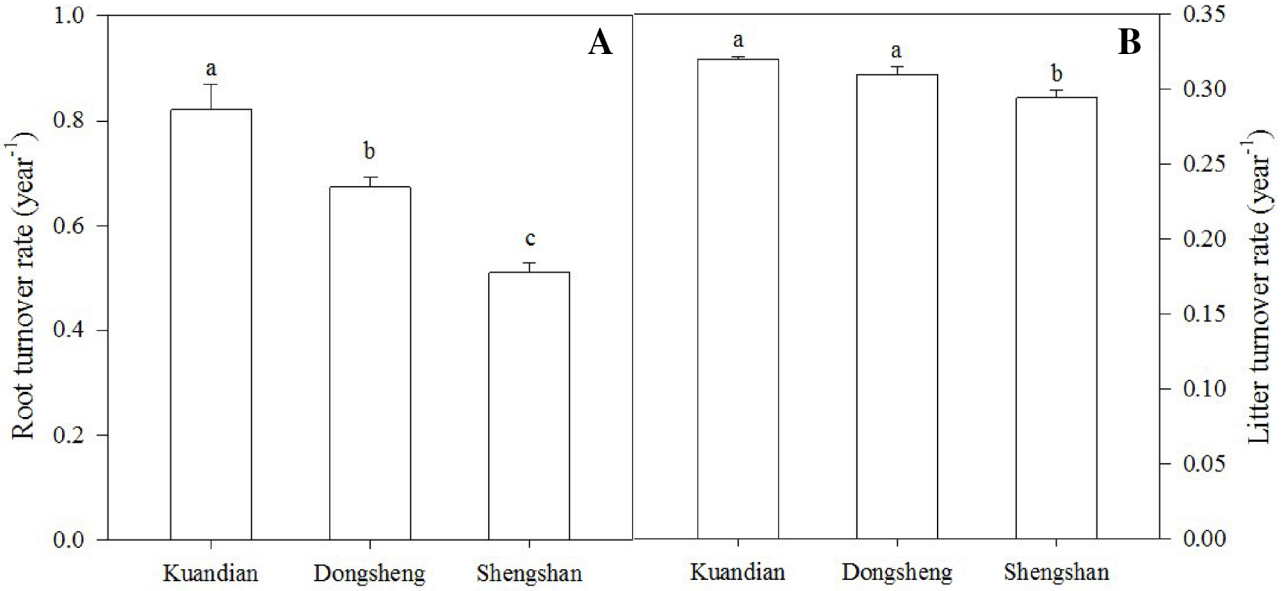

FIGURE 8 | Fine-root turnover rate **(A)** and litter turnover rate **(B)** of three mixed coniferous–deciduous forests. Vertical bars indicate standard errors of means (n = 3). Different letters are significantly different ($P < 0.05$) according to the Duncan *post hoc* test.

TABLE 5 | Results of the stepwise regression on annual decay constant.

	Model	Unstandardized coefficients		Standardized coefficients	*t*	*P*
		B	Std. Error	β		
Fine-root k	(Constant)	1.098	0.073		15.097	0.000
	Root litter C:P	–0.001	0.000	–0.301	–6.339	0.003
	Soil C:N	–0.016	0.002	–0.310	–10.229	0.001
	Soil water content	0.002	0.000	0.294	5.859	0.004
	Root litter C:N	–0.003	0.001	–0.143	–3.132	0.035
Leaf litter k	(Constant)	1.685	0.013		127.315	0.000
	Leaf litter C:N	–0.013	0.001	–0.385	–19.975	0.000
	Soil C:N	–0.028	0.001	–0.481	–25.652	0.000
	Korean pine basal area	–0.004	0.000	–0.226	–31.918	0.000
	Tree density	–2.749E–5	0.000	–0.061	–8.106	0.004
	Fungi:bacteria	–0.899	0.205	–0.057	–4.383	0.022

The main drivers of decomposition were related to climate and litter chemistry, but also partially controlled by soil microbial characteristics, in particular the microbial biomass and its F:B ratio. The F:B ratio in the three Korean pine forests decreased along the latitudinal gradient, but we also observed that the fine-root and litter decomposition drivers were controlled by other environmental factors.

Finally, we showed that litter stocks (including aboveground and belowground litter) were different among the three study sites, even though they were all dominated by Korean pine. We further showed that the litter stock dynamics are the result of the balance between primary production and dead plant material decay. Both are controlled by stand characteristics and soil properties that may go into different directions. It is, nevertheless, difficult to clearly separate the effects of all the drivers. Manipulative experimental sites modifying, for instance, soil temperature or soil moisture are quite powerful tools to test ecological hypotheses (Genardzielinski et al., 2018), and we strongly advocate their use in future research, but also a combination with manipulated stand structure would be of great interest.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

CX and BG formulated the original idea and developed methodology. FY, XS and CX performed sample processing. LL, FY and YW performed statistical analysis. CX, BG and IJ wrote the first draft of the manuscript.

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Disentangling Drought and Nutrient Effects on Soil Carbon Dioxide and Methane Fluxes in a Tropical Forest

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Tropical soils are a major contributor to the balance of greenhouse gas (GHG) fluxes in the atmosphere. Models of tropical GHG fluxes predict that both the frequency of drought events and changes in atmospheric deposition of nitrogen (N) will significantly affect dynamics of soil carbon dioxide (CO_2) and methane (CH_4) production and consumption. In this study, we examined the combined effect of a reduction in precipitation and an increase in nutrient availability on soil CO_2 and CH_4 fluxes in a primary French Guiana tropical forest. Drought conditions were simulated by intercepting precipitation falling through the forest canopy with tarpaulin roofs. Nutrient availability was manipulated through application of granular N and/or phosphorus (P) fertilizer to the soil. Soil water content (SWC) below the roofs decreased rapidly and stayed at continuously low values until roof removal, which as a consequence roughly doubled the duration of the dry season. After roof removal, SWC slowly increased but remained lower than in the control soils even after 2.5 months of wet-season precipitation. We showed that drought-imposed reduction in SWC decreased the CO_2 emissions (i.e., CO_2 efflux), but strongly increased the CH_4 emissions. N, P, and N × P (i.e., NP) additions all significantly increased CO_2 emission but had no effect on CH_4 fluxes. In treatments where both fertilization and drought were applied, the positive effect of N, P, and NP fertilization on CO_2 efflux was reduced. After roof removal, soil CO_2 efflux was more resilient in the control plots than in the fertilized plots while there was only a modest effect of roof removal on soil CH_4 fluxes. Our results suggest that a combined increase in drought and nutrient availability in soil can locally increase the emissions of both CO_2 and CH_4 from tropical soils, for a long term.

Keywords: carbon dioxide, drought, fertilization, methane, nitrogen, phosphorus, soil GHG fluxes, tropical forest

INTRODUCTION

Climate models predict a range of changes in weather patterns in tropical forest regions (Feng et al., 2013). Across Amazonia, a drier and warmer climate is expected for the coming century, which is predicted to result in an increased frequency of drought events, and can put a strain on the Amazon's ecological functioning (Duffy et al., 2015). In addition, atmospheric deposition of

nitrogen (N) can result in a stoichiometric imbalance of carbon (C) and N relative to phosphorus (P) in tropical biomes (Peñuelas et al., 2013). These global changes can have a broad impact on tropical ecosystem functioning and are predicted to lead to a severe disturbance of the Amazonian forest biome (Huntingford et al., 2004). Even though the exact magnitude of this projection is still debated (Cook et al., 2012), modeling studies report that mature tropical forests are highly vulnerable to extreme drought events (Phillips et al., 2009) and to changes in precipitation regimes in combination with modifications in nutrient stoichiometry (Malhi et al., 2008). However, soil biological processes, which are an important determinant of forest functioning, remain poorly described in models focusing on forest ecosystems.

Soils are an important source and sink of radiatively active trace-gases, i.e., carbon dioxide (CO_2) and methane (CH_4). Soil trace gas production and consumption are highly sensitive to soil moisture (Davidson et al., 2004, 2008) through its effect on soil redox state, diffusion, nutrient pools, and microbial activity (Conrad, 1996; Davidson et al., 2004, 2008). Several studies have explored the effect of precipitation regimes on soil trace-gas emissions either through experimental throughfall exclusion (Davidson et al., 2004; Cleveland et al., 2010; Wood and Silver, 2012; Meir et al., 2015) or comparisons of annual emissions of years differing in throughfall (Bonal et al., 2008; Rowland et al., 2014; Doughty et al., 2015; O'Connell et al., 2018). These studies report contrasting results with either a reduction (Bonal et al., 2008; Wood and Silver, 2012; Rowland et al., 2014), an increase (Cleveland et al., 2010; O'Connell et al., 2018), or no change (Davidson et al., 2008) in soil CO_2 emissions (i.e., soil CO_2 efflux; soil respiration) with drought. Discrepancies in the direction, and especially extent, of fluxes are related to differences in forest or soil types (Meir et al., 2015) or the structure of microbial communities (Schimel et al., 2007). The few studies that have investigated the effect of drought on soil CH_4 fluxes report a net reduction in CH_4 emissions (Davidson et al., 2004, 2008; Wood and Silver, 2012; O'Connell et al., 2018).

Apart from drought, changes in nutrient availability also impact soil gas fluxes in tropical forests. It is generally acknowledged that P rather than N is limiting in lowland tropical forests (Martinelli et al., 1999; Camenzind et al., 2018; Wright et al., 2018; Wright, 2019), and therefore we might expect stronger effects of P addition rather than N addition. In agreement with this, in several tropical forests around the world it has been shown that P addition tends to result in a strong increase in microbial biomass (Cleveland et al., 2002; Kaspari et al., 2008; Liu et al., 2013; Fanin et al., 2015). Fewer data are available on the effect of P addition on fluxes of trace gases, but P addition tended to increase soil CO_2 efflux (Cleveland and Townsend, 2006) and CH_4 consumption (Zhang et al., 2011). Nitrogen addition also tended to increase soil CO_2 efflux in a field experiment in Costa Rica (Cleveland and Townsend, 2006) while N had no effect or induced a decrease in soil CO_2 efflux in China (Mo et al., 2008). Such discrepancies are likely related to multiple factors including microbial community composition, soil properties, precipitation, and differences in initial N availability among tropical forests. Nitrogen addition alone tends to have an inhibitive effect on methanotrophs and thus lower the consumption of CH_4 by soils (Zhang et al., 2011). Several studies have shown that simultaneous additions of both N and P did not impact soil microbial structure or functioning (Liu et al., 2013; Fanin et al., 2015), possibly because increased P availability mitigates the inhibitive effect of N addition on soil CH_4 consumption. Together, these studies indicate that we are far from predicting the combined effect of nutrient addition and drought on trace-gas fluxes in soils.

In a temperate ecosystem, Aronson et al. (2012) showed positive effects of soil water content (SWC) and N addition on soil CH_4 fluxes, but it is unsure whether such results will extrapolate to tropical forest ecosystems. Moreover, little is known about the capacity of soils to recover from one or multiple stressors, such as drought and changes in nutrient availability. Previous results in mature tropical forests suggest that during post-drought periods, soil CO_2 efflux (as a result of both root/autotrophic respiration and microbial/heterotrophic respiration) were stimulated by the return of water leading to both renewed root growth and a large increase of microbial activity (Bonal et al., 2008; O'Connell et al., 2018). O'Connell et al. (2018) showed a large peak of CH_4 emissions after drought, higher than the pre-drought period, a decrease of soil aeration, and a change of organic and inorganic P content. Prolonged dry periods can be associated with soil nutrient accumulation which is then made available for microorganisms during soil rewetting (Fierer and Schimel, 2002). Moreover, increased root exudation (Preece and Penuelas, 2016), litterfall (Wagner et al., 2013), or microbial mortality during or after drought can also contribute to an increase in heterotrophic respiration once SWC is restored (Bengough et al., 2011).

To our knowledge, no study to date has addressed the combined effect of nutrient availability and drought on soil GHG fluxes in tropical forests. Field-based throughfall exclusion experiments combined with fertilization are therefore urgently needed to improve model-based simulations, which are currently not accounting for these effects.

In the current experiment, we asked the following question: how quickly and to what extent do soil GHG fluxes (CO_2 and CH_4) respond to changes in nutrient content and water content in a tropical forest soil? To answer this question, we conducted a full factorial drought and NP fertilization experiment during 1 year in a mature tropical forest of the Guiana shield. We hypothesized a strong positive effect of SWC on soil CO_2 effluxes and a negative effect on CH_4 fluxes. We hypothesized a positive effect of nutrients on CO_2 production, but we had no a priori expectation on the effect on soil CH_4 fluxes as both CH_4 production and CH_4 consumption could be stimulated with unknown resulting flux. Furthermore, we expected any effect of nutrients to be conditional on SWC such that its effect was less pronounced when water is limiting.

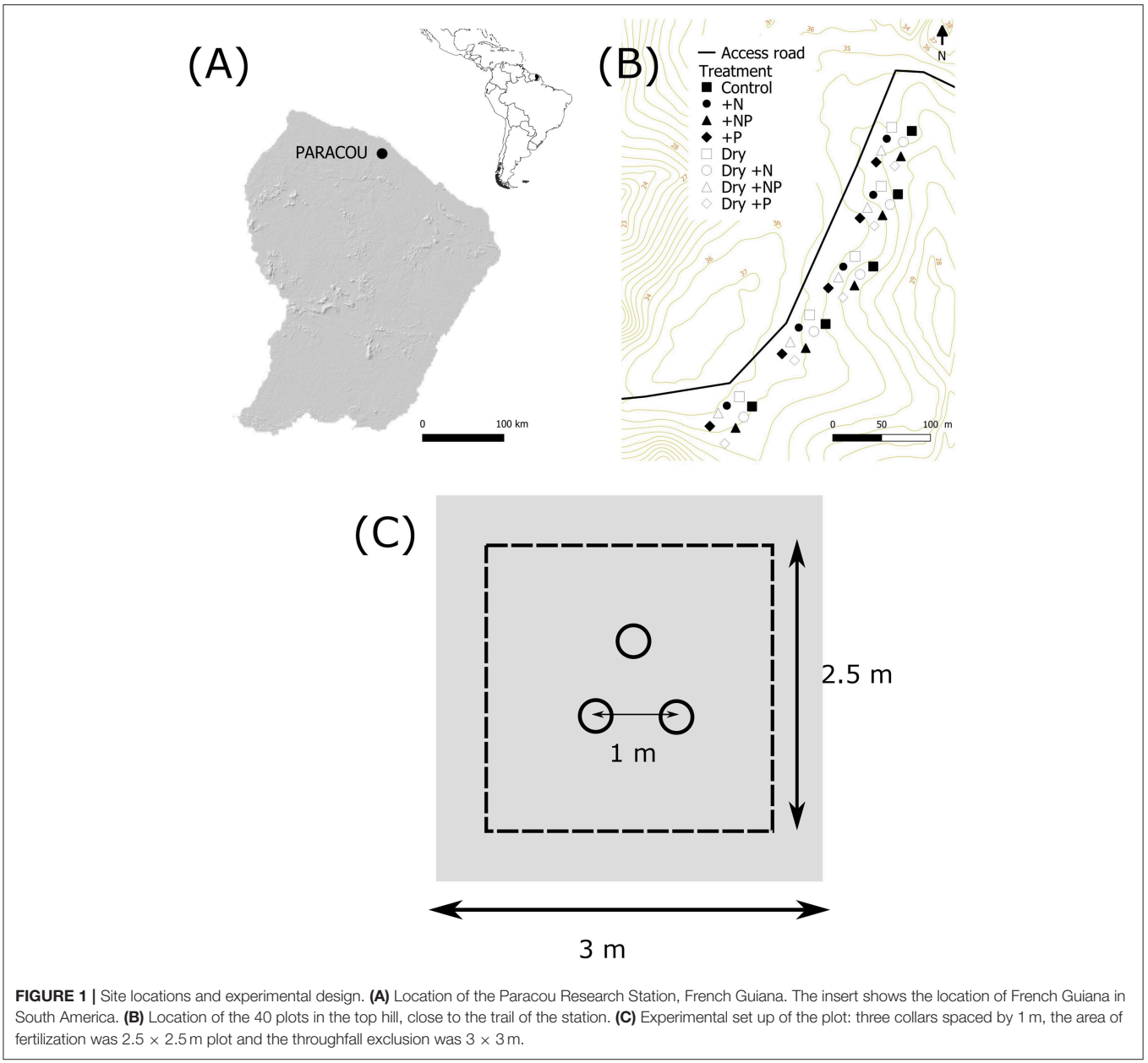

FIGURE 1 | Site locations and experimental design. **(A)** Location of the Paracou Research Station, French Guiana. The insert shows the location of French Guiana in South America. **(B)** Location of the 40 plots in the top hill, close to the trail of the station. **(C)** Experimental set up of the plot: three collars spaced by 1 m, the area of fertilization was 2.5 × 2.5 m plot and the throughfall exclusion was 3 × 3 m.

MATERIALS AND METHODS

Study Site

The study was conducted in French Guiana, South America, at the Paracou research station (**Figure 1A**, 05°16′54″N, 52°54′44″W). Decadal average annual rainfall at the study site was 3,102 mm ± 70 mm and average annual air temperature was 25.7°C ± 0.1°C (from 2004 to 2014, Aguilos et al., 2019). The tropical wet climate of French Guiana is highly seasonal due to the north/south movement of the Inter-Tropical Convergence Zone. This zone brings heavy rains from December to July (wet season, with precipitation between 300 and 500 mm mo^{-1}) and a long dry season from mid-August to mid-November. Precipitation during the dry period is typically <100 mm mo^{-1} (Aguilos et al., 2019). Soils at Paracou are predominantly schist soils with veins of pegmatite along a Precambrian metamorphic formation called the Bonidoro series (Sabatier et al., 1997). The soils are mostly nutrient-poor acrisols (FAO et al., 1998).

Experimental Design

The study zone covered ∼2.4 ha (400 m by 60 m, **Figure 1B**). A full factorial experimental design was applied with drought (two levels: ambient or "Dry") and fertilization (four levels: "Control," "+N," "+P," or "+NP") treatments crossed. Forty plots (2.5 × 2.5 m) were represented by three PVC soil collars, each at least 1.0 m apart (i.e., 120 collars in total, **Figure 1C**). The collars (20 cm in diameter, 7 cm in height) were installed to a depth of

∼4 cm into the soil 3 months prior to the first measurement and were left in the field during the full study period.

Drought Treatment

In order to simulate a prolonged drought period, 20 plots ("Dry") were covered by translucent plastic tarpaulins, creating a roof 1.50 m above the soil surface. Edge effects were avoided by using tarpaulins (i.e., 3 × 3 m) larger than the plot surface area. The tarpaulins were installed in October 2017 (dry season) before the return of the rainy season and removed in April 2018 during the rainy season. Plots were not trenched to minimize soil disturbance and allow natural nutrient cycling and water movements between the roots and microorganisms responsible for CO_2 and CH_4 fluxes, following the design of Wood and Silver (2012). Nearby the plots, litter traps of the same size as the tarpaulins were set up at 1.50 m above the soil surface. Once every 2 weeks, the litter was collected from the litter traps and homogeneously spread on the soil surface below the tarpaulin of the corresponding plot. No trees were cut or damaged during the treatment application.

Fertilization Treatments

In January 2018 (3 months after starting the drought treatment), a fertilization treatment was applied to 30 different 2.5 × 2.5 m plots either with N (125 kg N ha^{-1} as coated urea [$(NH_2)_2CO$]), P (50 kg P ha^{-1} as triple superphosphate [$Ca(H_2PO_4)_2\ H_2O$]) or with a mixture of these two elements. In total, five replicate plots with 15 sample points (three collars per plots) per combination of treatments (drought/fertilization) were therefore available. Previous studies conducted at the same site (Paracou research station) showed an average natural annual inputs of N and P via leaf litterfall of 65 kg N ha^{-1} y^{-1} and 1.4 kg P ha^{-1} y^{-1}, respectively. The amount of nutrients added in this experiment therefore correspond to twice the annual N input and 35 times the annual P input from leaf litter decomposition (Bonal et al., 2008; Hättenschwiler et al., 2008; Barantal et al., 2012). Our nutrient addition is similar to the amount of nutrients applied in other fertilization experiments in tropical forests (Wright et al., 2018; Wright, 2019).

Soil Characteristics

Soil samples for chemical and physical characterization were collected at the start of the experiment (September 2017, dry season). A composite soil sample of three topsoil cores of 8 cm by 15 cm (width/depth; Bi-partite root auger, Eijkelkamp, The Netherlands) in each plot was analyzed to determine the amount of clay, silt, sand, organic C, total N, aluminum (Al), iron (Fe), total P, and cation exchange capacity (CEC). Analyses were carried out by the soil analysis laboratory of INRA (Arras, France). Soil bulk density (BD) to a depth of 5 cm was measured with 100-cm^3 cylinders. Soil pH (KCl) was measured by mixing 10 g of moist soil with 1 M KCl in a 1:2.5 ratio. The resulting slurry was stirred for 1 h, then allowed to sit for another hour before measuring with a pH probe. After fertilization (January 2018), available P (ppm) was measured by Bray-P acid fluoride extraction (Bray and Kurtz, 1945) of soil dried at 60°C for 48 h; the resulting solution was analyzed on an iCAP 6300 Duo ICP optical emission spectrometer (Thermo Fisher Scientific, USA). Available N (ppm), defined as the sum of the NH_4^+ and NO_3^- concentrations, was measured by extracting moist soil with 1 M KCl, after which the concentrations of NH_4^+ and NO_3^- were determined colorimetrically (San++ continuous flow analyzer, Skalar Inc., Breda, The Netherlands).

Soil Gas Measurements

Soil gas flux measurement campaigns were carried out once a month from September to December 2017, every week from January to March 2018 (following fertilization), and every 2 weeks from March to July 2018.

Soil CO_2 and CH_4 fluxes were measured between 10:00 and 15:00 local time with two Ultraportable Greenhouse Gas Analyzers (UGGA, Los Gatos Research Inc., Mountain View, USA) connected to two home-made PVC chambers of 2.564 L operating in closed circuit and including an internal fan for mixing air inside each chamber. The volumes of each collar were summed, corresponding to a total volume of 3.882 ± 0.193 L for the UGGA/chamber system. The fluxes were measured during 5 min measurement periods (CO_2 and CH_4 concentration logged every 20 s), this period was sufficient to detect fluxes of each gas (Courtois et al., 2019). Two consecutive days (hereafter referred to as a sampling campaign) were necessary to measure the soil gas fluxes, because of the restricted time period of measurement. Fluxes were computed with the HMR package (Pedersen, 2011) for the two gases using linear regression (LR), or revised Hutchinson/Mosier (HMR) methods following recommendations from Pedersen (2011). SWC and soil surface temperature were recorded next to each collar during each sampling campaign using a dielectric soil moisture sensor, with general mineral soil calibration, at a depth of 5 cm (SM150T, Delta-T Devices, England) and a thermometer at a depth of 10 cm (HI 98501, Hanna instruments, USA), respectively.

Data Analysis

We used mean values per plot for all variables and data were first evaluated for assumptions of normality and homoscedasticity prior to further statistical analyses. To meet these assumptions, the soil CH_4 flux data were log transformed with a base of 10 (gas flux + most negative gas flux in dataset + 1). A Tukey-Kramer test was used to compare the changes in nutrient availabilities in the soil after fertilization. In addition, a principal component analysis (PCA, **Figure S1**) was performed to identify main relationships between the different soil properties and plots later assigned to treatments.

To assess how the drought and fertilization treatments affected CO_2 and CH_4 fluxes in the soil before and after roof removal, linear mixed-effects models were run, where fertilization treatment (+N, +P, +NP, Control), drought treatment (Dry, Control) and sampling campaign were treated as fixed factors and plot as random factor. Models were selected minimizing AIC.

Linear regression models were used to investigate the relationships between soil fluxes, i.e., CO_2 and CH_4, and SWC. For CO_2 and CH_4 fluxes in the dry plots, the relationships were

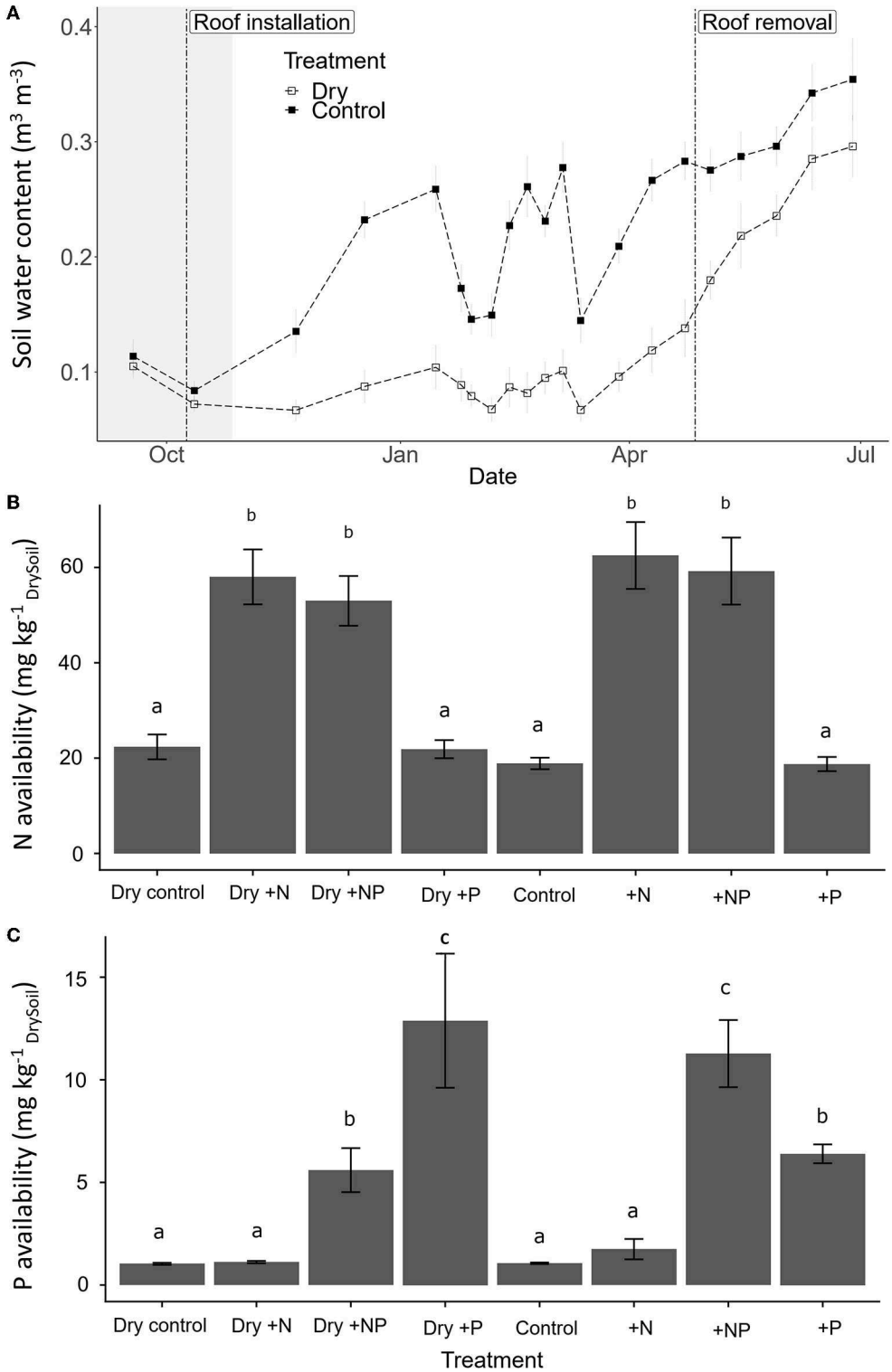

FIGURE 2 | (A) Temporal change in mean soil water content (SWC; ± SE; 0–5 cm; $n = 20$) in the controls (close square) and "dry-control" for the throughfall exclusion plots (open square) over the study period, French Guiana. Vertical dashed line indicate the dates of the roof installation (09/10/2017) and roof removal (27/04/2018) in the throughfall exclusion plots. The shaded area represents the dry season 2017. **(B)** Effect of fertilization on available N and **(C)** on available P in soils 2 weeks after fertilization. Different lowercase letters indicate significant differences among treatments; error bars indicate standard error of the mean ($n = 5$).

TABLE 1 | Results from the linear mixed models for CO_2 and CH_4 fluxes of the soil, in a tropical forest, French Guiana.

	Model 1	Model 2	Model 3	Model 4
CO_2				
Intercept	158.51	131.93	149.81	146.47
Sampling Campaign (SC)	+	+	+	+
Drought Treatment (DT)	+	+	+	+
Fertilization Treatment (FT)		+	+	+
SC × FT			+	+
SC × DT			+	+
DT × FT			+	+
SC × DT × FT				+
AICc	4,009	3,971	3,705	3,500
CH_4				
Intercept	0.099	0.092	0.089	0.081
Sampling Campaign (SC)	+	+	+	+
Drought Treatment (DT)	+	+	+	+
Fertilization Treatment (FT)		+	+	+
SC × FT			+	+
SC × DT			+	+
DT × FT			+	+
SC × DT × FT				+
AICc	−1,456	−1,431	−1,091	−887

Four models were tested for each gas: Model 1 including (symbolized by "+" sign in the table, in opposition to empty cell for not included in the model) only Sampling Campaign (SC) and Drought Treatment (DT); Model 2 including SC, DT, and Fertilization Treatment (FT); Model 3 including SC, DT, FT, and their first order interactions and Model 4 with the addition of the second order interactions. Models were selected minimizing AIC. P-values of each variable or interaction of the selected models were highly significant ($p < 0.001$), excepted for DT × FT for CO_2 (Model 4).

best described by:

$$SoilFluxes = aSWC^2 + bSWC + c \quad (1)$$

And, for CH_4 fluxes in the Control plots by:

$$SoilFluxes = aSWC + b \quad (2)$$

where SWC is the soil water content ($m^3\ m^{-3}$) and a, b, and c the constants fitted in each regression model.

Resilience indices, from Orwin and Wardle (2004), were calculated to assess the recovery of soil CO_2 and CH_4 fluxes after removing the roof, characterizing the end of the drought treatment. This resilience index, which compares the absolute difference that exists between post-drought and control treatment relative to the initial absolute effect of the drought, ranges from −1 to +1, with +1 indicating maximum resilience (being complete recovery after rewetting).

All statistical analyses were conducted and figures produced using R statistical software (R Core Team, 2018) using the packages dplyr (Wickham et al., 2015), nlme (Lindstrom and Bates, 1990), multcomp (Bretz et al., 2016), MuMIn (Barton, 2009), and FactoMineR (Husson et al., 2013).

RESULTS

Soil samples had a high sand content (77.84% of sand), a low nutrient concentration (1.71% of C, 0.12% of N, and 0.022% of P), a low nutrient availability (18.87 mg $kg^{-1}_{DrySoil}$ of N and 1.06 mg $kg^{-1}_{DrySoil}$ of P) and a low pH (3.88). Soil characteristics were very similar between plots (**Table S1**, **Figure S1**).

One month after initiation of the throughfall exclusion treatment (i.e., prolonged drought initiated with roof installation), SWC of dry plots was roughly half that of control plots, and this difference remained over the course of the experiment (**Figure 2A**). As a consequence, the throughfall exclusion treatment extended the duration of the dry season by at least a factor 2 maintaining a SWC of ∼10%, whereas control plots followed precipitation events resulted in SWC between 15 and 35% (**Figure 2A**). After roof removal, differences in SWC decreased but control plots remained wetter until the end of the experiment. The respective fertilization treatments significantly increased N and P availability in soil (**Figures 2B,C**; 3 fold for N under N addition and 10 fold for P under P addition).

Both drought, fertilization and the combination of both had a significant impact on CO_2 efflux (**Table 1**, **Figure 3A**). The effect of fertilization with N only and P only on CO_2 fluxes was transient where no throughfall exclusion was applied; CO_2 efflux started to increase 19 days (i.e., sampling campaign #3) after the fertilization, reached a peak (62% increased as compared to control plots) at 34 days (i.e., sampling campaign #5) and returned to the same range of magnitude of the control plots 55 days after fertilization (**Figures 4A,B**). In the combined drought and fertilization (Dry +N, Dry +P, and Dry +NP) however, Dry +N had no effect on CO_2 efflux (**Figure 4A**), while Dry +P steadily increased CO_2 efflux from the first measurement until about day 82 (i.e., sampling campaign #10) after fertilization (**Figure 4B**). Addition of both N and P (+NP) on all plots immediately increased CO_2 efflux (86% increased as compared to control), which kept increasing until 26 and 82 days (for no throughfall exclusion and throughfall exclusion, respectively) and remained steady until the end of the experiment (**Figure 4C**). This increase in CO_2 efflux was stronger and earlier in +NP plots than in +N or +P plots. The increase 26 days after the fertilization was stronger in plots with no throughfall exclusion than in plots where rain was excluded (**Figure 4C**).

Overall, resilience of soil CO_2 efflux to drought was greater in unfertilized than in fertilized plots (**Figure 5A**). Over a two-month period after roof removal, resilience of CO_2 efflux was high and steady in the control plots, low and steady in +P plots or strongly decreased in +N and +NP plots.

Only throughfall exclusion had an impact on CH_4 fluxes (**Table 1**, **Figure 3B**). All plots where rain was excluded, regardless of the fertilization treatments, displayed higher CH_4 fluxes than plots where rain was not excluded. This effect resulted in a change of the relationships between CH_4 fluxes and SWC (**Figure 6**). In control plots (without roof), soils shifted from a sink under dryer conditions to a source under wetter conditions (**Figure 6**). In plots where rain was excluded, there was a strong

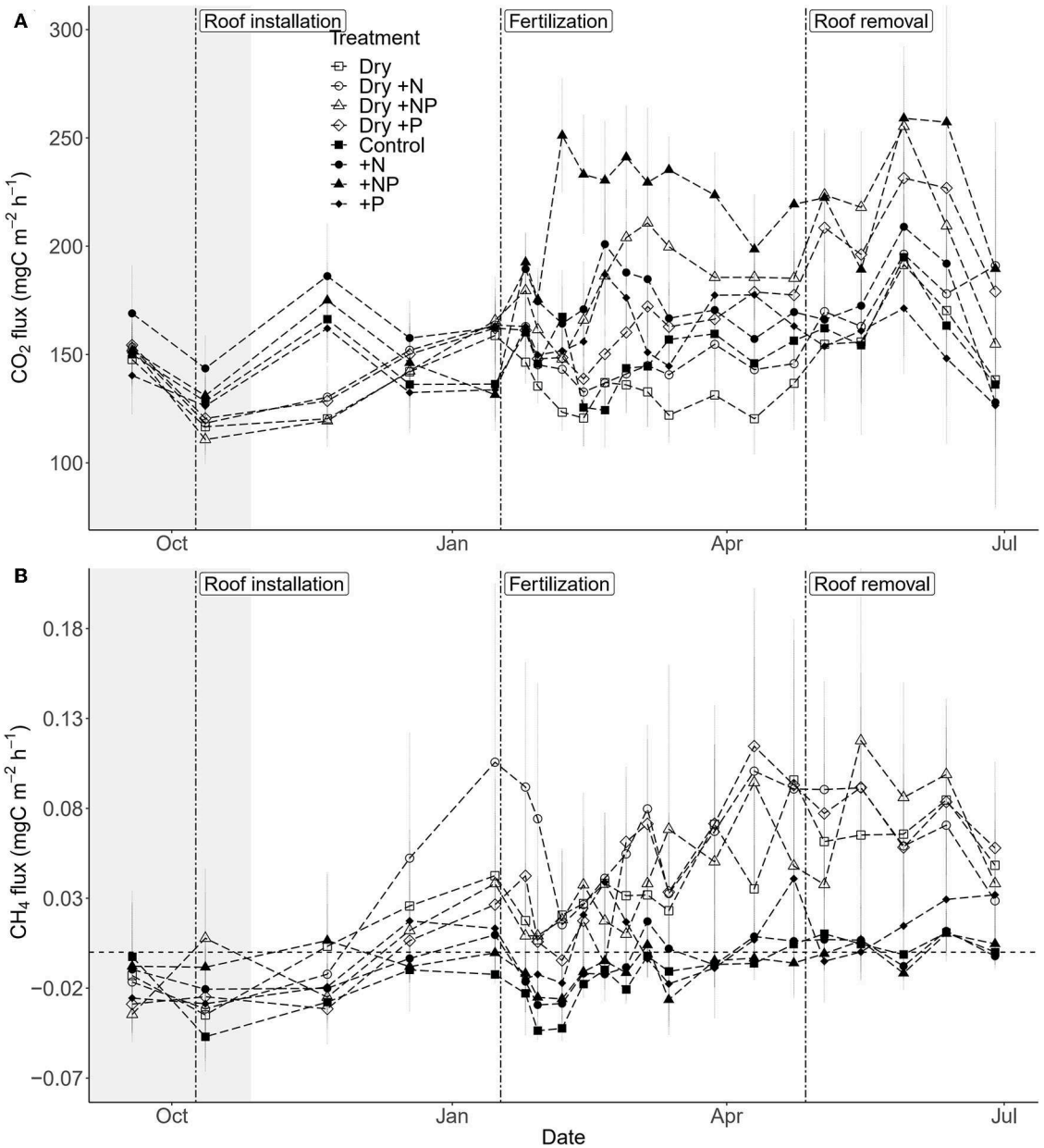

FIGURE 3 | Temporal change in **(A)** mean soil CO_2 efflux and **(B)** mean soil CH_4 flux over the study period (± SE; $n = 5$ plots per treatment) in a tropical forest, French Guiana. Vertical dashed lines indicate the dates of the roof installation (09/10/2017), roof removal (27/04/2018), and fertilization (17/01/2018). The shaded area represents the dry season 2017. Drought treatment is symbolized by open symbols, fertilization by circles for +N, triangles for +NP, and diamonds for +P and squares for controls.

increase in CH_4 fluxes, even under dryer conditions (SWC < 15%, **Figure 6**).

Resilience of soil CH_4 fluxes to drought was in the same order of magnitude for unfertilized and fertilized plots. Fifty days after roof removal, resilience of soil CH_4 fluxes slightly increased in all plots (**Figure 5B**). As SWC gradually increased in plots after roof removal, the shape of the relationship between SWC and CH_4 displayed a negative trend toward lower CH_4 fluxes with higher SWC.

DISCUSSION

In this study, we report on an *in situ* manipulation of N and P fertilization combined with a drought treatment on two soil GHG fluxes (CO_2 and CH_4), in a tropical rainforest. These treatments had complex but marked effects on both soil GHG fluxes. Soil CO_2 efflux consistently increased with fertilization but was reduced by drought-imposed reductions in SWC. In contrast to CO_2 fluxes, none of the nutrient additions (i.e., +N, +P, and

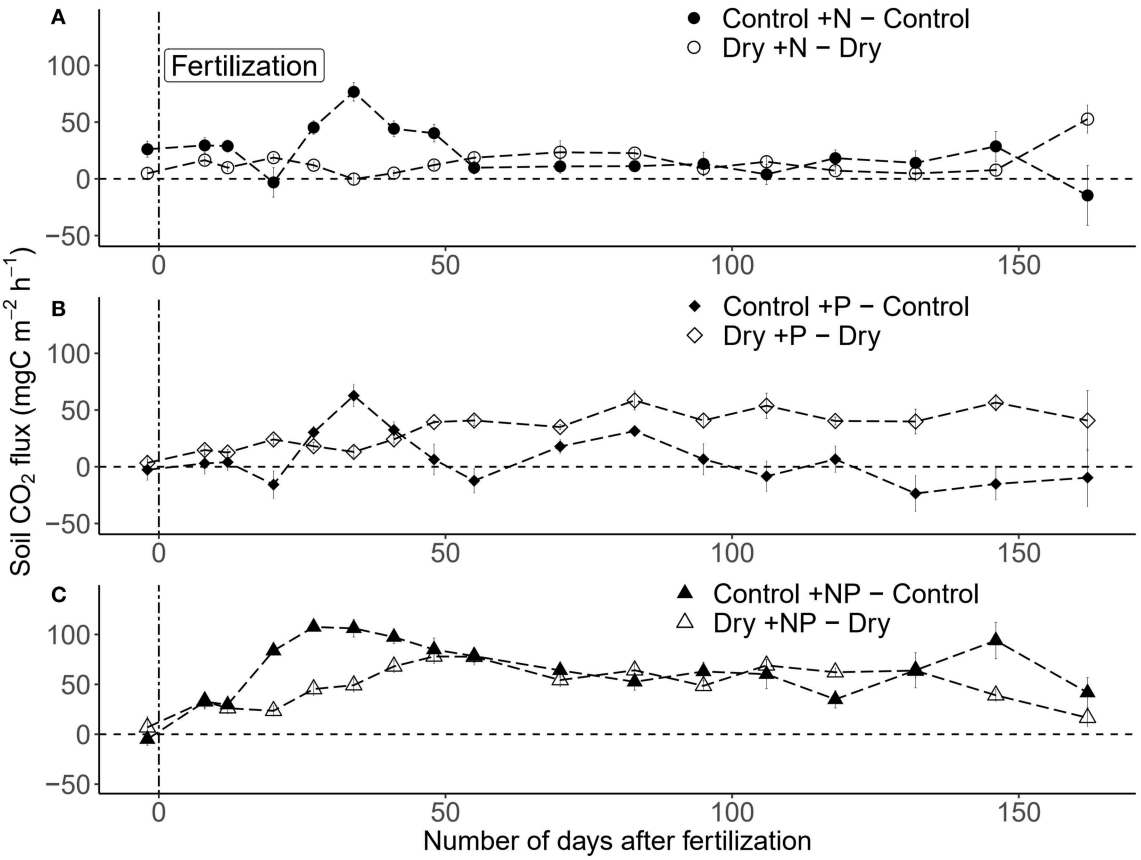

FIGURE 4 | Differences among treatments for **(A)** +N, **(B)** +P, and **(C)** +NP over the study period (± SE; $n = 5$ plots per treatment) in a tropical forest, French Guiana. The vertical dashed line indicates the date of the fertilization (17/01/2018).

+NP) had an effect on soil CH_4 flux, but the drought treatment led to an increase in CH_4 emission. Below we will further discuss each of these main findings.

Combined Effects of Fertilization and Drought on Soil CO_2 Efflux

Our experiment showed a positive effect of fertilization on soil CO_2 efflux, which was modulated by drought. A low soil CO_2 efflux, but not lower than the pretreatment period, was maintained during the artificial drought period, which lasted for at least two times the regular duration of the dry season.

The response to the fertilization treatments was rapid (≈1 month; **Figures 3A**, **4**) but relatively transient for +N and +P, compared to +NP. Although soils in French Guiana have very low concentrations in available P (Grau et al., 2017; Van Langenhove et al., 2019) compared with many southern and western Amazonian soils [i.e., 10 times lower for Acrisols (Quesada et al., 2010)], our results showed a higher CO_2 efflux in +NP fertilized soils than in +N or +P alone, suggesting co-limitation of N and P. In concordance, previous studies on the same tropical French Guianese soils have demonstrated the positive impact of N and P additions on faunal decomposers and litter decomposition rates (Barantal et al., 2012; Fanin et al., 2015, 2016) but no effects when N or P were added alone. This co-limitation of soil respiration contrasts with findings from fertilization experiments in tropical lowland forests in Panama (Kaspari et al., 2008), Costa Rica (Cleveland and Townsend, 2006), and Hawaii (Hobbie and Vitousek, 2000), which showed a significant and positive effect of N or P addition on soil organic matter decomposition. One possible explanation for the different soil responses is the difference between soils across these tropical sites, where all study sites mentioned above had soils that were richer in N and P compared to our site in French Guiana [i.e., 0.02% vs. 0.10% P content and 0.12% vs. 0.50% N content for this study and a soil in Costa Rica (Cleveland and Townsend, 2006), respectively]. The strong and rapid effects of NP fertilization and to a lesser extent N and P fertilization alone on CO_2 efflux observed here may in part be attributed to the extremely nutrient-poor soil at our site. This is further corroborated by results from Soong et al. (2018) who showed that CO_2 efflux was most increased with a combined application of NPK in an incubation experiment with soil from the same site. The modest effects of the singly added N on CO_2 efflux could however be due to acidification, a known side-effect of adding Urea (Chen et al., 2016; Riggs and Hobbie, 2016); a decrease in pH can negatively affect microbial activities and mask microbial N limitation. If this were the case, it was however overcome when P was added along with N as evidenced by the high CO_2 efflux in the +NP treatment.

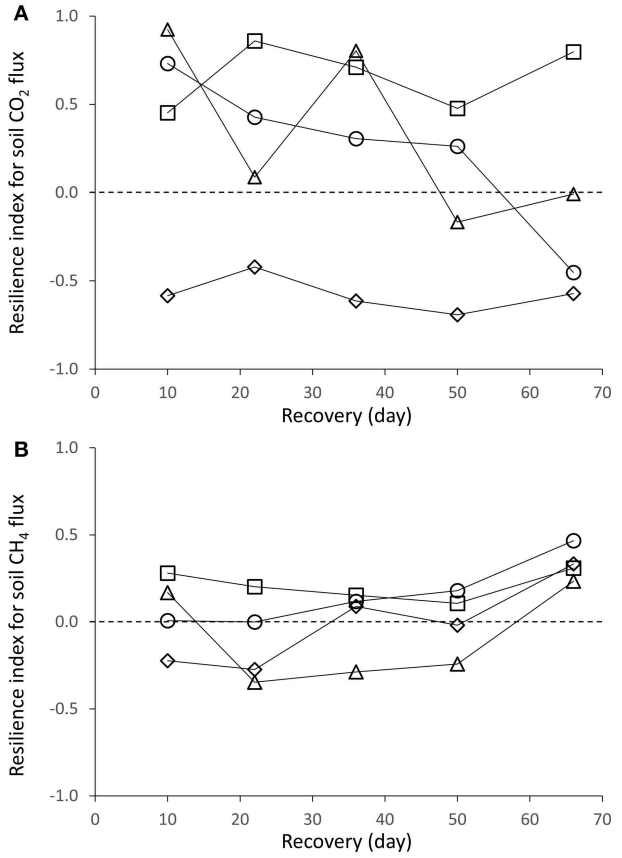

FIGURE 5 | Change in the resilience index for **(A)** CO_2 and **(B)** CH_4 fluxes of the soil, in a tropical forest, French Guiana; where control plots are symbolized by squares, +N by circles, +NP by triangles, and +P by diamonds.

A comparison between fertilized plots and dry fertilized plots showed that a decrease in SWC tended to mitigate the fertilization effects on CO_2 efflux. The effect of our throughfall exclusion experiment on soil CO_2 efflux was in the same order of magnitude as a synthesis of eight throughfall exclusion experiments in humid tropical forests (Wood and Silver, 2012). That study showed a reduction of CO_2 of ∼20% (in our study ∼16% for the February–April period and ∼25% in November). A strong relationship between soil CO_2 efflux and natural changes in SWC evidences the predominant soil water control on temporal variations in C fluxes, especially during the dry to wet-season transition (October 2017–January 2018), compared with low variation in CO_2 efflux in the dry plots. Precipitation, driving SWC, can modify biophysical and biogeochemical soil conditions and thereby soil CO_2 efflux in complex ways, to which root and microbial respiration contribute approximately equally in tropical forests (Hanson et al., 2000). In our study, the highest SWC for CO_2 efflux was ∼28% in the control plots (**Figure S2**), regardless of fertilization regime. Above or below this optimum, soil CO_2 efflux decreased by a maximum factor of 1.4. This finding is in agreement with previous studies performed in the same site (Rowland et al., 2014; Courtois et al., 2019). On the one hand, excess SWC can create a physical barrier for gas diffusion between the soil and the atmosphere, and a biological inhibitor for root and microbial activities because of the decrease, or even absence, of O_2 (Wood et al., 2013). On the other hand, low SWC in dry soil can strongly reduce nutrient availability and impose water limitation and thereby decrease soil CO_2 efflux. Therefore, CO_2 efflux rates remained low in the dry plots, where SWC was rarely above 10%. In contrast to bio-physical expectations, O'Connell et al. (2018) observed an increase in soil CO_2 effluxes under natural drought conditions, which can be explained by different soil conditions such as higher nutrient content, lower bulk density, and texture. In our study, soil CO_2 dynamics were driven by SWC and soil nutrient contents, necessary for nutrient exchanges and nutrient uptake by roots and micro-organisms. However, additional measurements on soil and plant properties would be necessary to better understand the mechanism behind the interactions of fertilization and drought and partitioning between microbial and root CO_2 efflux.

Our results show that soil CO_2 effluxes in the control plots were more resilient to drought than those in the fertilized plots. We explain the increase to initial levels of soil CO_2 efflux (heterotrophic and autotrophic respiration) after roof removal by both renewed root growth and a pulse of microbial activity as described in previous studies (Fierer and Schimel, 2002; O'Connell et al., 2018). However, this resilience of soil CO_2 effluxes to drought was particularly low and more variable in the fertilized plots, suggesting a long-lasting effects of the N, NP, and P addition treatments (over a 105-day period) on soil C dynamics. The possible rapid reversible effect of a long drought, and its interaction with nutrient levels and limitation, should be of interest to further improve C cycling models of tropical forests.

No Combined Effects of Fertilization and Drought on Soil CH_4 Flux

Soils shifted from sinks of CH_4 during the dry season (beginning of the experiment, shaded area in **Figure 3B**) to sources of CH_4 during the wet season in the control plots, which is in agreement with other studies in tropical forests (Keller and Reiners, 1994; Keller et al., 2005; Davidson et al., 2008; Teh et al., 2014; Courtois et al., 2018). Increased precipitation is likely to decrease rates of O_2 diffusion into the soil (Silver et al., 1999; Teh et al., 2005; Liptzin et al., 2011), which potentially increases CH_4 production. Furthermore, soil water saturation can create a physical barrier for gas diffusion, and potentially stop the root and microbial activities because of a lack of available O_2. Studies on drought effects on soil CH_4 flux however remain sparse, although all showed an increase in CH_4 consumption (Davidson et al., 2004, 2008) with extremely high spatial heterogeneity (Wood and Silver, 2012; Courtois et al., 2019). In our study however, soils are mainly a source under drought treatment, with highest CH_4 emissions when SWC was about 20% in the dry plots.

In contrast to our hypothesis, relationships between SWC and CH_4 flux were complex in that its relationship differed between dry and control plots, where dry plot soils were consistent sources of CH_4 even when where SWC levels overlapped

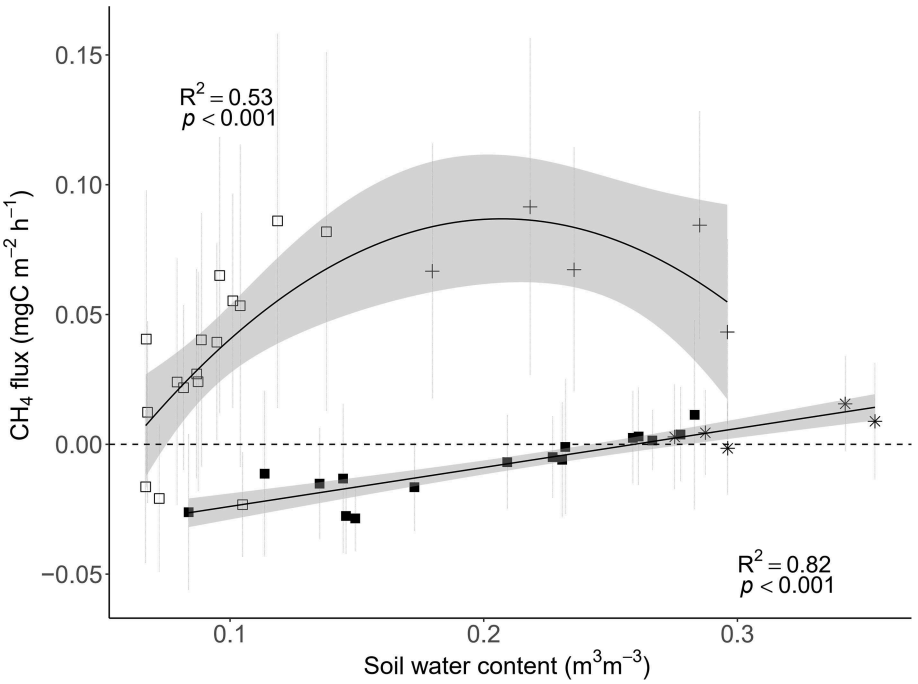

FIGURE 6 | Relationships between soil CH_4 fluxes and soil water content (SWC) in the control (closed square and black star; $SoilFluxes = SWC - 0.04$) and dry plots (open square and black cross; $SoilFluxes = SWC^2 + 0.02\ SWC - 0.09$) for the entire study period ($n = 21$ sampling campaigns; 18/09/2017–28/06/2018) in a tropical forest, French Guiana. The roof removal period is symbolized by the black stars and crosses. For both functions, the values of the slopes are very closed to 0.

between treatments. Importantly, this positive effect of drought persisted during the entire study period of throughfall exclusion, with no clear reason. A possible explanation can be distinct responses of the CH_4 producers and consumers to soil water conditions. In their study, Benstead and King (1997) reported that methanotrophs are particularly sensitive to water stress, creating larger emissions of CH_4 in dry soils due to lower consumption of CH_4. Also, it is possible that belowground substrate availability to methanogens is reduced in the dry season but not in the wet season, which could cause variation in topsoil SWC to differentially affect methane fluxes between natural and imposed drought conditions. Therefore, more information about methanogen behavior to soil water limitation is necessary. Soil water conditions can control the gas diffusion as well as the distribution of CH_4 producers and consumers along the soil profile as shown by Koehler et al. (2012) in a tropical lowland forest in Panama. Finally, soil fauna (e.g., termites) can also significantly contribute to the CH_4 emissions but no particular changes in the presence of the soil macro-fauna (e.g., termites ant ants) was observed in our plots during the study period.

Fertilization with N and P alone or combined did not change the CH_4 flux. These results did not corroborate with findings by Hanson and Hanson (1996) who reported a decrease in CH_4 uptake due to a shift from CH_4 oxidation to ammonia oxidation by methanotrophs with added ammonium resulting from urea hydrolysis. Our results also did not support a previous study by Zhang et al. (2011) that reported an increase in CH_4 consumption after P fertilization most likely due to an increase in methanotrophic abundance. Phosphorus supply in soils can increase pH, water and nutrient uptake by plant roots, which can lead to higher O_2 diffusivity and O_2 availability in soil pore spaces for methanotrophs (Zhang et al., 2011). Also, P addition can induce a significant increase in microbial biomass (Cleveland et al., 2002; Kaspari et al., 2008; Liu et al., 2012, 2013; Fanin et al., 2015) even if these effects were transient and disappeared over longer periods. However, several studies showed that simultaneous addition of both N and P did not impact soil microbial structure and functioning (Liu et al., 2013; Fanin et al., 2015), but an increase in P availability might mitigate the inhibitive effect of N addition on soil CH_4 consumption. These studies highlight that the relationships between biophysical processes behind CH_4 fluxes and soil nutrient contents are still unknown and possibly site-specific.

During 50 days after roof removal, natural precipitation had a modest effect on soil CH_4 fluxes, which was corroborated by low resilience indices (**Figure 5B**). However, differences in soil CH_4 fluxes between dry and control plots tended to disappear once rain was allowed back into the dry plots (**Figure 6**), suggesting a possible reversible effect in the longer term, regardless of fertilization regime. Davidson et al. (2008) also reported a recovery capacity of the soils for the N_2O and CH_4 fluxes after a drought treatment. They suggested that changes in the balance of the gaseous production and consumption via nitrification, denitrification, methanogenesis, and methanotrophy recovered by the return of the precipitation where a change in the soil aeration was instrumental in this recovery.

CONCLUSION

In this *in situ* experimental study, we show marked differences in the responses of soil C dynamics depending on whether nutrients and water were applied or withheld, respectively, as single or combined treatments. Differences were (i) a positive effect of N and P additions, mitigated by SWC via imposed drought conditions, for CO_2 efflux, and (ii) a positive and strong effect of SWC for CH_4 flux. Surprisingly, fertilization only affected soil CO_2 efflux, and drought caused soil to become sources of CH_4 instead of sinks. These results suggested that changes in nutrients and water contents in soils most likely influence the complex processes of CO_2 and CH_4 exchanges, which are controlled by multiple biophysical and biogeochemical conditions, e.g., methanotroph activities. Studies of soil microbial properties are now needed to disentangle the effects of the fertilization and drought treatments on CO_2 and CH_4 fluxes. In this study, we also find that resilience of soil CO_2 efflux after a long dry period, equal to at least twice the duration of a regular dry season, is high for unfertilized plots. While, although slow, the resilience for soil CH_4 flux is consistent for all fertilization treatments. For both soil fluxes, we observe that resilience is slow, and should thus be examined over a long period of time. Finally, we highlight here that in tropical forest ecosystems nutrient and water availabilities, both expected to be affected by climate change, can dramatically affect soil GHG fluxes.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://zenodo.org/record/3372722.

AUTHOR CONTRIBUTIONS

CS and EC conceived and designed the experiments. TS-G, CS, EC, LB, and LV carried out the field and lab-work. TS-G, CS, EC, and LB analyzed the data. LB, EC, and CS wrote the paper. All authors commented on the analysis, interpretation, and presentation of the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2019.00180/full#supplementary-material

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REVIEW
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Salt-Tolerant Plant Growth Promoting Rhizobacteria for Enhancing Crop Productivity of Saline Soils

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Soil salinity has emerged as a serious issue for global food security. It is estimated that currently about 62 million hectares or 20 percent of the world's irrigated land is affected by salinity. The deposition of an excess amount of soluble salt in cultivable land directly affects crop yields. The uptake of high amount of salt inhibits diverse physiological and metabolic processes of plants even impacting their survival. The conventional methods of reclamation of saline soil which involve scraping, flushing, leaching or adding an amendment (e.g., gypsum, $CaCl_2$, etc.) are of limited success and also adversely affect the agro-ecosystems. In this context, developing sustainable methods which increase the productivity of saline soil without harming the environment are necessary. Since long, breeding of salt-tolerant plants and development of salt-resistant crop varieties have also been tried, but these and aforesaid conventional approaches are not able to solve the problem. Salt tolerance and dependence are the characteristics of some microbes. Salt-tolerant microbes can survive in osmotic and ionic stress. Various genera of salt-tolerant plant growth promoting rhizobacteria (ST-PGPR) have been isolated from extreme alkaline, saline, and sodic soils. Many of them are also known to mitigate various biotic and abiotic stresses in plants. In the last few years, potential PGPR enhancing the productivity of plants facing salt-stress have been researched upon suggesting that ST-PGPR can be exploited for the reclamation of saline agro-ecosystems. In this review, ST-PGPR and their potential in enhancing the productivity of saline agro-ecosystems will be discussed. Apart from this, PGPR mediated mechanisms of salt tolerance in different crop plants and future research trends of using ST-PGPR for reclamation of saline soils will also be highlighted.

Keywords: salinity, climate change, PGPR, crop productivity, agro-ecosystem

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INTRODUCTION

Since the inception of agricultural practices, the question that how to enhance crop productivity to feed the growing population has been challenging. As highlighted in 2018 Global Agricultural Productivity (GAP) Index the current growth rate of agricultural production is not enough to meet the projected food demand of 10 billion people in 2050 (GAP Report, 2018). The report also stated that under such circumstances GAP must be increased by 1.75% annually. Enhancing crop

productivity in agro-ecosystems is intricate and greatly influenced by pedo-climatic conditions, farming systems and management techniques (Nemecek and Gaillard, 2010). A number of abiotic factors including temperature, salinity, drought, pesticides and fertilizer application, soil pH, and heavy metal contamination also hamper crop productivity (Ahmad, 2014). Amongst all these, salinization of arable land is being considered as a real menace for agricultural production. Recently, the United Nations' Food and Agriculture Organization (FAO and ITPS, 2015) report "The status of the world's soil resources" has identified nine major threats to soil functions and soil salinization is one of them. Soil salinization is globally expanding and in the past few years, the accelerated rate of salinization has also created food insecurity in several countries. The delta regions of India, Myanmar and Bangladesh which majorly contribute in world rice production are facing serious threats to food security due to salinization of coastal soil (Abedin et al., 2014; Szabo et al., 2016). According to Ghassemi et al. (1995) salt-affected, irrigated areas caused annual income losses in terms of $12 billion globally. In the United States, the central California region is losing approximately $3.7 billion in agricultural crop yield every year due to salinity (Dove, 2017). In Alberta, western province of Canada, on an average, 25 percent of crop yields are reduced annually due to salinity. Sindh region of Pakistan is suffering 31 percent crop loss annually due to waterlogging and salinity (Ilyas, 2017). These are just a few examples of this global menace.

In excess, any form of salt is deleterious for plant health. Soils with high salinity levels interfere with plants' physiological process. The high salt concentration adversely affects important soil processes such as respiration, residue decomposition, nitrification, denitrification, soil biodiversity and microbial activity (Schirawski and Perlin, 2018). The loss of crop productivity and high salinity is also noticed where fertilizer input is too high in soil (Rütting et al., 2018). Application of high salt index fertilizers impose an osmotic effect which causes difficulty in extraction of water required for plant growth (Herger et al., 2015). It has also been observed that farming practices also affect crop production in saline soils. In such cases plowing down or tilling deeper increases the evaporation of water from the soil surface and deposit more salts. Apart from this, salts present in irrigation water may also increase soil salinity that results in productivity loss (Rengasamy, 2010; Arora et al., 2018). Earlier it was estimated that around 20 to 50 percent of irrigated lands worldwide were salt-affected (Szabolcs, 1992). It has also been projected that a huge proportion of the globe i.e., 24% or 60 million ha is being affected due to inappropriate irrigation practices (WWAP, 2012). Removal of salt from saline soil is an intensive process and requires too much time and money (Qadir et al., 2014). However, since long, reclamation of saline soils is mainly performed by physical and chemical processes. In a physical process, soluble salts in the root zone are removed by scraping, flushing and leaching methods (Ayyam et al., 2019). However, in chemical methods use of gypsum and lime as neutralizing agents is commonly done (Keren, 2005). But these methods are not sustainable and also considered inefficient when the salt concentration is too high.

Planting salt-tolerant crop varieties such as barley and canola on saline soils is common practice (Fita et al., 2015). However, due to normal salt tolerance profile, these crops have limited global reach and can't be used in the soil with moderate or high electrical conductivity (EC) levels. Morton et al. (2019) also highlighted that despite vigorous efforts from the research community, only few salt tolerance genes have been identified having real applications in improving productivity of saline soils.

Hence, securing attainable crop yield in saline soils is the need of the hour and aside from using salt-tolerant varieties or amelioration methods involving chemical neutralizers, sustainable approaches should also be employed. In the last few years, research showed that the use of salt-tolerant plant growth promoting rhizobacteria (ST-PGPR) in saline agriculture can be harnessed for enhancing productivity and improving soil fertility as well (Grover et al., 2011). Their adaptive responses toward salt stress are related to the ability to produce osmoprotectants, compatible solutes, and specialized transporters. ST-PGPR are now being used as bioinoculants for enhancing crop yields, protection from phytopathogens and improving soil health. The present review targets the use of ST-PGPR for improving the productivity of crops grown in salt-affected soil. Their application in the form of bioinoculants for enhancing crop yield is also targeted. Apart from this, ST-PGPR mediated mechanisms, including the new insights and perspectives on productivity enhancement of crops facing the salinity stress are also discussed.

SALINE SOILS: GLOBAL DISTRIBUTION

Although, it has been realized that all continents on the globe are facing the problem of soil salinity (**Figure 1**) yet accurate estimation of the locations and distribution of saline soils is missing. The Food and Agriculture Organization (FAO), United Nations Educational, Scientific and Cultural Organization – United Nations Environment Programme (UNESCO-UNEP), and International Society of Soil Science (ISSS) are the leading world agencies that paid attention in gathering data on quality of soil across the globe. The Soil Map of the World (FAO, 1971–1981) documented that a total area of 953 Million hectares (Mha) is salt-affected. According to FAO report on "Status of the World's Soil Resources" soil of more than 100 countries with an estimated area of approximately one billion hectares is afflicted with the problem of salinity (FAO and ITPS, 2015). Due to very high amount of soluble salts (NaCl and Na_2SO_4) the EC of these soils exceeds 4 dSm^{-1}. Currently, the soil classification system is governed by the World Reference Base for Soil Resources (WRB) which is endorsed by the International Union of Soil Sciences (IUSS) and it replaced the FAO/UNESCO Legend for the Soil Map of the World. The legend of the soil map described salt-affected soil as solonchak and solonetz. Solonchaks are characterized by the accumulation of high soluble salts. The salic horizon starts within $\leq$50 cm from the soil surface and are largely distributed to the arid and semi-arid coastal regions in all climatic zones in the world. The estimated global area of solonchaks is around 260 million hectares (IUSS Working Group WRB, 2015).

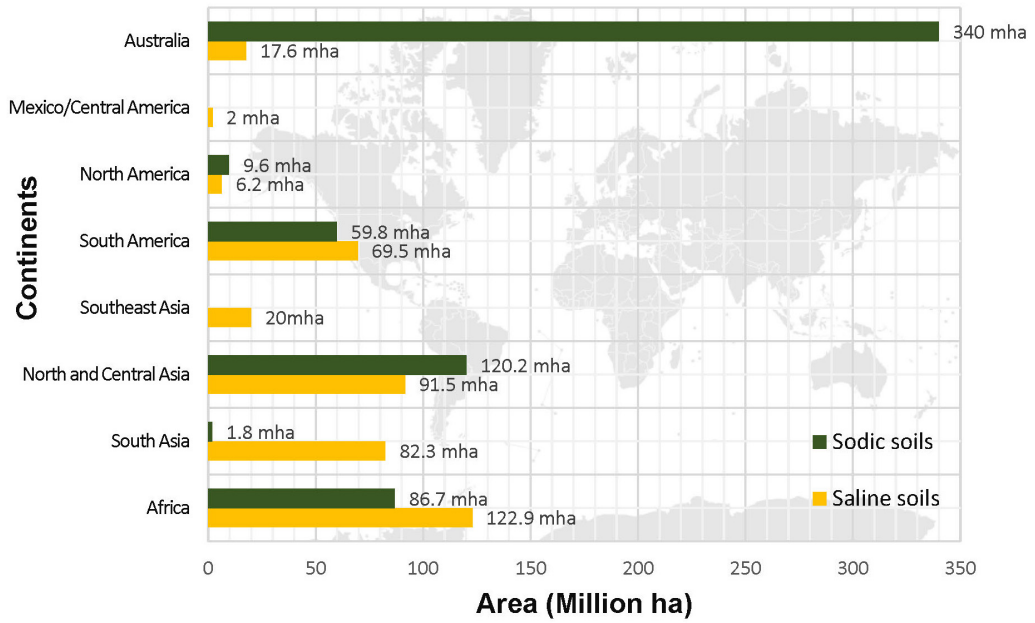

FIGURE 1 | Global distribution of saline and sodic soil (Source: FAO and ITPS, 2015).

According to national soil classification systems, solonchaks belong to Halomorphic Soils (Russia), Halosols (China) and Salids (United States of America) and EC may range from 8 to 15 dSm^{-1}. On the basis of salt precipitation, there may be external solonchaks (deposition at the surface) or internal solonchaks (deposition at depth). Whereas solonetz which are commonly known as alkaline soils and sodic soils contain a large proportion of adsorbed Na and in some cases Mg ions also. However, solonetz with free Na_2O_3 are considered highly alkaline and their pH is recorded to be higher than 8.5. Globally, they are distributed in the semi-arid temperate continental climate. Area-wide, a total of 135 Mha solonetz are found in Ukraine, the Russian Federation, Kazakhstan, Hungary, Bulgaria, Romania, China, the United States of America, Canada, South Africa, Argentina, and Australia (IUSS Working Group WRB, 2015).

THE DIVERSITY OF SALT-TOLERANT PLANT GROWTH PROMOTING RHIZOBACTERIA (ST-PGPR)

Soil has a huge versatility of microorganisms, belonging to different groups of bacteria, fungi, and archaea. Amongst microbes, some are now well known for their inherent capability to tolerate different concentrations of salt and to promote plant growth as well. These salt-tolerant plant beneficial microbes have great importance in agriculture. They have shown their potential in improving crop productivity in arid and semiarid regions (Niu et al., 2018). The genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Agrobacterium*, *Streptomyces*, *Klebsiella*, and *Ochromobacter* are best reported for improving the productivity of diverse crops under saline conditions (Sharma et al., 2016; Singh and Jha, 2016; Sarkar et al., 2018). The diazotrophic salt-tolerant bacterial strains of *Klebsiella*, *Agrobacterium*, *Pseudomonas*, and *Ochrobactrum* isolated from the roots of a halophytic plant, *Arthrocnemum indicum* showed salinity tolerance ranging from 4 to 8% NaCl, and improved the productivity of peanut in saline as well as in control conditions (Sharma et al., 2016). *Planococcus rifietoensis*, an alkaliphilic bacterium is reported to enhance growth and yield of wheat crop under salinity stress (Rajput et al., 2013). Upadhyay et al. (2009) explored the genetic diversity of ST-PGPR isolated from the wheat rhizosphere. They found that most of the isolates were able to tolerate up to 8% NaCl and belong to the genus *Bacillus*. The diversity of salt-tolerant bacteria isolated from paddy rhizosphere in Taoyuan, China was reported by Zhang et al. (2018). They isolated 305 bacterial strains, and amongst them, 162 were tested for salt tolerance up to 150 g/l NaCl concentration. Phylogenic analysis of 74 of these salt-tolerant strains showed that they belong to orders Bacillales (72%), Actinomycetales (22%), Rhizobiales (1%), and Oceanospirillales (4%). Most of the isolates also showed their potential in improving salt tolerance, growth, and yield of rice under salt-stress conditions. ST-PGPR strain *Bacillus licheniformis* SA03 isolated from *Chrysanthemum* plants grown in saline-alkaline soil of China conferred increased salt tolerance in *Chrysanthemum* (Zhou et al., 2017).

The diversity of salt-tolerant bacilli was also deciphered in the soil of eastern Indo Gangetic plains of India by Sharma et al. (2015). They isolated 95 bacterial strains and amongst them, 55 showed plant growth promoting characteristics and salt-tolerance to more than 4% NaCl. Several researchers also report the diversity of ST-PGPR in the coastal areas. For example, in Tsunami affected regions in Andaman and Nicobar Islands of India, 121 bacterial strains were isolated, and amongst them 23

showed salt tolerance up to 10% NaCl with PGP characteristics including production of indole acetic acid (IAA), siderophore, extracellular enzymes and phosphate solubilization (Amaresan et al., 2016). The study revealed that the majority of isolates were *Bacillus* spp. and rest were *Alcaligenes faecalis*, *Microbacterium resistance*, *Enterobacter* sp., *Lysinibacillus* sp. A recent study claimed the presence of a novel salt-tolerant bacterial strain *Pseudomonas* sp. M30-35 from the rhizosphere of *Haloxylon ammodendron*, a C_4 perennial succulent xerohalophyte with outstanding drought and salt tolerance capabilities. *Pseudomonas* sp. M30-35 was found to contain 34 genes possessing homology with certain genes associated with PGP traits and abiotic stress tolerance (He et al., 2018). Recently, the genome of many ST-PGPR have also been sequenced which envisaged information about their salt tolerance and plant growth promoting attributes. Kothari et al. (2013) reported that *Bacillus safensis* VK from the desert of Gujrat, India, showed salt tolerance up to 14% NaCl and pH ranging from 4 to 8. Further, study of this *B. safensis* strain deciphered that its genome harbors several genes associated with PGP traits functioning in conditions of high salt concentrations, drought, heavy metals, and polyaromatic hydrocarbons (PAHs) contamination. ST-PGPR *Klebsiella* sp. IG 3 isolated from the rhizosphere of wheat showed salt tolerance up to 20%. This strain positively modulated the expression profile of *rbcL* (codes for the ribulose-1,5-bisphosphate carboxylase/oxygenase RuBisCo) and *WRKY1* (transcription factor dealing with plants reaction to biotic stress) genes under salt-stress conditions (Sapre et al., 2018). In another study, whole genome analysis of a halotolerant PGPR *Klebsiella* sp. D5A revealed the presence of salt tolerance genes with a wide range of pH adaptability and PGP traits including phosphate solubilization, IAA biosynthesis, acetoin, and 2,3-butanediol synthesis, siderophore production, and N_2 fixation (Liu et al., 2016). *Pseudomonas putida* and *Novosphingobium* sp. are reported to reduce salt-stress induced damage in citrus plants by lowering the level of abscisic acid (ABA) and salicylic acid (SA), reducing the efficiency of photosystem II (Fv/Fm), increasing accumulation of IAA in the leaf and inhibiting accumulation of root chloride and proline during salt stress (Vives-Peris et al., 2018). A *Pseudomonas* strain isolated from halophilic grass *Distichlis spicata* also improved the growth of different crops under salt stress (Palacio-Rodríguez et al., 2017). A ST-PGPR strain *Enterobacter* sp. UPMR18 with ability to produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase showed improvement in crop productivity through induction of reactive oxygen species (ROS) scavenging enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) and upregulating to ROS pathway genes (Habib et al., 2016a). Recently, functional metagenomics provided a magnificent way of identification of various genes responsible for salt resistance in microorganisms.

EFFECT OF SOIL SALINIZATION ON CROPS

Soil salinity has an overall detrimental effect on plants' health (**Figure 2**). Salinity affects flowering and fruiting pattern, aberration in reproductive physiology, which ultimately influences crop yields and biomass. Salinity may cause up to 50% reduction in flowering of pigeon pea (*Cajanus cajan* L. Mill) (Promila and Kumar, 1982). In tomato, high salt stress (150 mm NaCl) is reported to affect flowering transition time and causes delay in the first inflorescence along with reduction in the growth of shoot and root (Ghanem et al., 2009). In chickpea (*Cicer arietinum* L.), delayed flowering was directly linked with the higher concentrations of Na^+ in the laminae of completely expanded leaves (Pushpavalli et al., 2016). Salt Overly Sensitive (SOS) pathway is a major defense pathway involved in Na^+ extrusion and maintaining ion homeostasis at the cellular level (Zhu et al., 1998; Zhu, 2003; Ji et al., 2013). There are several reports where both SOS and photoperiodical and circadian clock switch proteins related with flowering are deactivated by salt stress (Kim et al., 2013; Park et al., 2013, 2016; Ryu et al., 2014).

Salt stress remarkably affects plant reproductive physiology. Ghanem et al. (2009) reported that in tomato the exposure of salinity stress results in Na^+ accumulation in style, ovaries, and anther intermediate layers which caused an increase in the rate of flower abortion, reduction of pollen number and viability of the plant. Läuchli and Grattan (2007) reported that salt stress decelerates reproductive growth of wheat by inhibiting spike development and decreasing the yield potential, whereas in salt-sensitive rice, lowering of yield by the reduction in tillers, and formation of sterile spikelet is also experienced. The effect of salinity on *Arabidopsis* was explored in hydroponic solution which induced many symptoms including reduced fertility, decline in fruit length, transient wilting and fruits with predominantly aborted ovules and embryos which were narrower and reduced in size (Sun et al., 2004). Similarly, the effect of salt on early flowering and male gametophyte of canola (*Brassica napus*) plant showed a reduction in pollen grain numbers and abnormal growth of anthers. All these symptoms indirectly lead to reduced crop yield (Mahmoodzadeh and Bemani, 2008).

The drastic effect of salt stress can be seen in terms of yield loss. The primary effects related to crop yield can be in terms of germination which either decreases or sometimes ceases under extreme saline conditions. A study by Ali Khan et al. (2012) showed that under saline conditions growth, yield, and biomass of pearl millet is adversely affected in terms of germination percentage, plant height, leaf area, total biomass and grain yield $plant^{-1}$. Impact of salinity on pea was also found to adversely affect growth, yield and biomass (Wolde and Adamu, 2018). Farooq et al. (2017) also reviewed the effects of salt stress on grain legumes, and they described that in different legumes salinity may reduce crop yield by 12–100%. Salt tolerance of black cumin (*Nigella sativa* L.) and its effect on seed emergence and germination, and yield were studied by Faravani et al. (2013). They showed that an increase in salinity level from 0.3 to 9 dS m^{-1} reduced the average seed and biological yield. Similarly, the effect of different levels of salinity on a weed plant *Portulaca oleracea* L. which is of nutritional importance and being utilized in same ways as spinach and lettuce in many countries, showed a reduction in biomass and yield, changes in physiological attributes, and alteration in stem and root structure (Amirul Alam et al., 2015). Salinity has thus a wide level of impacts on

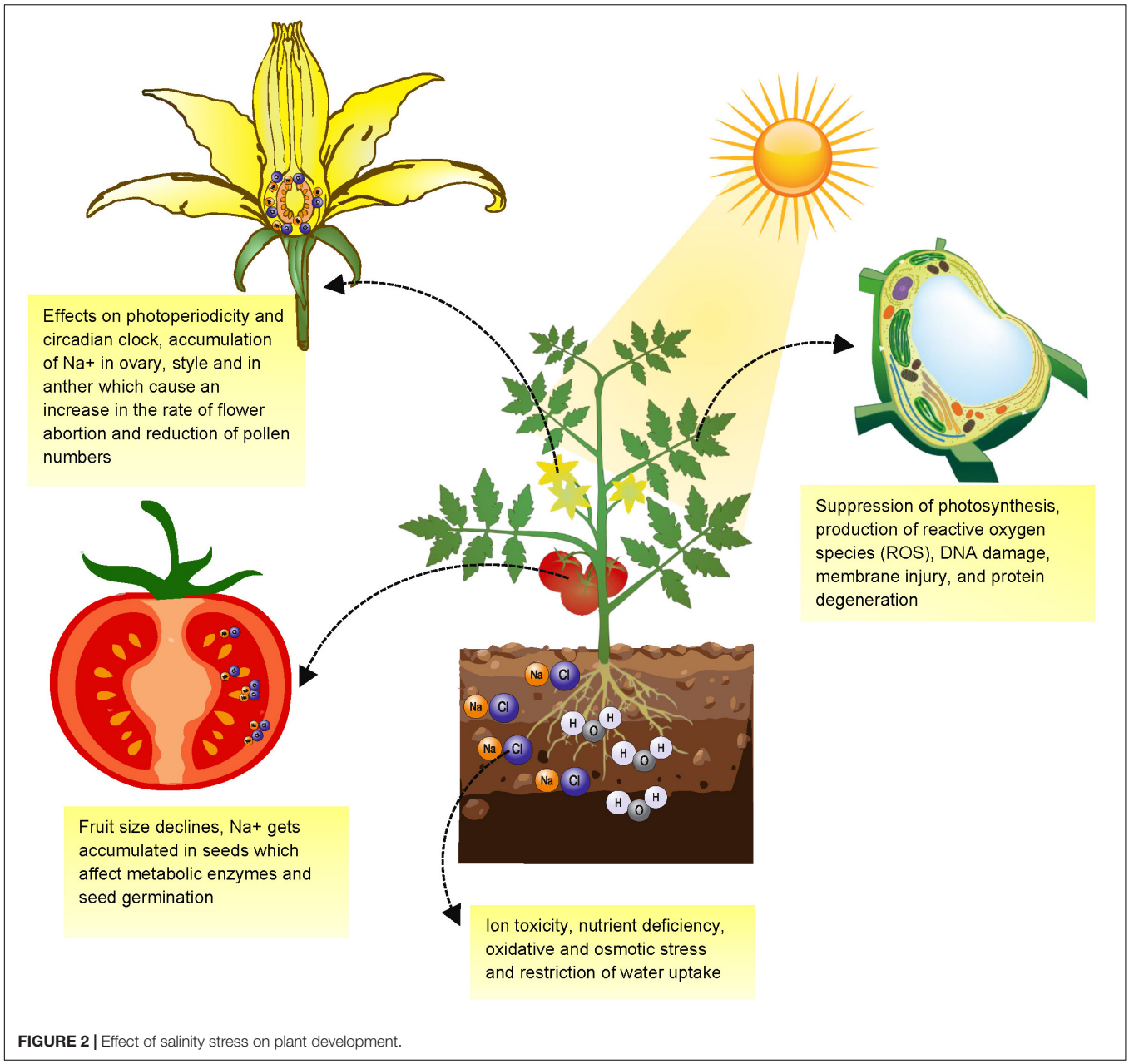

FIGURE 2 | Effect of salinity stress on plant development.

different crops, including even complete loss of yields. This also has impact on soil quality, greenhouse gas (GHG) emissions and food security.

MECHANISMS OF PGPR MEDIATED SALT STRESS TOLERANCE

The ST-PGPR utilize an array of mechanisms (**Figure 3**) which directly or indirectly take part in amelioration of salt stress in crop plants (Egamberdieva et al., 2016; Hashem et al., 2016). Studies confirmed that ST-PGPR produce various types of phytohormones, such as auxins, gibberellins, cytokinins (Dodd et al., 2010), synthesize ACC deaminase (Glick et al., 2007), produce secondary compounds such as exopolysaccharides (Upadhyay et al., 2012; Timmusk et al., 2014) and osmolytes (proline, trehalose, and glycine betaines) (Bano and Fatima, 2009; Upadhyay and Singh, 2015), regulate plant defense systems and activate plant's antioxidative enzymes under salt stress (Hashem et al., 2016).

Plant Growth Regulators

Phytohormones produced by ST-PGPR play an essential role in modulation of plant physiology under salt-stress (Egamberdieva and Kucharova, 2009). The ST-PGPR produce IAA which is required for cell division and elongation in plants coping with salt stress. Some of the best-known ST-PGPR producing IAA

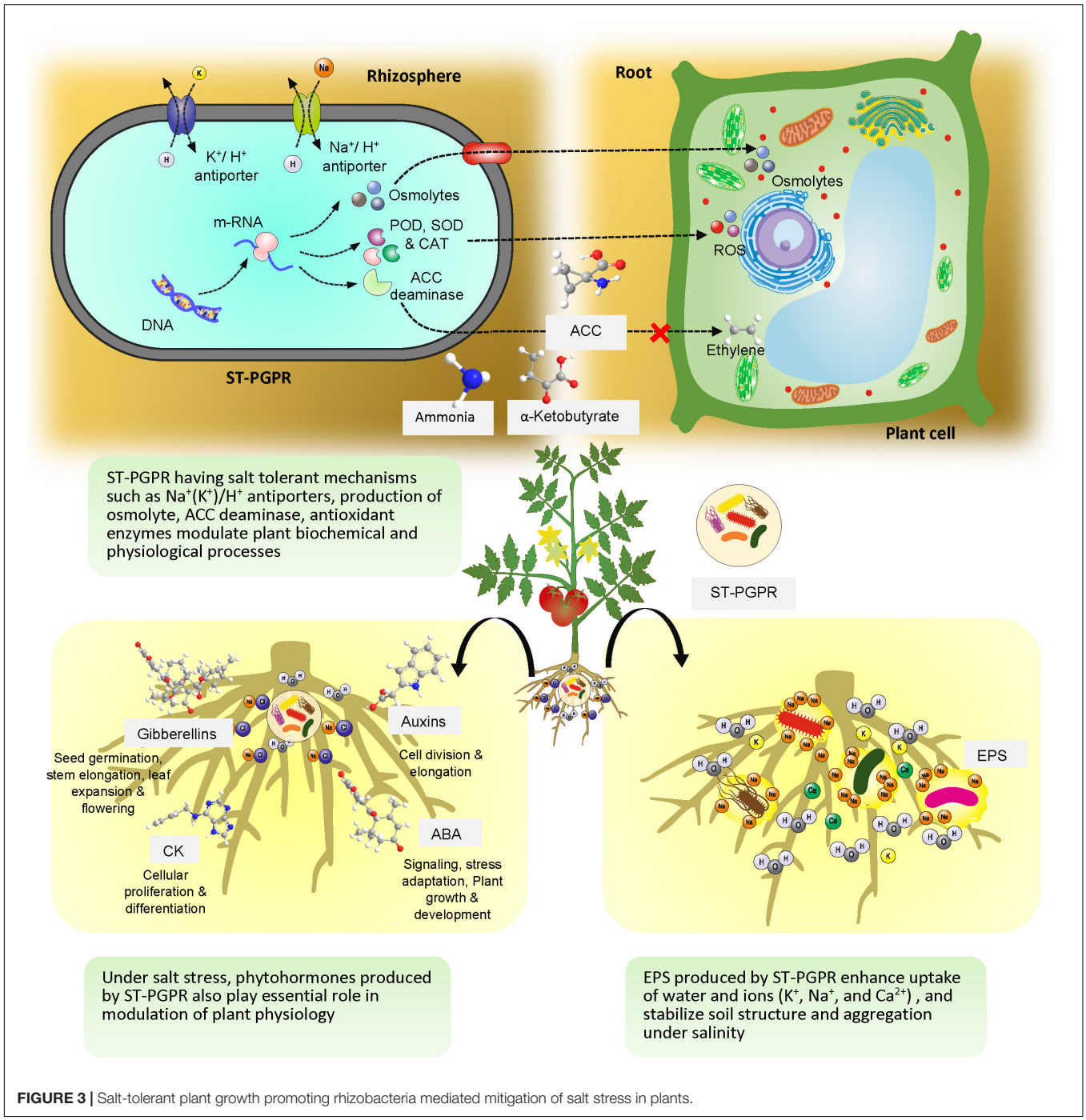

FIGURE 3 | Salt-tolerant plant growth promoting rhizobacteria mediated mitigation of salt stress in plants.

under salt stress are *Azotobacter, Arthrobacter*, *Azospirillum*, *Pseudomonas*, *Stenotrophomonas*, and *Rahnella* (Egamberdieva et al., 2008, 2018; Piccoli et al., 2011; Abd_Allah et al., 2017). Studies have confirmed that under salt stress yield loss in crops can be minimized by the application of phytohormone producing ST-PGPR. Under saline conditions, *P. putida* modulated IAA synthesis in plant tissue and increased the growth parameters of cotton (Yao et al., 2010). It has also been observed that inoculation of ST-PGPR increased the uptake of minerals, protected plants from ion toxicity and enhanced root and shoot growth under saline conditions (Egamberdieva et al., 2017a). Sadeghi et al. (2012) reported that salt-tolerant *Streptomyces* isolates with IAA producing ability improved the root system of wheat under salt stress. Recently, Kang et al. (2019) reported that IAA produced by a ST-PGPR *Leclercia adecarboxylata* MO1 has linkages with sugar synthesis, organic acid production and chlorophyll fluorescence improvement (Fv/Fm) in tomato. Apart from auxins other phytohormones are also reported

to alleviate the effect of salt stress in plants. For example, production of cytokinins (CK), which are important for cellular proliferation and differentiation have been reported in salt-tolerant *Arthrobacter*, *Bacillus*, *Halomonas*, *Azospirillum*, and *Pseudomonas* species (García de Salamone et al., 2001; Karadeniz et al., 2006; Naz et al., 2009; TrParray et al., 2016). ABA is also synthesized by strains of ST-PGPR including *Proteus mirabilis*, *Bacillus megaterium*, *B. licheniformis*, *Pseudomonas fluorescens*, and *Achromobacter xylosoxidans* (Karadeniz et al., 2006; Forchetti et al., 2007; Salomon et al., 2014). Gibberellin producing bacterial strains such as *Bacillus pumilus*, *B. licheniformis*, *Azospirillium* sp. were also reported by Bottini et al. (2004). There are reports where ST-PGPR are known to produce more than one type of phytohormones. Patel and Saraf (2017) reported that bacterial strains of *Pseuodomonas stutzeri*, *Stenotrophomonas maltophilia*, and *P. putida* isolated from the *Coleus* rhizosphere produced IAA, gibberellic acid, and CK under saline conditions. Recently, Tewari and Arora (2018) reported the role of SA in ameliorating salinity stress and enhancing the growth of sunflower in saline soils.

ACC Deaminase Activity

The presence of ACC deaminase activity in ST-PGPR is a common phenomenon especially when exposed to high salt stress. The enzyme ACC deaminase cleaves ACC into ammonia and α-ketobutyrate which are consumed by the bacteria as nitrogen (N) and carbon sources (Glick, 2014). The presence of ST-PGPR dramatically lowers the level of stress ethylene and prevents inhibition of down-regulated genes involved in ethylene-induced plant stress and up-regulates genes involved in plant growth (Glick et al., 2007). There are many examples where ACC deaminase activity by ST-PGPR not only improved plant survival in saline soils but also enhanced productivity. *Stenotrophomonas rhizophila* synthesized ACC deaminase and stimulated growth of cucumber in saline soil (Egamberdieva et al., 2011). Ali et al. (2014) reported improvement in physiological properties of plants under salt stress by ACC deaminase producing *P. fluorescens* and *Pseudomonas migulae* strains. In another study, halotolerant bacterial strains, *Brachybacterium saurashtrense* (JG-06), *Brevibacterium casei* (JG-08), and *Haererohalobacter* (JG-11), producing ACC deaminase, improved salt tolerance in peanut grown in saline soils (Shukla et al., 2012). It has been found that ACC deaminase producing ST-PGPR have effects on other biochemical properties of plant cell, including membrane stability, biocompatible solutes formation and photosynthetic pigment production under drought and salt stress (Tiwari et al., 2018). Aslam and Ali (2018) also reported that ACC-deaminase activity in halolerant bacterial genera of *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Gracilibacillus*, *Virgibacillus*, *Salinicoccus*, and *Pseudomonas*, *Exiguobacterium* isolated from the rhizosphere and phytoplane of *Suaeda fruticosa* (L.) Forssk stimulated growth of maize under saline conditions. The effect of ACC deaminase by ST-PGPR on nodule formation in legume crops is also well documented (Ahmad et al., 2011; Barnawal et al., 2014). In nodulation process, ACC deaminase is found to play an essential role to enhance persistence of infection threads which is negatively affected by ethylene level and thus help in nodule formation under saline conditions (Nascimento et al., 2016).

Osmoprotectants

Plants accumulate organic osmolytes such as proline, glycine, betaine, polyamines, quaternary ammonium compounds, and other amino acids in response to various abiotic stresses (Sandhya et al., 2010). ST-PGPR also employ this mechanism for protection against osmotic stress which is more common in saline soils (Mishra et al., 2018). While exposed to salt stress, salt-tolerant bacteria may temporarily increase their cytoplasmic content of K^+, but the accumulation of osmolytes is a more sustained stress response to prevent water loss (Bremer and Kramer, 2019). During salt stress, internal concentration of organic osmolytes may reach up to 1 M in certain halophilic bacteria and has a major role in destabilization of the double helix and lower the Tm of DNA (Rajendrakumar et al., 1997). In moderately halophilic bacteria salinity induced expression of proline biosynthesis genes *proH*, *proJ,* and *proA* was reported at 2.5 M NaCl which lead to the highest accumulation of proline (Saum and Müller, 2007). Recently, Kushwaha et al. (2019) explored osmoprotection in *Halomonas* sp. SBS 10 and found that at low NaCl, betaine accumulation suppresses the *de novo* synthesis of ectoine whereas at a high NaCl concentration, the ectoine concentration increases abruptly as compared to the betaine. Further, they concluded that ectoine accumulation is transcriptionally up-regulated by the salinity stress. In another study it was observed that *Azospirillum* spp. accumulate proline, glycine betaine, and trehalose, supporting the plant to withstand osmotic stress (Rodríguez-Salazar et al., 2009). A ST-PGPR strain of *Bacillus* sp. increased growth and development of maize under drought and salinity through accumulation of proline and soluble sugars (Vardharajula et al., 2011). Role of the trehalose as an osmoprotectant under salt-stress is also well documented and a large number of ST-PGPR have been discovered having genes for trehalose biosynthetic pathways (Qin et al., 2018; Orozco-Mosqueda et al., 2019; Shim et al., 2019).

Exopolysaccharides (EPS)

Production of EPS or surface polysaccharides is a general characteristic of some rhizosphere bacteria. Although, the composition and amount of EPS may vary in different ST-PGPR strains, copious amount of EPS is formed in adverse conditions (Bomfeti et al., 2011; Tewari and Arora, 2014a; Khan and Bano, 2019). EPS work as physical barrier around roots and support plant growth in high salinity stress (Vaishnav et al., 2016). Inoculation of EPS producing ST-PGPR also showed ameliorative effects on the uptake of K^+, Na^+, and Ca^{2+} in plants (Ashraf et al., 2004; Kohler et al., 2006). Qurashi and Sabri (2012a) showed that EPS producing ST-PGPR *Halomonas variabilis* (HT1) and *P. rifietoensis* (RT4) improved chickpea growth and stabilization of soil structure and aggregation under salinity. The presence of EPS in the biofilm also has positive effects on root colonization by ST-PGPR (Qurashi and Sabri, 2012b). In the context of yield improvement the role of EPS producing ST-PGPR is very significant as they are being used as priming agents of seeds and help in enhancing germination

(Tewari and Arora, 2014a). Atouei et al. (2019) found that salt-tolerant EPS producing *B. subtilis* subsp. *inaquosorum* and *Marinobacter lipolyticus* SM19 reduced adverse effects of salinity and drought stresses in wheat. Recently, Chu et al. (2019) showed possible role of EPS producing salt-tolerant *Pseudomonas* PS01 strain in regulation of genes related to stress tolerance in *Arabidopsis thaliana*. They found that a LOX2 gene which encodes a lipoxygenase that constitutes an essential component of the jasmonic acid (JA) synthesis pathway was up-regulated. As JA itself is a positive regulator and accumulates rapidly in plants (under salt stress), bacterial EPS provide additional benefits to survive under salt stress.

Antioxidant Enzymes

Inoculation of plants with ST-PGPR decreases the negative effects of oxidative stress by producing antioxidative enzymes (Manaf and Zayed, 2015; Islam et al., 2016). There are evidences where ST-PGPR produced high concentration of antioxidant enzymes including POD, SOD, CAT, and nitrate reductase (NR), glutathione reductase (GR) under salinity stress (Jha and Subramanian, 2013; Sen and Chandrasekhar, 2015; Ansari et al., 2019). El-Esawi et al. (2019) observed that *Azospirillum lipoferum* FK1 inoculated plants demonstrated a higher expression level of the antioxidant genes and thus improved antioxidant enzymes and non-enzymatic metabolites, nutrient uptake, phenols and flavonoids content, growth, and development of chickpea. Similarly, Kohler et al. (2009) observed an increase in CAT activity in tissue of lettuce, reducing oxidative damage under saline conditions. Some ST-PGPR such as *Enterobacter clocae*, *Pseudomonas pseudoalcaligenes* and *Bacillus* sp., increased levels of APX and CAT in Jatropha leaves in response to salt stress and also stimulated the roots, increased biomass, N, phosphorus (P), potassium (K) uptake and chlorophyll content in the vegetative parts of the plant (Patel and Saraf, 2013). The salt-tolerant plant *Sulla carnosa* showed better growth and stress tolerance after inoculation with strains of *Pseudomonas* sp. and *Bacillus* sp. in saline Tunisian soils. The bacterial inoculants increased root and shoot biomass, and nutrient acquisition under salt stress by reducing stomatal conductance, and modulated antioxidant activities involved in plant stress responses (Hidri et al., 2016).

Taken together, these findings clearly suggest the important role of ST-PGPR in plant stress tolerance by modulating plant physiological and biochemical processes such as stress-related genes, osmolytes, and enzymatic and non-enzymatic antioxidants.

ST-PGPR FOR IMPROVING CROP PRODUCTIVITY

It is now widely accepted that ST-PGPR are endowed with the inherent capability to cope with high concentration of salts in the soil. Their presence in the soil provides a direct benefit to plants. Their application in the form of bioinoculants/bioformulations not only improves crop productivity but also makes the survival of plants easier under extreme saline conditions (**Table 1**). In this section, the role of ST-PGPR in enhancing productivity of various crops is discussed.

Cereal Crops

Cereal crops are the main source of energy and protein in the human diet. Worldwide, in comparison to other crops, cereal crops are cultivated in much higher quantity. Wheat, maize, rice, barley, oats, sorghum, and millet are known as major cereal crops. However, very few of them are reported as salt-tolerant. Since decades, most common approaches of enhancing yield of saline soils, which include conventional breeding, marker-assisted selection, and genetic engineering have also proven to be successful, but only for wheat and rice (Shahbaz and Ashraf, 2013; Roy et al., 2014). It has been realized that application of ST-PGPR in salt-affected soil not only assisted in the survival of crop but also improved yield in a wide range of cereal crops (Singh and Jha, 2016). In a study, ST-PGPR *B. subtilis* enhanced wheat yield by around 18% in salt-affected soil (EC 5.2 dSm^{-1}) (Upadhyay and Singh, 2015). Research shows that application of ST-PGPR in rice may increase germination, promote seedling growth, induce antioxidative enzymes against ROS, favor osmolyte accumulation and modulate expression of genes related to salt stress (Nautiyal et al., 2013; Paul and Lade, 2014; Rima et al., 2018; Sarkar et al., 2018). The effect of various ST-PGPR in enhancing the productivity of salt-tolerant rice and wheat cultivated on sodic soils was explored by Damodaran et al. (2019) and they found that *Lysinibacillus* sp. was most effective in ameliorating the adverse effect of salinity. Similarly, a comprehensive research on the diversity of ST-PGPR in different agro-climatic zones was conducted by Misra et al. (2017) and they revealed that amongst all, *Bacillus* sp. with ACC deaminase activity were most dominant in amelioration of salt stress and enhancing biomass of rice. A ST-PGPR, *Pseudomonas* strain 002 was found to improve root formation in maize under salinity (150 mM NaCl) stress (Zerrouk et al., 2016). The effect of ST-PGPR *S. sciuri* SAT-17 strain on anti-oxidative defense mechanisms and modulation of maize growth under salt stress was studied by Akram et al. (2016). They reported that inoculation of maize with SAT-17 improved plant growth and decreased the ROS levels by increasing the cellular antioxidant enzyme activities (CAT, POD, and proline) under salinity treatments (75 and 150 mM NaCl).

Legumes and Oil Yielding Crops

Along with the cereals, legumes reserve their position as important source of protein in the human diet. Salinity hampers production of grain and food legumes in many regions around the globe (Manchanda and Garg, 2008). In legumes, salt-stress adversely effects root-nodule formation, symbiotic relation and finally the nitrogen fixation capacity (Manchanda and Garg, 2008). Symbiotic association of rhizobia with legumes under salinity stress is still a broad area of research (Zahran, 1991, 1999; Graham, 1992). Most of the findings reveal that application of salt-tolerant rhizobia is a sustainable solution for enhancing the productivity of legume crops grown under salinity stress (Abiala et al., 2018). Some workers have demonstrated that negative impacts of salinity on legumes including soybean, pigeon pea, common bean, mung bean, groundnut and even tree legumes can

TABLE 1 | Salt-tolerant plant growth promoting rhizobacteria and their possible roles in enhancing yield and growth of diverse plants/crops.

Crops	ST-PGPR	Mechanism	References
Sunflower	*P. fluorescens*	IAA production, siderophore production and K^+/Na^+ ratio	Shilev et al., 2012
	Pseudomonas aeruginosa	EPS production	Tewari and Arora, 2014b
	Pseudomonas sp.	IAA production, phosphate solubilization, siderophore, nitrogen fixation, HCN, chitinase and β-1-3 glucanase activity	Tewari and Arora, 2016
Soybean	*P. putida* H-2-3	ABA, salicylic acid and JA and gibberellins	Kang et al., 2014
	Bradyrhizobium japonicum USDA 110 and *P. putida* TSAU1	Root system physiology, nitrogen and phosphorus acquisition and nodule formation	Egamberdieva et al., 2017a
	Bacillus firmus SW5	Antioxidant enzyme and alternation in root system architecture	El-Esawi et al., 2018a
	Bradyrhizobium japonicum, Bacillus subtilis SU-12 and *Serratia proteamaculans*	Antioxidant enzyme and Proline content	Han and Lee, 2005
	Pseudomonas simiae	IAA production, phosphate solubilization and siderophore production	Vaishnav et al., 2016
	B. japonicum and *B. subtilis*	EPS production, antioxidant activity and concentration of proline	Han and Lee, 2005
	P. fluorescens	CK production	Bhattacharyya and Jha, 2012
Groundnut	*P. aeruginosa* AMAAS57 and *P. aeruginosa* BM6	IAA production, HCN, ammonia, phosphate solubilization, production of phenol and free amino acids	Ghorai et al., 2015
	B. saurashtrense, B. casei, Haererohalobacter	Osmotic stress and proline	Shukla et al., 2012
	P. fluorescens	ACC deaminase	Saravanakumar and Samiyappan, 2007
	Klebsiella, Pseudomonas, Agrobacterium, and *Ochrobactrum*	IAA production, phosphate solubilization, ACC deaminase	Sharma et al., 2016
Rape seed	*Rhizobium* sp.	ACC deaminase, IAA production and phosphate solubilization	Saghafi et al., 2018
	P. fluorescens and *P. putida*	ACC deaminase, IAA and hydrogen cyanide	Jalili et al., 2009
	P. putida UW4	Photosynthesis, antioxidant enzyme, membrane transportation and pathogenesis-related responses	Cheng et al., 2012
Cotton seed	*P. putida R4* and *Pseudomonas chlororaphis* R5	IA production	Egamberdieva et al., 2015
	P. putida	Germination rate and biomass	Yao et al., 2010
	Bacillus amyloliquefaciens, Curtobacterium oceanosedimentum and *Pseudomonas oryzihabitans*	Seed germination	Irizarry and White, 2017
	Klebsiella oxytoca Rs-5, *Bacillus* sp. SL-13, *Bacillus* sp. SL-14 and *Bacillus* sp. SL-44	Antioxidant enzymes and photosynthetic pigment content	Wu et al., 2012
Rice	*Alcaligens* sp., *Bacillus* sp. and *Ochrobactrum* sp.	ACC deaminase	Bal et al., 2013
	Serratia sp. and *Pseudomonas* sp.	IAA production, nitrogen fixation, and phosphate solubilization	Nakbanpote et al., 2014
	P. pseudoalcaligenes and *B. pumilus*	Reduced the toxicity of ROS by reducing plant cell membrane index, cell caspase-like protease activity, and programmed cell death	Jha and Subramanian, 2013
	Bacillus aryabhattai MS3	Nitrogen fixation, IAA production, phosphorus solubilization and siderophore production	Sultana et al., 2018
	Enterobacter sp. P23	Phosphate solubilization, IAA production, siderophore production, HCN production	Sarkar et al., 2018
	Halobacillus dabanensis strain SB-26, *Halobacillus* sp. GSP 34	Nitrogen fixation and IAA production	Rima et al., 2018
	Pseudomonas strains PF1 and TDK1	Antioxidant enzyme	Sen and Chandrasekhar, 2015
	B. stratosphericus (NBRI 5Q and NBRI 7A)	Phosphate solubilization, ACC deaminase activity IAA production	Misra et al., 2017
	B. amyloliquefaciens NBRISN13 (SN13)	Betaine, sucrose and trehalose	Nautiyal et al., 2013

(Continued)

TABLE 1 | Continued

Crops	ST-PGPR	Mechanism	References
	Bacillus and *Citrobacter*	Nitrogen fixation, phosphate solubilization and IAA production	Habib et al., 2016b
	B. pumilus	Antioxidative enzymes	Khan et al., 2016
Chick pea	*P. pseudoalcaligens*	Phosphate solubilization, siderophore and IAA production	Patel et al., 2012
	Mezorhizobium ciceri	Nodulation and Nitrogen fixation	Egamberdieva et al., 2014
	H. variabilis (HT1) and *P. rifietoensis (RT4)*	Biofilm formation and EPS	Qurashi and Sabri, 2012a
	Mesorhizobium strains	ACC deaminase activity improved nodulation and reduced level of ethylene	Chaudhary and Sindhu, 2015
Mung bean	*Rhizobium* and *pseudomonas*	Photosynthetic rate, chlorophyll content and water use efficiency	Ahmad et al., 2013
	Rhizobium sp.	ACC deaminase	Aamir et al., 2013
	P. fluorescens (Mk20) and *Rhizobium phaseoli*	ACC deaminase	Ahmad et al., 2011
Lentil	*Rhizobium leguminosarum* bv.viciae	IAA production and phosphate solubilization	Jida and Assefa, 2011
	Pseudomonas jessenii and *R. leguminosarum* (P10Z22)	ACC deaminase	Zahir et al., 2011
	Rhizobium sp. BCKV MU5 and BCKV MU2	Nodulation efficiency	Halder et al., 2016
Pea	*Arthrobacter protophormiae* with *R. leguminosarum* and *Glomus mosseae*	Reduction of ethylene stress through ACC deaminase	Barnawal et al., 2014
Pigeon pea	*Bradyrhizobium* (RA-5) and *Burkholderia cepacia* (RRE-5)	Enhancement of root nodulation and N_2 fixation	Bano et al., 2015
	P. putida, P. fluorescens, Bacillus cereus with Rhizobium strain	Nodulation and N_2 fixation	Tilak et al., 2006
Black gram	*P. fluorescens* SA8 with kinetin (10 μM)	Improvement in water relation, gas exchange, and photosynthetic content	Yasin et al., 2018
Faba bean	*P. putida*, *P. fluorescens* and *B. subtilis*	Increase in growth traits of plant	Metwali et al., 2015
	Pseudomonas anguilliseptica SAW 24	Biofilm production and EPS production	Mohammed, 2018
Maize	*Pseudomonas syringae*, *Enterobacter aerogenes* and *P. fluorescens*	ACC deaminase	Nadeem et al., 2007
	Azospirillum brasilense	Ion toxicity, NOR and nitrogenase activity	Hamdia et al., 2004
	Pseudomonas and *Enterobacter* spp.	ACC deaminase	Nadeem et al., 2009
	Azotobacter chroococcum	Improved K^+/Na^+ ratio, polyphenol content and proline concentration	Rojas-Tapias et al., 2012
	Proteus penneri, *P. aeruginosa*, and *A. faecalis*	EPS	Naseem and Bano, 2014
	B. amyloliquefaciens	Soluble sugar content and antioxidant enzymes	Chen et al., 2016
	Pantoea agglomerans	Up-regulation of aquaporin genes	Gond et al., 2015
	Rhizobium and *Pseudomonas*	Osmotic regulation	Bano and Fatima, 2009
	Staphylococcus sciuri	Antioxidant enzymes	Akram et al., 2016
	Bacillus spp. and *Arthrobacter pascens*	phosphate solubilization, osmotic regulation and antioxidant enzymes	Ullah and Bano, 2015
	Bacillus aquimaris DY-3	Chlorophyll content, osmotic regulation and antioxidant enzymes	Li and Jiang, 2017
	P. syringae and *P. fluorescens*	ACC deaminase	Zafar-ul-Hye et al., 2014
	A. brasilense strains Ab-V5 and Ab-V6 and Rhizobium tropici strain CIAT 899	Antioxidant enzymes and proline contents	Fukami et al., 2018
	Gracilibacillus, *Staphylococcus*, *Virgibacillus*, *Salinicoccus*, *Bacillus*, *Zhihengliuella*, *Brevibacterium*, *Oceanobacillus*, *Exiguobacterium*, *Pseudomonas*, *Arthrobacter*, and *Halomonas* spp.	IAA production, ACC deaminase, phosphate solubilization and biofilm formation	Aslam and Ali, 2018
	Serratia liquefaciens KM4	Facilitated gas exchange, osmoregulation, antioxidant enzymes, nutrient uptake and downregulation of ABA biosynthesis	El-Esawi et al., 2018b

be minimized by the application of salt-tolerant rhizobial strains (Bashan and Holguin, 1997; Kumar et al., 1999; Dobbelaere et al., 2001; Bashan et al., 2004; Dardanelli et al., 2008; Meena et al., 2017; Yasin et al., 2018). Salt-tolerant species of *Pseudomonas* are reported to improve the health of groundnut (Saravanakumar and Samiyappan, 2007), common beans (Egamberdieva, 2011), faba beans (Metwali et al., 2015), and chickpea (Jatan et al., 2019). In a study, application of ST-PGPR *B. firmus* SW5 on soybean grown under salt stress showed its beneficial effect on nutrient uptake, chlorophyll synthesis, osmolyte levels, gas exchange parameters, total phenolics, flavonoid contents, and antioxidant enzyme activities, in comparison to control (El-Esawi et al., 2018a). Wang et al. (2016) found that ACC-deaminase containing rhizobacterium *Variovorax paradoxus* 5C-2 increased total biomass of pea by 25 and 54% under salinity levels of 70 and 130mM NaCl, respectively. In a study, Yanni et al. (2016) used four strains of *Rhizobium* as bioinoculants for common bean in saline and drought stressed fields and found that inoculation increased seed yield considerably. A combined effect of *R. phaseoli* with L-tryptophan on the mung bean grown under salinity stress was evaluated by Zahir et al. (2010) and it was found that the application induced more pronounced effects and increased the plant height, number of nodules per plant, plant biomass, grain yield, and grain N concentration as compared with the untreated control. In another study, Ahmad et al. (2011) evaluated the combined application of salt-tolerant *Rhizobium* and *Pseudomonas* under salt-stressed conditions for improving the productivity of mung bean. They found that co-inoculation increased all the growth parameters including seed yield by 150% as compared with un-inoculated control. *B. japonicum* USDA 110 and *P. putida* TSAU1 were reported with salt tolerance activity and abilities to work synergistically with each other to enhance growth and productivity of soybean under high salinity (Egamberdieva et al., 2017b).

Salinity halts the growth and affects the oil yield in several legumes. Field application of ST- PGPR in oil-yielding crops has proven beneficial in remediation of salt stress (Tewari and Arora, 2014a,b). In other studies fluorescent *Pseudomonas* improved root and shoot length of sunflower under saline conditions (Tewari and Arora, 2016) and ST-PGPR *Klebsiella*, *Agrobacterium*, *Pseudomonas*, and *Ochrobactrum* enhanced salt-tolerance in groundnut crop (Sharma et al., 2016). Similarly, salt-tolerant *P. fluorescens* strain TDK1 with ACC deaminase activity also enhanced the salt resistance in groundnut plants, which in turn enhanced yields (Saravanakumar and Samiyappan, 2007). Furthermore, co-inoculation of *B. japonicum* USDA 110 and salt-tolerant *P. putida* TSAU1 were found to improve growth parameters, protein content, N, and P uptake and root system architecture of soybean under salt stress (Egamberdieva et al., 2017a).

Vegetable Crops

Globally, about 1.1 percent of the total agricultural area is covered with vegetables[1] and China and India are the leading countries in this regard. The threshold of salinity for most vegetable crops is $\leq$2.5 dS m^{-1} hence compared with others, vegetable crops are more prone to damage caused due to salinity stress (Shannon and Grieve, 1998). Unfortunately, research on the role of ST-PGPR in alleviating salt stress of vegetable crops is very scarce. However, there are some reports about the role of ST-PGPR in enhancing the performance of vegetable crops under salinity stress. Mayak et al. (2004) isolated an *Achromobacter piechaudii* bacterium from Arava region of southern Israel which significantly increased the fresh and dry weights of tomato seedlings at high salt concentration. Similarly, Tank and Saraf (2010) also found that the ST-PGPR *P. stutzeri* improved salt tolerance in tomato plants. Recently, Van Oosten et al. (2018) reported that inoculation of tomato plants with *A. chroococcum 76A* under both moderate (50 mM NaCl) and severe (100 mM NaCl) salt stresses increased growth parameters i.e., shoot dry weight, fresh fruit weight and fruit number per plant as compared to the control. Abd El-Azeem et al. (2012) reported that ST-PGPR strains of *Xanthobacter autotrophicus* BM13, *E. aerogenes* BM10, and *Bacillus brevis* FK2 alleviated negative effects of salt and improved growth and yield in egg plant. Furthermore, Ge and Zhang (2019) showed that under salt stress (3% NaCl), *Rhodopseudomonas palustris* G5 increased shoot height, root length, fresh weight, dry weight, total chlorophyll content and soluble sugar content of cucumber seedlings. Recently, Tahir et al. (2019) conferred that consortium of salt-tolerant *Bacillus* strains enhanced potato tuber yield by the production of auxin, antioxidant enzymes and regulating uptake of Na^+, K^+, and Ca^{+2} in normal and salt affected soils. ST-PGPR have proven to be beneficial for cultivation of vegetables in saline soils, however, this needs to be explored and utilized further.

FUTURE PROSPECTS

Although much progress has been made in understanding the effect of salinity on plants, little success is achieved in the sustainable management of productivity losses. The potential of ST-PGPR can be harnessed for the improvement of crop yield of saline soils. According to Pan et al. (2019) physiological roles played by ST-PGPR could improve plant performance under saline conditions. It has been realized that elucidation of mechanisms of osmo-adaptation in ST-PGPR may contribute to the long-term goal of improvement of productivity in crops grown in saline agro-ecosystems (Paul, 2013). Knowledge of salt tolerance mechanisms in ST-PGPR is still not up to mark, particularly about the involvement of bacterial genes in osmotic regulation and plant-microbe interactions (under saline conditions). Although, in the last few years, molecular mechanisms of salt tolerance have been studied in several halophilic bacteria. This revealed that amongst various mechanisms, bacterial salt-related antiporters such as Na^+/H^+ play specific roles in salinity tolerance in plants as well (Zhong et al., 2012). Ma et al. (2019) stated that the understanding of regulatory networks of ST-PGPR in inducing salt tolerance in plants, could serve as a promising measure to alleviate salt stress and improve global food production. However, a deeper investigation of microbial responses to

[1] http://www.fao.org/3/i3138e/i3138e05.pdf

soil salinity are required for their better utilization in the reclamation of saline soils. Kim et al. (2019) suggested that identification of the dominant indigenous microflora from the highly saline soil and their possible adaptation mechanisms may provide a better understanding for exploring ecological and evolutionary responses in ecosystems. The role of metagenomic and metabolomic approaches becomes very important in case of harnessing and identifying novel ST-PGPR, along with the key genes and metabolites involved in salt tolerance.

Crop specific field trials (**Table 1**) have already suggested strong ability of ST-PGPR in mitigation of salt stress (in diverse crops). However, replication of these research findings with same vigor in different geographical regions and on different plants has remained a challenge. This is why, in some of the instances, erratic results are obtained while using PGPR inoculants at field level (Souza et al., 2015; Ambrosini et al., 2016). Recently, Kumar et al. (2019) suggested that salinity related research on PGPR should follow certain protocols and screening is an important issue to maintain the quality and accuracy. They also addressed that use of indigenous ST-PGPR strains should be preferred in bioinoculant development as they can easily adapt to the local field conditions. Similarly, development of ST-PGPR based bioinoculant exclusively designed for saline soils could be more efficient approach in managing productivity loss in several crops (Mishra et al., 2018). Whereas prospects on future research of developing novel bioinoculants providing metabolites (osmolytes, biosurfactants, precursor of phytohormones and stress enzymes) along with efficient ST-PGPR strains (including consortia) should also be explored. Consortia based bioinoculants have recently gained importance because by involving diverse ST-PGPR not only salinity stress can be managed but phytopathogens can also be controlled and nutrients be assimilated simultaneously (Woo and Pepe, 2018). It has also been suggested that use of the diverse microbes such as PGPR, mycorrhiza and endosymbionts in consortial formulations can be a very promising strategy for combating stress in plants (Ilangumaran and Smith, 2017). At present there is lack of products of ST-PGPR for specific use in saline agro-ecosystems and available bioinoculants do not work in such conditions. This makes the end-user skeptical about the use of biological alternatives. The use of metabolites such as EPS has already been suggested and explored in some latest bio-products (Tewari and Arora, 2014b; Arora and Mishra, 2016). EPS can be cheaply produced at lab and industrial levels and can be added to bioinoculants prepared for saline soils. This metabolite can act as protectant for both the inoculated PGPR and the plant. Similarly other metabolites and carriers can also be explored for development of tailor-made bioformulations for saline soils.

Nowadays, several researchers are vigorously engaged to enhance the efficacy and applicability of ST-PGPR containing bioinoculants in field conditions (Ambrosini et al., 2016). Furthermore, a major part of research has to be devoted to improve the issues related with formulation development (Mishra et al., 2018). In recent past, development in the field of polymeric science devised amalgamated use of biopolymers and PGPR in agriculture (Raj et al., 2011; Sharif et al., 2018). For example, chitosan-immobilized aggregated *Methylobacterium oryzae* CBMB20 has been used as a bioinoculant to promote growth of tomato under salt stress conditions (Chanratana et al., 2019). Li et al. (2019) also showed synergistic use of a Super Absorbent Polymer (SAP) along with PGPR strains of *Paenibacillus beijingensis* BJ-18 and *Bacillus* sp. L-56 to overcome salinity stress in wheat and cucumber. Enhancing the productivity of saline soils will not only help in achieving food security but also result in enhanced content and quality of soil organic matter in these nutritionally poor agro-systems. This can help in reducing the carbon footprint and combating climate change as well (Arora, 2019; SDG, 2019).

CONCLUSION

Salt-tolerant plant growth promoting rhizobacteria have evolved several mechanisms to cope with salinity stress. They can easily withstand high salinity stress through various mechanisms such as efflux systems, formation and accumulation of compatible solutes for balancing external osmotic pressure, formation of ROS, secondary metabolites and other means. Several genes and metabolites are involved in maintaining the cell integrity and plant-microbe interactions under salinity stress. Still a lot is yet to be explored at molecular and biochemical level on how the ST-PGPR support themselves and their symbiotic partner under salinity stress which has multi-dimensional impacts on the cell (of both bacteria and plant). Research on ST-PGPR also indicates their vast potential in remediation and productivity enhancement of agro-ecosystems suffering from problems of salinity. However, in-depth studies targeting gene level expression and functional characteristics of ST-PGPR involved in plant growth promotion under salinity stress have to be conducted in the near future to design tailor-made bioformulations for saline soil systems, which are increasing worldwide, day by day. Utilization of this green biotechnology will have multi-faceted positive impacts on agro-ecosystems and rural environment.

AUTHOR CONTRIBUTIONS

DE and NA conceptualized the idea. JM and NA prepared the illustrations. All authors equally contributed in writing of the manuscript.

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Effects of Soil Microbes on Functional Traits of Loblolly Pine (*Pinus taeda*) Seedling Families From Contrasting Climates

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Examining factors that influence seedling establishment is essential for predicting the impacts of climate change on tree species' distributions. Seedlings originating from contrasting climates differentially express functional traits related to water and nutrient uptake and drought resistance that reflect their climate of origin and influence their responses to drought. Soil microbes may improve seedling establishment because they can enhance water and nutrient uptake and drought resistance. However, the relative influence of soil microbes on the expression of these functional traits between seedling families or populations from contrasting climates is unknown. To determine if soil microbes may differentially alter functional traits to enhance water and nutrient uptake and drought resistance between dry and wet families, seeds of loblolly pine families from the driest and wettest ends of its geographic range (dry, wet) were planted in sterilized sand (controls) or in sterilized sand inoculated with a soil microbial community (inoculated). Functional traits related to seedling establishment (germination), water and nutrient uptake and C allocation (root:shoot biomass ratio, root exudate concentration, leaf C:N, leaf N isotope composition ($\delta^{15}N$)), and drought resistance (turgor loss point, leaf carbon isotope composition ($\delta^{13}C$)) were measured. Then, plants were exposed to a drought treatment and possible shifts in photosynthetic performance were monitored using chlorophyll fluorescence. Inoculated plants exhibited significantly greater germination than controls regardless of family. The inoculation treatment significantly increased root:shoot biomass ratio in the wet family but not in the dry family, suggesting soil microbes alter functional traits that improve water and nutrient uptake more so in a family originating from a wetter climate than in a family originating from a drier climate. Microbial effects on photosynthetic performance during drought also differed between families, as photosynthetic performance of the dry inoculated group declined fastest. Regardless of treatment, the dry family exhibited a greater root:shoot biomass ratio, root exudate concentration, and leaf $\delta^{15}N$ than the wet family. This indicates that the dry family allocated more resources belowground than the wet and the two family may have used different

sources of plant available N, which may be related to their contrasting climates of origin and influence their drought resistance. Examination of variation in impacts of soil microbes on seedling physiology improves efforts to enhance seedling establishment and beneficial plant-microbe interactions under climate change.

Keywords: soil microbes, loblolly pine, seedling physiology, genetic variation, drought, turgor loss point, growth, root exudates

INTRODUCTION

Increased intensity and frequency of heat waves, drought, and wildfire (IPCC, 2018) have led to widespread forest mortality in recent decades (Allen et al., 2010; Hartmann et al., 2018). To sustain forests for ecosystem services and atmospheric CO_2 sequestration, it is essential to understand vegetation responses to changing climate that determine forest species' geographic distributions under future climate regimes. Successful seedling establishment is a key determinant of future tree species' distributions (Harper, 1977; Jackson et al., 2009; Zhu et al., 2012; Davis et al., 2016; Simeone et al., 2019). However, the seedling stage is the most vulnerable developmental stage of plants because seedlings are small and delicate with limited access to water and nutrients, which exacerbates their susceptibility to climate change stresses like drought (Johnson et al., 2011; García de la Serrana et al., 2015; Chen et al., 2016; Yao et al., 2018). Therefore, examining factors that influence seedling establishment and mortality (Kursar et al., 2009; Sapes et al., 2019) are crucial for predicting future species' distributions.

Intraspecific (within-species) variation in the expression of functional traits related to water and nutrient uptake influences seedling establishment and physiological responses to drought (Sultan, 2000; Howe et al., 2003; Kawecki and Ebert, 2004; Isaac-Renton et al., 2018; Ramírez-Valiente et al., 2018; Roches et al., 2018; Chauvin et al., 2019; Roskilly et al., 2019). Intraspecific adaptations to their climate of origin collectively enable a species to survive in diverse climates and span a large geographic range. As regions shift to more arid conditions under climate change, there is great research interest in identifying populations and families that will thrive under more arid conditions to facilitate adaptation and reforestation efforts (O'Neill et al., 2008; Grady et al., 2015; Moran et al., 2017). Provenance, greenhouse, and common garden studies have been used to examine how the differential expression of functional traits in seedling populations and families from contrasting climates influences physiological responses to drought. Populations and families from drier climates often exhibit functional traits that enable them to enhance water and nutrient uptake and resist drought more effectively than populations and families from wetter climates (Fernández et al., 1999; Cregg and Zhang, 2001; Nguyen-Queyrens and Bouchet-Lannat, 2003; Gratani, 2014; Kerr et al., 2015; Carvalho et al., 2017; Marias et al., 2017; Moran et al., 2017). Drought resistance is defined as the capacity of plants to avoid or tolerate drought, which is achieved through diverse physiological mechanisms (Levitt, 1980; Khanna-Chopra and Singh, 2015; Polle et al., 2019). Compared to seedling populations and families from wetter climates, seedling populations and families from drier climates can exhibit increased resource allocation to root growth which enhances water and nutrient uptake. Seedling populations and families from drier climates also can exhibit greater leaf carbon isotope ratios which indicate greater intrinsic water use efficiency and greater stomatal constraints on gas exchange, and lower leaf turgor loss point which can indicate greater drought tolerance (Grossnickle et al., 1996; Cregg and Zhang, 2001; Nguyen-Queyrens and Bouchet-Lannat, 2003; López et al., 2009; Bartlett et al., 2014; Kerr et al., 2015; Carvalho et al., 2017; Marias et al., 2017).

Soil microbial communities of bacteria and fungi have been suggested as a solution to improve seedling establishment because they can alter functional traits related to water and nutrient uptake and drought resistance (Kim et al., 2012). However, we do not know if and when soil microbial impacts on seedling function are positive or negative. Soil microbial communities and host-specific microbial associates can manipulate plant hormone signaling to stimulate root growth and water uptake (Bent et al., 2001; Vonderwell et al., 2001; Verbon and Liberman, 2016), increase nutrient availability to enhance nutrient uptake (Yang et al., 2009), and alter soil moisture conditions to delay the onset of drought (Gehring et al., 2017; Kannenberg and Phillips, 2017) and promote germination (Ulrich et al., 2019). Therefore, beneficial plant-microbe interactions can improve seedling establishment and adaptation to new conditions (Compant et al., 2010). However, soil microbes can also negatively influence plant function through pathogenesis and disease (Rodriguez et al., 2008; Mendes et al., 2013; Jacoby et al., 2017; Schirawski and Perlin, 2018). Furthermore, sustaining microbial symbionts comes at a significant C cost to plant hosts *via* the release of root exudates (Bais et al., 2006). The positive and negative impacts of soil microbes on seedlings, plus the overwhelming diversity of microbes in the soil, complicates prediction of soil microbial influences on plant physiological responses to drought (Allison and Martiny, 2008; Mendes et al., 2013; Finkel et al., 2017; Sánchez-Cañizares et al., 2017). Therefore, investigating how soil microbes influence seedling physiological response to drought in diverse systems is greatly needed (Dimkpa et al., 2009; de Zelicourt et al., 2013; Baldrian, 2017).

Given the ability of seedling populations and families from contrasting climates to differentially respond to drought, populations and families from contrasting climates may also differentially interact with soil microbes, which may influence plant water and nutrient acquisition. For example, drought tolerant and drought intolerant *Pinus edulis* associated with

distinct ectomycorrhizal fungal communities in the field (Gehring et al., 2017). This suggests that microbial communities can vary by plant population, family, and genotype (Schweitzer et al., 2008; İnceoğlu et al., 2010; Berendsen et al., 2012; Whitham et al., 2012; Lamit et al., 2016) due to differential gene expression in functional traits such as the quantity and quality of root exudates used to shape the soil and root microbiome (Micallef et al., 2009; Mendes et al., 2013; Patel et al., 2015; Bakker et al., 2018). Variation in root exudate quantity and quality can attract beneficial microbes and repel harmful microbes to increase water and nutrient uptake (e.g., root growth) and drought resistance (Mendes et al., 2013; Coskun et al., 2017; Jacoby et al., 2017). One of the nutrients important to plant drought resistance is nitrogen (N) as numerous proteins underlie plant functional responses to stress. RUBISCO is the main protein that determines photosynthetic capacity, and thus contributes to the ability to maintain gas exchange during stress (Field and Mooney, 1986; Evans, 1989). Drought stress can inhibit RUBISCO activity *via* reductions in ribulose-1,5-bisphosphate (RuBP) (Gimenez et al., 1992) and reductions in the large subunit of RUBISCO (Majumdar et al., 1991). Different soil microbes are responsible for converting organic N into forms that are accessible to plants: either ammonium (NH_4^+) or nitrate (NO_3^-) (Hayatsu et al., 2008; Ohyama, 2010; Jacoby et al., 2017). Therefore, within-species populations and families can use root exudates to recruit and repel different soil microbes (Haney et al., 2015; Wille et al., 2019) that cause the plant populations and families to use different forms of N (i.e., NH_4^+, NO_3^-). The form of N used by the plant is reflected in leaf N isotope ratios ($\delta^{15}N$) (Kahmen et al., 2008; Temperton et al., 2012). Therefore, within-species variation in functional traits like leaf $\delta^{15}N$, %N, and root exudates suggests that within-species populations and families may differentially interact with soil microbes, altering nutrient acquisition among populations and families.

Intraspecific variation in plant-microbe interactions suggest that soil microbes may differentially affect the performance of plant populations and families under drought by differentially altering functional traits related to water and nutrient uptake and drought resistance. Indeed, the direction and magnitude of the effects of soil microbes on plant performance can vary among within-species groups (O'Brien et al., 2018). For example, during drought, *Ostrya virginiana* and *Betula nigra* seedling populations grew more biomass when grown with soil microbes originating from drier sites than when grown with soil microbes from wetter sites (Allsup and Lankau, 2019). This suggests dry-adapted soil microbes may drive greater improvements in plant productivity in populations and families from wetter climates than drier climates. However, this hypothesis has only been tested on plant biomass and needs to be tested on functional traits more directly related to water and nutrient uptake and drought resistance in diverse systems. Extending this research beyond just plant biomass and focusing on functional traits that elucidate the impact of soil microbes on plant physiology improves efforts to engineer beneficial plant-microbe interactions under climate change. Identifying specific plant populations and families that may gain the greatest physiological benefits from soil microbes facilitates seedling adaptation and reforestation efforts.

Loblolly pine (*Pinus taeda* L.) is the most widely planted and the most economically valuable species in the southern USA (Schultz, 1997). Its geographic distribution spans a wide range of moisture conditions from its driest edge in eastern Texas to its wettest edge on the mid-Atlantic coast, USA (97.5W to 75.0W). Studies have shown that loblolly pine populations and families can vary in functional traits related to drought resistance including growth (Sierra-Lucero et al., 2002) and physiology, where populations and families from drier locations were often, but not always, more drought resistant (Buijtenen et al., 1976; Wells, 1983; Lambeth et al., 1984; but see (Bongarten and Teskey, 1986; Meier et al., 1992; Yang et al., 2002). Mycorrhizal fungi can improve water and nutrient uptake in loblolly pine (Ford et al., 1985; Constable et al., 2001) and plant growth promoting bacteria can have both positive and negative effects on loblolly pine seedling growth (Enebak et al., 1998).

Here, our objective was to determine if and how soil microbes differentially influence functional traits and photosynthetic performance under drought in loblolly pine families from contrasting climates. We grew seeds of loblolly pine families from the driest and wettest ends of its geographic range (dry, wet) and inoculated seeds of both families with a dry-adapted soil microbial community (inoculated). We measured functional traits related to seedling establishment (germination), water and nutrient uptake, and carbon allocation (root:shoot biomass ratio, root exudate concentration, leaf C:N, leaf N isotope composition), and drought resistance (turgor loss point, leaf C isotope composition) before drought. We imposed a drought by completely withholding water and monitored photosynthetic performance using chlorophyll fluorescence. We hypothesized that soil microbes would differentially alter functional traits between the dry and wet families where soil microbes would alter functional traits to enhance water and nutrient uptake and drought resistance to a greater extent in a family originating from a wetter climate than in a family originating from a drier climate.

MATERIALS AND METHODS

Plant Material and Experimental Set-Up

To test our hypothesis, we used a controlled greenhouse experiment where loblolly pine seedlings from dry and wet climates of origin were grown from seed in sterilized sand and exposed to an inoculation treatment with a soil microbial community from an arid region. Single family, open-pollinated, geographically distinct (Schultz, 1997) loblolly pine seed originating from Bastrop county in Texas ("dry") and Orangeburg county in South Carolina ("wet") were provided by the Western Gulf Forest Tree Improvement Program and International Forest Genetics & Seed Company, respectively. These families originated from climates that represent loblolly pine's wettest and driest ends of its geographic range (Schultz, 1997). Mean annual precipitation of the wet family's climate of origin was 33% greater than that of the dry family (**Table 1**; PRISM, 2018).

TABLE 1 | Location and climate information (1960–2017) for the dry and wet families and the drought-adapted soil microbial community used for inoculation treatment.

	Dry	Wet	Soil microbial community
Region	Interior Texas, USA ("lost pines")	Coastal South Carolina, USA	Interior New Mexico, USA
Latitude (°N)	30.1036	33.4390	35.5194
Longitude (°W)	–97.3120	–80.8003	–106.2277
Elevation (m)	131	54	1750
MAT (°C)	20.0	17.8	12.1
T_{min}	13.5	11.3	3.8
T_{max}	26.4	17.8	20.5
MAP (mm)	917	1215	306
MVPD (hPa)	10.9	9.2	12.2

Mean annual temperature (MAT), minimum temperature (T_{min}), maximum temperature (T_{max}), mean annual precipitation (MAP), mean vapor pressure deficit (MVPD).

Inoculated plants were inoculated with a microbial community from a soil sample collected 0–5 cm deep in north central New Mexico, USA (35.5194, –106.2277), a site that receives 67% less mean annual precipitation than the dry family and 75% less mean annual precipitation than the wet family (**Table 1**). Because we aimed to focus on how seedling families from contrasting climates may differentially interact with any kind of soil microbial community, we selected a soil microbial community outside the contemporary range of loblolly pine. By choosing a soil microbial community to which neither family has been exposed enabled us to isolate contrasting seedling family responses to a novel microbial community, not confounded by previous exposure to such a microbial community. The microbial community was sequenced as in (Ulrich et al., 2019) to determine its bacterial and fungal composition. Briefly, DNA extractions were performed using the DNeasy PowerSoil DNA extraction kit (Qiagen, Hilden, Germany). DNA samples were quantified with an Invitrogen Quant-iTTM ds DNA Assay Kit on a BioTek Synergy HI Hybrid Reader. PCR templates were prepared by diluting an aliquot of each DNA stock in sterile water to 1 ng/µl. The bacterial (and archaeal) 16 S rRNA gene (V3-V4 region) was amplified using primers 515f-806r (Mueller et al., 2016). The fungal 28 S rRNA gene (D2 hypervariable region) was amplified using the LR22R and the reverse LR3. Amplicons were cleaned using a Mobio UltraClean PCR clean-up kit, quantified using the same procedure as for the extracted DNA, and then pooled at a concentration of 10 ng each. A bioanalyzer was used to assess DNA quality, concentration was verified using qPCR, and paired-end 250 bp reads were obtained using an Illumina MiSeq sequencer at the Los Alamos National Laboratory, NM, USA.

To create the soil inoculum for the microbial inoculation treatment, microbes from the soil were extracted by suspension in sterile DI water to create a 1:20 dilution; this isolates the microbial community and reduces potential biogeochemical effects of the original soil. Before planting, stratified seeds (moistened then stored at 35°C for 45 days) were surface sterilized for 10 min in 10% bleach and rinsed for 15 min in sterile DI water twice. Seeds were then soaked in the soil inoculum (inoculated) or sterile DI water (controls) for 10 min. Seeds were each planted in 2.65 L pots (10.2 cm × 10.2 cm × 34.3 cm) of sterilized sand (1 seed per pot) on 23 August 2017 (day 0). Five ml of soil inoculum (inoculated) or sterile DI water (controls) was applied to each pot once during initial planting and also a second time 13 days (5 September 2017) after planting to ensure effects of soil microbial communities (e.g., Corkidi et al., 2002). Both the soil inoculum and water-only treatments each were applied to 15 pots that formed the inoculated and control groups, respectively (N = 15 in each group and family). Clear plastic was placed over all pots to maintain high humidity to promote germination and then was removed after 13 days when most of the plants had germinated. A fertilizer treatment of 5 ml of ammonium nitrate (1 mg/ml) was applied evenly to each pot 19 days after planting (11 September 2017). All plants were well-watered every 2–3 days to field capacity using reverse osmosis (RO) water filtered with a 0.2-µm filter, until drought treatment during which water was completely withheld. Drought was imposed by completely withholding water beginning on day 395 after planting (22 September 2018).

Plants were grown under a 14-h photoperiod in a temperature-controlled greenhouse at the New Mexico Consortium in Los Alamos, New Mexico, USA. Average daytime temperature in the greenhouse was 22.6°C, average nighttime temperature 20.6°C, average daytime relative humidity 47.5%, and average daily maximum photosynthetic photon flux density (PPFD) was 382.4 umol m^{-2} s^{-1}.

Measurements

Germination was determined by counting the total number of individuals that germinated per group (inoculated, control) in each family (dry, wet) 14 days after planting (6 September 2017). Shoot height of all 15 individuals per group in each family was measured 287 days after planting (6 June 2018).

To examine family and treatment effects on gas exchange, photosynthesis (A) and stomatal conductance (g_s) were measured on four randomly selected individuals per group in each family using a portable photosynthesis system with an infrared gas analyzer (LI-6400 XT, Licor, Lincoln, NE, USA) on day 307 (26 June 2018) between 08h00 and 11h00. In the cuvette, flow rate was set to 500 µmol s^{-1}, reference [CO_2] 400 µmol mol^{-1}, quantum flux 2,000 µmol m^{-2} s^{-1} to avoid any light limitation of A, and leaf temperature 20°C. Needles in the cuvette (eventually collected for biomass measurements; see below) were scanned using ImageJ image processing software (Schindelin et al., 2012; Schneider et al., 2012) to determine leaf area and normalize gas exchange values by leaf area.

After gas exchange was measured, root exudate concentration was measured in the same four individuals per group in each family used for gas exchange measurements. Total organic carbon (TOC) released by roots (hereafter referred to as root

exudates) was collected using methods adapted from (Phillips et al., 2008; Karst et al., 2017; Preece et al., 2018). Loblolly seedlings were carefully dug up to keep roots intact, and roots were dipped in an antimicrobial solution (10,000 units penicillin, 10 mg streptomycin, and 25 µg amphotericin B per ml) to halt microbial activity. Seedlings were then transplanted into pots of glass beads (500-µm diameter). Pots were wrapped in aluminum foil to exclude light and seedlings were allowed to acclimate for 3 days. For root exudate collection, seedlings were first flushed with 150 ml of sterile DI H_2O using a vacuum pump. Another 150 ml of sterile DI H_2O was added and seedlings were left to release exudates for 24 h. Root exudates were then collected in vials with a vacuum pump. TOC concentration of the collected root exudates was measured using a microplate reader with a UV-visible absorbance detector (Synergy H1 Hybrid Reader, BioTek, Winooski, VT, USA) at a wavelength of 254 nm. Root exudate concentration was determined by converting absorbance (x) to concentration (y) using the linear relationship: $y = 6.3259x + 2.5901$ ($R^2 = 0.6$). This relationship was determined using 92 liquid TOC samples that had been measured with both UV-visible absorbance and a wet oxidation TOC analyzer (OI Analytical model 1010, Xylem Inc., Rye Brook, NJ, USA) as in (Jones et al., 2014). This method is advantageous because soil water TOC concentrations can be rapidly and cheaply estimated from spectral properties, yet may be limited because variability in concentration within a soil type was observed (Jones et al., 2014). Root exudate concentration values were normalized by dry root biomass. Roots and shoots from the same four plants per group in each family were then harvested, dried, and weighed for root:shoot biomass measurements. While roots were excavated for root exudate collection, we observed that seedlings were not root bound.

To evaluate intraspecific differences in the effect of inoculation treatment on nutrient uptake and drought resistance, dried leaves from the same four plants per group in each family were then analyzed for C and N content (%) and C and N isotope ratios ($\delta^{13}C$, δ. Approximately 0.8 mg of dried needle powder was packed in tin capsules for combustion for subsequent elemental analysis using a stable isotope ratio mass spectrometer (Finnigan MAT253, Thermo Electron Corporation, Waltham, MA, USA) coupled to an elemental analyzer (Costech Analytical Technologies, Inc., Valencia, CA, USA). The δ and^{15}N were recorded as deviations per thousand (‰) and were calibrated using International Atomic Energy Agency (IAEA) standards C3, C6, C8, N1, and N2. The $\delta^{13}C$ of leaf tissue ($\delta^{13}C_{leaf}$) reflects the $\delta^{13}C$ of CO_2 in the atmosphere ($\delta^{13}C_{air}$), the fractionation against the heavier carbon isotope (^{13}C) due to physiological processes and the ratio of the concentration of CO_2 inside the leaf (c_i) to that in the ambient air (c_a):

$$\delta^{13}C_{leaf} = \delta^{13}C_{air} - a - (b - a)\frac{c_i}{c_a} \quad (1)$$

where a is the fractionation effect of diffusion of CO_2 through stomata (4.4‰), and b is the fractionation effect (27‰) associated with discrimination against ^{13}C by the enzyme RUBISCO during carbon fixation (Farquhar et al., 1982; Farquhar and Richards, 1984). $\delta^{13}C_{leaf}$ is also an integrated measure of intrinsic water-use efficiency (iWUE) at the time the tissue was formed where greater $\delta^{13}C_{leaf}$ (i.e., less negative) indicates greater iWUE (Farquhar et al., 1989). Like $\delta^{13}C_{leaf}$, the $\delta^{15}N$ of leaf tissue reflects sources of N taken up by the plant and the possible discrimination against ^{15}N during the assimilation of each source (Shearer and Kohl, 1986). Discrimination for the reduction of nitrate to nitrate can occur *via* the nitrate reductase enzyme (Comstock, 2001; Cernusak et al., 2009)

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Before drought treatment, we measured leaf drought tolerance using the water potential at turgor loss or turgor loss point (Ψ_{TLP}; Bartlett et al., 2014) on four new individuals randomly selected per group (inoculated, control) in each family (dry, wet; 16 curves total) not used for the aforementioned measurements. Ψ_{TLP} was determined from pressure-volume (*P-V*) curves as in (Meinzer et al., 2014). P-V curves plot leaf water potential in response to changes in water volume as leaves dry and are used to determine bulk leaf parameters related to leaf cellular composition and structural properties such as Ψ_{TLP}. Species with lower (more negative) Ψ_{TLP} are more tolerant of drought because they are able to maintain turgor and function under more negative soil water potentials (Bucci et al., 2004; Blackman et al., 2010). *P-V* curves were measured over five days (days 338-343, 27 July–1 August 2018). Seedlings were cut at predawn and were then placed in beakers of water to rehydrate for 2-3 hours. No rehydration-induced plateau was detected (Kubiske and Abrams, 1991). Shoots were allowed to dry out slowly on the laboratory bench. Measurements of shoot mass taken with a balance, and water potential taken with a pressure chamber (PMS Instruments, Albany, OR, USA) were recorded as shoots dried out. Data were plotted with relative water deficit on the x-axis and $1/\Psi$ on the y-axis. Data were checked during measurement to ensure at least 3–5 points were recorded along the linear portion of the curve. Ψ_{TLP} was estimated from the intersection of the linear portion of the curve with a negative exponential function fitted to the nonlinear portion as in (Meinzer et al., 2014).

To compare the effects of drought on inoculated and control groups in both families, drought was imposed by completely withholding water beginning on day 395 after planting (22 September 2018). To detect family and treatment differences in drought effects on seedling physiology related to photosynthetic performance, dark-adapted chlorophyll fluorescence was measured on the remaining seven individuals per group in each family the day before drought began and then weekly until photosynthetic performance declined to zero. Chlorophyll fluorescence measurements were made on mature, fully

expanded needles at ambient temperature using a field portable pulse-modulated chlorophyll fluorometer (FMS2, Hansatech, Norfolk, UK) at predawn to ensure plants were dark-adapted. Chlorophyll fluorescence was measured as the ratio of variable to maximum fluorescence (F_V/F_M) in the convention of Maxwell and Johnson (2000). F_V/F_M measures the maximum quantum efficiency of PSII photochemistry (Genty et al., 1989) and is calculated as:

$$\frac{F_V}{F_M} = \frac{F_M - F_O}{F_M} = 1 - \frac{F_O}{F_M},$$

where F_V is variable fluorescence, F_M is maximum fluorescence, and F_O is the minimum level of fluorescence. F_O was induced using a measuring light (red light-emitting diode, 650 nm, 0.15 μmol m^{-2} s^{-1} PAR) with a pulse-width of 3 μs and a pulse modulation frequency of 0.6 kHz. F_V/F_M was then determined by applying a 0.8 s saturating pulse of white light (18,000 μmol photons m^{-2} s^{-1} PAR), which transiently closed all PSII reaction centers (preventing any photochemical processes from occurring), minimized heat dissipation (since leaves were dark-adapted), and induced maximum and variable fluorescence. F_V/F_M is considered a sensitive indicator of photosynthetic performance (Maxwell and Johnson, 2000) and has been used to monitor photosynthetic function during severe drought stress (e.g. Ings et al., 2013; García de la Serrana et al., 2015; Chen et al., 2016; Yao et al., 2018). Because F_V/F_M is most affected by severe drought, we focused on relative F_V/F_M differences among groups under severe drought. Optimal F_V/F_M values are ~0.8 (Björkman and Demmig, 1987); however, our plants before drought had F_V/F_M values ~0.6 because plants were grown in pure, fast-draining sand with low nutrients. F_V/F_M before drought did not significantly differ among families or treatment ($P > 0.05$). We used F_V/F_M as a rapid, nondestructive method to compare relative differences in effects of severe drought among groups. We did not measure gas exchange because it requires destructively harvesting the leaf area (to correct gas exchange measurements for leaf area) and we did not want needle loss to influence the functions of the remaining needles (for example, compensatory gas exchange that can occur in response to defoliation).

Statistical Analyses

A two-way ANOVA was used to evaluate the significance of the main effects of family and treatment on functional traits (i.e. response variables): germination, height, root:shoot biomass ratio, root exudates, turgor loss point, leaf %C, %N and C:N, leaf $\delta^{13}C$ and $\delta^{15}N$, photosynthesis (A), and stomatal conductance (g_s). Assumptions of equal variance and normality were checked using residual and quantile-quantile plots. Root:shoot biomass was log-transformed to meet the normality assumption. Tukey's posthoc test was used to identify statistically significant differences in means. To compare differences in inoculation treatment effects between families, we determined the treatment effect size and the 95% confidence interval on the aforementioned functional traits for both the dry and wet families. Effect size was calculated as the mean difference in functional trait between the control and inoculated groups divided by the pooled standard deviation within each family. We corrected the effect size for bias using the standard correction that accounts for small sample averages according to (Hedges et al., 1985; Lakens, 2013; Hedges and Olkin, 2014). Effect size was considered to be significant if the 95% confidence interval did not overlap with an effect size of 0. Effect size was considered to be moderately significant if the 95% confidence interval overlapped an effect size of 0 by less than ± 0.1. A positive effect size indicates an increase in the functional trait.

A linear mixed effects model was used to determine differences in F_V/F_M between inoculated and control groups through time during the drought treatment. Fixed effects were treatment (inoculated, control), family (dry, wet), and week and the random effect was individual. A linear mixed effects model, as opposed to the two-way ANOVA, enables us to account for repeated measurements through time by fitting models with different correlation structures. The model of best fit was selected based on Akaike information criterion (AIC) values. Assumptions of constant variance and normality were checked using residual and quantile-quantile plots. All interactive and main effects of factors on the response were tested using marginal F-tests (also known as type III tests). Post-hoc comparisons were made using a 95% confidence interval and $P \leq 0.05$. All statistical analyses were conducted in R version 3.4.2 (R Core Team, C. T., 2018). The linear mixed effects model was conducted using the *nlme* and *gmodels* R packages (Warnes et al., 2018; Pinheiro et al., 2019).

RESULTS

The microbial inoculation treatment affected functional traits of both families similarly for the majority of traits we measured (germination, height, Ψ_{TLP}, root exudate concentration, %C, $\delta^{13}C$, photosynthesis (A), and stomatal conductance (g_s)) but the effect size was only significant for one trait: germination (**Figure 1**, **Supplementary Table 1**), where inoculated plants exhibited significantly greater germination than controls regardless of family (ANOVA; $P < 0.001$; **Figures 1** and **2A**, **Supplementary Table 2**). In contrast, the inoculation treatment affected the remaining traits (root:shoot biomass ratio, leaf $\delta^{15}N$, %N, C:N) of the dry and wet families in opposite directions but the effect size was only significant or moderately significant for two traits: root:shoot biomass ratio and leaf $\delta^{15}N$ (**Figure 1**, **Supplementary Table 1**). The inoculation treatment significantly increased the root:shoot biomass ratio in the wet family but not the dry family, as indicated by only the wet family's significant, positive effect size of 1.7. The inoculation treatment increased leaf $\delta^{15}N$ in the dry family but not the wet family, as indicated by only the dry family's moderately significant, positive effect size of 1.5 (i.e., 95% CI = –0.05 to 3.1, **Supplementary Table 1**). Despite these significant and moderately significant effect sizes on root:shoot biomass ratio and leaf $\delta^{15}N$, the ANOVA did not result in significant effects of treatment or the interaction on root:shoot biomass ratio and leaf $\delta^{15}N$ in either family (ANOVA; $P = 0.78, 0.12$; $P = 0.30, 0.23$; **Figures 2C, D**, **Supplementary Table 2**), possibly due to sample size ($N = 4$).

Despite the variation in the direction and magnitude of treatment effect size on measured functional traits between

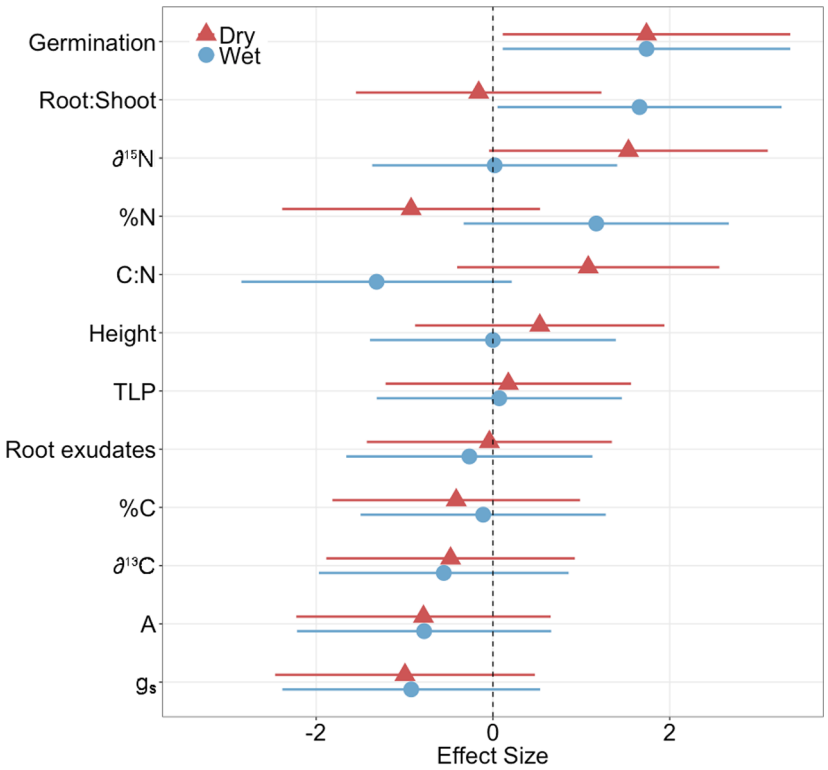

FIGURE 1 | Effect size of inoculation treatment on physiological measurements in dry and wet families (germination, root:shoot biomass ratio, leaf N isotope ratio ($\delta^{15}N$), leaf N content (%N), leaf C:N ratio (C:N), height, turgor loss point (Ψ_{TLP}), root exudate concentration, leaf C content (%C), leaf C isotope ratio ($\delta^{13}C$), photosynthesis (A), stomatal conductance (g_s)). A positive effect size indicates an increase in the physiological measurement. Bars represent 95% confident intervals. Effect sizes were considered significant if the 95% confidence intervals did not overlap with an effect size of zero. N = 4. Seedlings were ~11 months old for all measurements except germination (14 days old) and height (~9 months old).

plant families, the dry family exhibited functional traits indicative of a greater capacity to withstand drought than the wet family regardless of treatment. Regardless of treatment, the dry family exhibited a significantly greater root:shoot biomass ratio ($P = 0.047$; **Figure 2C**), was significantly shorter ($P = 0.030$, **Figure 2B**), exhibited significantly greater leaf $\delta^{15}N$ ($P < 0.001$, **Figure 2D**), and released a greater root exudate concentration compared to the wet family ($P = 0.031$, **Figure 2E**).

Leaf $\delta^{13}C$, %C, %N, C:N, A, g_s, and Ψ_{TLP} were not significantly influenced by treatment, family, or the interaction between treatment and family ($P > 0.05$; **Table 2**, **Supplementary Table 2**). Germination was not affected by family or the interaction between treatment and family ($P = 0.27$, 0.55, respectively; **Figure 2A**, **Supplementary Table 2**). Height was not significantly affected by treatment or the interaction ($P = 0.35$, 0.51, respectively; **Figure 2B**, **Supplementary Table 2**). Root exudate concentration was not affected by treatment or the interaction ($P = 0.19$, 0.080, respectively; **Figure 2E**, **Supplementary Table 2**). In addition to Ψ_{TLP}, other parameters derived from pressure-volume curves were not significantly influenced by treatment, family, or the interaction between treatment and family ($P > 0.05$; **Supplementary Table 3**). Representative pressure-volume curves for each family and treatment are included in **Supplementary Figure 1**.

The effect of the inoculation treatment on the time course of photosynthetic performance during drought as measured with F_V/F_M differed between dry and wet families. During drought at week 3, F_V/F_M of the inoculated group declined the fastest and was significantly lower than that of controls in the dry family ($P < 0.05$), but not the wet family (**Figure 3**). No statistically significant differences in F_V/F_M existed between groups before drought or at other weeks ($P > 0.05$). Model selection parameters for F_V/F_M are contained in **Supplementary Table 4**.

The taxonomic profile of the soil microbial community used for the inoculation treatment is presented in **Supplementary Tables 1** and **2**.

DISCUSSION

Intraspecific Variation in Microbial Treatment Effects on Functional Traits

Consistent with our hypothesis, we found evidence that soil microbes can alter functional traits such as root:shoot biomass ratio that improve water and nutrient uptake and drought resistance more so in the wet family than in the dry family. The taxonomic profile of the soil microbial community used for the inoculation treatment contained drought-adapted bacteria (**Supplementary Table 5**), where the majority (60%) of the top ten most abundant fungal taxa was identified as Pleosporales in Ascomycota, which is dominant in arid soils (Porras-Alfaro

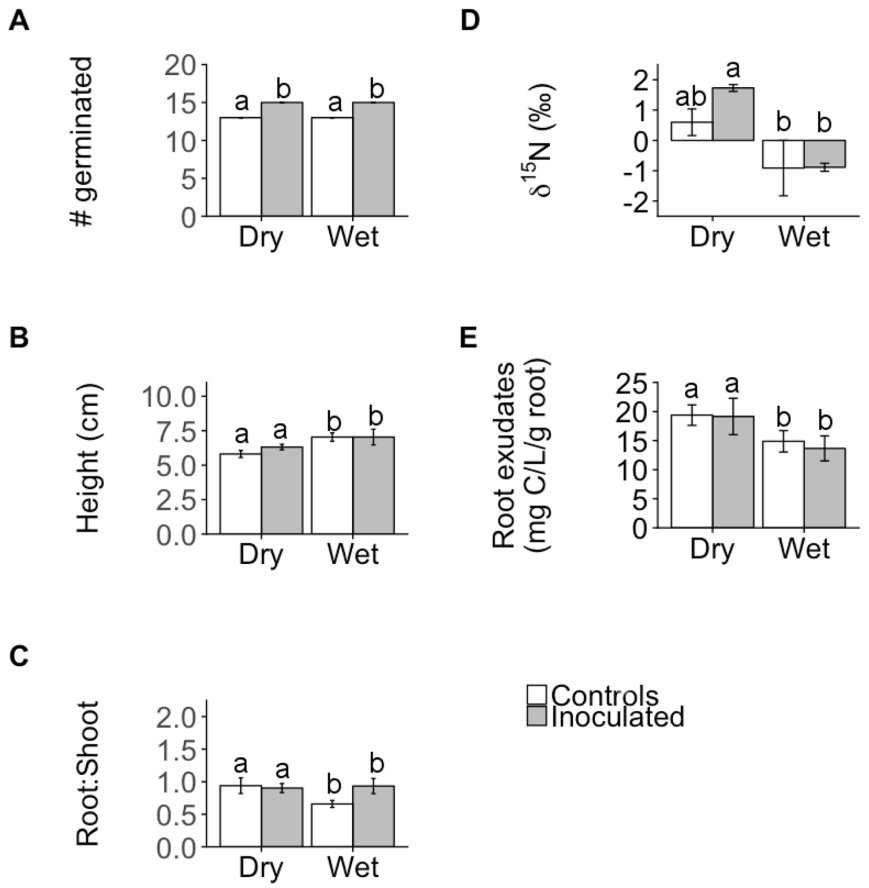

FIGURE 2 | Mean number of individuals germinated **(A)**, shoot height **(B)**, root:shoot biomass ratio (root:shoot; **C**), leaf N isotope ratios ($\delta^{15}N$; **D**), and root exudate concentration **(E)** of control and inoculated groups in dry and wet families. Letters indicate statistically significant differences among the four groups at $P \leq 0.05$. Error bars represent ± SE. N = 4. Seedlings were ~11 months old for all measurements except germination (14 days old) and height (~9 months old).

TABLE 2 | Physiological measurements of control and inoculated groups in dry and wet families (leaf C content, N content, C:N ratio, C isotope ratio ($\delta^{13}C$), photosynthesis (*A*), stomatal conductance (g_s), turgor loss point (Ψ_{TLP})).

	Dry		Wet	
	Controls	**Inoculated**	**Controls**	**Inoculated**
C content (%)	45.42 ± 0.28 a	45.10 ± 0.39 a	45.77 ± 0.46 a	45.67 ± 0.39 a
N content (%)	0.50 ± 0.062 a	0.41 ± 0.0090 a	0.40 ± 0.011 a	0.47 ± 0.036 a
C:N	93.52 ± 9.0 a	110.41 ± 3.41 a	114.88 ± 2.06 a	98.84 ± 7.3 a
$\delta^{13}C$ (‰)	−29.9 ± 0.8 a	−30.5 ± 0.4 a	−29.8 ± 0.2 a	−30.5 ± 0.7 a
***A* (μmol m^{-2} s^{-1})**	1.67 ± 0.83 a	0.74 ± 0.078 a	1.37 ± 0.63 a	0.82 ± 0.069 a
g_s (mol m^{-2} s^{-1})	0.038 ± 0.008 a	0.018 ± 0.002 a	0.033 ± 0.008 a	0.030 ± 0.006 a
Ψ_{TLP} (MPa)	−1.36 ± 0.08 a	−1.34 ± 0.07 a	−1.36 ± 0.11 a	−1.34 ± 0.12 a

Letters indicate statistically significant differences among groups at $P \leq 0.05$. All values are expressed as means ± SE. N = 4. Seedlings were ~11 months old.

et al., 2008; Porras-Alfaro et al., 2011). This supports its potential to benefit the wet family more than the dry family (Allsup and Lankau, 2019). The wet family's significant and positive treatment effect size on root:shoot biomass ratio and the dry family nonsignificant effect size on root:shoot biomass ratio suggest that the inoculated group in the wet family allocated more resources belowground to increase root growth, which enhances plant water and nutrient acquisition, drought resistance, and the capacity to

withstand drought (Brunner et al., 2015; Hagedorn et al., 2016). The inoculation treatment also differentially altered leaf %N, C:N, and $\delta^{15}N$ between families where the effect sizes were opposite in sign between families.

Similar to root:shoot biomass ratio, F_V/F_M was also differentially affected by the inoculation treatment between families. F_V/F_M of the inoculated group declined faster than controls during the imposed drought only in the dry family and not the wet. One proposed mechanism underlying this observation is that the slightly greater height of the dry inoculated group compared to dry control group (albeit not significant) resulted in increased leaf area which may have contributed to faster desiccation and functional decline during drought. Another possible mechanism underlying the dry inoculated group's F_V/F_M decline may be related to the opposite (albeit not significant) effect sizes between families for %N and consequently C:N. In the wet family, we observed a positive treatment effect size on leaf %N and a negative effect size on C:N, while in contrast, the dry family exhibited the opposite: a negative effect size on leaf %N and a positive effect size on C:N. Increased C:N, as we observed in the dry inoculated group, can indicate greater drought stress (Chen et al., 2015; Ma et al., 2016). This may possibly be due to a negative interaction between the soil microbes and the dry family. (Enebak et al., 1998) also observed a negative effect of plant growth-promoting bacteria on loblolly pine seedling growth.

In addition to leaf %N and C:N, the family difference in inoculation treatment effect on F_V/F_M may also be related to

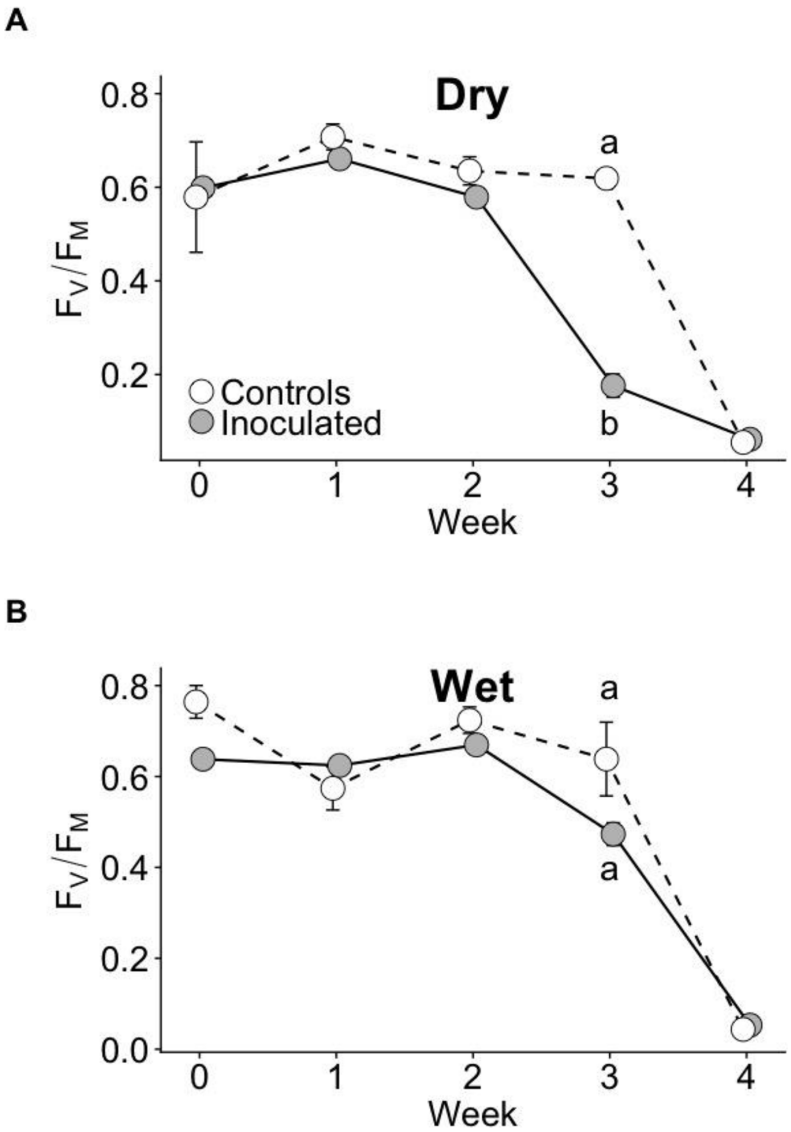

FIGURE 3 | F_V/F_M time courses of control and inoculated groups in dry **(A)** and wet **(B)** families measured weekly before and throughout drought. Drought was imposed after week 0 (when seedlings were ~13 months old). Error bars represent ± SE. At week 3, letters indicate statistically significant differences among the four groups at $P \leq 0.05$. No significant differences among groups were detected at other time points. N = 7.

family differences in inoculation treatment effects on N uptake (e.g. NO_3^-, NH_4^+), as indicated by the moderately significant, positive treatment effect size on leaf $\delta^{15}N$ in the dry family and the nonsignificant effect size in the wet family. Leaf $\delta^{15}N$ reflects the ^{15}N abundance of N sources available to plants (Shearer and Kohl, 1986). Our leaf $\delta^{15}N$ results suggest that soil microbes may influence N uptake in the dry family differently than the wet family. This may occur because within-species families can interact differently with soil microbes by altering root exudate composition and quantity to recruit and repel different microbes (Haney et al., 2015). Because different soil microbes are responsible for transforming organic N to plant-accessible NH_4^+ or NO_3^- (Hayatsu et al., 2008), the form of plant available N and thus leaf $\delta^{15}N$ can differ between within-species families. The family differences in inoculation treatment effect sizes on leaf $\delta^{15}N$ resulted in the dry inoculated group exhibiting the greatest leaf $\delta^{15}N$, which may indicate higher uptake of NH_4^+ (Miller and Bowman, 2002; Falkengren-Grerup et al., 2004), the primary form of N for loblolly (Lucash et al., 2008) and coniferous systems (Alexander, 1983). At NH_4^+ levels of 0.1 to 0.5 mmol/L, NH_4^+can be toxic to plants but the level at which it becomes toxic varies among species (Britto and Kronzucker, 2002). Therefore, it is possible that this could have contributed to the dry inoculated group's decline in F_V/F_M during drought at week 3 if the microbes in the inoculation treatment transformed N into relatively more NH_4^+ in the dry family compared to in the wet family. However, we present this only as a possibility because we cannot determine the quantity of NH_4^+ present given only the leaf $\delta^{15}N$ data in this study. The low F_V/F_M values of ~0.6 before drought suggest that seedlings may have been nutrient-deficient because they were grown in pure, fast-draining sand.

The taxonomic profile of the soil microbial community used for the inoculation treatment supports the possibility that the dry inoculated group's F_V/F_M decline may have been related to microbial effects on plant N availability due to the presence of fungi involved in plant N acquisition (**Supplementary Table 6**), which may have interacted with each seedling family differently. The majority of the top most abundant fungi was in Pleosporales, which contains dark septate fungi that can colonize *Pinus* species and are found in nutrient-stressed environments (Barrow, 2003), such as in our experiment using fast-draining sand. Dark septate fungi can form mutualistic relationships with plants by obtaining C from the plant in return for making nutrients available to the plant (Usuki and Narisawa, 2007; Wagg et al., 2008; Vergara et al., 2018), specifically transforming organic N into plant available forms (Alberton et al., 2010; Newsham, 2011; Bueno de Mesquita et al., 2018). The most abundant fungal genus in the soil microbial community used for the inoculation treatment was *Alternaria* of Pleosporales, which has been shown to vary with soil N availability (Porras-Alfaro et al., 2011). Given the presence of fungi involved in plant N acquisition, it is possible that the dry family's significantly greater concentration of root exudates may have differentially affected the microbial community and resulted in different N types available compared to that of the wet family. This may have contributed to the observed variation in treatment effect size on leaf %N, C:N, and $\delta^{15}N$ between families. Additionally, contrary to our hypothesis, the soil microbe treatment did not always influence functional traits that increased water and nutrient uptake and drought resistance (e.g. root:shoot biomass ratio) more in the wet family than in the dry family. This suggests that significant effects of soil microbes on plant function can vary depending on the trait of interest (Rincón et al., 2008). Regardless of family, inoculated plants exhibited significantly greater germination than controls, suggesting that soil microbes may improve seedling establishment regardless of family by directly or indirectly enhancing seed germination (Madhaiyan et al., 2005; Balshor et al., 2016). Soil microbes can directly enhance germination *via* the production of plant growth hormones (Wu et al., 2016), and also indirectly by increasing the soil water holding capacity of soil (Roberson and Firestone, 1992; Kaci et al., 2005; Or et al., 2007; Ulrich et al., 2019) and maintaining high soil moisture required for germination (Schultz, 1997), as well as increasing nutrient acquisition.

The taxonomic profile of the soil microbial community used for the inoculation treatment reflects its arid environment of origin and indicates the presence of fungi involved in plant N acquisition (**Supplementary Tables 5** and **6**). The majority (60%) of the top ten most abundant bacterial taxa was composed of Rubrobacterales in Actinobacteria, known to be thermophilic, radiation-resistant, and common in arid, desert soils (Holmes et al., 2000).

Intraspecific Variation in Functional Traits Related to Seedling Drought Resistance and N Use

The dry family's significantly greater root:shoot biomass ratio and root exudate concentration than that of the wet family suggests that the dry family may be more drought-adapted than the wet family. The dry family's significantly greater root:shoot biomass ratio and concentration of root exudates supports its greater capacity to resist drought and reflects its drier climate of origin compared to the wet family (regardless of treatment). This greater belowground allocation of resources to root biomass enables the dry family to access deep water sources during limited water availability, a trait observed in families and populations adapted to drier climates (Brunner et al., 2015; Hagedorn et al., 2016). Greater allocation to root growth also enhances nutrient acquisition (López-Bucio et al., 2003; Hodge, 2004; Maire et al., 2009; Chapman et al., 2012), which can vary among loblolly pine families and populations (Li et al., 1991). The dry family's greater allocation to belowground growth may also underlie why we observed a significant treatment effect size on root:shoot biomass in only the wet family, as the dry family was already allocating more resources belowground independent of treatment. The dry family was also significantly shorter than the wet family, reducing leaf area through which water can be lost, another mechanism of drought resistance.

The dry family's significantly greater concentration of root exudates compared to the wet family may be used to shape the soil microbial community by attracting or repelling different microbes (Bever et al., 2012; Sasse et al., 2018; Zhalnina et al.,

2018), another strategy to increase water and nutrient uptake, enhance drought resistance (Badri and Vivanco, 2009; Lareen et al., 2016; Jacoby et al., 2017), and alter the water holding capacity of the soil (Young, 1995; Angers and Caron, 1998; Walker et al., 2003). Families adapted to drier or wetter climates can differ in their root exudation quantity and quality (Whitham et al., 2003) because root exudate quantity can increase during drought (Liese et al., 2018; Preece et al., 2018) and root exudate composition can also change as a result of dry conditions (Gargallo-Garriga et al., 2018). Furthermore, root exudate rates and composition have been shown to vary across genotypes (Cieslinski et al., 1997; Hoekenga et al., 2003; Badri and Vivanco, 2009) and influence the genotype-specific ability to recruit beneficial soil microbes (Haney et al., 2015).

The dry and wet families may have used different sources of plant available N (e.g. NO_3^-, NH_4^+), as suggested by the dry family's significantly greater leaf $\delta^{15}N$ than the wet family regardless of treatment. N use is driven by the quantity of N forms available in the soil and the capacity of plants to take up different N forms (Vasquez et al., 2008). Within-species pine populations and families can differ in their use of NH_4^+ or NO_3^- (Miller and Hawkins, 2007; Maire et al., 2009) because plants can switch their N source (Louahlia et al., 2000) depending on precipitation (Houlton et al., 2007) and season (Lucash et al., 2008), suggesting that plasticity exists in the uptake of different N sources between within-species populations and families from contrasting climates. Plasticity in N use can be driven by within-species variation in root morphology, preferential expression of N transporters (NO_3^- versus NH_4^+) (Maire et al., 2009), and/or activity patterns of soil enzymes involved in the acquisition of N (Purahong et al., 2016). However, it remains unclear if the relationship between shoot $\delta^{15}N$ and N source is universal (Kahmen et al., 2008; Temperton et al., 2012) because the $\delta^{15}N$ of NO_3^- and NH_4^+ can vary substantially depending on relative mineralization, nitrification, and denitrification (Shearer et al., 1974; Mariotti et al., 1981; Handley et al., 1999).

The family differences in N use may be related to the family differences in root exudates because the dry family both released greater root exudates and exhibited greater leaf $\delta^{15}N$ than the wet family. Root exudates can alter the availability of plant available N sources by stimulating microbial growth and activity (Marschner, 2012). An increase in microbial activity can increase microbial N transformations and influence the quantity and type of plant available N (Yin et al., 2013). Root exudates may also affect the N cycling function of the soil microbial community by attracting or repelling specific microbes that may promote the plant availability of NO_3^- or NH_4^+ and alter the NO_3^-:NH_4^+ ratio (Hayatsu et al., 2008). Root exudates can also alter N availability by influencing different steps of the N cycle such as inhibiting nitrification (Subbarao et al., 2006; Coskun et al., 2017).

Despite significant family differences in root:shoot biomass, root exudates, and $\delta^{15}N$, we did not observe significant family or treatment differences in functional traits related to leaf drought tolerance, intrinsic water use efficiency, and stomatal constraints on gas exchange as indicated by Ψ_{TLP}, $\delta^{13}C$, A, and g_s, respectively. This suggests that Ψ_{TLP}, a metric of drought tolerance (Bartlett et al., 2014), can reflect the conditions under which plant tissues develop and can outweigh ecotypic differences between families and populations. Meier et al. (1992) also did not find significant differences in Ψ_{TLP} between *P. taeda* families and populations from contrasting climates. For leaf $\delta^{13}C$, a metric of intrinsic water use efficiency (Farquhar et al., 1989), some studies of conifer species have observed intraspecific variation in leaf $\delta^{13}C$ reflecting climate of origin (Yang et al., 2002; Kerr et al., 2015; Marias et al., 2017) while others have not (Zhang et al., 1993; Zhang et al., 1997). For A, and g_s, others also have not observed significant differences in gas exchange parameters among within-species loblolly pine families and populations (Yang et al., 2002; Aspinwall et al., 2011). Given these mixed results, future provenance studies have been urged to focus on growth and survival traits rather than $\delta^{13}C$ as a proxy for drought resistance (Moran et al., 2017).

CONCLUSION

Our results showed that soil microbial inoculation treatment effects varied in direction and magnitude by trait and family. Soil microbes may alter functional traits that improve water and nutrient uptake and drought resistance such as the root:shoot biomass ratio more so in a family originating from a wetter climate than in a family originating from a drier climate. Regardless of treatment, the dry family exhibited a greater root:shoot biomass ratio, root exudate concentration, and leaf $\delta^{15}N$ than the wet family. This suggests that the dry family allocated more resources belowground than the wet, and that within-species families may have used different sources of plant available N, which may be related to their climate and soil of origin. Together, this work highlights the need to further investigate in diverse systems how abiotic factors like drought and biotic factors like soil microbes influence diverse functional traits that influence seedling establishment of families and populations from contrasting climates. Given the mix of positive and negative microbial treatment effects on the dry family (e.g. increased germination but reduced F_V/F_M), more research is needed to inform plant-microbe interactions by identifying potential physiological tradeoffs due to negative interactions with soil microbes. Examination of intraspecific variation in plant physiological impacts of soil microbes informs species' distributions, improves efforts to engineer beneficial plant-microbe interactions, and facilitates seedling adaptation and reforestation under future climate regimes.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Supplementary Information of this article.

AUTHOR CONTRIBUTIONS

DU, SS, and JD conceived and designed the study. DU, MR, and SP set up the experiment and made all measurements. DU conducted data analysis and wrote the first draft of the manuscript. All authors revised and approved of the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2019.01643/full#supplementary-material

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Effects of Litterfall on the Accumulation of Extracted Soil Humic Substances in Subalpine Forests

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Plant litter is one of the main sources of soil humus, but which can also promote primary humus degradation by increasing microbial activity due to the higher availability of energy released, resulting in a confusing relationship between litterfall and soil humus. Therefore, an *in situ* incubation experiment was carried out in three subalpine forests (coniferous, mixed and broadleaved forests) on the eastern Qinghai-Tibetan Plateau. We set up two treatments. One that allowed litterfall to enter the soil normally and the other prevented litterfall to enter the soil. Soils were sampled in October (the end of the growing season), January (the onset of the freezing season), March (the end of the freezing season), and May (the start of the growing season) from May 2017 to May 2018. By assessing the litterfall production, the content of total extracted humus, humic acid (HA) and fulvic acid (FA) in the topsoil (0–20 cm) in each incubation period, we determined the impact of litterfall on the content of humus extracted from the soil during the freezing and the growing season. Over 1-year incubation, soil total extracted humus and HA showed considerable decreases in the treatment of retained litterfall in the mixed forest but not in the coniferous or broadleaved forests. Moreover, litterfall significantly reduced the contents of soil total extracted humus and HA during the growing season in all three forests, while only reduced soil HA content in the broadleaved forest in the freezing season. The relationship between litterfall and soil extracted humic substances was greatly regulated by the seasonal dynamics of litter types and litter production in all forest types. The larger the amount of litterfall was, the more litterfall could promote the reduction of soil extracted humic substances. Compared with a single type of broadleaf or needle litter, mixed litterfall could promote a higher degradation of soil humic substances. However, broadleaf litter might lead to much greater decreases in soil humic substance than needle litter because it is more decomposable. These results indicate that the effect of litterfall on soil humic substances are mainly regulated by litter types and litter production. Moreover, the effects of litterfall on soil humic substances are

more significant during the growing season than winter. This suggests that the longer growing season and a shorter winter caused by ongoing global warming may alter the relationships between litterfall and extracted humic substances, further disrupting the carbon balance of forest ecosystems in the subalpine forests.

Keywords: litterfall, humic substances, humic acid, fulvic acid, soil organic matter, subalpine forest

INTRODUCTION

Humus is the main component of soil organic matter (Kogel-Knabner, 2000) and is important for soil fertility and nutrient cycling (Ponge, 1999; Ono et al., 2011; Chertov and Komarov, 2013). As a basic carrier of nutrients and carbon, forest litterfall plays an essential role in the formation of soil humus (Stevenson, 1994; Prescott et al., 2000; Neumann et al., 2018). Different kinds of litterfall have different chemical compositions and qualities that might determine the development processes of humus in soil (Wei et al., 2018; Zanella et al., 2018a,b). However, there is evidence that shows that the incorporation of fresh organic matter into soil can increase microbial activity due to the higher availability of energy released, then exacerbate soil humus mineralization as named "priming effect" (Broadbent and Nakashima, 1974; Wu et al., 1993; Liljeroth et al., 1994). As such, it is still unclear whether the input of litterfall promotes the synthesis or degradation of soil humic substances. Moreover, both climatic factors (Prescott et al., 2000; Ponge, 2013) and forest litter type (Langenbruch et al., 2012) significantly affect litter humification and soil humus development, which further complicates the relationship between litterfall and soil humic substances.

Litterfall production often varies seasonally (Ma and Wang, 1993; Yang et al., 2017). Different litterfall production has different effects on soil humic substance content. Increasing fresh litterfall inputs increases the amount of CO_2 released from the soil, indicating that there is a positive correlation between the decomposition of soil organic matter and the amount of litterfall (Jenkinson, 1977; Leff et al., 2012; Xu et al., 2013). Many studies have also shown that the relationship between soil organic matter and nutrient availability is related to the litter quality (e.g., C/N ratio) (Kuzyakov, 2002; Cheng, 2009). When the C/N ratio of twig litter is high (Yang et al., 2007), microorganisms are more likely to mine N from soil organic matter and thus increase its decomposition (Kuzyakov, 2010). Studies also found that the relative proportions of different litterfall components change with time, resulting in the accumulation or decomposition of soil humic substances content (Fontaine et al., 2003; Kuzyakov, 2010). Litterfall is often dominated by foliar litter during the growing period and by twigs in winter. Foliar litter, which is rich in liable components and nutrients, is more likely to stimulate microbial activity and further promote soil humification and "old humus" degradation (Gonet and Debska, 1998). In contrast, the addition of twig litter, which is rich in refractory substances such as lignin, could stay more readily in soil as the components of soil humus than easily decomposable compounds (Fontaine et al., 2003; Kuzyakov, 2010). Therefore, the effects of litterfall on soil humus content may vary at different seasons. In addition, global warming not only increases litter production (Delucia, 1999) but also increases the litter decomposition rate (Chapin and Shaver, 1996; McHale et al., 1998), which complicates the relationship between litterfall and soil humic substances content.

Extracted humic substances are mainly divided into humic acid (HA) and fulvic acid (FA) (Abakumov et al., 2013; Kõlli and Rannik, 2018); these substances are of great significance to soil fertility, nutrient cycling, and the sustainability of ecosystem productivity (Komarov et al., 2016). FAs are a group of substances that are more active than HAs due to their small molecular weight, aromaticity, and stronger acidity (Wen, 1984). However, FA is easily lost through physical leaching by rainfall and snowmelt water (Elliott, 2013; Ni et al., 2015). High temperatures and year-round low temperatures will prevent the accumulation of soil HA (Wen, 1984). Studies have shown that climate, substrate quality, and the soil environment determine whether the formation of humic substances is dominated by HA or FA (Stevenson, 1994) due to their special properties of acid solubility and alkali solubility (Prescott et al., 2000; Ponge and Chevalier, 2006). However, there are still many unclear factors regarding the formation and transformation of humic substances.

As an important ecosystem in southwestern China (Yang et al., 2005), the subalpine forests in western Sichuan play an important role in the regional and national economy as well as in regulating the climate and conserving water and soil (Zhang et al., 2004; Sun et al., 2005; Wu et al., 2010). The accumulation of soil humic substances is essential for soil fertility and maintaining the stability of subalpine forest ecosystems, but it is closely related to litterfall. However, frequent freeze-thaw cycles and long-term low temperatures in winter can cause physical damage and chemical changes in litterfall further inhibiting the accumulation of humic substances by providing a fast-turn-over substrate for living microorganisms (Deng et al., 2010; Wetterstedt et al., 2010), and freeze-thaw cycles can even destroy the structure of newly formed humus (Dou, 2010) in subalpine forests. These processes could offset the contribution of litter to soil humus. Therefore, the effects of litter on the content of soil humus in subalpine forests remain uncertain.

We hypothesized that retained litter may decrease soil humic substances content, but this effect may be regulated by litter types and litter production in different periods. To test this hypothesis, we selected three representative forests (including coniferous, mixed and broadleaved forests) that dominate the subalpine forests on the eastern Tibetan Plateau, and check the effects of litter input on soil humic substances content as affected by seasonal freeze-thaw from May 2017 to May 2018. Our objective is to assess the accumulation or loss of soil humic substances with and without litter inputs.

MATERIALS AND METHODS

Study Site

The study site was located in the Wanglang National Nature Reserve (103°55′−104°10′ E, 32°49′−33°02′ N, 2540–2600 m), Pingwu County, Sichuan Province, China. The mean annual temperature ranges from 2.5 to 2.9 °C, and the maximum and minimum temperatures are 26°C (July) and −18°C (January), respectively. The annual mean precipitation is approximately 826 mm, with most falling between May and August. The winter normally extends from late October to late April (Yang et al., 2004). The soil type at the experimental sites is dark brown forest soil according to the Chinese soil genetic classification (Gong et al., 2007) and is classified as a type of Cambisol in the world reference base for soil resources [FAO, 2006 (2007)].

The geological characteristics, dominant arboreal species and representative shrubs of the sites are shown in **Table 1**.

Experimental Design

This study was conducted in coniferous, mixed and broadleaved forests with similar elevations and age structures (**Table 1**). Three plots were established in each forest type at similar altitude, slope and aspect. In May 2017, we set twelve *in situ* incubation boxes with lengths of 70 cm, widths of 51 cm and heights of 43 cm in each plot, a total of 108 boxes (12 × 3 plots × 3 sites = 108). We dug to about 50 cm depth, removed litterfall, stones, impurities, etc., from the soil and then added the soil to the corresponding *in situ* incubation boxes. Small holes were drilled in the bottoms of the boxes to ensure that water flow could permeate without removing soil. We set eleven boxes as the litterfall removal boxes and one box as the permanent litterfall input box in each plot. For the permanent litterfall input boxes, no interception net was set above the box. This allowed continuous accumulation of natural input of litter. The litterfall removal boxes meant that the boxes did not accept input of litter. In the litterfall removal boxes, nylon net was fixed 50 cm above the soil surface with brackets. This net captured all falling litterfall and prevented it from reaching the soil. We collected all the litterfall on the nylon net on each sampling date. The daily mean air temperature, air humidity and soil temperature were measured to quantitatively assess the environmental factors. The air temperature and humidity at the three study sites were measured using air temperature and humidity recorders (LITE5032P-RH, Fourtec-Fourier Technologies, Israel) and shown in **Figure 1**. Button thermometers (iButton DS1923-F5, Maxim/Dallas Semiconductor, Sunnyvale, United States) were randomly buried in the boxes at the three study sites to record the soil temperature.

Based on temperature detection data and previous studies conducted by our group (Wu et al., 2010), the period between two adjacent sampling dates were named the growing season (May 11, 2017–October 29, 2017), early freezing season (October 29, 2017–January 16, 2018), deep freezing season (January 16, 2018–March 24, 2018), and early growing season (March 24, 2018–May 30, 2018).

Sample Collection and Treatment

From May 2017 to May 2018, samples of soil and litterfall were collected at the end of the growing season (October), the onset of the freezing season (January), the end of the freezing season (March), and the start of the growing season (May). First, all the litterfall on the nylon net of the litter removal boxes was collected on each sampling date. All the litterfall from the same plot was evenly mixed and brought back to laboratory to calculate the average litterfall production (**Figure 2**). Second, the nylon net was removed from one randomly selected litterfall removal box in each plot on each sampling date to allow that box to re-receive litterfall from that time onward. Third, separate soil samples were collected from all permanent litterfall input boxes in every plot on each sampling date. Finally, we collected soil samples from the boxes that were just removed from the nylon net on the sampling date. When collecting the soil samples, the upper litterfall was removed, and the topsoil (0–20 cm, the average depth of soil organic layer in the forest) was collected randomly and mixed uniformity in each *in situ* incubation box. All litter materials and soil samples were air-dried at room temperature for 1 week. Then, the litter materials were classified as needle leaf, broad leaf, twig litter, flower or fruit litter, and any unrecognized residue was classified as other (including unidentifiable plant residues and animal waste). All soil samples were ground and passed

TABLE 1 | Altitude, slope, dominant arboreal species and representative shrubs of the coniferous forest, mixed forest and broadleaved forest in the study site.

Forest type	Altitude(m)	Slope(°)	Dominant species	Major understory vegetation
Coniferous forest	2600	25	*Picea purpurea*	*Lonicera japonica*
				Rubia cordifolia
				Adiantum capillus-veneris
Mixed forest	2580	30	*Abies faxoniana*	*Rhododendron lapponicum*
			Picea purpurea	*Fargesia denudata*
			Betula albosinensis	*Artemisia lactiflora*
Broadleaved forest	2540	22	*Tilia tuan*	*Ribes nigrum*
			Padus racemosa	*Fargesia denudata*
			Salix paraplesia	*Elaeagnus pungens*
				Rubia cordifolia
				Lonicera japonica

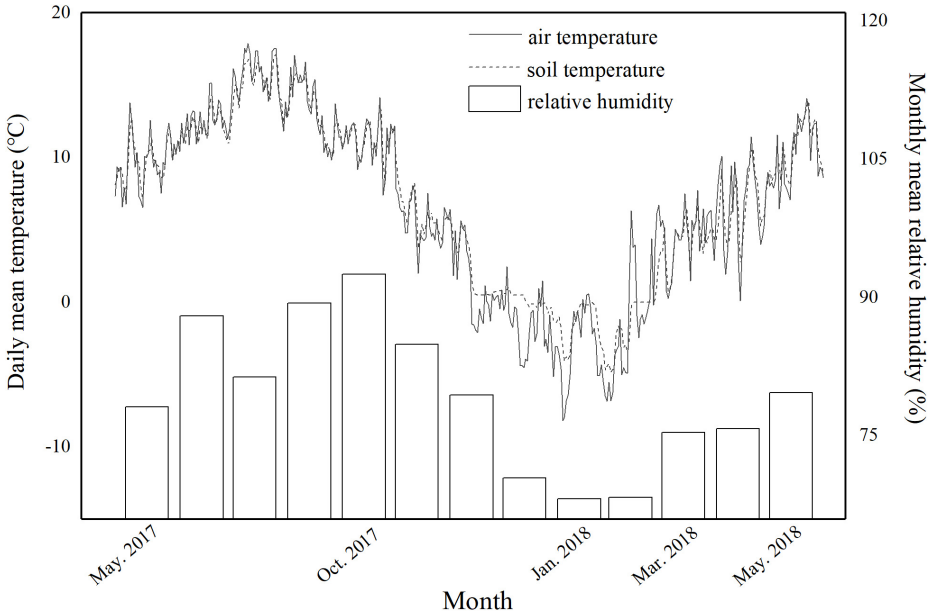

FIGURE 1 | Daily mean temperature of the soil and air and the monthly mean relative humidity of the air in a subalpine forest on the eastern Tibetan Plateau from May 11, 2017 to May 30, 2018.

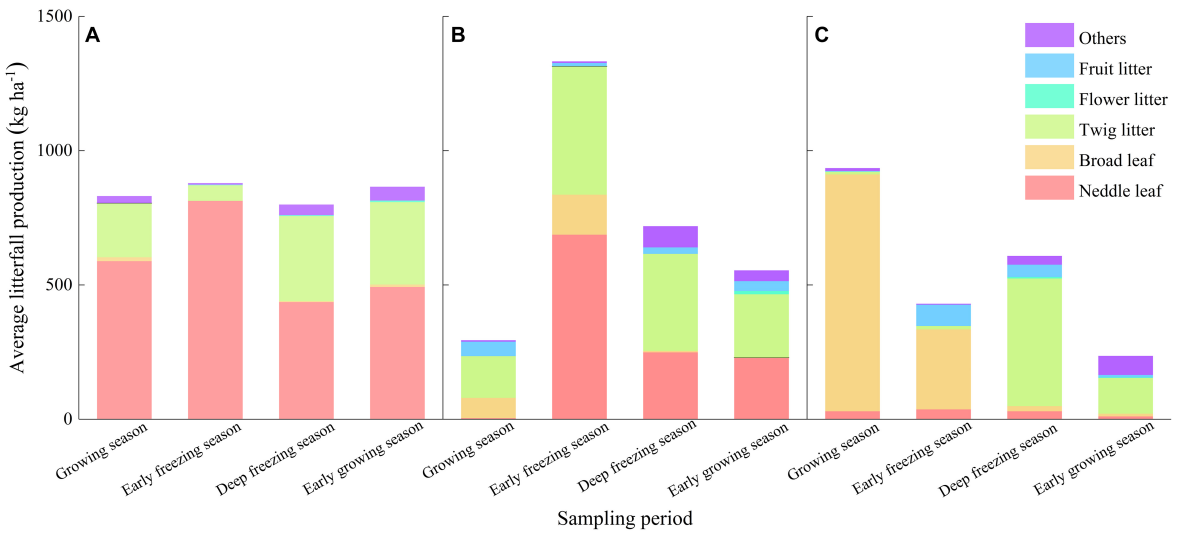

FIGURE 2 | Average production of different litter components in the three forests in the different sampling periods on the eastern Tibetan Plateau: **(A)** coniferous forest, **(B)** mixed forest, and **(C)** broadleaved forest.

through a 0.25 mm sieve in preparation for the extraction of humic substances.

Sample Analysis

The extraction and separation methods of soil extractable total humus, HA and FA were as follows. The air-dried samples (0.500 g) were shaken with a mixed solution of 100 mL of 0.1 mol L^{-1} NaOH+0.1 mol L^{-1} $Na_4P_2O_7$ for 10 min and heated at 100°C in boiling water for 1 h (Adani and Ricca, 2004; Wang et al., 2010). The extracted liquid was filtered, and the filtrate was used to analyze the soil extractable total humus. Simultaneously, 20 mL of extracted liquid was collected, and 0.5 mol L^{-1} H_2SO_4 was used to separate the HA and FA fractions at 80°C. Then, the HA was dissolved with a hot 0.05 mol L^{-1} NaOH solution (Kumada et al., 1967). The humus and HA fractions were passed through a 0.45 μm filter and then analyzed for the concentrations of humus and HA using a total organic carbon (TOC) analyzer (vario TOC cube/vario TOC select, Elementar Analysensysteme GmbH, Hanau, Germany).

Calculations and Statistical Analysis

The concentrations of humus and HA were analyzed, and the concentration of FA was calculated as follows (Gigliotti et al., 1999):

$$C_{FA}(gkg^{-1}) = C_{humus} - C_{HA} \quad (1)$$

where C_{humus}, C_{HA} and C_{FA} are the contents of total extracted humus, HA, and FA, respectively.

The HA/FA ratios were calculated from the current measured HA and FA contents on each sampling date (Abakumov et al., 2013).

The daily average temperature, number of freeze-thaw cycles were calculated for the different sampling periods based on the temperature data. Freeze-thaw cycles were defines as periods where the temperature was above or below freezing for 3 h until it changed to below or above freezing again (Konestabo et al., 2007).

An analysis of variance (ANOVA) was used to test for significant ($P < 0.05$) differences in the contents of soil total extracted humus, HA and FA and the HA/FA ratios between the litter retained and removed plots at each period. Correlations between the contents of total extracted humus, HA and FA and the HA/FA ratios and environmental factors under different litter treatments were analyzed by Pearson correlation analysis. The above analyses were performed using SPSS 20.0 (IBM SPSS Statistics Inc., Chicago, IL, United States), and the figures were drawn with Origin Pro9.0 (OriginLab, Northampton, MA, United States).

RESULTS

Total Extracted Humus

The litterfall input of subalpine forests significantly affected the content of soil total extracted humus ($F = 27.35$, $P < 0.01$, **Table 2**), and there were significant differences among forest types and incubation periods (**Table 2**). Over the 1-year incubation, litterfall significantly decreased the soil total extracted humus content in the mixed forest (**Figure 3B**), while there were non-significant effects in the coniferous and broadleaved forests (**Figures 3A,C**). Continuous litterfall input reduced the soil total extracted humus content by 17.5% in the mixed forest. However, litterfall significantly decreased the content of soil total extracted humus in all three forests during the early growing season and the growing season rather than in the other periods (**Figure 3**). The inhibitory effects of litterfall on the soil total extracted humus in the early growing season was 20.4% for broadleaved forest, 17.5% for coniferous forest, and 6.5% for mixed forest. In contrast, litterfall reduced the soil total extracted humus by 34.5, 31.2 and 6.1% in the growing season in the mixed forest, broadleaved forest, and coniferous forest, respectively.

Humic Acid

Similar to the total extracted humus, litterfall significantly affected the HA content in the soil ($F = 25.25$, $P < 0.01$, **Table 2**), and there were significant differences between different forests and seasons (**Table 2**). The content of HA in the retained litterfall soil was considerably decreased by 26.3% in the mixed forest but not in the coniferous and broadleaved forests after the 1-year incubation. Litter input significantly reduced by 24.8% soil HA content in the coniferous forest during the early growing season (**Figure 4A**). Litter input also significantly reduced the soil HA content in the mixed forest during the growing season and the early growing season, with a reduction of 36.3 and 7.4%, respectively (**Figure 4B**). Moreover, litterfall significantly decreased the soil HA content of the broadleaved forest in the growing season and deep freezing season, resulting in a 40.1 and 11.4% reduction, respectively (**Figure 4C**).

Fulvic Acid

The results showed that litterfall significantly affected the content of soil FA ($F = 11.36$, $P < 0.05$, **Table 2**), and there were significant differences between different seasons (**Table 2**). The continuous input of litter for 1 year had no significant effect on the content of FA in these three forests (**Figure 5**). In addition, seasonal litterfall input significantly reduced the content of soil FA in the broadleaved forest only during the early growing season and the growing season (**Figure 5C**), with a reduction of 63.0 and 23.4%, respectively; there were no significant effects in the other periods and forests.

HA/FA Ratios

The effects of continuous litterfall input for 1 year on the HA/FA ratios of the three types of forest differed. The HA/FA ratio of the mixed forest (**Figure 6B**) was reduced significantly, although non-significant effects were detected in the coniferous and broadleaved forests (**Figures 6A,C**). Seasonal litterfall significantly reduced the HA/FA ratio of the broadleaved forest in the growing season and increased the ratio during the early growing season. Litterfall did not have statistically significant effect on the soil HA/FA ratios in the coniferous and mixed forests in all periods, and the values were always less than 1 (**Figures 6A,B**).

Correlations Between the Extracted Soil Humic Substances and the Environment

The analysis showed that litterfall changed the relationships between the soil humic substances and the selected environmental factors. There was no significant correlation between the soil humic substances and the environmental factors in the litter retained plots in all three forests (**Table 3**). However, the content of humic substances in the removed litterfall soil was significantly positively correlated with the relative humidity, daily mean temperature, positive accumulated temperature and negative accumulated temperature but was negatively correlated with the number of freeze-thaw cycles in all studied forest types (**Table 3**).

TABLE 2 | Repeated measures ANOVA results for the effects of incubation period, forest type, litter, and their interactions on soil total extracted humus, humic acid, and fulvic acid.

Factors	df	Total extracted humus		HA		FA	
		F	sig.	F	sig.	F	sig.
Period	3	68.36	0.00**	37.80	0.00**	16.02	0.00**
Type	2	5.82	0.02*	11.79	0.00**	2.24	0.15
Litter	1	27.35	0.00**	25.25	0.00**	11.36	0.01*
Period × Litter	2	19.04	0.00**	12.41	0.00**	8.52	0.00**
Period × Type	6	3.00	0.04*	6.54	0.00**	3.27	0.03*
Type × Litter	3	0.48	0.63	1.60	0.24	1.12	0.36
Period × Type × Litter	6	8.99	0.00**	12.60	0.00**	5.65	0.00**

*$P < 0.05$, **$P < 0.01$. $n = 36$.

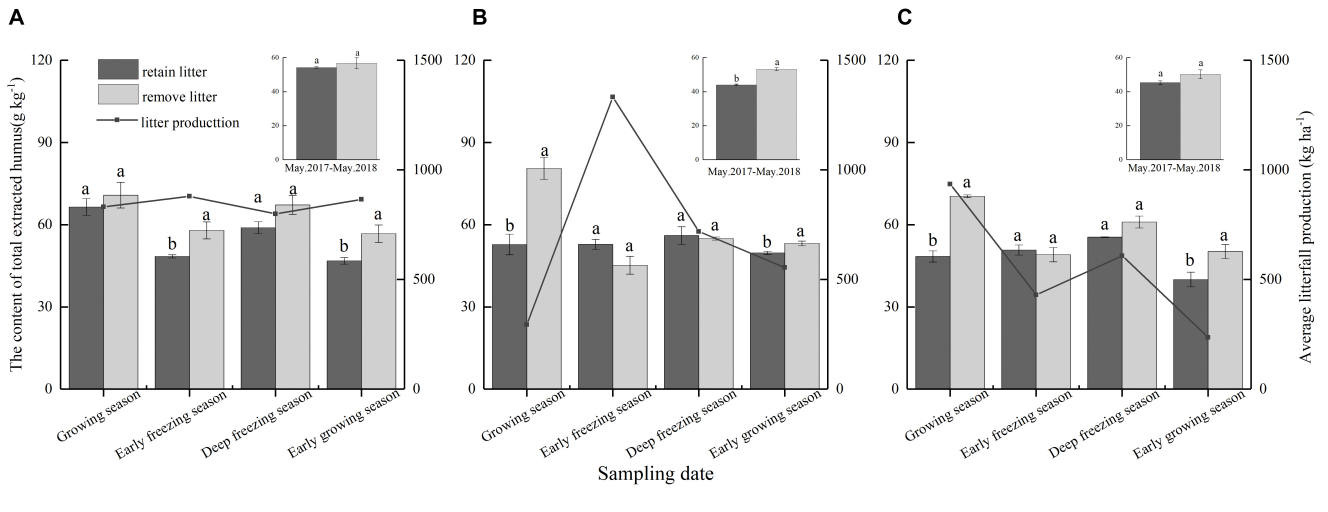

FIGURE 3 | Content of soil total extracted humus was affected by litter removal and average litterfall production at different periods in the **(A)** coniferous forest, **(B)** mixed forest, and **(C)** broadleaved forest. The histograms show the averaged values over 1 year. The lines show the averaged litterfall production in each forest. The presented content values are the means of 3 observations; the error bars represent standard errors. Lowercase letters represent significant differences in the contents of total extracted humus between the different litterfall treatments.

DISCUSSION

Litterfall transfers approximately one third of the global annual C uptake (approximately 18 Pg·C·year^{-1}) to soil surface (Grace, 2004; Malhi et al., 2011), although the amount of this C that is sequestered in soils is not fully understood. Humic substances in the soil are in a dynamic process of continuous decomposition and synthesis (Stevenson, 1994), and the amount depends on the relative amounts of the formation and degradation of humic substances (Dou et al., 2007). Our results partly supported our hypothesis that retained litter may decrease soil humic substances content, but this effect may be regulated by litter types and litter production in different periods. Our results show that litterfall promoted a reduction in soil humic substances in the mixed forest but had insignificant effect in the coniferous forest and broadleaved forest. In addition, seasonal litterfall input significantly promoted a reduction in soil total extracted humus in the three forests during the growing season. This finding suggests that the effects of litterfall on soil humic substances were related to the litterfall types. Moreover, a greater amount of seasonal litterfall corresponded to a greater reduction in soil total extracted humus.

Total Extracted Humus

Over 1 year continuous litterfall input, we found that the soil total extracted humus in the mixed forest decreased significantly, indicating that the degradation rate was higher than the synthesis rate in the 1-year incubation. However, litterfall did not have a significant effect on soil extracted humus in coniferous or broadleaved forest. The formation and degradation of humic substances mainly depend on the action of microorganisms (Cotrufo et al., 2013). Litterfall input increases the available carbon source of soil microorganisms (Nadelhoffer et al., 2004), which provides important energy for decomposer activities and stimulates the decomposition or mineralization of soil humic substances that originally existed in the soil (Kuzyakov, 2010; Cotrufo et al., 2013). Moreover, the decomposition rate of mixed forest litterfall has been demonstrated to be faster than that of single litter type (Briones and Ineson, 1996; Hooper, 1998; Chapman and Koch, 2007), and it can enrich the microbial

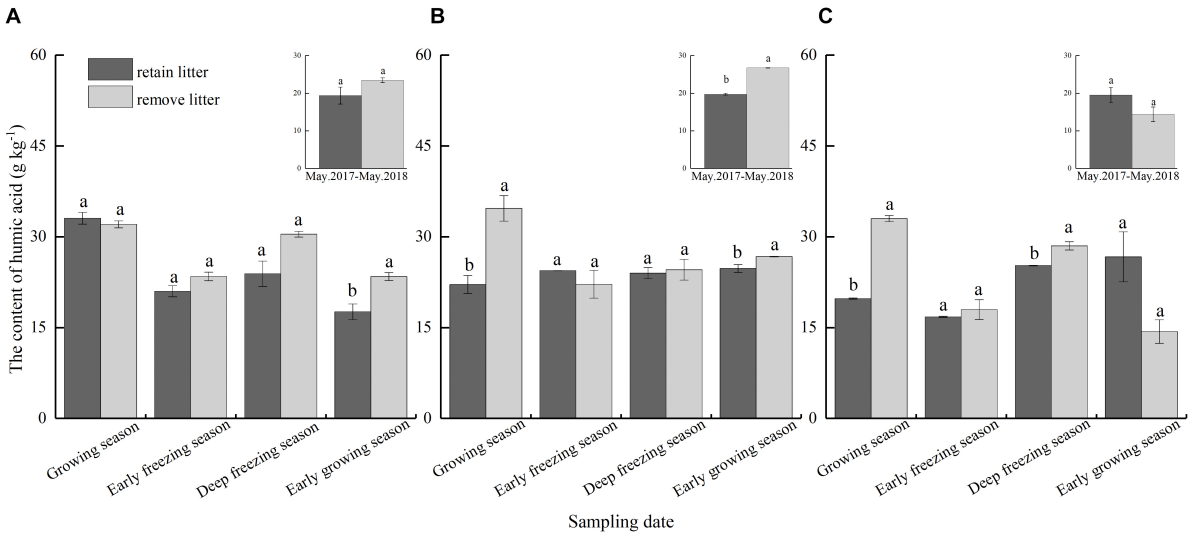

FIGURE 4 | Content of soil humic acid as affected by litter removal at different periods in the **(A)** coniferous forest, **(B)** mixed forest, and **(C)** broadleaved forest. The histograms show the averaged values over 1 year. The presented content values are the means of 3 observations; the error bars represent standard errors. Lowercase letters represent significant differences in the contents of total extracted humus between the different litterfall treatments.

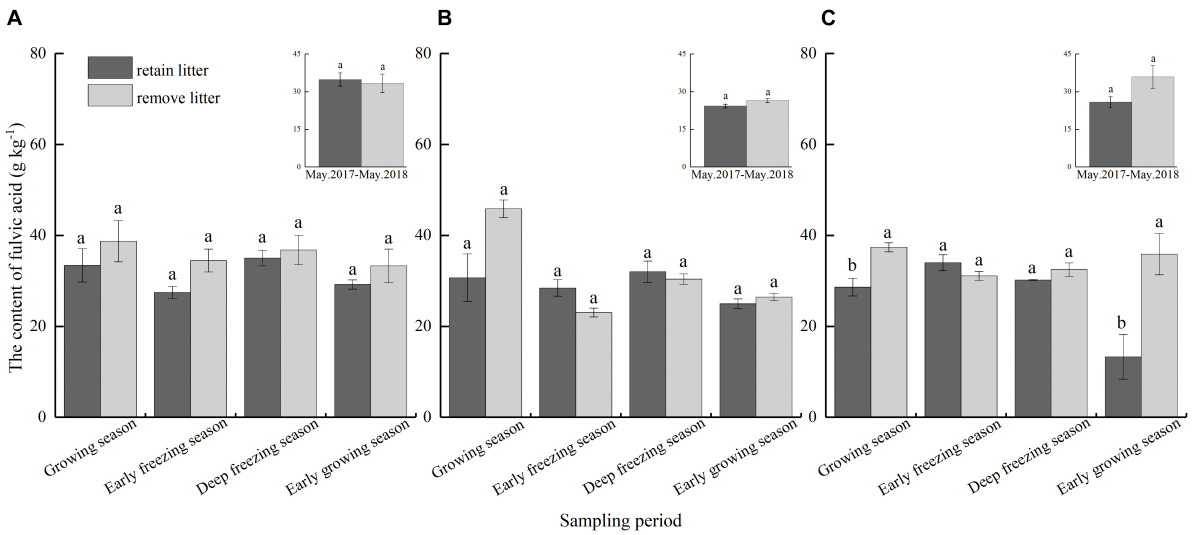

FIGURE 5 | Content of soil fulvic acid as affected by litter removal at different periods in the **(A)** coniferous forest, **(B)** mixed forest, and **(C)** broadleaved forest. The histograms show the averaged values over 1 year. The presented content values are the means of 3 observations; the error bars represent standard errors. Lowercase letters represent significant differences in the contents of total extracted humus between the different litterfall treatments.

community structure and provide more nutrients for soil microbes to promote their activity (Nadelhoffer et al., 2004), thereby promoting the reduction of soil humic substances. The input of litterfall also provides raw materials for the synthesis of humic substances and promotes their synthesis. Single litter type, such as needle leaves in the coniferous forest and broad leaves in broadleaved forest, decompose slowly and accumulate more refractory substances (Ponge, 2013), which can promote the synthesis of humus and balance the decomposition of humic substances to a certain extent (Gregorich et al., 1996). These results indicate that the effect of litterfall on the soil humic substances is closely related to the litterfall types (Wei et al., 2018).

In the present study, the total extracted humus in the three forests was decreased because of litterfall during the early growing season and the growing season. During the growing season, the greater the amount of litterfall was, the greater the reduction amount of soil total extracted humus, suggesting that the degradation of humus was affected by the amount of litterfall. However, the amount of litterfall during the early growing season

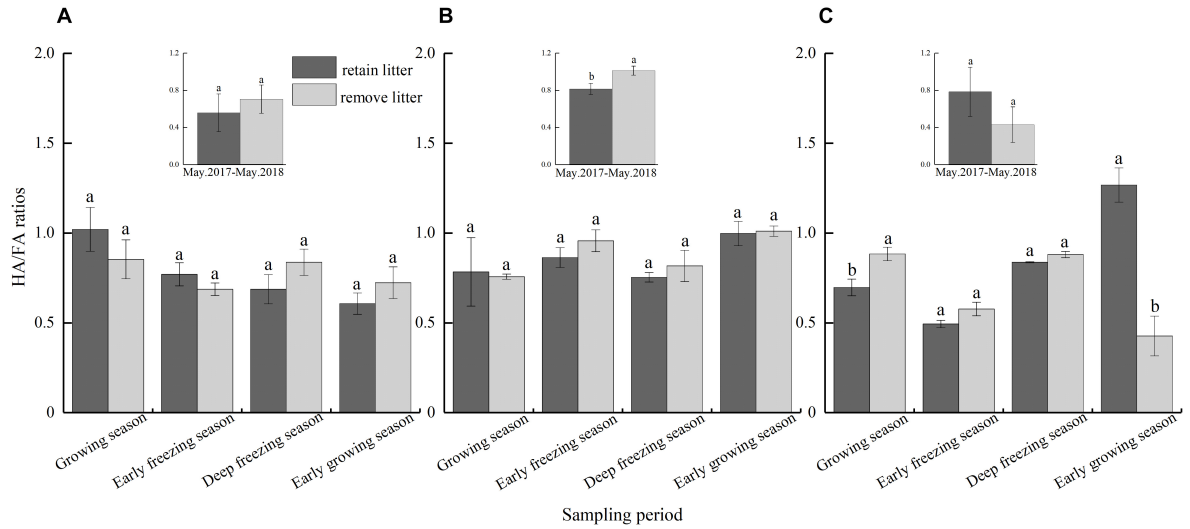

FIGURE 6 | The HA/FA ratios as affected by litter removal at different periods in the **(A)** coniferous forest, **(B)** mixed forest, and **(C)** broadleaved forest. The histograms show the averaged values over 1 year. The presented content values are the means of 3 observations; the error bars represent standard errors. Lowercase letters represent significant differences in the contents of total extracted humus between the different litterfall treatments.

TABLE 3 | Correlation analyses between the total extracted humus, HA, FA, HA/FA ratios and environmental factors with different litterfall treatments.

	Soil retained litter				Soil removed litter			
	Total extracted humus	HA	FA	HA/FA	Total extracted humus	HA	FA	HA/FA
Relative humidity	0.033	0.108	−0.041	0.129	0.537**	0.431**	0.468**	0.021
Daily mean temperature	−0.067	0.199	−0.212	0.297	0.523**	0.405*	0.470**	0.018
Positive accumulated temperature	0.188	0.217	0.047	0.137	0.715**	0.624**	0.577**	0.112
Negative accumulated temperature	−0.178	0.184	−0.32	0.356*	0.392*	0.272	0.380*	−0.022
Number of freeze-thaw cycles	0.058	−0.216	0.214	−0.308	−0.531**	−0.416*	−0.472**	−0.028

*$P < 0.05$, **$P < 0.01$. $n = 36$.

occurred in the order of coniferous forest > mixed forest > broadleaved forest (**Figure 2**), but the amount of reduction occurred in the order of broadleaved forest > coniferous forest > mixed forest (**Figure 3**). This result may be because the needle litter contained less simple and soluble substrates (Berg, 2000) and was high in C/N, cellulose and lignin (Dai et al., 2001), which have difficulty decomposing and form humus easily (Taylor, 1989; Cotrufo and Ineson, 1995).

Humic Acid, Fulvic Acid and HA/FA Ratios

Soil HA and FA are the main components of soil humic substances, but these two acids have different stability and formation processes. We found that soil HA content and HA/FA ratio significantly decreased in mixed forests after 1 year of continuous litterfall input, but FA content was not affected by litter input. Moreover, the reduction in the soil HA content of mixed forest was more than total extracted humus, which may be due to the conversion of HA and FA (Stevenson, 1994) or the priority synthesis of FA in the synthesis of humus (Dou et al., 2007; Ni et al., 2014, 2016). The effect of seasonal litterfall input on the soil HA was similar to that of the total extracted humus (**Figure 4**). However, seasonal litterfall significantly reduced the content of HA only in the broadleaved forest during the growing season and early growing season and had no significant effect in the coniferous and mixed forests (**Figure 5**), which may be related to the litterfall quality and components. The litterfall of the coniferous and mixed forests contained needle litter (**Figures 2A,B**), which contained terpenoids and phenolic substances (Wu et al., 1993; Shen and Bartha, 1997), and was more likely to cause acidic environments during decomposition (Yang et al., 2007). Acidic environments are more conducive to the synthesis of FA (Dou et al., 2010), and therefore the reductions in soil FA in the coniferous and mixed forests were non-significant. The HA/FA ratios of these forests were less than 1 (**Figure 6**), suggesting that the synthesis rate of FA was always higher than that of HA. Litterfall had no significant effect on the HA/FA ratios in the coniferous and mixed forests among the different periods, while litterfall significantly decreased the ratio in the broadleaved forest in the early growing season. This result might be due to the fact that litterfall promoted the conversion between HA and FA (Stevenson, 1994). Furthermore, litterfall had no significant effect on the content of soil humic substances in winter.

This finding may be because low temperatures and soil freezing inhibited soil microbial activity (Bokhorst et al., 2013) and hindered the physiological metabolism of the microorganisms involved in the formation and degradation of humic substances (Cotrufo et al., 2013).

Litterfall promoted a reduction in soil humic substances mainly in the growing season. Moreover, seasonal litterfall input had a more significant effect on the soil humic substances than continuous litterfall input. The seasonal litterfall input mainly relied on the rapid input of available carbon sources to promote soil microbial activity (Kuzyakov, 2010). After the rapid consumption of soluble carbon sources, continuous litterfall input was mainly dependent on the decomposition of components that are not easily decomposed, such as cellulose and lignin, to provide nutrients to the soil (Kumada et al., 1967). Therefore, we suspected that the input of easily decomposable substances mainly promoted the decomposition of soil humic substances while the input of substances that had difficultly decomposing mainly promoted the synthesis of humus (Fontaine et al., 2003). However, the decomposition of substances that have difficultly decomposing and the synthesis of humic substances are slow processes; therefore, they were not shown in this study.

Correlations Between the Extracted Soil Humic Substances and the Environment

A significant difference was observed between the content of humic substances in the removed litter soil and the environment, although few significant correlations were observed in the retained litter soil (**Table 3**). This result indicates that litterfall protected or buffered the soil humic substances, making them less susceptible to climatic factors. Furthermore, the content of humic substances in the removed-litter soil might be regulated by biological factors, such as microbial activity. Furthermore, the content of humic substances was significantly positively correlated with the relative humidity, daily mean temperature, positive accumulated temperature and negative accumulated temperature but negatively correlated with the number of freeze-thaw cycles (**Table 3**). This finding indicates that an increase in temperature affected the content of soil humic substances, and the frequent freeze-thaw cycles in winter may inhibit the formation of humus and even destroy newly formed humus to cause degradation (Dou, 2010). Therefore, under the background of global warming, litterfall can weaken the effects of frequent freeze-thaw cycles (Sahin et al., 2008) and temperature increases (Easterling et al., 1997) on the content of soil humic substances and play a protective role for soil humus.

CONCLUSION

Litterfall significantly decreased the soil humic substances and HA content of three forests during the growing season but showed insignificant effects in freezing season, implying that a longer growing season and shorter winter caused by global warming may promote the degradation of soil humic substances and the potential loss of soil organic matter. We also found that the relationship between litterfall and soil humus is related to the amount of litterfall during 1-year incubation, displaying a greater increase in the amount of litterfall corresponding to a greater decrease in the content of soil humic substances. Furthermore, a lack of litterfall could increase the sensitivity of soil humic substances to environmental factors. These results showed that, the less litterfall input in a short term, the more conducive to the accumulation of soil humic substances, but the effect of long-term litterfall input on soil humic substances content required further research. This study provided some basic evidence for understanding plant-soil interactions in the subalpine forests.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

LZ and FW conceived the study, designed the experiments, and supervised the whole study. XW, YY, YS, ZC, and YD performed the experiments. XW, LZ, and FW wrote the manuscript.

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Ectomycorrhizal and Dark Septate Fungal Associations of Pinyon Pine Are Differentially Affected by Experimental Drought and Warming

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Changing climates can cause shifts in temperature and precipitation, resulting in warming and drought in some regions. Although each of these factors has been shown to detrimentally affect forest ecosystems worldwide, information on the impacts of the combined effects of warming and drought is lacking. Forest trees rely on mutualistic root-associated fungi that contribute significantly to plant health and protection against climate stresses. We used a six-year, ecosystem-scale temperature and precipitation manipulation experiment targeted to simulate the climate in 2100 in the Southwestern United States to quantify the effects of drought, warming and combined drought and warming on the root colonization (abundance), species composition and diversity of ectomycorrhizal fungi (EMF), and dark septate fungal endophytes in a widespread woodland tree, pinyon pine (*Pinus edulis* E.). Our results show that pinyon shoot growth after 6 years of these treatments was reduced more by drought than warming. The combined drought and warming treatment reduced the abundance and diversity of EMF more than either treatment alone. Individual ectomycorrhizal fungal taxa, including the drought tolerant *Cenococcum geophilum*, were present in all treatments but the combined drought and warming treatment. The combined drought and warming treatment also reduced the abundance of dark septate endophytes (DSE), but did not affect their diversity or species composition. The current year shoot growth of the trees correlated positively with ectomycorrhizal fungal diversity, highlighting the importance of diversity in mutualistic relationships to plant growth. Our results suggest that EMF may be more important than DSE to aboveground growth in *P. edulis*, but also more susceptible to the negative effects of combined climate stressors.

Keywords: climate change, dark septate endophytes, dryland ecosystems, ectomycorrhizal fungi, fungal diversity, pinyon pine, root-associated fungi, tree drought response

INTRODUCTION

Changes in climate, including the combined effects of increased drought and warming temperatures, are significantly affecting temperate forest ecosystems (Allen C. D. et al., 2010). These stressors have already resulted in widespread tree mortality across the western United States (Breshears et al., 2005, 2009; Van Mantgem et al., 2009; Anderegg et al., 2013; Williams et al., 2013) and there is concern that significant shifts in the spatial extent and distribution of numerous tree species are imminent (e.g., Iversone and Prasad, 1998; Morin et al., 2018). However, there is also evidence that trees can acclimate to warming and drying conditions (Nicotra et al., 2010; Way and Yamori, 2014; Grossiord et al., 2017a, 2018a,b). Based on niche models, intraspecific differences among trees in morphological and physiological traits can be substantial enough to alter predictions of future plant distributions (Ikeda et al., 2017).

Microbial plant mutualists, such as root-associated fungi, significantly affect plant responses to climate change (reviewed by Kivlin et al., 2013; Mohan et al., 2014; Bennett and Classen, 2020). Many dominant temperate tree species form associations with ectomycorrhizal fungi (EMF), a diverse assemblage of ascomycete and basidiomycete fungi that improve host plant access to soil nutrients and water and provide protection from some pathogens in exchange for fixed carbon (Smith and Read, 2008). These fungi may buffer plants against climate change, but their activities and buffering ability can be affected by hot and dry conditions. Therefore, it is important to understand how root-colonizing fungi respond to environmental changes and to link those responses to the growth and survival of their plant hosts.

Ectomycorrhizal fungal responses to drought or warming have been studied in several ecosystems, but studies examining the combined effects of drought and heat stress on EMF and EMF-host plant relationships remain rare. Improvement of host plant drought tolerance by EMF has been widely documented and reviewed (Lehto and Zwiazek, 2010; Kivlin et al., 2013; Mohan et al., 2014; Gehring et al., 2017) with the strongest support for an indirect mode of action through improved host nutrition (Lehto and Zwiazek, 2010). However, drought has also been documented to lead to changes in EMF abundance, biomass, community composition and activity in pines (Karst et al., 2014). The effects of experimental warming on EMF have been less studied with an emphasis on temperate and arctic ecosystems with variable results (Mohan et al., 2014). However, temperature can be an important force structuring EMF communities, even when differences among sites in host species and associated plant communities are taken into account (Miyamoto et al., 2018; Koizumi and Nara, 2019).

The roots of many plant species, including some of those that host EMF, also are colonized by dark septate endophytes (DSE), ascomycete fungi grouped by the morphology of their highly melanized hyphae within host roots (Jumpponen and Trappe, 1998). Unlike EMF, DSE appear to lack a particular materials-exchange interface with the plant, however they may increase host plant resource uptake, particularly of organic nutrient sources (Newsham, 2011). DSE are also hypothesized to be tolerant of environmental stresses such as heat, cold, drought and salinity (Berthelot et al., 2019) and may play a role in the "fungal loop" that is thought to reduce carbon and nutrient losses in arid ecosystems by cycling them within biotic pools (Collins et al., 2008). However, there has been little research on the function of DSE in a climate change context. Kivlin et al. (2013) noted significant negative effects of inoculation with DSE on plant responses to warming in a meta-analysis but acknowledged that the results were heavily influenced by a single study of one fungal species (*Phialocephala fortinii*) and two plant species (*Picea abies* and *Betula pendula*). On the other hand, inoculation with DSE improved host plant responses to drought in the studies reviewed by Kivlin et al. (2013) and both positive and negative effects on plant biomass have been observed in more recent work on a species of arid land grass (Li et al., 2018). As with EMF, few studies have assessed the consequences of multiple climate changes on DSE-host plant relationships.

In this study, we used an ecosystem-scale field manipulation experiment to examine the consequences of drought and warming temperatures, alone and in combination, for the EMF and DSE communities associated with pinyon pine, *Pinus edulis*, a western United States tree species that occupies a large area of semi-arid landscape where it occurs with co-dominant members of the genus *Juniperus*. Warm temperatures combined with extreme drought resulted in significant *P. edulis* mortality across 12,000 km^2 of the southwestern United States in 2002–2003 (Breshears et al., 2005). Thus, *P. edulis* has become a model for studies of the physiological basis of plant drought susceptibility (McDowell et al., 2008, 2016; Adams et al., 2009; Plaut et al., 2012; Limousin et al., 2013; Dickman et al., 2014; Sevanto et al., 2014), intraspecific variation in drought tolerance (Sthultz et al., 2009a), the biotic and abiotic legacy effects of drought induced mortality (Peltier et al., 2016; Mueller et al., 2019), and the contribution of EMF to survival and growth during drought (Gehring et al., 2014, 2017). However, the individual and combined effect of warming and drought stresses on EMF communities have not been examined and DSE have not been studied in *P. edulis*. *Pinus edulis* is often the only associate for EMF across most of its distribution in the southwestern United States (Gehring et al., 2016), while juniper and many grass and shrub species that occupy pinyon-juniper woodlands are colonized by DSE (Gehring, unpublished data).

We tested the following hypotheses: **H1**: The combined effects of drought and warming on EMF abundance, diversity and community composition will exceed the effect of either drought or heat stress alone. Warming temperatures are expected to exacerbate the effects of drought on trees in the coming years and we expect negative impacts of these combined stressors on plant symbionts. **H2**: Drought and/or warming stress will have greater negative effects on EMF than DSE because DSE are well known for their ability to tolerate stressful conditions (Berthelot et al., 2019). **H3**: Declines in EMF diversity with drought and warming will be strongly negatively associated with *P. edulis* aboveground growth. Species of EMF vary in their functional characteristics including the environmental conditions they can tolerate (Sthultz et al., 2009b; Miyamoto et al., 2018),

the extent to which they colonize the soil [e.g., different hyphal exploration types (Tedersoo and Smith, 2013)], and the types of soil resources they are able to utilize (Frey, 2019). We predict that loss of EMF diversity will result in reduced functional diversity of EMFs and consequently lower plant growth because of reduced resource access capacity. We do not make a similar prediction for DSE because of their uncertain function in *P. edulis*.

MATERIALS AND METHODS

Experimental Methods and Sampling

To examine the effects of drought and warming on EMF and DSE in *P. edulis*, roots were sampled from trees that had been under ambient (control), drought (~50% reduction in precipitation), warming (temperature 5°C above ambient) and a combination of drought and warming treatments for 6 years at the Los Alamos Survival-Mortality (SUMO) experiment located in Los Alamos County, New Mexico (35.49°N, 106.18°W, 2175 m a.s.l). The SUMO site, established in summer 2012, consists of five treatments with 5–6 trees per treatment. These treatments are: control with trees experiencing ambient temperature and precipitation, heat with trees inside open-top chambers where temperature was maintained constantly at 4.8°C above ambient temperature, drought with trees located within a precipitation exclusion structure constructed of polyethylene troughs about 1.5 m above the soil surface covering ~50% of the ground area and directing ~45% of the precipitation off the site, a combined drought and heat treatment, and a chamber control treatment with open-top chambers kept at ambient temperature (not used in this study which thus has four treatments and 20 trees total; see Pangle et al., 2012; Adams et al., 2015).

The site is located in a native pinyon-juniper woodland close to the transition zone to Ponderosa pine forest, with vegetation dominated by pinyon pine (*P. edulis* Engelm.) and one-seed juniper [*Juniperus. monosperma* (Engelm.) Sarg.], with shrubby Gambel oak (*Quercus gambelii* Nutt.) and an occasional ponderosa pine (*Pinus ponderosa* C. Lawson) occurring in the vicinity. The climate is semi-arid, with a mean annual temperature of 10.4°C (1987–2017) and a mean annual precipitation of 358 mm (1987–2017) of which about 50% falls during the North American Monsoon season from July to September (Los Alamos Weather Machine[1]). The year of our root sampling, 2018, was warmer (average temperature 12.5°C) and drier (annual precipitation 255 mm) than the 30-year average with the monsoon precipitation prior to our sampling accounting for 42% (106 mm) of the total annual precipitation, and the average temperature of June and July at the typical range of 20–21°C. The soils are Hackroy clay loam derived from volcanic tuff with a typical profile of 0–8 cm of sandy loam, 8–40 cm of clay loam and 40–150 cm bedrock. Soil depth at the site ranges from 40 to 80 cm (Soil Survey Staff, Natural Resources Conservation Service, United States Department of Agriculture[2]).

[1]https://weathermachine.lanl.gov/
[2]http://websoilsurvey.nrcs.usda.gov

Mature *P. edulis* trees, were randomly selected for the treatments. All of the trees were >3 cm in diameter and averaged 56 ± 5 years of age based on tree cores (Grossiord et al., 2017b). The selected trees in the drought treatment were located at least 10 m from the border of the precipitation exclusion structure (equivalent to two times the height of the tallest tree in the drought treatment). In the heat treatment, the footprints of the open-top chambers ranged from 6 to 20 m^2, and contained between one and five trees located at a minimum distance of 1.5 m from the chamber boundary and at least 5 m from any target trees in other treatments. The drought and ambient treatments form two different plots with closest target trees >80 m apart. While some root outgrowth from target trees in the combined drought and heat treatment to drought treatment or from warming treatment to ambient might have occurred, any mixing between other treatments is highly unlikely because of the distances, and most of the root system of each tree can be expected to have resided with the assigned treatment. Both *P. edulis* and *J. monosperma* were included in the experiment, and sometimes shared a chamber, but we present data only on *P. edulis* here. Previous studies conducted at this site found no differences in physiological responses between trees in the control and chamber control treatments, suggesting no indirect effect of the chambers on plant function (Adams et al., 2015; Garcia-Forner et al., 2016; Grossiord et al., 2017a,b). Therefore, we focused our sample collection only on control (n = 5), heat (n = 4), drought (n = 6) and combined drought and heat treatments (n = 5). In addition to the effects on precipitation and air temperature, the treatments influence soil temperature measured continuously at the base of all target trees with thermocouples installed at 5, 10, 15 and 30 cm depths. The drought treatment alone had negligible effect (<0.1°C) on soil temperature while the warming treatment increased soil temperature on average by 3.6°C. In April 2016, the coverage of the precipitation exclusion structure was briefly increased to 90% by adding additional clear polymer troughs to increase the stress experienced by the trees. To prevent excessive heating of the soil surface and airspace below the troughs, thermal bubble insulation was installed underneath the polymer troughs, and portable blower fans (TE-CF2421, Triangle, Jacksonville, AR, United States) were placed throughout the drought and drought and heat treatments. To ensure the effectiveness of the cooling, soil temperature was additionally measured continuously (RT-1, Decagon Devices Inc., Pullman, WA, United States) over a 0–30 cm depth at the base of each tree. Mean daily soil temperature under the structure was on average 1.4 ± 0.9°C higher than ambient conditions (see Grossiord et al., 2017a), which was clearly cooler than in the heated treatment (3.6°C above ambient). The additional precipitation exclusion was removed in April 2017, and the precipitation exclusion returned to the original ~45% coverage prior to our sampling. With this change the soil temperatures under the drought structure were similar to ambient as before.

In August of 2018, we assessed plant growth, and harvested roots from four to six pinyons from each treatment for root colonization analysis. Plant growth was determined by measuring the length of the current year shoot of ten randomly selected

branches per tree using calipers. For root analyses, we collected a minimum of 200 cm fine roots (<2 mm in diameter) at a depth of 0–30 cm, pooled from two locations per tree. Roots were collected right at the tree base and well within each treatment footprint, traced to the focal tree, carefully excavated using a trowel, and placed in a cooler prior to transport to Northern Arizona University where they were stored at −20°C until processing. Root colonization by EMF was measured on each sample by counting the number of living ectomycorrhizal root tips relative to non-colonized root tips based on differences in their morphology as described in Gehring and Whitham (1991). Living ectomycorrhizal root tips (~75/tree) were then removed and examined under a dissecting microscope at 20X magnification to categorize them morphologically based on color, texture, hyphal quantity and structure (Agerer, 1991). Hyphal exploration type was assessed by observing each morphotype for emanating hyphae and presence of rhizomorphs (Agerer, 1991; Tedersoo and Smith, 2013), in addition to utilizing the Agerer (2006) categorization of EMF genera. Two morphotypes had not been observed in previous studies of *P. edulis* in the Gehring lab and were hand sectioned to look for a Hartig net, the specialized exchange structure characteristic of EMF (Smith and Read, 2008). Root tips were stored in separate tubes by morpohotype/tree at −20°C until molecular analysis of fungal communities.

To assess DSE colonization, a sample of the remaining fine roots from each sample (~50 cm, lacking EMF colonized root tips) was cleared for 20 min in boiling 10% KOH and then left an additional 12 h at room temperature in fresh 10% KOH followed by several rinses in tapwater. Around 10-1 cm segments of root were mounted on glass slides, and observed using a compound microscope at 400× magnification. The presence of melanized, septate hyphae and microsclerotia were used as indicators of DSE and quantified using the grid-line intersect method (McGonigle et al., 1990) using ~100 intersections per sample. Root samples were not stained as melanized hyphae were clearly visible without this step as observed in other study systems (Liu et al., 2017; Hughes et al., 2020). The remaining fine roots were stored at −20°C until molecular analysis of fungal communities.

Molecular Characterization of Fungal Communities

Standard methods for DNA extraction, PCR, and Sanger sequencing for EMF root tips were used (e.g., Gehring et al., 2017; Patterson et al., 2018). Briefly, we extracted DNA from one to five root tips (depending on availability) of every fungal morphotype found on every tree using the High Molecular Weight DNA Extraction protocol of Mayjonade et al. (2016). We performed polymerase chain reaction (PCR) under conditions described by White et al. (1990) and Gardes and Bruns (1993), to amplify the internal transcribed spacer (ITS) region of the rRNA of the fungal genome with the ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC) primer pair as in White et al. (1990) and Gardes and Bruns (1993), using KAPA Taq Hotstart (Kapa Biosystems, Wilmington, MA 01887, United States). Successfully amplified PCR product was purified and then cycle sequenced using BigDye Terminator Mix 3.1 (Thermo Fisher Scientific Inc.). Sequencing was performed on an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, California, United States) at the Environmental Genetics and Genomics Laboratory at Northern Arizona University. When amplification or sequencing of a morphotype was unsuccessful, an additional root tip from that morphotype from that tree was processed.

We sequenced the fine roots described above to assess DSE community characteristics using the Illumina platform. We extracted DNA from 2.0 g wet mass samples (one per tree) using DNeasy Plant Extraction Kits (Qiagen, Valencia, CA, United States). PCR was performed using primers and conditions described by Taylor et al. (2016) to amplify the ITS region of the rRNA of the fungal genome with the ITS4-FUN and 5.8S-FUN primer pair (Taylor et al., 2016) using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, United States). PCR products were checked on a 1% agarose gel, pooled, diluted 10-fold, and used as the template in the subsequent tailing reaction with region-specific primers including the Illumina flow cell adapter sequences and an eight-nucleotide barcode. Products of the tailing reaction were purified with carboxylated SeraMag Speed Beads (Sigma-Aldrich, St. Louis, Missouri, United States) at a 1:1 v/v ratio as described in (Rohland and Reich, 2012), and quantified by PicoGreen fluorescence. Equal quantities of the reaction products were then pooled. The library was bead-purified once again (1:1 ratio), quantified by qPCR using the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, Massachussetts, United States), and loaded at 9 pM (including a 30% PhiX control) onto an Illumina MiSeq instrument (Illumina, San Diego, California, United States) using 2 × 150 paired-end read chemistry.

Data Analysis

DNA sequences of EMF root tips were aligned and trimmed in Bioedit (Hall, 1999) and identified to the genus or species level using the Basic Logical Alignment Search Tool (BLAST; Altschul et al., 1990) and UNITE (Kõljalg et al., 2013) databases. We considered sequence similarity of ≥98% to published sequences indicative of species-level identity and 95–97% indicative of genus-level identity (Kõljalg et al., 2013).

For the DSE data set, the forward and reverse reads of ITS sequences were stitched using FastqJoin (Aronesty, 2011) and quality filtered using the software package Quantitative Insights into Microbial Ecology v 1.9 (QIIME; Caporaso et al., 2010) using a Phred score cut-off value of 20. DNA sequences were extracted using ITSx (Bengtsson-Palme et al., 2013), and OTUs were picked using SWARM (Mahé et al., 2014) with a local clustering threshold value of 3. The most abundant sequence for each operational taxonomic unit (OTU) was aligned with PyNAST (Caporaso et al., 2010) against the UNITE (ITS; Nilsson et al., 2019) database using a 97% similarity cutoff, and taxonomy was assigned using BLAST (Altschul et al., 1990). Community composition data generated from amplicon counts were CSS-normalized and OTU tables were filtered to putative DSE taxa including

the following orders: Helotiales, Xylariales, Pleosporales, Sordariales, Hypocreales and Chaetosphaeriales (Grünig et al., 2008).

Community composition of EMF and DSE was compared among treatments using separate Permutational MANOVAs (PERMANOVA) with the Bray-Curtis dissimilarity index in Primer 7 (Primer-e Ltd., Ivybridge, United Kingdom). The Shannon diversity (H′ log base e) was calculated for EMF and DSE using Primer 7 and compared among treatments using a one-way ANOVA in SPSS (IBM SPSS v. 20) followed by a Tukey's test to locate treatment differences. Data on EMF colonization, DSE colonization, and shoot growth also were analyzed using one-way ANOVAs followed by Tukey's tests. Hyphal exploration type was evaluated using a MANOVA in SPSS.

RESULTS

EMF Colonization and Community Composition

Root colonization by EMF was, on average, 50% lower in trees that experienced both drought and warming than in trees that experienced ambient conditions ($F_{3,16}$ = 3.573, p = 0.038; **Figure 1A**). Colonization by EMF was intermediate in the drought only or warming only treatments (**Figure 1A**). Similar patterns were observed with Shannon diversity which was, on average, 4.4X greater on control trees than trees in the combined drought and warming treatment ($F_{3,16}$ = 4.389, p = 0.02; **Figure 1B**). Again, trees that experienced only drought or warming were intermediate, but closer to the ambient treatment than to the combined treatment (**Figure 1B**).

The root tip EMF community consisted of 12 species, seven members of the Phylum Ascomycota and five members of the phylum Basidiomycota (**Table 1**; **Figure 2**). Low species richness and dominance by fungi in the Ascomycota is typical of *P. edulis* (Gehring et al., 2014; Patterson et al., 2018). The two members of the Ascomycota not observed in previous studies of *P. edulis* (e.g., Patterson et al., 2018; Mueller et al., 2019), *Cercophora* sp. and Helotiales sp. produced consistent morphotypes with obvious fungal mantles, but microscopy indicated poorly formed Hartig nets. These fungi were rare. They were observed in only one treatment each where they made up less than 2% of the community, but they were included in subsequent statistical analyses despite the poorly formed Hartig net. Recent research indicates that EM fungi can still carry out critical functions even without a functional Hartig net (Sa et al., 2019). Members of the Heliotiales can form associations with ectomycorrhizas (Nakamura et al., 2018) and it is possible that this is what we observed.

While overall EMF community composition was similar among treatments (pseudo $F_{3,19}$ = 1.35, p = 0.163), individual taxa were significantly affected by the combined drought and warming treatment (**Figure 2**). The relative abundance of both *Cenococcum* sp. and *Tomentella* sp. varied among treatments owing to their absence from the combined drought and warming treatment (*Cenococcum* sp. pseudo $F_{3,19}$ = 1.72, p = 0.021, *Tomentella* sp. pseudo $F_{3,19}$ = 1.97, p = 0.005; **Figure 2**). Only contact and short hyphal exploration types were observed, with short exploration type dominating in all treatments [mean (S.E.) % short exploration type for control trees = 79.3 (10.48) for drought only trees = 100 (0.0), for heat only trees = 90.6 (8.04) and for drought and heat combined = 86.3 (13.6); $F_{3,16}$ = 0.982, p = 0.462].

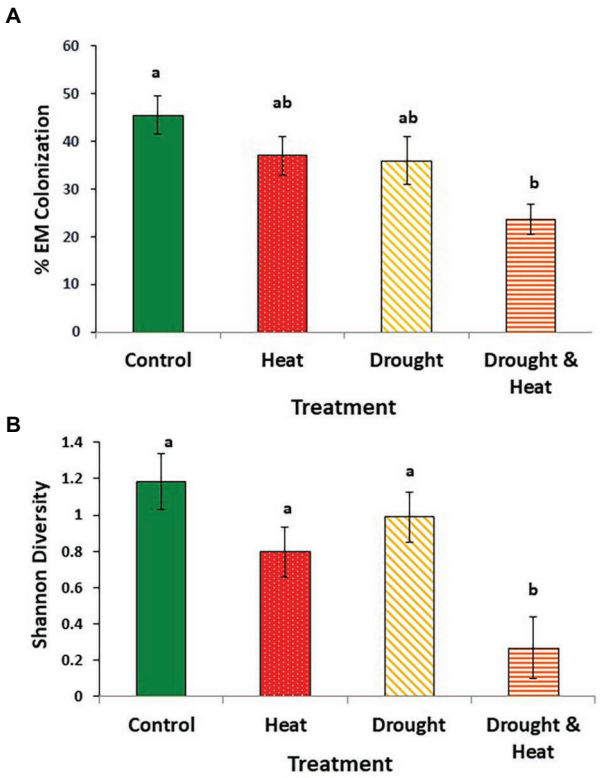

FIGURE 1 | Mean (+/– 1 S.E.) ectomycorrhizal fungal (EMF) colonization **(A)**, and Shannon diversity index of the EMF communities **(B)** found in the roots of pinyon pine trees grown under ambient (control), warming (+4.8°C compared to ambient), drought (–50% of precipitation) and combined heat and drought treatments for 6 years. Different letters above the bars denote differences among groups at $p < 0.05$.

DSE Colonization and Community Composition

As with EMF, root colonization by DSE was negatively affected by the combined drought and warming treatment. Colonization by DSE was high (~75%, on average) in the ambient, drought and warming treatments, but was ~20% lower in the combined drought and warming treatment ($F_{3,16}$ = 4.532, p = 0.018; **Figure 3A**).

The root DSE community consisted of 101 OTUs, with most of these (57%) occurring at less than 1% relative abundance in any treatment group. Thirty-two percent of the OTUs were identified to species, 31% to genus, 9% to family, 20% to order, and 8% to phylum (Ascomycota). The genus *Cladophialophora* had the most OTUs (n = 11) followed by *Paraphoma* (n = 6), while the most common OTUs identified at the ordinal level were found in the Pleosporales and Helotiales, with six OTUs each.

In contrast to observations with EMF, Shannon diversity at the OTU level was similar in all four treatments ($F_{3,16}$ = 0.397,

TABLE 1 | Ectomycorrhizal fungal taxa identified on *Pinus edulis* using ITS sequences.

ID	Fungal phylum[1]	Hyphal exploration type[2]	Matching GenBank accession number[3]	Query coverage%[4]	Identity%[5]	GenBank accession number[6]
Cenococcum geophilum	A	Short	MK131420.1	100	99	n/a
Cercophora sp.	A	Short	KX171944.1	95	96	MW026419
Clavulina sp.	B	Short	MK627472.1	88	99	MW026416
Geopora pinyonensis	A	Short	KF546493.1	99	99	n/a
Geopora 1	A	Short	KF546490.1	98	99	n/a
Geopora 2	A	Short	KF546492.1	98	99	n/a
Helotiales sp.	A	Short	HM488537.1	99	99	n/a
Helvellosebacina sp.	B	Short	KF000456.1	96	97	MW026417
Inocybe sp.	B	Short	MG833870.1	96	97	MW026420
Pezizaceae sp.	A	Short	AJ633598.1	100	97	MW026421
Russula sp.	B	Contact	KM402893.1	97	98	MW026415
Tomentella sp.	B	Short	EU444541.1	92	98	MW026418

[1]*Indicates Ascomycota (A) or Basidiomycota (B).*
[2]*Hyphal exploration type based on our observational measurements, Agerer (2006) and Tedersoo and Smith (2013).*
[3]*Accession number in NCBI GenBank (https://www.ncbi.nlm.nih.gov/genbank/) that most closely matches the sequences generated in this study.*
[4]*Query coverage indicates the percentage of the query sequence that overlaps the reference sequence.*
[5]*Identity percent indicates the similarity of the query sequence and the reference sequence across the length of the coverage area.*
[6]*DNA sequences of taxa in bold type did not match GenBank sequences at >99% identity with >95% query coverage. These sequences were submitted to GenBank and their accession numbers provided.*

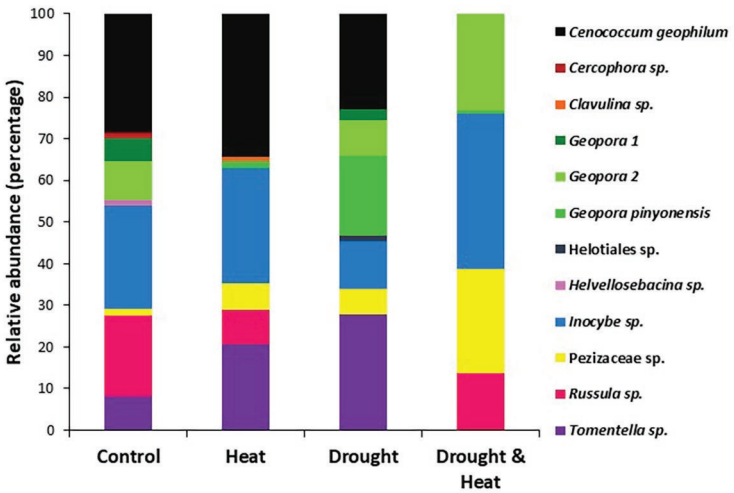

FIGURE 2 | Relative abundance of the EMF species found in the roots of pinyon pine trees grown under ambient (control), heat (+4.8°C compared to ambient), drought (–50% of precipitation) and combined heat and drought treatments for 6 years. The species in control and heat treatments resembled each other, while significantly fewer species were found in the combined drought and heat treatment.

p = 0.757; **Figure 3B**). DSE community composition was also similar among groups (pseudo $F_{3,16}$ = 1.35, p = 0.163). This similarity is illustrated by the relative abundance of the 10 most common OTUs which make up between 36 and 40% of the community in all four treatments (**Figure 4**).

Shoot Growth and Relationships to Fungal Colonization and Diversity

Pinyons growing in ambient conditions had the greatest mean shoot elongation during the year fungi were sampled, followed by the warming only treatment ($F_{3,16}$ = 40.325, p < 0.001; **Figure 5A**). Pinyons experiencing drought only or drought and warming had similar mean shoot lengths, which were approximately 50% lower than those of pinyons in the ambient treatment and approximately 40% lower than pinyons in the warming only treatment (**Figure 5A**).

Mean shoot length during the growing season in which fungi were sampled was most strongly positively correlated with EMF diversity (R^2 = 0.3252, $F_{1,18}$ = 7.022, p < 0.01, **Figure 5B**), but also positively correlated with EMF colonization (R^2 = 0.224, $F_{1,18}$ = 5.128, p = 0.035). There was no association between shoot growth and DSE colonization (R^2 = 0.07, $F_{1,18}$ = 1.486, p = 0.239) or DSE diversity (R^2 = 0.006, $F_{1,18}$ = 0.104, p = 0.751, data not shown).

DISCUSSION

Declines in Ectomycorrhizal Fungal Colonization and Diversity

Consistent with our first hypothesis, root colonization by EMF was most negatively affected in the combined drought and warming treatment, but also reduced in the drought or warming only treatments. Drought alone has been shown to cause reductions in EM colonization in many of the studies reviewed by Mohan et al. (2014) and Karst et al. (2014) and in a more recent study of beech (*Fagus sylvatica*; Köhler et al., 2018). The main effect of the drought treatment we implemented was to reduce precipitation reaching the ground by ~45%. This reduced the capacity of small precipitation events to replenish soil moisture reserves so that the relative water content extractable by plants at the top 30 cm of the soil remained on average ~50% lower compared to control and heat treatments throughout the experiment. It did not change the absolute maximum soil moisture content measured during snow melt or after the heaviest monsoon rains, or the minimum soil moisture content measured in the end of the dry season each year.

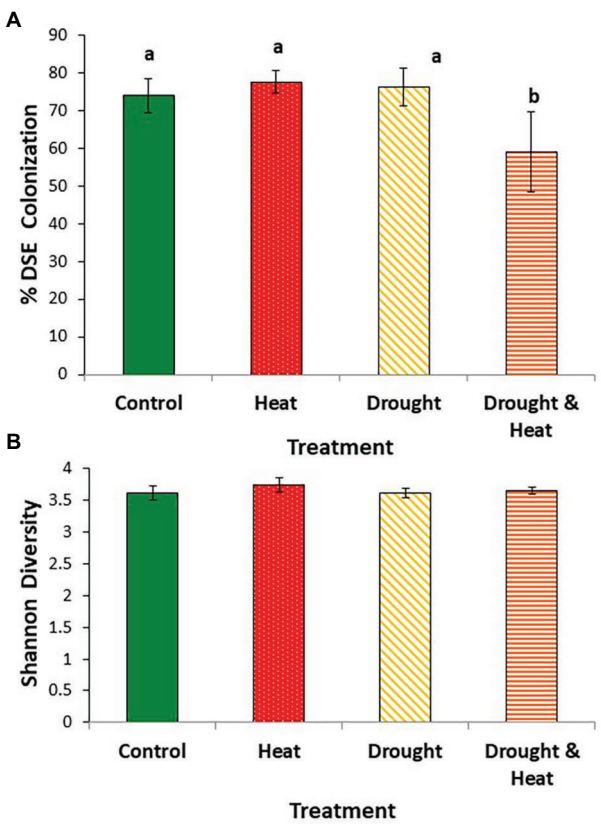

FIGURE 3 | Mean (+/– 1 S.E.) root colonization by dark septate endophytes (DSE) **(A)**, and Shannon diversity index of the DSE communities **(B)** found in the roots of pinyon pine trees grown under ambient (control), warming (+4.8°C compared to ambient), drought (–50% of precipitation) and combined heat and drought treatments for 6 years. Different letters above the bars denote differences among groups at $p < 0.05$.

These precipitation events and drought periods were strong enough to drive all the treatments to similar soil moisture content. But, during less extreme precipitation seasons, drought treatment caused plant extractable soil moisture content to fluctuate around 20% in the drought treatments compared to 40% in control and heat treatments (Grossiord et al., 2017a,b) significantly reducing water availability in the soil.

Our findings regarding warming temperatures are difficult to compare to other research as previous field studies have focused on arctic or boreal ecosystems where warming temperatures frequently led to increased EM colonization (Mohan et al., 2014; Bennett and Classen, 2020). At our site, the soil temperatures at 10 cm depth (the average depth from which roots were collected) can reach up to 60°C even without additional heating, exceeding the environmental tolerance of many species of fungi (Maheshwari et al., 2000). Experimental heating increased temperatures in our study system an average of 4.8°C (Adams et al., 2015; Garcia-Forner et al., 2016) which increased the peak temperatures proportionally and reduced the time spent at below freezing temperatures in the winter by roughly 50% compared to the ambient and drought treatments. The large reduction in EM colonization in the combined heat and drought treatment suggests that sustained warm temperatures or heat waves during periods of drought may limit the ectomycorrhizal symbiosis in semi-arid environments. A critical question that remains is if the reductions in EM colonization we observed also limit EMF propagule production and viability, and thus has a lasting effect on the inoculum potential of the soil.

In addition to a sharp decline in the abundance of EMF in the combined heat and drought treatment, EMF diversity dropped by more than 75% relative to the ambient control. Previous studies have documented that EMF diversity declines with drought (Karst et al., 2014; Mohan et al., 2014) while studies of warming temperatures have again focused largely on arctic or boreal systems where results have been mixed (Mohan et al., 2014; Fernandez et al., 2017). In pinyon pine, long-term drought resulted in reduced EMF diversity (Sthultz et al., 2009b; Gehring et al., 2014). Restoring moister conditions to pinyon pines in the same study area with experimental watering during drought did not increase EMF diversity, suggesting that reductions in diversity with drought may be long term (Patterson et al., 2018). While warming experiments (Fernandez et al., 2017) and drought (Gehring et al., 2014; Karst et al., 2014) appear to favor members of the Ascomycota, their dominance did not differ among treatments in our study. In fact, one of the more drought tolerant species of fungi, the ascomycete, *Cenococcum geophilum* (Pigott, 1982; Jany et al., 2003), was common (average 31% relative abundance) in the control, heat and drought treatments, but absent from the combined heat and drought treatment (**Figure 2**). However, members of the genus *Geopora*, documented to promote drought tolerance in *P. edulis* (Gehring et al., 2017) had their highest abundance in the drought only treatment but also were present (~25% relative abundance) in the combined heat and drought treatment. Members of this genus are found in numerous stressful environments including mine spoils (Hrynkiewicz et al., 2009) and post-fire landscapes (Fujimura et al., 2004) but the mechanisms

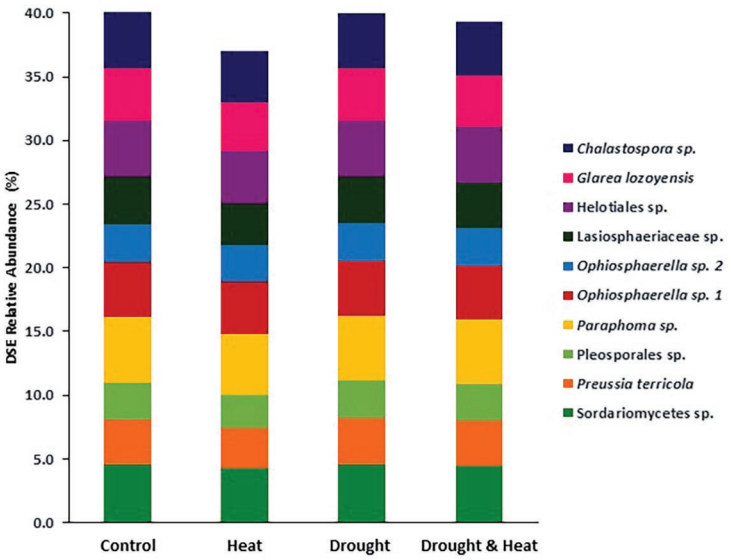

FIGURE 4 | Relative abundance of the 10 most abundant taxa of DSE found in the roots of pinyon pine trees grown under ambient (control), heat (+4.8°C compared to ambient), drought (–50% of precipitation) and combined heat and drought treatments for 6 years. There were no significant differences in DSE community composition among treatments.

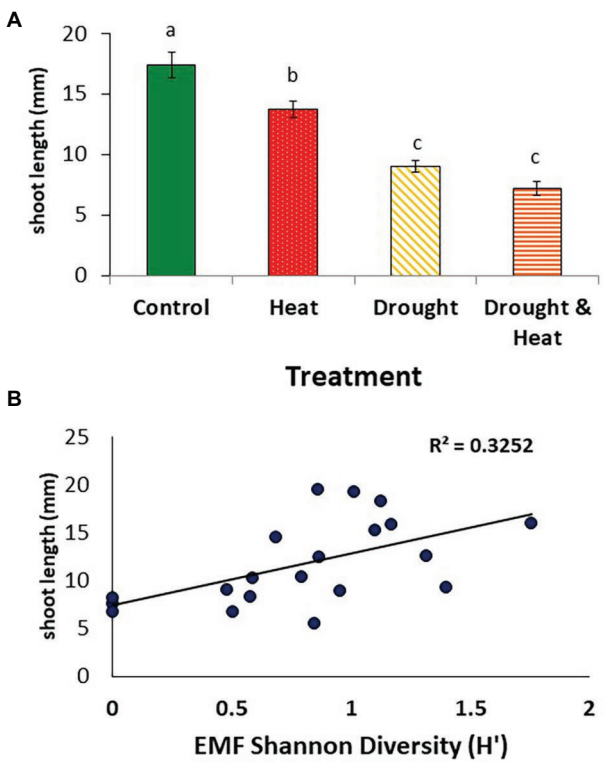

FIGURE 5 | Mean (+/– 1 S.E.) length of the current year shoots observed in pinyon pine trees in the year of root collection after growing under the control, heat, drought, and combined drought and heat treatment for 6 years **(A)**. The length of the current year shoots in these trees correlated positively with the Shannon diversity of the root ectomycorrhizal fungal communities **(B)**.

contributing to their success in these challenging environments are unknown. Studies in cooler, wetter ecosystems, have reported that warming increased EMF taxa with presumably less energetically expensive short distance hyphal exploration types (Fernandez et al., 2017), but EMF taxa with short exploration types dominated in all treatments in our study, consistent with previous studies of *P. edulis* (Patterson et al., 2018).

Small Effect of Treatments on DSE

Colonization by DSE was high in all groups, exceeding 50%, and only declined slightly in the combined drought and warming treatment. DSE diversity and species composition was unaffected by any of the temperature and precipitation reduction treatments. This lack of change relative to the large reductions in diversity and colonization observed in EMF is consistent with our second hypothesis. DSE are well known for their high abundance in stressful environments, including arid lands (Porras-Alfaro et al., 2008; Porras-Alfaro and Bayman, 2011), and were previously observed to be less responsive to changes in the abiotic environment than mycorrhizal fungi (Bueno de Mesquita et al., 2018). DSE may have been affected to a lesser extent than EMF because of the stress tolerance of their highly melanized hyphae. One function of melanin in fungi is protection from harmful environmental conditions including ultraviolet radiation and temperature extremes (Butler and Day, 1998). Interestingly, *C. geophilum*, the EMF taxon that was common in all treatments but the heat and drought treatment is also heavily melanized. Melanin inhibition studies on *C. geophilum* isolates showed that fungal growth was negatively affected only when isolates were subjected to osmotic and desiccation stress (Fernandez and Koide, 2013). Comparative studies of DSE and EMF like *C. geophilum* would be helpful to

elucidate the importance of melanin to their stress tolerance and that of their host plants.

Although we observed DSE taxa commonly found in members of the Pinaceae like *Phialocephala fortinii* (Jumpponen and Trappe, 1998; Grünig et al., 2008), these taxa were less abundant than members of the genera *Chalastospora* and *Paraphoma* that are better known as plant pathogens than endophytes. One of the most common genera we observed, *Paraphoma*, made up ~5% of all sequences across treatments but we could not find reference to its occurrence in members of the Pinaceae. *Paraphoma* can cause root rot in crops such as alfalfa resulting in necrotic lesions (Cao et al., 2020). However, we did not observe damage to the roots we sequenced or observed microscopically. Our results highlight how much remains to be learned about DSE. Their high taxonomic diversity within the Ascomycota and function along the mutualism-parasitism axis are well documented (Berthelot et al., 2019), but also present challenges to understanding their influence on host plant growth and survival.

Fungal Relationships to Host Plant Growth

We observed significant associations between *P. edulis* aboveground growth and the abundance and diversity of EMF but not DSE, consistent with our third hypothesis. Similar to previous observations at our site (Grossiord et al., 2017b), current year shoot growth was reduced relative to controls in the drought and combined drought and heat treatment, but not in the heat treatment alone (**Figure 5**). Although the drought treatment slightly negatively affected EMF colonization but not diversity, there was a higher correlation between EMF diversity and growth than EMF colonization and growth with EMF diversity explaining 32.5% of the variation. In a study of the EMF communities of *P. edulis* that remained following host plant mortality, reduced diversity due to the absence of EMF in the genus *Tuber* was associated with reduced seedling size (Mueller et al., 2019). While our results suggest that EMF may be more important to aboveground growth than DSE, we did not measure belowground growth. DSE may have influenced pinyon root length or biomass, important contributors to the beneficial effects of DSE in other arid land plant species (Li et al., 2018).

Few studies have experimentally manipulated EMF diversity to understand mechanisms with mixed results (Baxter and Dighton, 2001; Jonsson et al., 2001; Hazard et al., 2017). Studies are even more limited in low moisture, high temperature environments, but phosphorus uptake efficiency was observed to decrease due to reductions in EMF diversity under low soil moisture conditions in European beech (Köhler et al., 2018). At our site, plant phosphorus uptake has not been studied, but warming increased both nitrification processes and the amount of nitrate in the root zone, while drought increased the amount of ammonium, and both these effects were present in the combined heat and drought treatment (Grossiord et al., 2018a). These shifts did not have any effect on the N content of plant tissues, plant N allocation or preference for using nitrite or ammonium suggesting that nitrogen was not limiting growth. But, these changes in N dynamics could contribute to the composition and function of the root-zone microbiome given the importance of N to EMF communities in many other ecosystems (Lilleskov et al., 2019). Nitrogen fertilization increased leaf production and reduced EMF abundance in *P. edulis* (Allen M. F. et al., 2010). However, it also was associated with increased tree mortality in *P. edulis* during drought, possibly due to a reduced role of EMF in water uptake (Allen M. F. et al., 2010). Thus changes in N dynamics in this study system could become significant to plant growth and vitality as drought and warming continue. In beech (*Fagus sylvatica* L.), moderate drought increased the importance of EMF to uptake of inorganic N, but this effect was EMF species specific, even differing among members of the same genus (Pena and Polle, 2014). *C. geophilum*, the taxon shared between our study and that of Pena and Polle (2014), did not improve N uptake under drought.

In our study, we cannot determine conclusively if changes in fungi influenced host plants or the reverse (or a combination), but previous studies utilizing the same experiment provide clues. Over the years, the drought, heat and combined drought and heat treatments have affected the carbon fixation and water uptake as well as growth of the *P. edulis* trees. Drought and combined drought and heat treatments have shown significantly lower average stomatal conductance and photosynthesis at saturation light (Grossiord et al., 2017a, 2018a). These changes were combined with delayed initiation of both shoot (Adams et al., 2015; Grossiord et al., 2017b) and stem growth (Manrique-Alba et al., 2018) in the combined heat and drought treatment, reduced needle elongation in both the drought and combined heat and drought treatments (Grossiord et al., 2017b), and reduced capacity to replenish stem water reserves in the combined drought and heat treatment (Grossiord et al., 2017c; Manrique-Alba et al., 2018). These observations suggest reduced plant productivity that could influence the ability to attract and maintain mutualistic fungi. While EMF can access nutrients in soil organic matter through a variety of mechanisms (Frey, 2019) they generally rely on photosynthates from their hosts, but potentially to varying degrees (Koide et al., 2008). For example, species richness in EMF associated with European beech trees was affected by stem girdling that reduced direct transport of photosynthates to the roots (Pena et al., 2010). In addition to the protection provided by melanin for DSE, our observed differences in drought and heat effects on EMF and DSE colonization and species richness could be explained by different degree of fungal dependency on plant-produced carbohydrates between these groups.

At our site, reductions in plant photosynthesis and growth were accompanied by reduction in leaf-area-specific plant hydraulic conductivity, but no change in the depth of main water sources used by the trees (Grossiord et al., 2017a), or the leaf area: sapwood area ratio (McBranch et al., 2019). These findings suggest that the trees adjusted their water demand to water availability without changing anatomical structure or rooting depth, even if the heat and drought treatment increased competition for water in the main water source layer by inducing a shift that brought the main water source for co-occurring grasses to the same layer (Grossiord et al., 2019). Whether this shift affected DSE communities differently from

EMF communities is unclear, nor do we understand how the two groups of fungi interact within roots or soil. There is evidence that DSE colonization has positive effects on AMF colonization of grass roots in arid grasslands (Menoyo et al., 2020), while interactions between EMF and DSE appear to be species and strain specific (Berthelot et al., 2019). In most pinyon-juniper woodlands, DSE have multiple hosts, while EMF are restricted to *P. edulis*; this difference also may contribute to the different sensitivities of the two groups to the combined stressors in our study.

CONCLUSION

Our experimental study of the effects of warming and drought on the fungal communities of an arid land conifer provides an important contrast to similar studies in cooler, wetter climates. Heating alone caused little change, but combined heat and drought had strong negative effects on root-associated fungi. Our results also indicate that EMF are more sensitive than DSE, with the former showing declines in both abundance and diversity. The differences among root symbionts could be due to differences in stress tolerance, host plant specificity, degree of dependence on plant hosts for carbon, or a combination of these factors. Our data on aboveground plant growth and EMF species diversity support the view that EMF are mutualists, and emphasizes the importance of community diversity rather than simple abundance to plant vitality. Less is known about the importance of DSE to plant performance in arid land trees or how DSE and EMF interact with one another and thereby affect their shared host. Obtaining this information is critical for understanding potential acclimation and adaptation of forest ecosystems to changing climate as well as for predicting bottle necks and tipping points that influence forest health.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

CG contributed to data collection and analysis and led the writing of the manuscript. SS helped to construct and maintain the experiment, contributed to data collection, and helped to draft the manuscript. AP and DU contributed to data collection and revised the manuscript. CK initiated the collaboration and revised the manuscript. All authors contributed to the article and approved the submitted version.

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