



Effects of 8-Year Nitrogen and Phosphorus Treatments on the Ecophysiological Traits of Two Key Species on Tibetan Plateau

Dan Wang^{1*}, Tianqi Ling¹, Pengpeng Wang¹, Panpan Jing¹, Jiazhi Fan¹, Hao Wang¹ and Yaoqi Zhang²

¹ International Center for Ecology, Meteorology and Environment, Jiangsu Key Laboratory of Agricultural Meteorology, Nanjing University of Information Science and Technology, Nanjing, China, ² School of Forestry and Wildlife Sciences, Auburn University, Auburn, AL, United States

OPEN ACCESS

Edited by:

Jeffrey M. Warren,
Oak Ridge National Laboratory (DOE),
United States

Reviewed by:

Fernando José Cebola Lidon,
Faculdade de Ciências e Tecnologia
da Universidade Nova de Lisboa,
Portugal
Anirban Guha,
Oak Ridge National Laboratory (DOE),
United States

*Correspondence:

Dan Wang
wangdan@nuist.edu.cn;
wangdan.nuist@outlook.com

Specialty section:

This article was submitted to
Plant Physiology,
a section of the journal
Frontiers in Plant Science

Received: 27 March 2018

Accepted: 16 August 2018

Published: 11 September 2018

Citation:

Wang D, Ling T, Wang P, Jing P,
Fan J, Wang H and Zhang Y (2018)
Effects of 8-Year Nitrogen
and Phosphorus Treatments on
the Ecophysiological Traits of Two Key
Species on Tibetan Plateau.
Front. Plant Sci. 9:1290.
doi: 10.3389/fpls.2018.01290

Understanding how nitrogen (N) and/or phosphorus (P) addition affects plants carbon- and water- related ecophysiological characteristics is essential for predicting the global change impact on the alpine meadow ecosystem structure and function in carbon and water cycling. The Qinghai-Tibetan Plateau (QTP) with the largest alpine meadow in the world is regarded as the third pole in the earth and has been experiencing increased atmospheric N deposition. In this project, we focused on two key species (*Elymus dahuricus* and *Gentiana straminea*) of the alpine meadow on the Tibetan Plateau and investigated the variability of photosynthetic and stomatal responses to 8-year N and/or P treatments through field measurements and modeling. We measured photosynthesis- and g_s -response curves to generate parameter estimates from individual leaves with two widely used stomatal models (the BWB model and MED model) for validation of growth and ecosystem models and to elucidate the physiological basis for observed differences in productivity and WUE. We assessed WUE by means of gas exchange measurements (WUE_i) and stable carbon isotope composition ($\Delta^{13}C$) to get the intrinsic and integrated estimates of WUE of the two species. P and N+P treatments, but not N, improved the photosynthetic capacity (A_{net} and V_{cmax}) for both species. Stomatal functions including instantaneous measurements of stomatal conductance, intrinsic water-use efficiency and stomatal slope parameters of the two widely used stomatal models were altered by the addition of P or N+P treatment, but the impact varied across years and species. The inconsistent responses across species suggest that an understanding of photosynthetic, stomatal functions and water-use should be evaluated on species separately. WUE estimated by $\Delta^{13}C$ values had a positive relationship with A_{net} and g_s and a negative relationship with WUE_i . Our findings should be useful for understanding the underlying mechanisms of the response of alpine plants growth and alpine meadow ecosystem to global change.

Keywords: photosynthesis, stomatal conductance, intrinsic water-use efficiency, integrated water-use efficiency, stomatal slope parameter

INTRODUCTION

Terrestrial ecosystems worldwide are limited or co-limited by nutrients, especially by nitrogen (N) and phosphorus (P) (Elser et al., 2007; LeBauer and Treseder, 2008; Harpole et al., 2011). While it is well-known that N and P addition typically increases plant growth, less is known about how N and P addition affects plants ecophysiological characteristics related to carbon and water acquisition. The Qinghai-Tibetan Plateau (QTP) is regarded as the third pole and has one of the largest alpine grasslands in the world. The QTP has been experiencing much greater than average global changes, such as increased atmospheric N deposition and climate warming (IPCC, 2013). The wet nitrogen deposition was estimated $6.96\text{--}7.55\text{ kg N hm}^{-2}\text{ y}^{-1}$ on the QTP (Lv and Tian, 2007). With the increase of global nitrogen deposition and the relative slow mineralization rate due to the low temperature at the high elevation, it is critical to investigate the ecophysiological responses of the alpine grasslands species to N and P addition. The information will be valuable in predicting the global change impact on the alpine meadow ecosystem structure and function in carbon and water cycling.

Alpine meadow system on the Tibetan Plateau is characterized as low N and P availability due to the slow mineralization processes at the low temperature. The addition of N and P is therefore anticipated to boost the growth of the alpine meadow species. Yang et al. (2014) reported that N and P additions both increased the aboveground biomass on QTP alpine meadow and the P effect was more evident than the N effect. Fu and Shen (2017) synthesized 51 studies on the QTP and confirmed that nitrogen addition significantly increased plant height and aboveground biomass. Photosynthetic carbon gain of leaves was mainly affected by N concentration and light availability (Field and Mooney, 1986). This observation is supported by the positive relationships between leaf N concentration and net photosynthesis observed in many different species (Turnbull et al., 2007; Wang et al., 2012). However, whether the alpine meadow species is photosynthetically N or P limited and whether different species respond to N/P addition differently remains unknown. Chlorophyll fluorescence parameters, stomatal conductance (g_s) and maximum rate of carboxylation (V_{cmax}) are important physiological parameters related to plant photosynthesis. All these physiological parameters are nutrient-dependent and probably affected under N and/or P addition conditions (Reich et al., 2009; Liu and Greaver, 2010). Measurements of photosynthetic and stomatal responses of the alpine meadow species to N and/or P addition are needed for validation of plant growth models and to elucidate the physiological basis for

observed differences in plant growth responses to the addition of N and/or P.

Successfully simulating canopy and ecosystem photosynthesis and transpiration requires understanding the rate-impacting factors in leaf photosynthesis and stomatal activities (Laisk et al., 2005). Understanding and predicting larger scale carbon, water, and energy cycles also requires accurate estimates of the leaf diffusive (stomatal) conductance to water vapor using stomatal conductance models. The regulatory role of stomata in photosynthetic CO_2 assimilation and water vapor loss to the atmosphere is arguably the most fundamental constraint on plant function and most critical process in simulating and predicting larger scale carbon, water and energy fluxes. Empirical and mechanical models have been incorporated into land surface models to simulate stomatal conductance. The Ball, Woodrow & Berry (BWB model) and Medlyn model (MED model) are two widely used stomatal models to describe the complex behavior of stomata at the leaf level (Medlyn et al., 2001; Wolz et al., 2017). The parameters of these models (m and g_0 from BB model, g_1 and g_0 from MED model) are valuable for large-scale simulations and represent important physiological traits that determine plant water-use efficiency. Compared with instant measurements, the changes in stomatal slope parameters (m and g_1) with plant's biophysical environment provide a simple but synthetic framework for studying climate-change related carbon and water cycling, because of its sensitivity to CO_2 , vapor pressure deficit, and photosynthesis, as well as its crucial information about climate change impacts on photosynthesis and water-use efficiency (Oren et al., 1999). How stomatal slope parameters of alpine meadow species varies among different species and at different fertilization conditions requires further study and analysis.

Through gas exchange measurements, WUE can be expressed as intrinsic WUE (WUE_i , the ratio of net photosynthesis to stomatal conductance, A_{net}/g_s). Integrative WUE ($\Delta^{13}\text{C}$) can be assessed indirectly with measurements of the stable carbon isotope composition ($\delta^{13}\text{C}$) of leaves or other plant materials. This latter method is based on the linear relationship between $\delta^{13}\text{C}$ and the ratio of the concentration of CO_2 inside and outside of the leaf (Farquhar et al., 1982). Using gas exchange measurements (WUE_i) and carbon isotope composition would provide both instantaneous and integrated estimates of WUE (Seibt et al., 2008). Whether the integrated measurements matches with the instantaneous measurements of water-use efficiency, stomatal slope, and photosynthetic parameters for the alpine meadow species requires further investigation and analysis.

Previous studies indicated that N and P additions increased the aboveground biomass of grass but decreased forb biomass (Yang et al., 2014; Fu and Shen, 2017). To identify the ecophysiological responses of different PFTs to N/P addition, we will select two key species (*Elymus dahuricus*, a C_3 perennial grass and *Gentiana straminea*, a C_3 perennial forb) of the alpine meadow on the Tibetan Plateau and investigate the variability of photosynthetic and stomatal responses to N or P additions and associated leaf traits through field measurements and modeling. We measured photosynthesis- and g_s -response

Abbreviation: A_{net} , net CO_2 assimilation rate ($\mu\text{mol m}^{-2}\text{ s}^{-1}$); F_v/F_m , the maximal photosystem II (PSII) efficiency in the light; g_s , stomatal conductance ($\text{mol m}^{-2}\text{ s}^{-1}$); J_{max} , maximum electron transport rate ($\mu\text{mol m}^{-2}\text{ s}^{-1}$); N_{mass} , leaf mass-based nitrogen concentration (%); PFTs, plant functional types; Φ_{PSII} , the actual PSII efficiency; RuBP, ribulose-1,5-bisphosphate carboxylase; V_{cmax} , maximum carboxylation rate ($\mu\text{mol m}^{-2}\text{ s}^{-1}$); WUE_i , intrinsic water-use efficiency ($\mu\text{mol CO}_2/\text{mol H}_2\text{O}$); $\Delta^{13}\text{C}$, stable isotope ^{13}C discrimination values (‰).

curves to generate parameter estimates from individual leaves for two widely used stomatal models (the BWB model and MED model). We assessed WUE by means of gas exchange measurements (WUE_i) and stable carbon isotope composition ($\Delta^{13}C$) to get the intrinsic and integrated estimates of WUE of the two key alpine meadow species. The objectives of this study were (1) to determine whether P or N or both was the nutrient more limiting to the photosynthesis of two alpine meadow species growing in the field; (2) to investigate whether long-term fertilization treatments changes the stomatal slope parameters; (3) to identify the relationship of leaf traits to integrated water-use efficiency ($\Delta^{13}C$). We hypothesized that (1) as the effect on the aboveground biomass, both P and N addition will improve the photosynthetic capacity of the two species and the P effect will be more evident than the N effect; (2) the addition of N or P will not change the stomatal regulating properties; (3) the integrated water-use efficiency ($\Delta^{13}C$) will be correlated with the instantaneous measurements of water-use efficiency (WUE_i and stomatal slope parameters).

MATERIALS AND METHODS

Site Description

The study site was established in an alpine grassland at the Haibei Alpine Meadow Ecosystem Research Station (37°37' N, 101°12' E, 3240 m above the sea level), located on the northeastern Tibetan Plateau in Qinghai Province, China (Yang et al., 2014; Song and Yu, 2015). The historic mean annual temperature is -1.7°C and annual precipitation is 560 mm, 85% of which occurs in the growing season from May to September. The PAR (photosynthetically active radiation) reaches $370\text{ W m}^{-2}\text{ s}^{-1}$ in the growing season, equivalent of $10\text{ MJ m}^{-2}\text{ d}^{-1}$. The mean annual temperature was 2.89 and -0.02°C and the mean annual precipitation was 601 and 453 mm in 2015 and 2016, respectively. The mean daily day- and night-temperature and maximal temperature was 11.6, 4.6, and 28.4°C and 13.6, 5.2, and 28.9°C in the growing season in 2015 and 2016, respectively. The soil is classified as Mat Cry-gelic Cambisols (Chinese Soil Taxonomy Research Group, 1995), corresponding to Gelic Cambisol. Topsoil (0–10 cm) has a pH value of 7.5, and contains 71.4 g kg^{-1} organic C, 7.8 g kg^{-1} total N, and 0.77 g kg^{-1} total P before nutrient treatments were applied in 2009 (Huang et al., 2014). The experimental site was fenced before the experiment plot was established. The plant community at the experimental site is dominated by *Kobresia humilis*, *Stipa aliena*, *Elymus nutans*, *E. dahuricus*, *G. straminea*, and *Festuca ovina*.

Experimental Design and Sampling

The experimental design followed the standard protocols of Nutrient Network (NutNet¹). In mid-May 2009, an experimental area of 1 ha was fenced to prevent grazing disturbance. Twenty-four plots of $6\text{ m} \times 6\text{ m}$ were randomly assigned to four treatments with six replicates (blocks) in a complete randomized

block design. The blocks were separated by a 2-m-wide buffer zone, and the plots within each block were separated by a 1-m-wide buffer zone to minimize disturbance from neighboring treatments. The four treatments consisted of the following: (1) Control (CK, no fertilizer was added); (2) N addition (in the form of urea, $100\text{ kg N ha}^{-1}\text{ year}^{-1}$); (3) P addition (in the form of triple superphosphate, $50\text{ kg ha}^{-1}\text{ year}^{-1}$); and (4) N+P addition (combined addition of N and P in the same amounts as the solo treatments). Pelletized fertilizer was evenly distributed by hand onto the plots after sunset in July from 2009 to 2016.

Photosynthetic Measurements

Gas exchange (including net photosynthetic rate and stomatal conductance) was measured with a portable infrared gas analyzer (LI-COR 6400LCF; LI-COR, Lincoln, NE, United States) on 1 randomly selected fully expanded healthy leaf from each plot of each treatment in August, 2015 and 2016. During measurements, leaves were exposed to a CO_2 concentration of $370\text{ }\mu\text{mol mol}^{-1}$, leaf temperature of 25°C , and airflow through the chamber of $300\text{ }\mu\text{mol s}^{-1}$. Leaves were acclimated to a photosynthetic photon flux (PPFD; $2000\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$) until photosynthetic rates stabilized. The rate of photosynthesis at a PPFD of $2000\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ was defined as the net photosynthetic rate (A_{net}). PSII efficiency in light-adapted leaves (F_v/F_m') and PSII operating efficiency (Φ_{PSII}) were also measured using a Licor 6400-40 Leaf Chamber Fluorometer. The photosynthesis- CO_2 response ($A-C_i$) curves were measured each year in the middle of the growing season (August). During measurement, leaves were acclimated for 30–60 min before adjusting the CO_2 concentrations. Thereafter, CO_2 concentration was decreased in five steps (400, 300, 200, 100, and 50 ppm CO_2) and then increased in four steps (400, 600, 800, and $1000\text{ }\mu\text{mol mol}^{-1}\text{ CO}_2$). $A-C_i$ curves were fit to the Farquhar-von Caemmerer-Berry model based on the methods developed by Miao et al. (2009). By using grid search and non-linear two-stage least square regression technique, the fitting model solves the $A-C_i$ parameters including maximum ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) carboxylation rate (V_{cmax} , $\mu\text{mol m}^{-2}\text{ s}^{-1}$) and potential light saturated electron transport rate (J_{max} , $\mu\text{mol m}^{-2}\text{ s}^{-1}$), respectively.

Immediately following gas-exchange measurements, leaf samples were oven-dried till constant weight. Leaf samples were then ground and N concentration (N_{mass} , mass based nitrogen concentration) were measured with a Perkin Elmer CHN Analyzer (Model 2400).

Integrated Water-Use Efficiency ($\Delta^{13}C$)

Leaves were oven-dried at 65°C for 2 weeks, then ground to fine powder. Approximately, 2 mg of homogenized leaves were weighed into tin capsules and analyzed with an elemental analyzer coupled to an isotope ratio mass spectrometer (Elemental combustion system 4010, Costech instruments). Carbon isotope ratios were expressed in conventional δ notation and referenced to the Pee Dee Belemnite (PDB) standard for $\delta^{13}C$. Measurement error was less than 0.3‰ for $\delta^{13}C$.

¹<http://nutnet.umn.edu>

The carbon isotope composition ($\delta^{13}\text{C}$) was calculated as the ratio (‰):

$$\delta^{13}\text{C} = \left[\left(\frac{R_{\text{sample}}}{R_{\text{standard}}} \right) - 1 \right] \times 1000$$

Carbon isotope ratio values were converted to discrimination values (Δ , ‰) by the equation (Farquhar et al., 1989):

$$\Delta = (\delta_a - \delta_p) / \left(1 + \frac{\delta_p}{1000} \right)$$

Where δ_a is the carbon isotope ratio of CO_2 in the atmosphere (assumed to be -8 ‰ per mil, Seibt et al., 2008) and δ_p is the measured carbon isotope ratio of the leaf tissue. Lower values of Δ indicate higher water-use efficiency values.

Stomatal Slope Parameter Calculations

The Ball et al. (1987) (Eq. 1) or Medlyn et al. (2001) (Eq. 2) models of g_s were used to calculate the stomatal slope parameters (m and g_1).

$$\text{Eq. 1} \quad g_s = g_0 + m \frac{Ah}{C_a}$$

where g_s is stomatal conductance ($\text{mol m}^{-2} \text{s}^{-1}$), A is the net rate of photosynthetic CO_2 uptake ($\mu\text{mol m}^{-2} \text{s}^{-1}$), h is atmospheric relative humidity (unitless), C_a is the atmospheric CO_2 concentration at the leaf surface ($\mu\text{mol mol}^{-1}$), g_0 is the y -axis intercept and m is the slope of the line.

$$\text{Eq. 2} \quad g_s = g_0 + 1.6 \left(1 + \frac{g_1}{\sqrt{D}} \right) \frac{A}{C_a}$$

where D is atmospheric vapor pressure deficit (kPa) and g_1 is the model parameter related to the slope of the line.

For each leaf, a linear least squares regression of Eq. 1 or Eq. 2 was used to estimate the intercept and slope parameters of the Ball et al. (1987) (3) model and Medlyn et al. (5) model, respectively. Biologically, the slope parameter of each model represents the sensitivity of g_s to changes in A_{net} , C_a and atmospheric water status and will be the focus of this analysis. A term for the y intercept of each model algorithm (g_0) can be used to describe variation in minimum g_s . Only leaves that provided a regression between modeled and observed stomatal conductance with an $R^2 > 0.8$ were included in further analyses (Wolz et al., 2017).

Statistical Analysis

Three-way analysis of variance (ANOVA) was used to test the fixed effects of year, species, fertilization treatment and their interaction on A_{net} , g_s , WUE_i , N_{mass} , F_v'/F_m' , Φ_{PSII} , V_{cmax} , J_{max} , and $\Delta^{13}\text{C}$. *Post hoc* Tukey HSD tests were conducted on specific contrasts to examine significant treatment effects among groups. General linear models (GLMs) were used to assess the relationship between $\Delta^{13}\text{C}$, WUE_i and other physiological parameters. For all tests, the normality of the residuals was tested using the Shapiro–Wilk test. All statistical tests were considered significant at $p \leq 0.05$. Mean values of each variable

were expressed with their standard error (SE). All analyses were conducted in R (R 2.14²).

RESULTS

The three-way ANOVA analysis revealed that effects of nutrient additions on photosynthetic traits varied among species, years, treatment and their interactions (Figure 1 and Table 1). Photosynthetic and leaf traits varied between years and among species, with *G. straminea* possessing higher A_{net} , g_s , F_v'/F_m' , Φ_{PSII} , V_{cmax} , J_{max} , N_{mass} , and $\Delta^{13}\text{C}$ and lower WUE_i and stomatal slope parameters compared with *E. dahuricus* (Table 1). A_{net} of plants with P and N+P treatments was significantly higher than those with N and CK treatments for *E. dahuricus* and *G. straminea* in 2015 and 2016 (Figure 1). Across species and years, the value of g_s of plants with P and N+P treatment was significantly higher than those with N and CK treatments. There were significant species, year and species * treatment effect on WUE_i . The value of WUE_i of *E. dahuricus* with N+P and P treatment was significantly higher than those with N and CK treatments.

Significant effects were detected among species, year, treatment and species \times treatment for F_v'/F_m' and Φ_{PSII} (Figure 2 and Table 1). For *E. dahuricus*, plants with P and N+P treatments had higher F_v'/F_m' and Φ_{PSII} than those with CK and N treatments. For *G. straminea*, P addition significantly increased F_v'/F_m' in 2016 and 2017 compared with CK treatments and Φ_{PSII} compared with CK, N and N+P treatments in 2017.

There were significant effects of species, year, treatment and their interactions for V_{cmax} and J_{max} (Figure 3 and Table 1). Across *E. dahuricus* and *G. straminea* in 2 years, plants with P and N+P treatments had higher V_{cmax} and J_{max} than those with N and CK treatments.

There were no treatment, but species, year and treatment \times year effects on stomatal slope parameters of m and g_1 (Figure 4 and Table 1). Variation in estimates of the g_1 slope parameter from the Medlyn et al. model mirrored that of m , both in species rank and treatment effects. In 2015, plants with nutrient treatment had higher values of m and g_1 than plants with CK treatments for both species. In 2016, plants with P and N+P treatments had lower values of m and g_1 than plants with CK treatment for *E. dahuricus*. There were no significant treatment effects for *G. straminea* in 2016.

Significant effects were detected among species, years, treatment and their interactions for N_{mass} and $\Delta^{13}\text{C}$ (Figure 5 and Table 1). Across the two species in the 2 years, plants with N and N+P treatment had higher N_{mass} than plants with P and CK treatments. The value of $\Delta^{13}\text{C}$ varied among species and treatments. Plants with N+P treatment had lower $\Delta^{13}\text{C}$ than plants with CK treatments across two species.

$\Delta^{13}\text{C}$ had a positive relationship with A_{net} and g_s , a negative relationship with WUE_i and no relationship with stomatal slope parameter of m or g_1 (Figure 6).

²<http://www.r-project.org/>

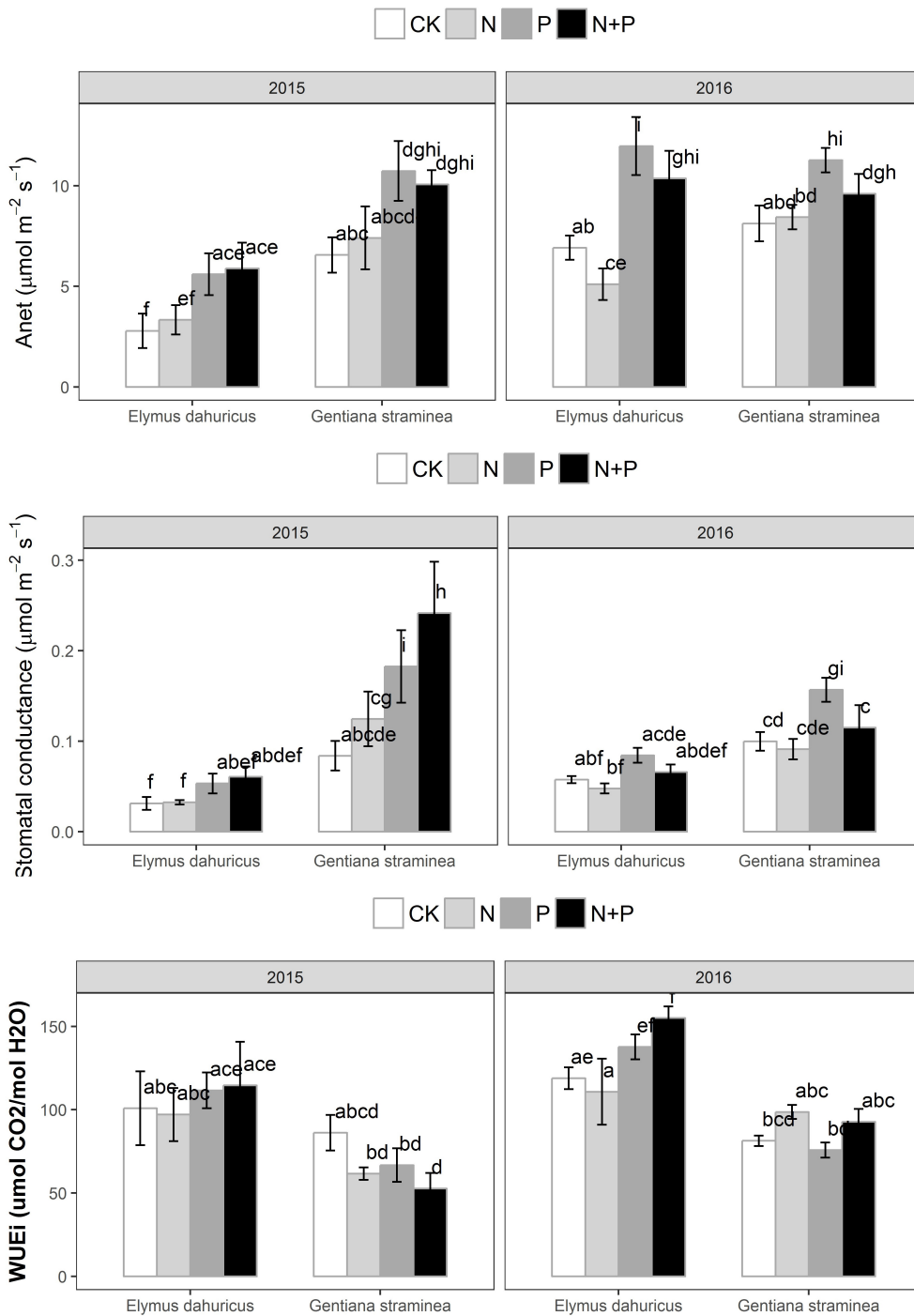


FIGURE 1 | The response of net photosynthetic CO₂ assimilation (A_{net}), stomatal conductance (g_s), and intrinsic water-use efficiency (WUE_i) to the nutrient addition for *Elymus dahuricus* and *Gentiana straminea*. Measurements were taken at CK (control), N (nitrogen), P (phosphorus), and N+P (nitrogen and phosphorus) treatments in the peak growing season in 2015 and 2016. Values are means \pm 1 SE; $n = 5$. Bars sharing the same letters are not significantly different.

DISCUSSION

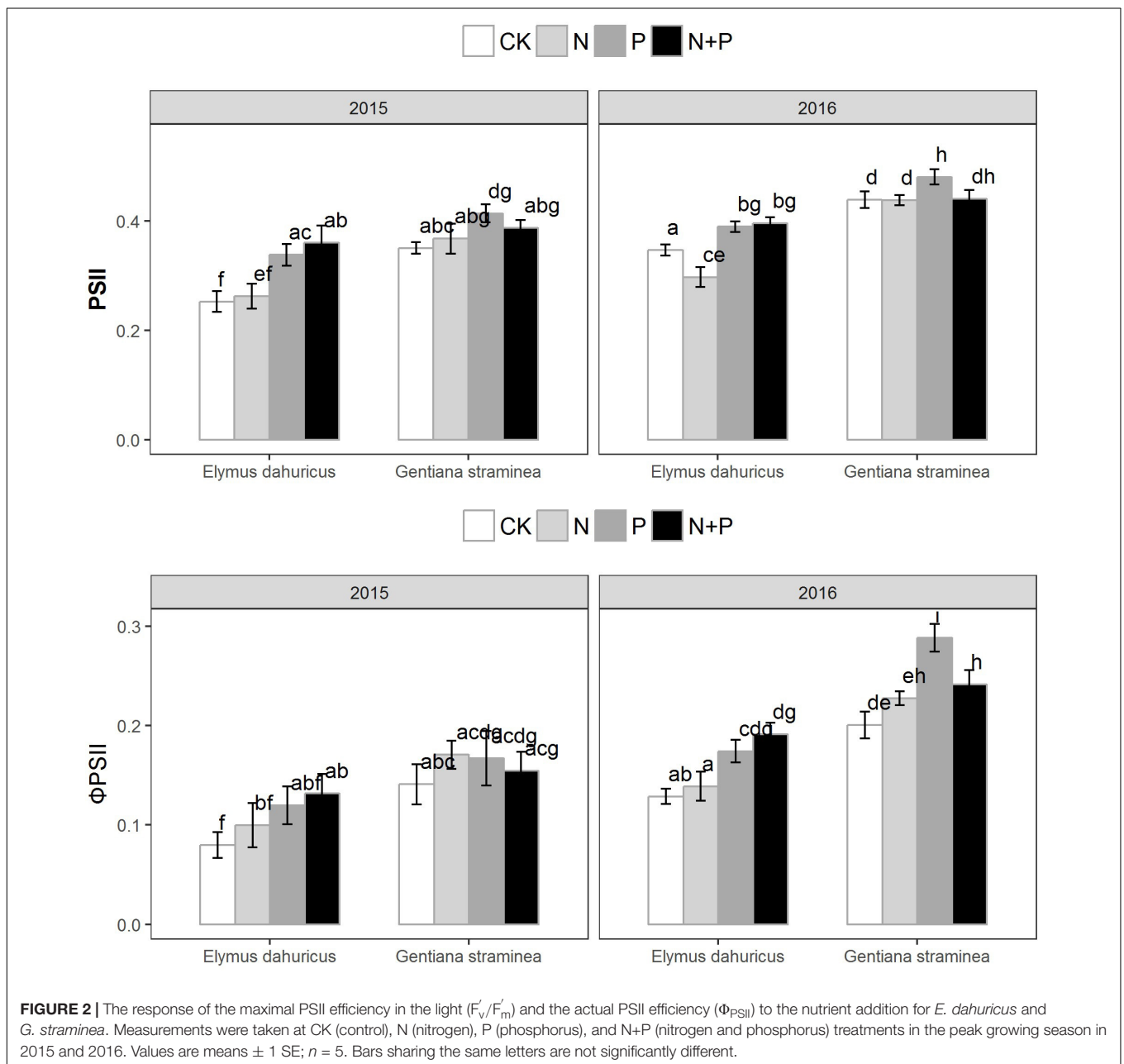
In order to investigate the variation in the ecophysiological responses to N and/or P treatment and detect whether there is stomatal acclimation with long-term fertilization treatment, we

assessed leaf traits of two key species on Tibet alpine grassland across two growing seasons. The systematic measurements of gas exchange after long-term fertilization treatment are essential for validation of plant growth and ecosystem models and to elucidate the physiological basis for observed differences

TABLE 1 | Analysis of variance (*F*-value) of the effects of species, treatment, year and their interactions on net photosynthetic rate (A_{net}), stomatal conductance (g_s), intrinsic WUE (WUE_i), leaf nitrogen concentration (N_{mass}), the maximal PSII efficiency in the light (F'_v/F'_m), the actual PSII efficiency (Φ_{PSII}), maximal carboxylation rate (V_{cmax}), potential light saturated electron transport rate (J_{max}), stomatal slope parameters m and g_1 , and $\Delta^{13}C$.

Variation	A_{net}	g_s	$iWUE$	F'_v/F'_m	Φ_{PSII}	V_{cmax}	J_{max}	g_1	m	N_{mass}	$\Delta^{13}C$
Species	8.9**	96.9***	48.7***	110.4***	79.3***	4.9*	4.1*	9.9***	12.3***	565.6***	264.7***
Treatment	19.8***	11.5***	1.6	17.8***	11.6***	7.5***	8.2**	1.0	0.8	10.7***	2.9*
Year	19.9***	0.8	12.2***	48.9***	61.2***	21.9***	14.9***	15.4***	27.3***	15.8***	0.1
Species × treatment	1.1	3.3*	3.2*	4.6**	2.0	2.8*	0.7	0.8	0.3	0.4	0.3
Species × year	6.9**	15.1***	0.0	1.4	4.5*	5.2*	6.8**	2.7	0.7	8.0**	167.7***
Treatment × year	0.7	3.6*	0.9	1.7	1.0	1.8	2.6*	4.1**	4.1**	1.5	0.4

*** Indicates significance level at 0.001, ** 0.01, * 0.05.



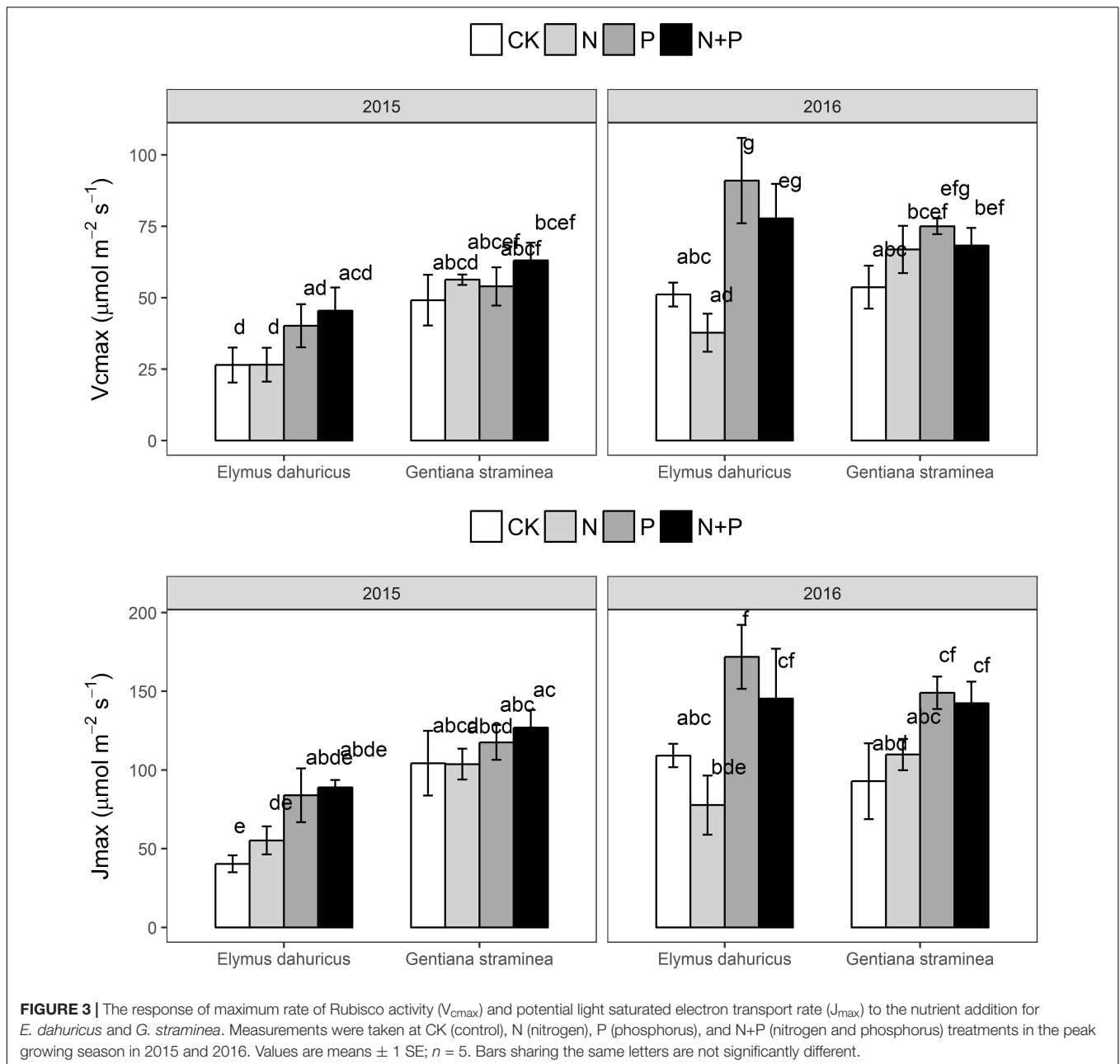


FIGURE 3 | The response of maximum rate of Rubisco activity (V_{cmax}) and potential light saturated electron transport rate (J_{max}) to the nutrient addition for *E. dahuricus* and *G. straminea*. Measurements were taken at CK (control), N (nitrogen), P (phosphorus), and N+P (nitrogen and phosphorus) treatments in the peak growing season in 2015 and 2016. Values are means \pm 1 SE; $n = 5$. Bars sharing the same letters are not significantly different.

in the response of growth and water-use to N and/or P additions.

In our study, photosynthetic and leaf traits varied among species and treatment, with *G. straminea* possessing higher A_{net} , g_s , F_v'/F_m' , Φ_{PSII} , V_{cmax} , J_{max} , N_{mass} , and $\Delta^{13}C$ and lower WUE_i and stomatal slope parameters compared with *E. dahuricus* (Table 1). Contrary to our first hypothesis, 8 years of N treatments had no effects on photosynthetic traits of either species, despite significant increases in foliar N for both species. No stimulation of photosynthetic traits by N treatment in the present study was not in line with other results from this experiment site demonstrating that N addition increased the aboveground biomass by 24% (Yang et al., 2014). Though nitrogen addition significantly increased

plant productivity in the alpine meadow (Bassin et al., 2012; Fu and Shen, 2017) and other grasslands (LeBauer and Treseder, 2008; Lee et al., 2010), the effect of nitrogen addition on plants' photosynthetic performance on alpine meadow has not been investigated widely. The results implied that the elevated foliar N might not have been partitioned to photosynthetic components, i.e., RuBP carboxylase (Rubisco) or chlorophylls (Bauer et al., 2004), suggesting a decoupling of photosynthesis and elevated foliar N. The proportion of N allocation to Rubisco may not increase, as shown by the maximum Rubisco carboxylation efficiency, which was not altered by N fertilization. The effect of N on plant growth is generally due to both an effect on photosynthesis and leaf growth, which was mostly confirmed

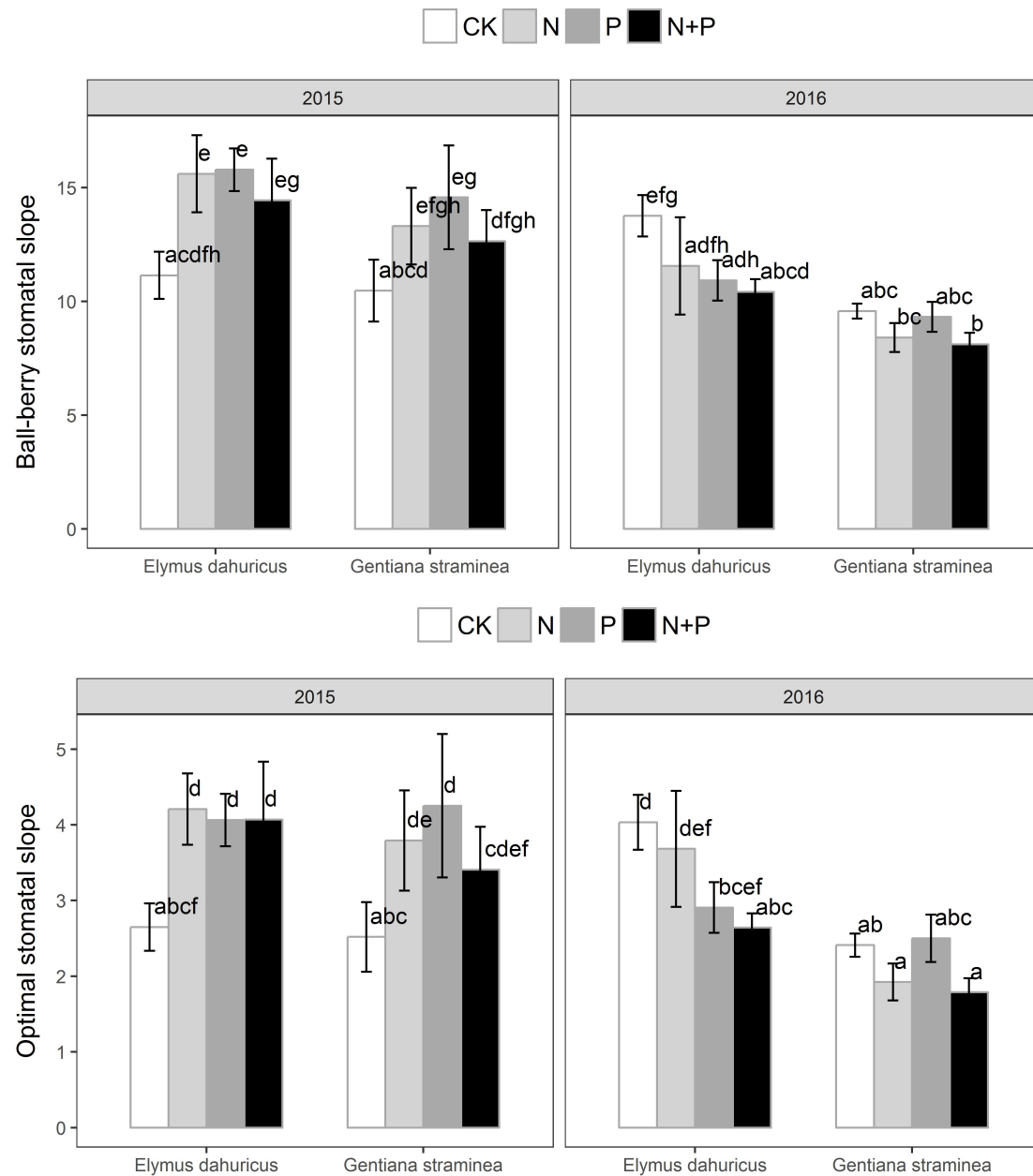
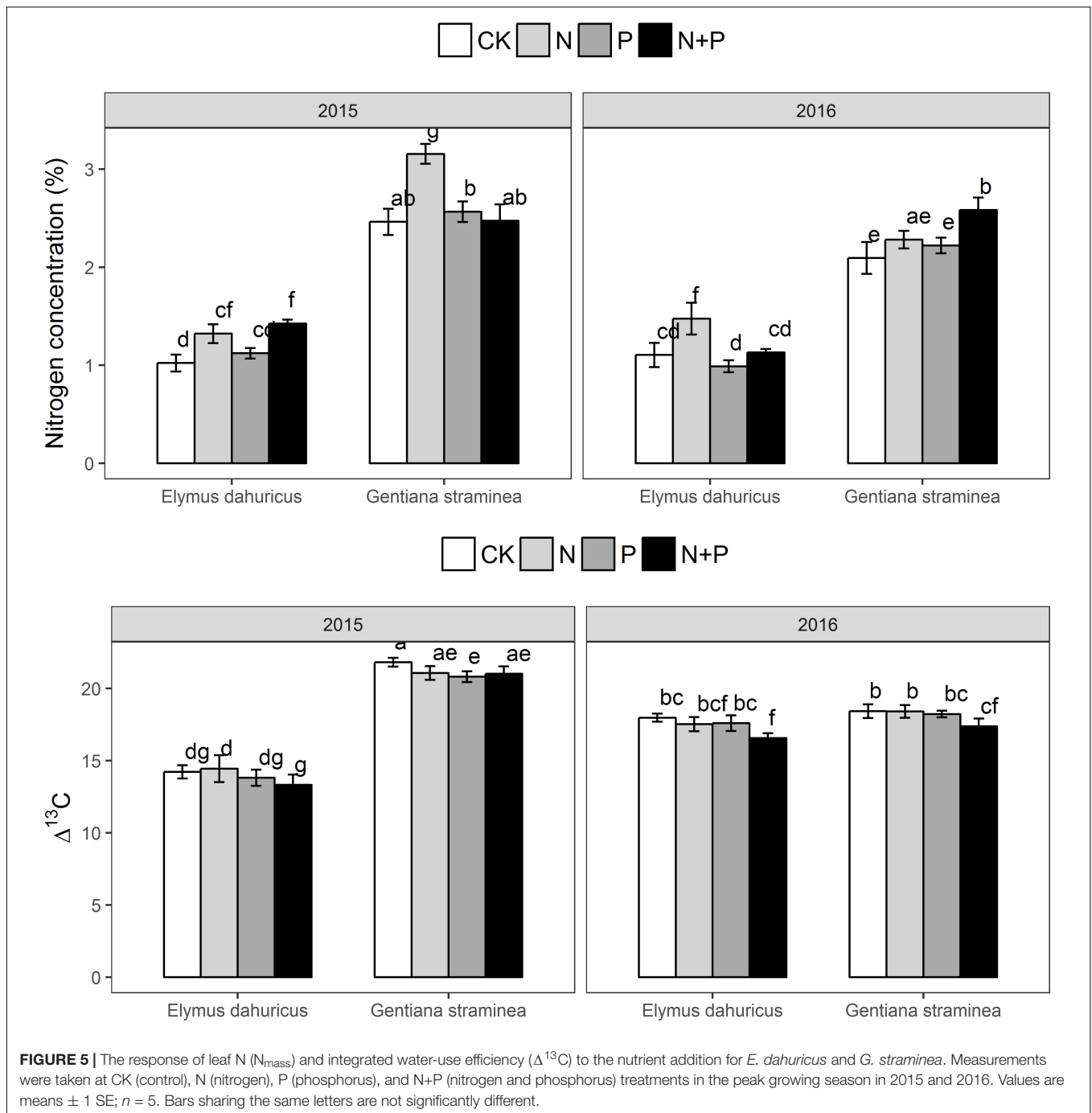


FIGURE 4 | The response of stomatal slope m from Ball-berry model and g_1 from Medlyn model to the nutrient addition for *E. dahuricus* and *G. straminea*. Measurements were taken at CK (control), N (nitrogen), P (phosphorus), and N+P (nitrogen and phosphorus) treatments in the peak growing season in 2015 and 2016. Values are means \pm 1 SE; $n = 5$. Bars sharing the same letters are not significantly different.

on C_3 species. Such long and high N treatments might have eliminated any N limitation to photosynthetic performances.

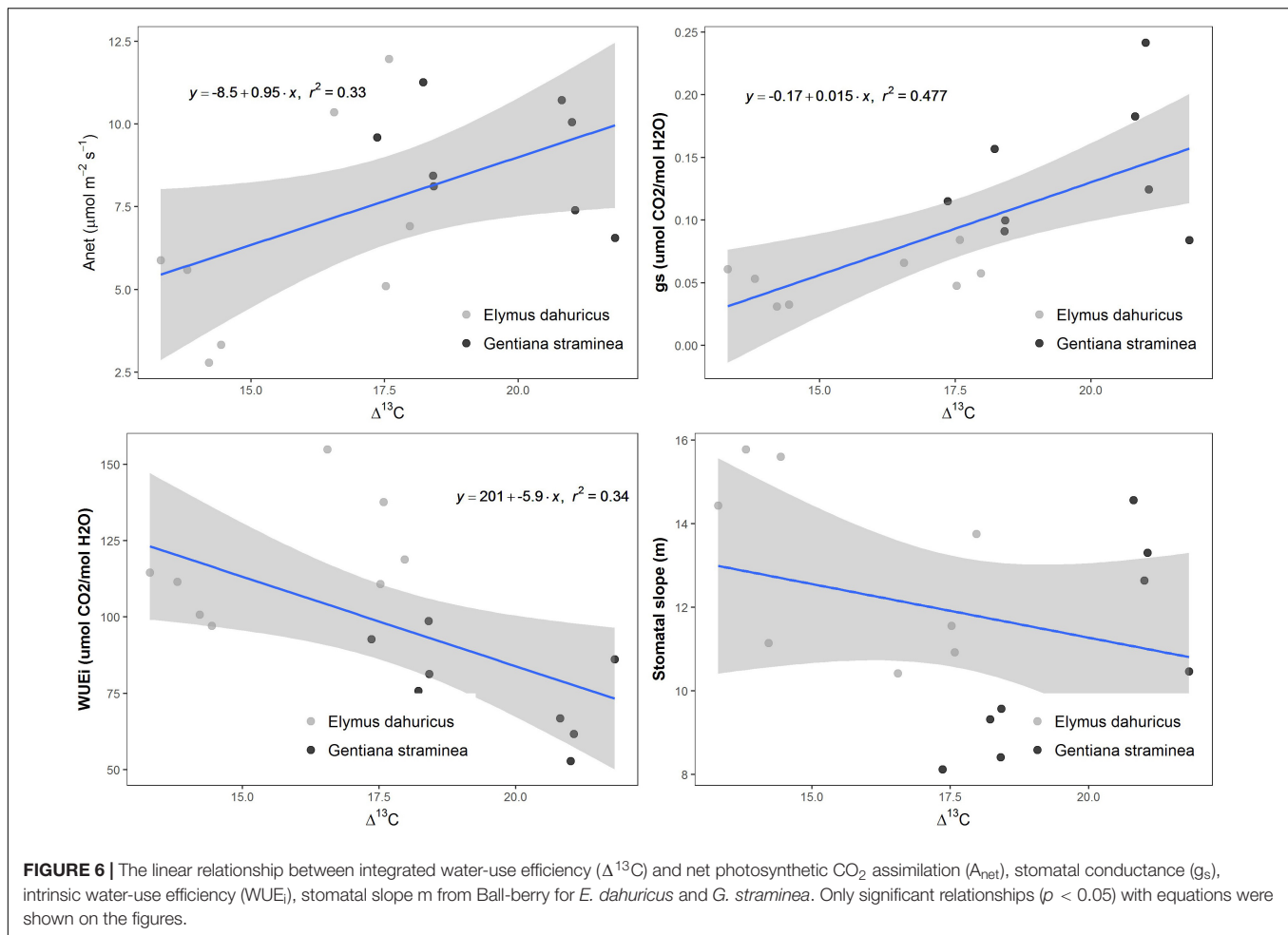
In consistent with earlier findings that P addition increased the aboveground biomass by 52% (Yang et al., 2014), P addition increased A_{net} for both species compared with CK treatment (Figure 1). The increase of A_{net} promoted by P addition may be attributed to increases in g_s , F_v'/F_m' , Φ_{PSII} and Rubisco activity (V_{cmax} and J_{max}) for both species

(Figures 1, 3). Phosphorus (P) nutrient is essential to a variety of plant functions and a major component of nucleic acids, sugar phosphates, ATP, and phospholipids, all of which play important roles in photosynthesis. Low leaf P is thought to limit A_{net} through reductions in ribulose-1,5-bisphosphate (RuBP) regeneration, carboxylation activity, light use efficiency, and stomatal conductance (Campbell and Sage, 2006; Thomas et al., 2006). It had been shown that P supply influenced partitioning of



N to Rubisco and important for RuBP regeneration, suggesting there might be interactive effects of N and P availability on A_{net} (Warren et al., 2005). In a cross-biome analysis of the influence of P on the linear relationship between photosynthetic capacity (A_{max}) and foliar N, the slope of such linear relationship increased with leaf P (Reich et al., 2009). The results in this study indicated there was no additive effect of N+P treatment on the photosynthetic capacity of these two species. In alpine ecosystems on the Tibetan Plateau, these two species were limited by P rather than N availability photosynthetically and P addition

will trigger a stronger positive response of plant photosynthesis than N addition. The findings here suggest that it is important to learn more about the physiology of P effects on A_{net} for modeling carbon and biogeochemical fluxes and vegetation-climate interactions, especially for regions where low P supply may play a role in limiting plant and ecosystem function. The significant year * species and year * treatment effects also suggested that the meteorological conditions might also play a significant effect on the ecophysiological responses of the two key species to the nutrient treatments.



Nutrient addition not only affected plants carbon gain of these two species, but also their stomatal functions in water relations. P addition significantly increased g_s for *E. dahuricus* and *G. straminea*. P stimulation on stomatal conductance indicated that nutrient availability may limit stomatal function and thus was important for maximizing carbon gain. Higher stomatal conductance and thus higher transpiration can enhance nutrient uptake. Variation across PFTs and environmental gradients in the g_1 and m parameters had been reported widely (Medlyn et al., 2011; Lin et al., 2015; Wolz et al., 2017). Consistent with our prediction, there was no altered stomatal sensitivity under different nutrient addition treatments. N, P or N+P treatment increased m and g_1 in 2015 ($p = 0.06$ for *E. dahuricus* and for *G. straminea*). The slope parameter g_1 and m (dimensionless) relating g_s to AH/cs was obtained by fitting the equation to leaf gas-exchange data (Leakey et al., 2006). The values of g_1 and m are largely representative of the ratio g_s/A , the reciprocal of intrinsic water-use efficiency (Franks et al., 2017). Therefore, it might be expected that plants with characteristically higher WUE_i will exhibit lower g_1 , which was the case for the stomatal slope parameter g_1 and m in 2015 and 2016 for both species. The case study of 15 temperate C_3 tree species revealed that long-held assumptions about stomatal function were a substantial

source of error in physiological models of carbon and water fluxes at the leaf scale (Wolz et al., 2017). Current modeling approaches assuming a universal stomatal slope parameter under different conditions could probably propagate the errors to simulations of crop performance, ecosystem function and global biogeochemical cycles.

The ratio C_i/C_a , measured under normal (light-saturated) conditions of leaf gas exchange or as a time-integrated value from carbon isotope discrimination ($\Delta^{13}\text{C}$) in plant material, has long been recognized as an index of plant water-use efficiency. A decline in C_i/C_a [and $\Delta^{13}\text{C}$] is equivalent to an increase in intrinsic water-use efficiency (Farquhar et al., 1989). This relationship can be difficult to resolve because the two variables integrate plant response over different time spans: WUE_i is an instantaneous measurement while $\Delta^{13}\text{C}$ is integrated over the growing season. There was evidence for a negative linear relationship between WUE_i and $\Delta^{13}\text{C}$ in different species and functional types (Roussel et al., 2009; Orchard et al., 2010; Wang et al., 2012). Recent analyses had suggested that using $\Delta^{13}\text{C}$ as an indicator of variation in WUE could be less effective when applied across species (Warren and Adams, 2006; Seibt et al., 2008; Wang et al., 2012; Cernusak et al., 2016). Our study showed that the diversity of $\Delta^{13}\text{C}$ had a negative relationship with WUE_i

of the two species, consistent with previous studies (Figure 6). Plants can increase WUE by increasing the efficiency of carbon fixation inside the leaf, either by increasing the efficiency of light harvesting or carboxylation processes. However, $\Delta^{13}\text{C}$ was positively correlated with A_{net} , implying that the decrease of WUE (increase of $\Delta^{13}\text{C}$) was more driven by the increase of g_s . The overall $\Delta^{13}\text{C}$ during carbon assimilation is dependent on the CO_2 concentration at the sites of carboxylation, which in turn is strongly dependent on mesophyll conductance (g_m). Many studies reported no significant relationship between $\Delta^{13}\text{C}$ and WUE_i (Seibt et al., 2008; McCarthy et al., 2011), claiming mesophyll conductance contributed to the observed variability of $\Delta^{13}\text{C}$.

CONCLUSION

This study provided an ecophysiological investigation of two alpine meadow species after 8-year N and/or P treatments with a systematic measurement of leaf traits across two growing seasons. P and N+P addition improved the photosynthetic capacity for both species. A_{net} of the two alpine species in this study responded similarly to N and/or P treatment and the P stimulation on the A_{net} was associated with increased g_s , F_v/F_m' and V_{cmax} for *E. dahuricus* and *G. straminea*. The stomatal functions including instantaneous measurements of stomatal conductance, intrinsic water-use efficiency and the stomatal slope parameters of the two widely used stomatal models were altered by the addition of P or N+P treatment, but the impact varied across years or species. This suggests that an understanding of photosynthesis, stomatal functions, and water-use should be

evaluated on species basis. The effectiveness of integrating $\Delta^{13}\text{C}$ and intrinsic water-use efficiency was confirmed. Our findings should be useful for understanding the underlying mechanisms of the response of alpine plants to global change.

AUTHOR CONTRIBUTIONS

DW came up with the idea and manage the experimental sites and wrote the paper. TL, PW, PJ, HW, and JF conducted the experiment and analyzed the data. YZ helped with the manuscript writing.

FUNDING

Funding for this research was provided by Nanjing University of Information Science and Technology (2013r115); Jiangsu Distinguished Professor Scholarship, Jiangsu six talent peaks (R2016L15); Jiangsu Natural Science Foundation (BK20150894); National Natural Science Foundation of China (31500503 and 31770485); Jiangsu Overseas Research & Training Program for University Prominent Young & Middle-aged Teachers and Presidents through DW; and International S&T Cooperation Program of China (2012DFA60830).

ACKNOWLEDGMENTS

We thank Dr. Jinsheng He, Dr. Zhenhua Zhang, and Zhiyuan Ma for their assistance with field sampling and measurements.

REFERENCES

- Ball, J., Woodrow, I., and Berry, J. (1987). "A model predicting stomatal conductance," in *Progress in Photosynthesis Research*, ed. J. Biggens (Dordrecht: Martinus Nijhoff), 221–224.
- Bassin, S., Schalajda, J., Vogel, A., and Suter, M. (2012). Different types of sub-alpine grassland respond similarly to elevated nitrogen deposition in terms of productivity and sedge abundance. *J. Veg. Sci.* 23, 1024–1034. doi: 10.1111/j.1654-1103.2012.01422.x
- Bauer, G. A., Bazzaz, F. A., Minocha, R., Long, S., Magill, A., Aber, J., et al. (2004). Effects of chronic N additions on tissue chemistry, photosynthetic capacity, and carbon sequestration potential of a red pine (*Pinus resinosa* Ait.) stand in the NE United States. *For. Ecol. Manage.* 196, 173–186. doi: 10.1016/j.foreco.2004.03.032
- Campbell, C. D., and Sage, R. F. (2006). Interactions between the effects of atmospheric CO_2 content and P nutrition on photosynthesis in white lupin (*Lupinus albus* L.). *Plant Cell Environ.* 29, 844–853. doi: 10.1111/j.1365-3040.2005.01464.x
- Cernusak, L. A., Barbour, M. M., Arndt, S. K., Cheesman, A. W., English, N. B., Feild, T. S., et al. (2016). Stable isotopes in leaf water of terrestrial plants. *Plant Cell Environ.* 39, 1087–1102. doi: 10.1111/pce.12703
- Chinese Soil Taxonomy Research Group (1995). *Chinese Soil Taxonomy*. New York, NY: Science Press, 58–147.
- Elser, J. J., Bracken, M. E., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., et al. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol. Lett.* 10, 1135–1142. doi: 10.1111/j.1461-0248.2007.01113.x
- Farquhar, G. D., Ehleringer, J. R., and Hubick, K. T. (1989). Carbon isotope discrimination and photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 40, 503–537. doi: 10.1146/annurev.pp.40.060189.002443
- Farquhar, G. D., O'Leary, M. H., and Berry, J. A. (1982). On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. *Funct. Plant Biol.* 9, 281–292.
- Field, C. B., and Mooney, H. A. (1986). "The photosynthesis-nitrogen relationship in wild plants," in *On the Economy of Plant Form and Function*, ed. T. J. Givnish (Cambridge: Cambridge University Press), 35–55.
- Franks, P. J., Berry, J. A., Lombardozzi, D. L., and Bonan, G. B. (2017). Stomatal function across temporal and spatial scales: deep-time trends, land-atmosphere coupling and global models. *Plant Physiol.* 174, 583–602. doi: 10.1104/pp.17.00287
- Fu, G., and Shen, Z. (2017). Response of alpine soils to nitrogen addition on the Tibetan plateau: a meta-analysis. *Appl. Soil Ecol.* 114, 99–104. doi: 10.1016/j.apsoil.2017.03.008
- Harpole, W. S., Ngai, J. T., Cleland, E. E., Seabloom, E. W., Borer, E. T., Bracken, M. E., et al. (2011). Nutrient co-limitation of primary producer communities. *Ecol. Lett.* 14, 852–862. doi: 10.1111/j.1461-0248.2011.01651.x
- Huang, Y., Olbrecht, L., Yang, X., and He, J. (2014). Effects of nutrient additions on the arbuscular mycorrhizal fungal colonization in the alpine meadow on the Tibetan Plateau. *Beijing Daxue Xuebao (Ziran Kexue Ban)*. *Acta Sci. Nat. Univ. Pekinensis* 50, 911–918.
- IPCC (2013). *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge: Cambridge University Press.

- Laisk, A., Eichelmann, H., Oja, V., Rasulov, B., Padu, E., Bichele, I., et al. (2005). Adjustment of leaf photosynthesis to shade in a natural canopy: rate parameters. *Plant Cell Environ.* 28, 375–388. doi: 10.1111/j.1365-3040.2004.01274.x
- Leakey, A. D. B., Bernacchi, C. J., Ort, D. R., and Long, S. P. (2006). Long-term growth of soybean at elevated CO₂ does not cause acclimation of stomatal conductance under fully open-air conditions. *Plant Cell Environ.* 29, 1794–1800. doi: 10.1111/j.1365-3040.2006.01556.x
- LeBauer, D. S., and Treseder, K. K. (2008). Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89, 371–379. doi: 10.1890/06-2057.1
- Lee, M., Manning, P., Rist, J., Power, S. A., and Marsh, C. (2010). A global comparison of grassland biomass responses to CO₂ and nitrogen enrichment. *Philos. Trans. R. Soc. B Biol. Sci.* 365, 2047–2056. doi: 10.1098/rstb.2010.0028
- Lin, Y. S., Medlyn, B. E., Duursma, R. A., Prentice, I. C., Wang, H., Baig, S., et al. (2015). Optimal stomatal behaviour around the world. *Nat. Clim. Change* 5, 459–464. doi: 10.1038/nclimate2550
- Liu, L., and Greaver, T. L. (2010). A global perspective on belowground carbon dynamics under nitrogen enrichment. *Ecol. Lett.* 13, 819–828. doi: 10.1111/j.1461-0248.2010.01482.x
- Lv, C., and Tian, H. (2007). Spatial and temporal patterns of nitrogen deposition in China: synthesis of observational data. *J. Geophys. Res.* 112:D22S05.
- McCarthy, H. R., Diane, P. E., and Jenerette, G. D. (2011). Plant water-use efficiency as a metric of urban ecosystem services. *Ecol. Appl.* 21, 3115–3127. doi: 10.1890/11-0048.1
- Medlyn, B. E., Barton, C. V. M., Broadmeadow, M. S. J., Ceulemans, R., De Angelis, P., Forstreuter, M., et al. (2001). Stomatal conductance of forest species after long-term exposure to elevated CO₂ concentration: a synthesis. *New Phytol.* 149, 247–264. doi: 10.1046/j.1469-8137.2001.00028.x
- Medlyn, B. E., Duursma, R. A., Eamus, D., Ellsworth, D. S., Prentice, I. C., Barton, C. V. M., et al. (2011). Reconciling the optimal and empirical approaches to modeling stomatal conductance. *Glob. Change Biol.* 17, 2134–2144. doi: 10.1111/j.1365-2486.2010.02375.x
- Miao, Z., Xu, M., Richard, G., Lathrop, J. R., and Wang, Y. (2009). Comparison of the A–C_i curve fitting methods in determining maximum ribulose 1,5-bisphosphate carboxylase/oxygenase carboxylation rate, potential light saturated electron transport rate and leaf dark respiration. *Plant Cell Environ.* 32, 109–122. doi: 10.1111/j.1365-3040.2008.01900.x
- Orchard, K. A., Cernusak, L. A., and Hutley, L. B. (2010). Photosynthesis and water-use efficiency of seedlings from northern Australian monsoon forest, savanna and swamp habitats grown in a common garden. *Funct. Plant Biol.* 37, 1050–1060. doi: 10.1071/FP09306
- Oren, R., Sperry, J. S., Katul, G. G., Pataki, D. E., Ewers, B. E., Phillips, N., et al. (1999). Survey and synthesis of intra- and interspecific variation in stomatal sensitivity to vapour pressure deficit. *Plant Cell Environ.* 22, 1515–1526. doi: 10.1046/j.1365-3040.1999.00513.x
- Reich, P. B., Oleksyn, J., and Wright, I. J. (2009). Leaf phosphorus influences the photosynthesis-nitrogen relation: a cross-biome analysis of 314 species. *Oecologia* 160, 207–212. doi: 10.1007/s00442-009-1291-3
- Roussel, M., Dreyer, E., Montpied, P., Le-Provost, G., Guehl, J. M., and Brendel, O. (2009). The diversity of (13)C isotope discrimination in a *Quercus robur* fullsib family is associated with differences in intrinsic water use efficiency, transpiration efficiency, and stomatal conductance. *J. Exp. Bot.* 60, 2419–2431. doi: 10.1093/jxb/erp100
- Seibt, U., Rajabi, A., Griffiths, H., and Berry, J. A. (2008). Carbon isotopes and water use efficiency: sense and sensitivity. *Oecologia* 155, 441–454. doi: 10.1007/s00442-007-0932-7
- Song, M. H., and Yu, F. H. (2015). Reduced compensatory effects explain the nitrogen mediated reduction in stability of an alpine meadow on the Tibetan Plateau. *New Phytol.* 207, 70–77. doi: 10.1111/nph.13329
- Thomas, D. S., Montagu, K. D., and Conroy, J. P. (2006). Leaf inorganic phosphorus as a potential indicator of phosphorus status, photosynthesis and growth of *Eucalyptus grandis* seedlings. *For. Ecol. Manage.* 223, 267–274. doi: 10.1016/j.foreco.2005.11.006
- Turnbull, T. L., Kelly, N., Adams, M. A., and Warren, C. R. (2007). Within-canopy nitrogen and photosynthetic gradients are unaffected by soil fertility in field-grown *Eucalyptus globulus*. *Tree Physiol.* 27, 1607–1617. doi: 10.1093/treephys/27.11.1607
- Wang, D., Maughan, M. W., Sun, J., Feng, X., Miguez, F., Lee, D., et al. (2012). Impact of nitrogen allocation on growth and photosynthesis of *Miscanthus (Miscanthus x giganteus)*. *Glob. Change Biol. Bioenergy* 4, 688–697. doi: 10.1111/j.1757-1707.2012.01167.x
- Warren, C. R., and Adams, M. A. (2006). Internal conductance does not scale with photosynthetic capacity: implications for carbon isotope discrimination and the economics of water and nitrogen use in photosynthesis. *Plant Cell Environ.* 29, 192–201. doi: 10.1111/j.1365-3040.2005.01412.x
- Warren, C. R., McGrath, J. F., and Adams, M. A. (2005). Differential effects of N, P and K on photosynthesis and partitioning of N in *Pinus pinaster* needles. *Annu. For. Sci.* 62, 1–8. doi: 10.1051/forest:2004088
- Wolz, K., Timothy, W., Mark, A., Wang, D., and Leakey, A. (2017). Diversity in stomatal function is integral to modelling plant carbon and water fluxes. *Nat. Ecol. Evol.* 1, 1292–1298. doi: 10.1038/s41559-017-0238-z
- Yang, X. X., Ren, F., Zhou, H. K., and He, J. S. (2014). Responses of plant community biomass to nitrogen and phosphorus additions in an alpine meadow on the Qinghai-Xizang Plateau. *Chin. J. Plant Ecol.* 38, 159–166. doi: 10.3724/SP.J.1258.2014.00014

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

The reviewer AG and handling Editor declared their shared affiliation.

Copyright © 2018 Wang, Ling, Wang, Jing, Fan, Wang and Zhang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.