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Effects of nitrogen deposition on ecosystems above and belowground

Edited by
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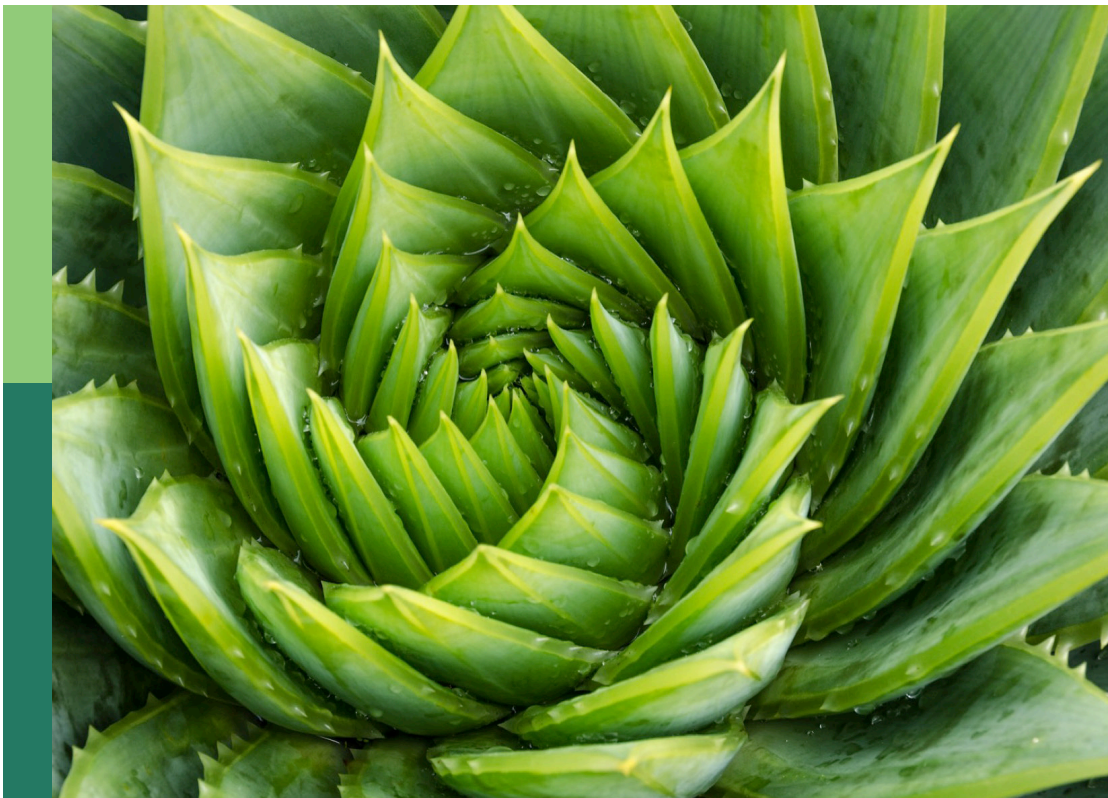

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Effects of nitrogen deposition on ecosystems above and belowground

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Differential Mechanisms Drive Species Loss Under Artificial Shade and Fertilization in the Alpine Meadow of the Tibetan Plateau

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Fertilization is an effective management strategy to promote community biomass but can simultaneously reduce species diversity in many grassland systems. Shifts in competition for resources have been proposed to explain the decline in plant species diversity due to fertilization, yet the underlying mechanism driving species loss remains controversial. This uncertainty may be driven by variation in aboveground and belowground resource availability. However, experiments simultaneously manipulating both light availability and soil nutrients are rare. Using a 6-year field experiment to manipulate light availability (*via* shade cloth) and soil nutrients (*via* fertilizer addition), we tested this resource competition hypothesis in a species-rich alpine meadow by examining the variation of species traits associated with the capacity of light acquisition within these treatments. Our results showed that artificial shade decreased community biomass accumulation whereas fertilization increased it. In contrast, both shade and fertilization reduced species diversity. Extinction of non-*Gramineae* species (e.g., *Fabaceae* and *Cyperaceae*) was the main reason for species diversity decline. Species loss can be explained by the limitation of light availability and predicted by species traits associated with light acquisition capability under fertilization and low light tolerance under artificial shade. Specifically, fertilization eliminated species with lower stature and artificial shade exterminated species with the higher light compensation point (LCP). The findings suggest that light availability is consistently important for plant growth and that low competitiveness for light under fertilization and intolerance of low light conditions under artificial shade trigger species loss process in the alpine meadow. Our experiment helps clarify the mechanisms of how artificial shade and fertilization decreased species diversity and highlight that LCP, which tends to be neglected by most of the studies, is one of the vital drivers in determining species coexistence.

Keywords: alpine meadow, fertilization, functional traits, artificial shade, species loss, resource competition

INTRODUCTION

Human activities, such as industrial agriculture, are altering terrestrial nutrient availability worldwide by widespread fertilizer application (Yang et al., 2013; IPCC, 2014; FAO et al., 2017). Fertilizer application is a common and efficient management practice to promote productivity but reduces plant species diversity in grassland ecosystems. However, understanding the specific mechanisms of species loss in response to nutrient enrichment remains a challenge for ecologists (Isbell et al., 2013; Harpole et al., 2016; DeMalach et al., 2017).

The decline of plant species diversity at high soil fertility or high productivity is thought to be a result of increased competition for light (Hautier et al., 2009; DeMalach et al., 2017). The light competition hypothesis predicts that competition may shift from belowground when soil resources are limited to aboveground when soil resources are abundant but shading is intense (Newman, 1973). This reduction in photosynthetically active radiation (PAR) may, over time, lead to the competitive exclusion of shade-intolerant species and improved fitness for light resource acquisitive species (Tilman, 1982; Hautier et al., 2009).

While previous studies have observed species decline due to a reduction in light availability after fertilizer application (Carson and Pickett, 1990; Hautier et al., 2009), there is strong evidence that an increase in nutrient availability after fertilization decreased plant species diversity even when the light was not limiting (Harpole and Tilman, 2007; Dickson and Foster, 2011; Borer et al., 2014; Yang et al., 2015; Harpole et al., 2016). This pattern was further supported by two short-term ($\leq$2 years) field experiments, which used shade cloth to reduce light availability without changing species diversity (Rajaniemi, 2002; Li et al., 2011).

While the majority of experimental approaches used to investigate the relationship between light competition and diversity loss are derived from community-level studies (Harpole et al., 2016; DeMalach et al., 2017; Li et al., 2017), fewer studies have focused on individual-level responses to nutrient enrichment and light limitation based on functional traits associated with light acquisition (Suding et al., 2005; Dickson et al., 2014; Yang et al., 2015). For example, plant height is a well-documented trait that relates to improve light acquisition (Grime, 2001), with taller plants having an advantage in low-light conditions driven by the increase of community biomass in response to fertilization (Hautier et al., 2009; Borer et al., 2014). Another key functional trait affecting the survival of plant species under light-limiting conditions is light compensation point (LCP), which is defined as the minimum light level required for plant survival and regular growth. LCP reflects the shade tolerance of a species (Horn, 1971), and species with low LCP typically correspond to an increase in carbon acquisition, higher shade tolerance, and high survival probability under limited light availability conditions (Kitao et al., 2016). Thus, functional traits associated with light capture and shade tolerance may dictate species performance in response to reduced light availability.

Alpine meadows within the Tibetan Plateau are a climate-sensitive ecosystem and currently face the selection pressures of increasing nutrient loading (IPCC, 2014; Zhang et al., 2019; Ma et al., 2021; Sun et al., 2021a,b). Understanding the mechanism controlling species diversity is essential for maintaining ecological equilibrium in this sensitive ecosystem. However, the underlying mechanisms driving species loss after nutrient addition remain controversial. In this study, we presented results of the field experiment of 6 consecutive years by manipulating light availability indirectly *via* increasing soil resource availability and directly *via* shade cloth in an alpine meadow of the Tibetan Plateau. We compared the effects of nutrient addition and artificial shade on species diversity and community composition and then, explored the relationships between species relative abundance (SRA) and functional traits to address the following three questions. (1) How does plant species diversity respond to changes in light and nutrient availability? (2) Do changes in community composition within the artificial shade and fertilization treatment convergent or divergent along a temporal scale? and (3) Are the functional traits associated with light acquisition capability and the minimum light level required for photosynthesis good indicators to predict species loss in the alpine meadow of the Tibetan Plateau?

MATERIALS AND METHODS

Study Site

This experiment was performed at the Research Station of Alpine Meadow and Wetland Ecosystems of Lanzhou University located in Maqu County (34°00′N, 102°00′E; 3,500 m above the sea level), eastern Tibetan Plateau, China. The annual average temperature during the sampling period (2008–2012) ranged from −8.2°C in winter to 11.7°C in summer, with the annual average precipitation of 706 mm, mainly distributed during the short, cool summer (**Supplementary Figure 1**). The vegetation, typical alpine meadows of the Tibetan Plateau, is dominated by perennial sedges (e.g., *Kobresia graminifolia*), grasses (e.g., *Poa botryoides and Elymus nutans*), species of *Compositae* (e.g., *Saussurea nigrescens*), and other broad-leaved species (e.g., *Anemone rivularis*). The average aboveground biomass within the experimental site (450 m × 220 m) ranges from 280 to 400 g m^{-2} dry weight, which corresponds to the median value of global grassland productivity; and the species richness ranges from 20 to 35 per 0.25 m^2, at the upper limit of species diversity among the global grassland ecosystems (Borer et al., 2014).

Experimental Design

To assess the impact of light and soil nutrient availability on species diversity, two blocks (25 m × 30 m) were established within the fenced exclosure (450 m × 220 m) to exclude herbivores. Light availability was manipulated by covering one block with a black polypropylene mesh shade cloth, which was permeable to air and water, to reduce 70 ± 2% (mean ± SD) of PAR. The shade cloth was suspended 1.3 m above the ground surface by wooden stakes attached to the corners of one block and fastened to the ground on all sides. The other block remained

uncovered, corresponding to ambient light availability. Nutrient availability was manipulated by two fertilizer addition levels: 0 g m^{-2} $year^{-1}$ (no fertilizer addition) and 45 g m^{-2} $year^{-1}$ of a slow-release pelletized fertilizer [$(NH_4)_2HPO_4$] manufactured by Tianjin International Trading Company, Tianjin, China, corresponding to 9.5 g N m^{-2} and 10.6 g P m^{-2}. The nutrient treatment was nested within the light availability treatment block, resulting in a total of four treatments: fertilizer addition and ambient light (F); no fertilizer addition and shade (S); synchronous fertilizer addition and shade (F+S); and no fertilizer addition and ambient light (CK). Thirty-two permanent 2 × 2 m plots were established with 16 plots under the shade shed and the other 16 plots outside the shed, arranged in a regular 4 × 4 matrix with a 2-m buffer zone between plots. Half of the plots both inside and outside of the shade shed were randomly selected to add fertilizer, which means that each treatment had eight replications (refer to **Supplementary Figure 2**, for more details). The experiment began with a background survey (n = 12) to evaluate the entire experimental community characteristics of the location site on August 25, 2007. Shade and fertilization treatments were applied annually at the beginning of each growing season (end of May) from 2008 to 2012. Artificial shade shed was dismissed annually during the nongrowing season (usually from October to next May) to avoid destruction by local strong wind and heavy snow.

Species Diversity and Aboveground Biomass

To measure species diversity, one quadrat (0.5 m × 0.5 m) was randomly selected in each plot at the end of each growing season (September), approximately 0.5 m away from the edge to avoid the edge effect. Within each quadrat, species were identified, and the number of all individuals was recorded. For clonal species, we regarded a ramet as an individual (Yang et al., 2013). To quantify aboveground biomass, the aboveground parts of all plant individuals rooted within each quadrat were harvested. All samples were dried at 60°C for 72 h and weighed (accuracy of 10^{-2} g). We separated all species into two functional groups: *Gramineae* and non-*Gramineae* groups, which generally correspond with their appearance in the canopy and understory within the quadrats, respectively. Within the non-*Gramineae* group, species belonging to *Cyperaceae* and *Fabaceae* families were analyzed as separate subgroups since these species are known to be sensitive to light resource availability (**Supplementary Table 1**; Li et al., 2011).

Belowground Biomass and Soil Nutrients

To assess belowground biomass and soil nutrients, we employed the root in-growth method (3.5 cm diameter × 20 cm deep) to estimate the belowground biomass from each quadrat post aboveground harvesting in 2012. Soil samples were brought to the laboratory in air-tight plastic bags, and then, roots were washed from the soil cores with a 2-mm mesh sieve after air-drying. Root samples were dried at 60°C for 72 h and weighed (accuracy of 10^{-2} g). Soil characteristics (including pH, total nitrogen, total phosphorus, total organic carbon, available nitrogen, and available phosphorus) were analyzed using the protocol described by Liu et al. (2015).

Light Availability

Light availability was measured in each plot using a Decagon Sunfleck Ceptometer (Decagon, Pullman, Washington, DC, United States) in August 2012. Light readings were taken on a cloudless day between 11:00 and 13:00 h. The ceptometer was placed north-south across each plot, and PAR was recorded at 0, 10, and 40 cm above the soil surface (below vegetation canopy). Additionally, PAR above the vegetation canopy was also recorded in each plot as a measure of full sunlight. The soil temperature was not monitored during the experimental period for the reason that the shade shed was permeable to air and water and suspended 1.3 m above the ground, resulting in no significant difference with fertilization plots in soil moisture and understory PAR (**Table 1**).

Trait Measurements

Prior to the community biomass collection in 2012, we selected 14 common species in fertilization plots, 17 species in shade plots, 7 species in synchronous shade and fertilization plots, and 21 species in control plots to measure functional traits (**Supplementary Table 1**). These species accounted for more than 92.4, 89.2, 94.7, and 82.5% of the aboveground biomass in fertilized, shaded, simultaneous shade and fertilization and the control communities, respectively. For each treatment, we measured the maximum height of 24 individuals (3 individuals per plot) of each species. The photosynthesis-light response curve of each species was measured for 3 fully expanded leaves using the LICO LI-6400 portable photosynthesis system. PAR levels of 0, 20, 50, 80, 100, 150, 200, 400, 800, 1,600, and 1,800 μmol m^{-2} s^{-1} were provided by red light-emitting diodes. Ambient air in the leaf chamber was maintained at 20°C, and the CO_2 concentration of the incoming air was controlled at 400 μmol L^{-1}. Parameters of photosynthesis-light response curve were simulated by the nonrectangular hyperbola equation using SPSS software (version 22.0). The model is given as follows:

$$A = \left(aPAR + Amax - \sqrt{\left((aPAR + Amax)^2 - 4kaPAR \times Amax\right)}\right)/2k - Rday$$

where A is the net leaf photosynthesis rate (μmol m^{-2} s^{-1}), Amax is the light-saturated net leaf photosynthesis rate (μmol m^{-2} s^{-1}), a is the light-limited quantum efficiency (μmol CO_2 μmol^{-1} photons), k is the curvature parameter, and Rday is the dark respiration rate. When A is zero, the PAR is the LCP.

Data Analysis

Two plant diversity indexes are calculated to describe the species diversity within each treatment plot. One is species richness, which is defined as the number of species present in a quadrat

TABLE 1 | Light intensity and soil property in different treatments and the results of two-way ANOVA for the effects of fertilization (F), shade (S), and their interaction.

Environmental variables									
	PAR (0 cm HAG) (μmol m^{-2} s^{-1})	PAR (10 cm HAG) (μmol m^{-2} s^{-1})	PAR (40 cm HAG) (μmol m^{-2} s^{-1})	Light availability score	Moisture (%)	pH	SOC (%)	SAN (mg kg^{-1})	SAP (mg kg^{-1})
Control (CK)	410.38 ± 34.72a	1212.13 ± 85.64a	5965.50 ± 102.15a	1.64 ± 0.13a	42.2 ± 1.45a	7.12 ± 0.02a	2.05 ± 0.11a	14.45 ± 0.19c	1.76 ± 0.07b
Fertilization (F)	107.13 ± 5.60b	332.00 ± 65.69b	3248.25 ± 112.89b	−0.24 ± 0.05b	42.4 ± 0.99a	6.91 ± 0.17a	1.97 ± 0.12a	17.94 ± 0.59b	63.68 ± 6.09a
Shade (S)	117.25 ± 7.57b	238.00 ± 16.32b	1441.50 ± 17.06c	−0.62 ± 0.02c	40.4 ± 2.92a	7.09 ± 0.12a	1.85 ± 0.04a	14.65 ± 0.24c	1.82 ± 0.24b
F+S	43.00 ± 4.30c	238.75 ± 14.83b	1472.25 ± 134.42c	−0.78 ± 0.03c	44.5 ± 1.33a	6.96 ± 0.07a	1.95 ± 0.08a	23.47 ± 1.07a	64.81 ± 1.65a
Summary of two-way ANOVA									
Effect of F	***F* = 108.54**	***F* = 63.72**	***F* = 173.76**	***F* = 205.95**	*F* = 1.38	*F* = 2.58	*F* = 0.03	***F* = 81.96**	***F* = 493.64**
	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	*P* = 0.258	*P* = 0.128	*P* = 0.878	***P* < 0.001**	***P* < 0.001**
Effect of S	***F* = 97.21**	***F* = 93.88**	***F* = 955.53**	***F* = 387.89**	*F* = 0.01	*F* = 0.01	*F* = 1.40	*F* = 1.77	*F* = 0.05
	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	*P* = 0.931	*P* = 0.949	*P* = 0.255	*P* = 0.284	*P* = 0.830
Effect of F × S	***F* = 39.94**	***F* = 63.94**	***F* = 181.80**	***F* = 145.36**	*F* = 1.13	*F* = 0.13	*F* = 1.01	***F* = 15.37**	*F* = 0.04
	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	***P* < 0.001**	*P* = 0.305	*P* = 0.722	*P* = 0.329	***P* = 0.001**	*P* = 0.850

Data were means ± SE (n = 8) collected from the last year of the experiment (2012). PAR, HAG, SOC, SAN, and SAP stand for photosynthetically active radiation, height of above the ground, soil organic carbon, soil available nitrogen, and soil available phosphorus, respectively. Significant differences among treatments within each variable were determined using the least-significant difference (LSD) test (P ≤ 0.05) and indicated by different letters. Significant effects are in bold. PCA ordinations showed that the variations of light (83.0%) could be well explained by the light availability score (PC1).

and the other one is the Shannon–Weiner index (H), which is calculated as:

$$H = -\sum_{i-1}^{s} (p_i)(\ln p_i)$$

where p_i is the relative abundance of species i in a quadrat and s is the species richness in the quadrat. We also calculated SRA (SRA = number of individuals of a given species in a quadrat/total number of individuals for all species in the quadrat) for each treatment plot.

Prior to analysis, all response variables were tested to meet the assumptions of normality and heterogeneity of variance. Any variable not meeting these assumptions was log-transformed. To account for differences in light resource availability at different levels of the canopy, the scores of the first principle component (PC1) of principal component analysis (PCA) were viewed as a proxy of light availability (Liu et al., 2015). The PC1 described 83% of the variation in light availability at different heights above the soil surface (**Supplementary Figure 3**). First, two-way ANOVA was performed to test the effects of fertilization and shade on belowground biomass and local abiotic conditions because all the above response variables were only measured in the last year of the experiment (2012). Second, we used repeated measure ANOVA to test for the significance of the effects of fertilization and shade on plant community characteristics across the 5 years (2008–2012). The years were used as within-subject factors, and fertilization and shade were used as between-subject factors. Third, the significant differences of each response variables among treatment plots were determined using the least significant difference (LSD) test at the 95% CI. Linear regression was used to assess the relationship between light availability and species diversity and plant functional traits and SRA. Fourth, we used paired-samples t-test (2-tailed) to test for differences of plant functional traits of *Gramineae* and non-*Gramineae* groups among the treatment plots at the community level. All the above statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, United States).

To assess the differences in community composition between the treatment plots, we computed the dissimilarities of plant species composition among different treatment plots along temporal scale by nonmetric multidimensional scaling (NMDS) with Bray-Curtis distance using the "metaMDS" function of the R package "Vegan" (Oksanen et al., 2010). The statistical method can depict community composition in multidimensions, and the variance of samples is maximized on the first dimension (Oksanen et al., 2010). Ordination was constructed in two dimensions and calculated with R-program using Windows version 3.4.2.

Structural equation modeling (SEM) was employed to examine the relationships of fertilization, shade, and species functional traits and SRA among *Gramineae* and non-*Gramineae* groups, respectively. The pathways through which fertilization and shade influence SRA were also assessed (Shipley, 2002). Two categorical variables were chosen to describe the treatments: fertilization (0 = no fertilizer addition; 1 = fertilization) and shade (0 = no shade cloth; 1 = shade). Results from the best fitness

SEM showed that fertilization and shade affect SRA by changing species height and LCP (indicated by one-way arrows in the path diagrams). The method has been widely used previously (Socher et al., 2012; Li et al., 2017). All SEMs were performed using AMOS 20.0 (SPSS Inc., Chicago, IL, United States).

RESULTS

Effects of Fertilization and Shade on Local Abiotic Variables

The light intensity varied among 0, 10, and 40 cm layers of the community in the control plots (F = 581.3, $P < 0.001$) and were significantly affected by fertilization, shade, and their interactions (all $P < 0.01$). On average, light intensities (0–40 cm heights above the ground) were 2,529 ± 74 μmol m^{-2} s^{-1} in the control plots. Fertilization, shade, and F+S treatments significantly reduced light intensity by 51.4, 76.3, and 76.9%, respectively (all $P < 0.001$, **Table 1**). Within the control plots, soil available nitrogen (SAN) and soil available phosphorous (SAP) were recorded as 14.45 ± 0.19 and 1.76 ± 0.07 mg kg^{-1}, respectively. Fertilization dramatically increased SAN and SAP by 24.2 and 3,518.2% (both $P < 0.001$), respectively. In addition, shade treatment had no significant effects on either SAN or SAP (both $P > 0.05$). There was a significant interactive effect of shade and fertilization on SAN ($P = 0.001$), yet the interaction effect was not detected on SAP ($P > 0.05$). Neither fertilization nor shade treatment significantly affected soil moisture, soil pH, and soil organic carbon (all $P > 0.05$, **Table 1**).

Effects of Fertilization and Shade on Species Diversity and Community Variables

Species Diversity

A total of 40 species belonging to 18 families were recorded, and no significant effect of year on species richness and Shannon–Weiner index was detected in the control plots across the 6 years (both $P > 0.05$, **Supplementary Table 1**). In contrast, species richness and Shannon–Weiner index were reduced by an average of 26.0 and 10.7% in fertilization plots, 33.9 and 11.7% in shade plots, 61.1 and 34.6% in F+S plots, respectively (**Figures 1A,B** and **Table 2**). The number of individuals per quadrat was also significantly reduced by an average of 33.4% in fertilization plots, 57.9% in shade plots, 72.1% in F+S plots, respectively (**Figure 1C** and **Table 2**).

Community Biomass

Fertilization significantly increased aboveground biomass by 53.0% but had no significant effect on belowground biomass, resulting in a 44.0% decrease in the root:shoot ratio, while shade significantly decreased aboveground by 50.2% and belowground biomass by 43.6%, resulting in a neutral influence on the root:shoot ratio. The effect F+S treatment significantly decreased aboveground by 31.2% and belowground biomass by 54.9%, resulting in a neutral influence on the root:shoot ratio as same as the shade treatment (**Figure 1D** and **Table 2**).

Species Relative Abundance

Compared with the control plots, the SRA of *Gramineae* showed a significant increase in response to fertilization (92.4%), shade (49.2%), and F+S treatment (142.8%), and the SRA of non-*Gramineae* inevitably decreased in response to fertilization (27.3%), shade (14.6%), and F+S treatment (42.2%) (**Figures 1E,F** and **Table 2**). The treatments performed a remarkable influence on the SRA of the two groups along the temporal scale, especially for the *Fabaceae* and *Cyperaceae* within the non-*Gramineae* group, (**Figures 1G,H**).

Community Composition

Nonmetric multidimensional scaling ordinations of plant communities revealed that prior to the implementation of treatments, there was no distinct separation of species composition among the initial communities (**Figure 2A**). Since 2008, significant separation trends among different treatment plots were gradually exhibited along the first two dimensions of the NMDS over time (**Figures 2B–F**). Plant community similarity in all treatment plots diverged from control plots, whereas composition did not significantly differ between fertilization plots and shade plots. Dominant species shifted from a mixture of *Trigonella ruthenica (Fabaceae), Kobresia capillifolia (Cyperaceae), and E. nutans (Gramineae)* in control plots to *Gramineae* species with high height (*Poa poophagorum* and *Koeleria cristata*) and non-*Gramineae* shade-tolerant species (*Anemone obtusiloba*) in fertilization plots and shade plots (**Supplementary Table 2**).

Effects of Fertilization and Shade on Species Functional Traits

Results of paired-samples t-test showed that shade significantly increased species height of *Gramineae* and non-*Gramineae*, while fertilization significantly increased species height of *Gramineae* but had no significant effect on non-*Gramineae* (**Table 3** and **Supplementary Table 2**). Meanwhile, fertilization significantly increased the LCP of *Gramineae* and non-*Gramineae* groups, while shade significantly decreased LCP of non-*Gramineae* but had no significant effect on the *Gramineae* group. Neither species height nor LCP was significantly affected by the F+S treatment (**Table 3** and **Supplementary Table 2**).

Relationships Among Abiotic Variables, Community Variables, and Functional Traits

Significantly positive correlations were detected between scores of light availability and species richness (R^2 = 0.76, $P < 0.001$, **Figure 3A**) and Shannon–Weiner index (R^2 = 0.55, $P < 0.001$, **Figure 3B**). There was a strong negative relationship between LCP and SRA in shade plots (R^2 = 0.64, $P < 0.001$), but the relationship was not detected in the control, fertilization, or F+S plots (**Figure 3C**). Species height was positively correlated with SRA in fertilization plots (R^2 = 0.58, $P < 0.001$), whereas no such relationship was observed in the control, shade, or F+S treatment plots (**Figure 3D**).

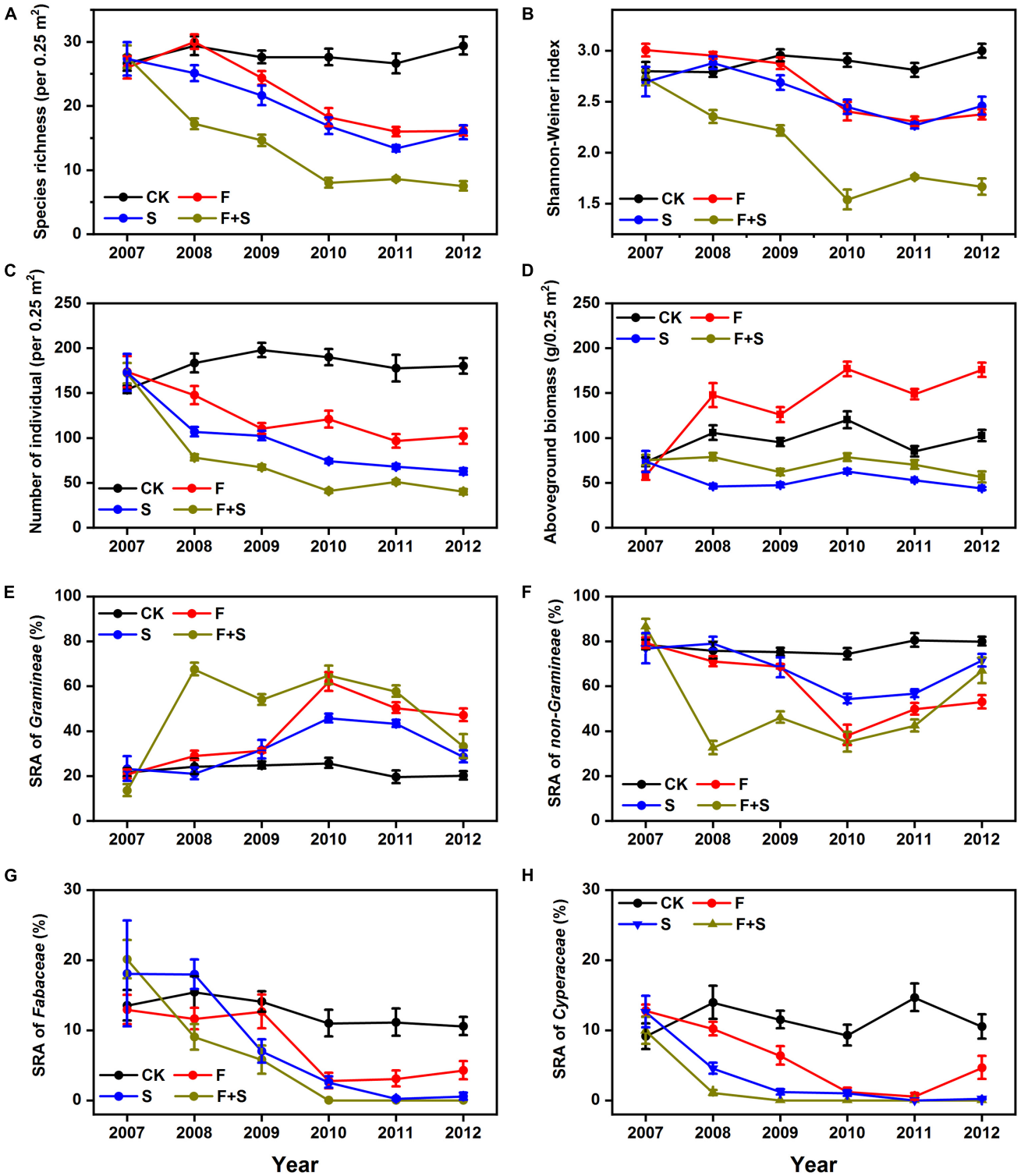

FIGURE 1 | The effect of fertilization (F, red line), shade (S, blue line), and interaction of fertilization and shade (F+S, brown line) on temporal trends of species richness **(A)**, Shannon–Weiner index **(B)**, number of individuals **(C)**, aboveground biomass **(D)**, species relative abundance (SRA) of *Gramineae* **(E)**, SRA of non-*Gramineae* **(F)**, SRA of *Fabaceae* **(G)**, and SRA of *Cyperaceae* **(H)**. Circular symbols represent means and SE (n = 8) across 6 years (2007–2012). The Control treatment (CK = ambient light and no fertilizer addition) is denoted by black lines. Means were connected by lines to illustrate the temporal patterns. Means and SE of 2007 were calculated using the baseline surveys prior to treatments (n = 3) (refer to the section "Materials and Methods" for more details).

TABLE 2 | Plant community characteristics (mean ± SE) in different treatments and the results of repeated measure ANOVA (*F*-value and degrees of freedom) for the effects of the fertilization and shade on plant community characteristics.

Plant variables	Community characteristics in different treatments				Summary of the results of repeated measure ANOVA					
	Control	Fertilization (F)	Shade (S)	F+S	Year (Y)	Fertilization (F)	Shade (S)	Y × F	Y × S	F × S	Y × F × S
d.f.					4	1	1	4	4	1	4
Species richness	28.35 ± 0.61a	20.98 ± 1.00b	18.75 ± 0.88b	11.03 ± 0.67c	53.27***	161.32***	270.42***	7.22***	2.06^{ns}	0.09^{ns}	10.60***
Shannon–Weiner index	2.89 ± 0.03a	2.58 ± 0.05b	2.55 ± 0.05b	1.89 ± 0.06c	49.48***	172.13***	189.92***	14.65***	7.19***	21.38***	6.20***
Number of individual	199.0 ± 5.9a	132.5 ± 8.9b	83.7 ± 3.6c	55.5 ± 2.8d	76.31***	114.21***	470.80***	5.28**	19.08***	18.71***	7.17***
Aboveground biomass	100.7 ± 3.6b	154.1 ± 5.3a	50.1 ± 1.4d	69.3 ± 2.5c	7.39***	202.37***	705.82***	2.87*	5.35**	44.72***	3.45*
Belowground biomass	736.1 ± 50.7a	745.1 ± 36.3a	414.8 ± 23.0b	332.1 ± 25.0b	–	1.08^{ns}	106.95***	–	–	1.67^{ns}	–
Root:Shoot ratio	7.71 ± 0.60a	4.32 ± 0.34b	8.00 ± 1.04a	7.66 ± 0.63a	–	7.12*	6.75*	–	–	4.76*	–
SRA (*Gramineae*)	22.82 ± 1.20d	43.90 ± 2.32b	34.05 ± 1.93c	55.41 ± 2.54a	21.34***	190.01***	54.56***	3.82**	7.32***	0.01^{ns}	20.68***
SRA (Non-*Gramineae*)	77.18 ± 1.20a	56.10 ± 2.32c	65.95 ± 1.93b	44.59 ± 2.54d	21.34***	190.02***	54.56***	3.82**	7.32***	0.01^{ns}	20.68***
SRA (*Fabaceae*)	11.96 ± 0.88a	4.58 ± 0.75b	1.34 ± 0.33c	0.21 ± 0.10c	6.45**	50.23***	155.50***	2.08^{ns}	2.42^{ns}	50.17***	3.94*
SRA (*Cyperaceae*)	12.41 ± 0.88a	6.86 ± 0.99b	5.65 ± 1.21bc	2.95 ± 0.82c	27.83***	21.71***	36.41***	1.40^{ns}	3.66*	38.42***	2.96*

*Community characteristics were collected and calculated with the data (n = 40) across 5 experimental years (2008–2012) except for that belowground biomass and root:shoot ratio were collected and calculated using the data (n = 8) from the last year of the experiment (2012). Significant differences of each community characteristic among treatments were determined using the least-significant difference (LSD) test ($P \leq 0.05$) and indicated by different letters. The value of species richness and number of individuals are numbers per 0.25 m^2 (a quadrat area), and biomass is denoted in grams per 0.25 m^2. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$; ns, not significant. SRA, species relative abundance; d.f., degree of freedom.*

We employed SEM to further estimate the contribution of four factors (i.e., fertilization, shade, species height, and LCP) on the variation of SRA and found that the optimal SEM model explained 59% of the variations in SRA of *Gramineae* and 33% of the variations in SRA of non-*Gramineae* (**Figure 4**). For *Gramineae* species, fertilization had significant direct and indirect effects on SRA which was mediated by increasing species height and LCP, whereas shade treatment had significant indirect effects on SRA *via* increasing species height and decreasing LCP (**Figure 4A**). For non-*Gramineae*, fertilizer application had no significant direct effects but displayed significant indirect effects on SRA *via* increasing species height, whereas shade treatment had significant indirect effects on SRA *via* increasing species height and decreasing LCP (**Figure 4B**).

DISCUSSION

Overall, our 6-year field experiment *via* simultaneously manipulating the light availability and soil nutrient conditions revealed that artificial shade could reduce species diversity as same as fertilization in the alpine meadow. Reduction in light availability triggered species loss process both in the artificial shade and fertilization plots but through different mechanisms. Specifically, fertilization facilitated the survival of species with tall stature, whereas artificial shade promoted the performance of species with low LCP. Our results confirmed that species height and LCP were effective indicators of species coexistence patterns in the light limitation habitat of the Tibetan Plateau.

Effects of Fertilization and Shade on Local Abiotic Conditions and Plant Diversity

The availability of light in the lower canopy layers influences conditions for species recruitment, growth, and reproduction, thus affecting plant diversity (Kotowski and van Diggelen, 2004). In our study, light intensity was significantly reduced directly by artificial shade and indirectly by fertilization *via* increasing aboveground biomass (**Figure 1D** and **Table 1**). As expected, artificial shade treatment had no influence on the abiotic conditions except for the light availability. In addition, fertilization significantly increased soil available nitrogen and soil available phosphorus (**Table 1**). These responses of environmental variables to experimental treatments verified our hypothesis assumption that fertilization decreased light availability in plant community and increased soil nutrient contents, while shade decreased light availability without changing soil nutrient contents (**Table 1**). Our results were consistent with the prediction of the light competition hypothesis (Rajaniemi, 2002), both fertilization and shade treatment significantly reduced species diversity (**Figures 1A,B** and **Table 2**), and species diversity was strongly positively correlated with light availability scores (**Figures 3A,B**), indicating that direct and indirect light limitation performed similarly negative effects on plant diversity regardless of aboveground biomass increased by fertilization or decreased by shade treatment (**Figure 1D**).

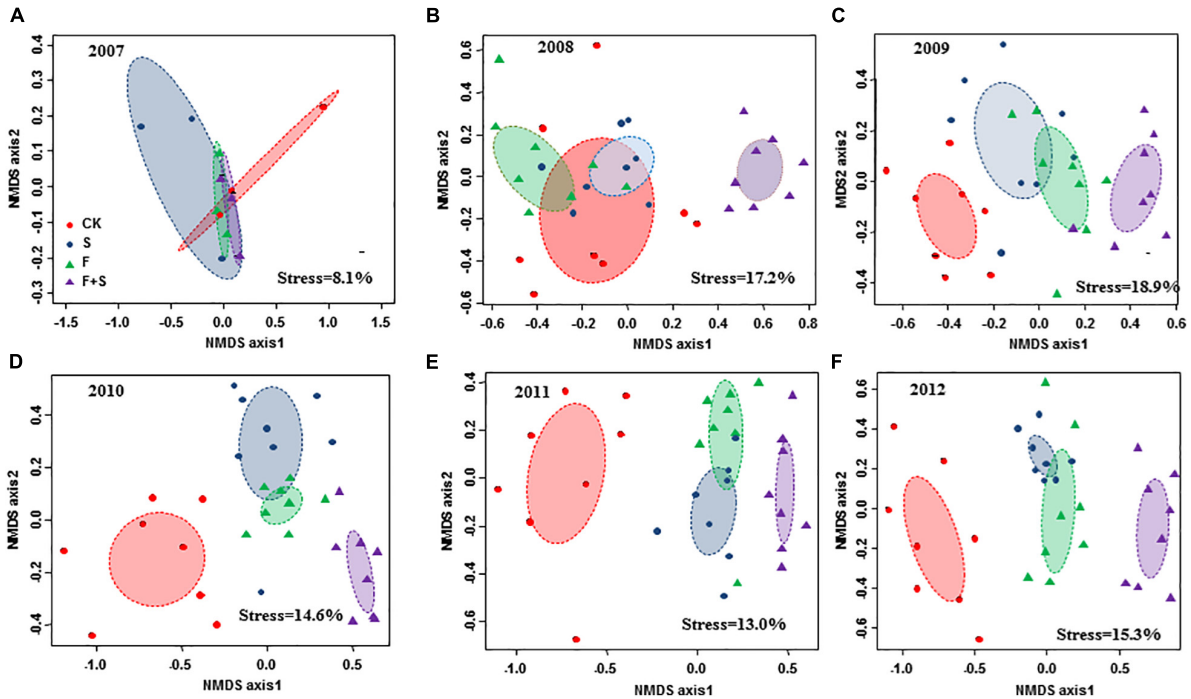

FIGURE 2 | Nonmetric multidimensional scaling (NMDS) patterns of community dissimilarities among treatments using each quadrat data (n = 8) of plant species composition along the temporal scale (**A**: 2007; **B**: 2008; **C**: 2009; **D**: 2010; **E**: 2011; **F**: 2012). Ellipses with different colors indicate 95% CI ellipses for centroids of the control (CK, red color), fertilization (F, green color), shade (S, blue color), and interaction with fertilization and shade (F+S, purple color). It is noted that quadrat data of 2007 (n = 3) were calculated using the baseline surveys prior to treatments (refer to the section "Materials and Methods" for more details).

TABLE 3 | Results of paired-samples t-test (2-tailed) for the effects of treatments on species height and light compensation point (LCP) of *Gramineae* and non-*Gramineae* species at the community level.

Treatment	*Gramineae*					Non-*Gramineae*				
	d.f.	Species height		LCP		d.f.	Species height		LCP	
		t	*p*	*t*	*p*		*t*	*p*	*t*	*p*
Fertilization (F)	4	**−5.651**	**0.005**	**−3.510**	**0.025**	8	−0.798	0.448	**−3.281**	**0.011**
Shade (S)	3	**−5.011**	**0.015**	2.736	0.072	12	**−5.210**	**0.001**	**5.502**	**0.001**
F+S	2	−3.332	0.079	2.205	0.158	3	−2.324	0.103	1.718	0.184

Significant differences are denoted in bold ($P < 0.05$).

Interestingly, changes in species diversity due to reductions in light intensity were only apparent after the 2nd year of exposure to artificial shade or fertilization and became more pronounced along the temporal scale (**Figures 1A,B**). The result was consistent with previous studies that short-term (1–2 years) artificial shade had a neutral effect on species diversity (Rajaniemi, 2002; Li et al., 2011). Tilman (1988) reported that short-term community dynamics following disturbance was merely an intermediate process during the succession. Moreover, another research proved that it took over 10 years for the shifts in species composition occurring after fertilization in northwestern Canada (Turkington et al., 2010).

Our results also mirrored the commonly observed negative relationship between plant diversity and nutrient availability (Rajaniemi et al., 2003; Suding et al., 2005; Harpole and Tilman, 2007; Bobbink et al., 2010; Duprè et al., 2010; Isbell et al., 2013, 2015). In this study, an increase in nutrient availability and subsequent enhancement for aboveground biomass may increase competition intensity for light (Hautier et al., 2009; Borer et al., 2014; Yang et al., 2015), conferring a competitive advantage to fast-growing resource acquisitive species at the expense of slower growing, resource conservative species (Dybzinski and Tilman, 2007; Hautier et al., 2009; Dickson and Foster, 2011; DeMalach et al., 2017). It was noteworthy that the soil pH was not affected by fertilization in our experiment, contrary to other study reports (Clark et al., 2010; Li et al., 2011, 2017; Yang et al., 2021; **Table 1**). One possible reason was that we employed ammonium phosphate [$(NH_4)_2HPO_4$], a slow-release pelletized fertilizer, to manipulate the soil nutrient input. The fertilizer is widely used in the local agricultural cultivation and

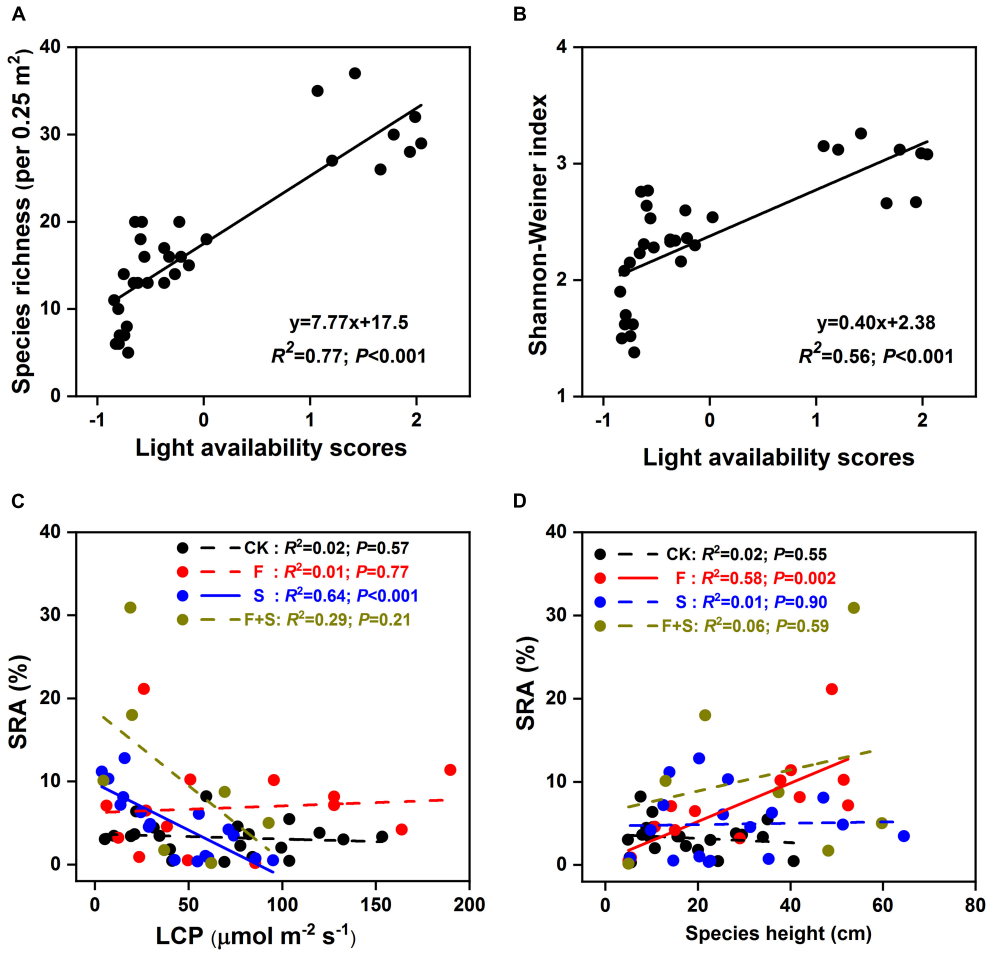

FIGURE 3 | Linear regressions of light availability scores vs. species richness **(A)**, Shannon–Weiner index **(B)** and light compensation point (LCP) **(C)**, species height **(D)** vs. SRA among the control (CK, black), fertilization (F, red), shade (S, blue), and synchronous fertilization and shade (F+S, brown) plots, respectively. Symbols were means of the SRA of 21 species in control plots, 17 species in shade plots, 14 species in fertilized plots, and 7 species in F+S plots for panels **(C,D)**, respectively. Solid and dashed arrows indicate significant ($P < 0.05$) and nonsignificant relationships ($P > 0.05$) between the variables (refer to the section "Materials and Methods" for more detail).

showed weak alkalinity, while other studies involving fertilization usually employed ammonium nitrate (NH_4NO_3), strong acid and weak base salt, as fertilizer and showed that fertilization induced soil acidification and reduced species diversity, which differed with our experimental design. Overall, to the best of our knowledge, the result that artificial shade performed the same negative effect on species diversity as fertilization had not been reported in previous studies. Our results provide novel evidence that artificial shade had the same negative effect on species diversity as increased nutrient availability and that species loss can be simply triggered by light limitation, independent of nutrient addition within alpine meadow systems.

Effects of Fertilization and Shade on Plant Community Composition

Species composition of the community in each treatment plot changed sequentially during the experimental period (2007–2012) (**Figure 2**). The reason may be that it takes time to shift community composition because some species gradually disappeared with fertilization and shade, while others gradually increased. For example, *Gramineae* species, such as *P. poophagorum* and *K. cristata,* rapidly increased and dominated in the fertilization plots and shade plots instead of the former dominant species (*T. ruthenica* and *K. capillifolia*) of the initial communities (**Supplementary Table 2**). *Fabaceae* and *Cyperaceae* species that inhabited in the understory of the community were very sensitive to light limitation and extincted in less than 3 years within the treatment plots (**Figures 1G,H** and **Table 2**).

Nonmetric multidimensional scaling ordination verified the low similarity of community composition between the control and the other treatment plots (**Figure 2**). One possible reason is that an average of 28 species was found in the control plots compared with 21 species in the fertilization plots and 19 species in the shade plots during the experimental period (**Table 2**). Moreover, the dominant species between the control and the

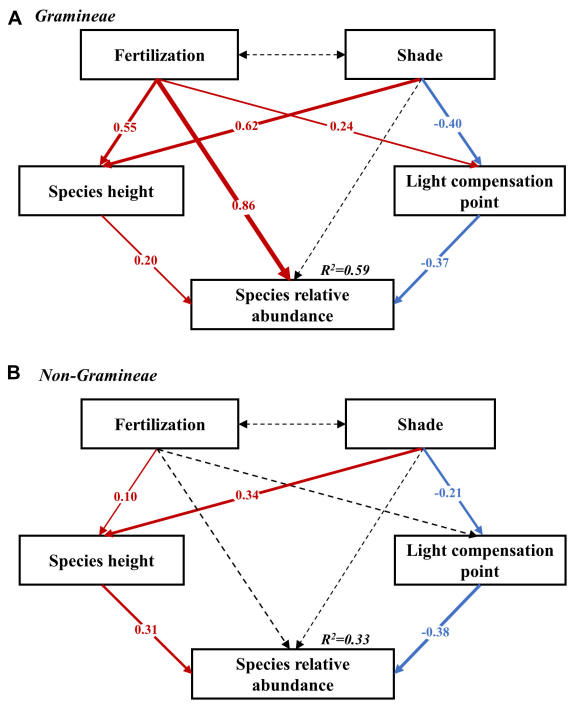

FIGURE 4 | The results of the best-fitting structural equation model (SEM) showing the causal relationships among fertilization, shade, species height, LCP, and species relative abundance of *Gramineae* species and non-*Gramineae* species. **(A)** *Gramineae* species and model fit statistics variables were $\chi^2 = 0.179$, $P = 0.672$, CFI = 1.000, root mean square error of approximation (RMSEA) = 0.000, Akaike information criterion (AIC) = 38.179. **(B)** Non-*gramineae* species and model fit statistics variables were $\chi^2 = 1.900$, $P = 0.167$, CFI = 0.935, RMSEA = 0.151, AIC = 39.907. Solid and dashed arrows indicate significant ($P < 0.05$) and nonsignificant relationships ($P > 0.05$) between the variable of the onset arrow and the variable of the terminated arrow, respectively. The thickness of the arrows reflects the degree of relationships, and red and blue arrows indicate positive and negative relationships, respectively. Numbers at arrows are standardized path coefficients. R^2 values indicate the variation of response variables explained by the models.

other treatment plots were also different. *T. ruthenica* (8.22%) dominated the control community but absent from fertilization plots and only covered 0.53% in the shade plots (**Supplementary Table 2**). There was a high similarity between the fertilization and the shade communities for the reason that some species such as *P. poophagorum* and *Anemone obtusiloba* species gradually became more dominant and some species such as *Fabaceae and Cyperaceae* gradually decreased in abundance both in fertilization plots and shade plots. Although fertilization and shade performed a similar negative effect on species diversity, there was no overlap between the F+S and fertilization or shade plots since only one-third of species were found to be survived in the F+S plots (**Table 2**).

Studies on the relationships between species diversity and ecosystem functioning often showed a unimodal pattern along the primary productivity gradient (O'Connor et al., 2017; Kimmel et al., 2020; Yue et al., 2020). In our experiment, species diversity reduced significantly regardless of aboveground biomass increase due to fertilization or decrease due to shade (**Figures 1A,B,D**). Interestingly, fertilization increased aboveground biomass with a neutral effect on belowground biomass, resulting in a significant decrease of the root:shoot ratio (**Figure 1D** and **Table 2**). The result implied that the coexisting plant species switched the strategy of biomass allocation tending to aboveground organs to promote vegetative growth in response to fertilization, especially for the species with rapid feedback on soil nutrients and light availability fluctuations (e.g., *Gramineae* species). In contrast, artificial shade decreased aboveground and belowground biomass synchronously, resulting in a neutral — change in the root:shoot ratio (**Figure 1D** and **Table 2**). The result suggested that the coexist plant species did not adjust the strategy of biomass allocation in response to light limitation due to shade treatment. Therefore, the species loss due to light limitation should have different underlying mechanisms between the fertilization and shade communities.

Previous studies have demonstrated that species loss due to long-term exposure to high nutrient availability was nonrandom (Zhou et al., 2021, 2022) and that the initial biomass increase trend after fertilizer application gradually diminished over time (Isbell et al., 2013; Kimmel et al., 2020; Zhao et al., 2021). Understory species might be severely constrained by light limitation and thus suffered a higher risk of localized exclusion compared with the canopy species (Dickson and Gross, 2013; DeMalach et al., 2017). Our results confirmed that most non-*Gramineae* species, which usually inhabited the understory, were very sensitive to light limitation (**Figures 1G,H**). Both fertilization and shade dramatically and disproportionally affected the SRA of understory species (**Figures 1G,H**). In contrast, fast-growing, nutrient-preferring, and taller *Gramineae* species dominated the canopy of the treated communities, contributing to the majority of the community biomass and competitively excluding slow-growing, nutrient-conservative species (Li et al., 2011, 2017; Liu et al., 2015). Furthermore, there were significant interaction effects between fertilization and shade on species diversity and species abundance, and the interaction effects enhanced along the temporal scale (**Figure 1** and **Table 2**), indicating that fertilization and shade performed roughly equal effects on plant communities.

Effects of Fertilization and Shade on Functional Traits and Their Relationships With Species Relative Abundance

Species traits are the results of functional trade-offs between different plant functions and from adaptive and plastic responses to its biotic and abiotic environments (Dickson et al., 2014; Li et al., 2021). Consistent with Clark et al. (2010) and Dickson et al. (2014), species loss under fertilization and shade in our study could be predicted by plant height and photosynthetic capacity traits, respectively (**Figures 3C,D**). For example, fertilization facilitated the performance of taller *Gramineae* species but suppressed non-*Gramineae* species in accordance with the previous study that *Gramineae* species had asymmetric access to the higher nitrogen supply and then eliminate non-*Gramineae* species through light competition due to their higher growth rate (**Figures 1E,F**; Dickson and Gross, 2013; Dickson et al., 2014). Moreover, our results

were in contrast to those from a meta-analysis of 37 studies by Suding et al. (2005), which recorded no relationship between species height and species loss. The negative effect of fertilization on non-*Gramineae* species could be attributed to their shorter, rosette life form. In addition, our results clearly showed that there was a strong positive correlation between species height and their SRA in the fertilized plots, with height being a strong predictor of species loss in these plots (**Figure 3D**). However, this relationship was not observed within communities in either the shade or control plots. This disparity suggested that enhanced plant vegetative growth due to fertilization was an important driver of species loss in the communities of alpine meadows.

Shade promoted species with lower LCP, with an increase in the relative abundance of these species in the shaded plots (**Figure 3C**). Species with low LCP typically exhibit strong tolerance to light limitation and usually were dominant species in low-light environments (Horn, 1971; Kitao et al., 2016). This pattern was evident in our study system, whereby the SRA of rare species with low LCP (e.g., *Gentiana macrophylla* and *Galium verum*) remarkably increased in shade plots (**Supplementary Table 2**). We also detected a strong negative correlation between species LCP and their SRA in the artificial shade plots, with LCP being an effective predictor of species loss in these plots (**Figure 3C**). However, this relationship was not observed within communities in either the fertilization or control plots, indicating that the tolerance capacity of light limitation was an important driver of community assembly for the alpine meadow. Results from the SEMs also supported our theory that both species height and LCP played decisive roles in the process of species loss due to light limitation.

Our previous 3-year field experiment at this study site indicated that fertilization influenced plant species richness by increasing aboveground biomass and livestock can neutralize or mask the negative effects of fertilization on plant species diversity *via* ingesting aboveground biomass (Li et al., 2017). Since competition for light is size asymmetric and increasing aboveground biomass due to fertilization aggravated the light limitation in the understory, we recommend that moderate grazing or mowing should be applied along with the increasing nitrogen deposition or fertilizer application to stabilize the local species diversity in the alpine meadow of the Tibetan Plateau (Sun et al., 2021a,b).

Overall, this study illustrates how artificial shade and fertilization influenced species diversity and community structure and verified the light competition theory as the main drivers of community assembly in the alpine meadow of the Tibetan Plateau. While the responses of community composition to fertilization and shade differed among experimental treatments, traits associated with light capture and shade tolerance determined the pattern of species coexistence in light limitation habitat. Furthermore, our results highlighted that species height and LCP are good indicators for predicting species loss and suggested that species diversity reduction due to fertilization may be minimized by reducing the individual height of canopy species in the alpine meadow (e.g., mowing or grazing).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

JL and ZY contributed to the conception and design of the study. JL organized the database and wrote the first draft of the manuscript. JL, ZY, and LC performed the statistical analysis and wrote the draft of the manuscript. GD and SF provided the funding and revised the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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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.2022.832473/full#supplementary-material

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Leaf Functional Traits of Two Species Affected by Nitrogen Addition Rate and Period Not Nitrogen Compound Type in a Meadow Grassland

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Plasticity of plant functional traits plays an important role in plant growth and survival under changing climate. However, knowledge about how leaf functional traits respond to the multi-level N addition rates, multiple N compound and duration of N application remains lacking. This study investigated the effects of 2-year and 7-year N addition on the leaf functional traits of *Leymus chinensis* and *Thermopsis lanceolata* in a meadow grassland. The results showed that the type of N compounds had no significant effect on leaf functional traits regardless of duration of N application. N addition significantly increased the leaf total N content (LN) and specific leaf area (SLA), and decreased the leaf total P content (LP) and leaf dry matter content (LDMC) of the two species. Compared with short-term N addition, long-term N addition increased LN, LP, SLA, and plant height, but decreased the LDMC. In addition, the traits of the two species were differentially responsive to N addition, LN and LP of *T. lanceolata* were consistently higher than those of *L. chinensis.* N addition would make *L. chinensis* and *T. lanceolata* tend to "quick investment-return" strategy. Our results provide more robust and comprehensive predictions of the effects of N deposition on leaf traits.

Keywords: leaf functional traits, meadow steppe, nitrogen compound type, nitrogen deposition, nitrogen addition period

INTRODUCTION

Nitrogen (N) deposition in the global atmosphere has increased dramatically as a result of increased human activities, particularly the burning of fossil fuels and the development of intensive agriculture (Galloway et al., 2008; Yu et al., 2019). Nitrogen is a major limiting nutrient for plant growth in terrestrial ecosystems (Elser et al., 2007), with plant community structure and composition usually affected by changes in N availability (Clair et al., 2010). At present, increases in atmospheric N deposition have attracted the attention of researchers, it has been shown that increased N deposition has led to various ecological problems (Suding et al., 2005; Tian et al., 2020).

For example, long-term N application can drive declines in plant diversity (Stevens et al., 2004; Clark and Tilman, 2008; Midolo et al., 2019), and long-term N addition is more likely to lead to other nutrient limitations [e.g., phosphorus (P)] in terrestrial ecosystems (Su et al., 2021).

Plant functional traits are important for exploring the relationship between plants and the environment, which provide a basis for predicting the response of ecosystems in the context of global change (Wright et al., 2010; Li et al., 2015; Liu et al., 2021b). Previous studies have suggested that plant functional traits are key to elucidating the mechanisms that drive the responses of plant communities and ecosystems to resource addition (McGill et al., 2006; Suding and Goldstein, 2008). Plant functional traits are often associated with the environment (Liu et al., 2020, 2021c). It is recognized that N deposition can alter leaf chemical, morphological, and physiological traits (Yan et al., 2012; Zhang et al., 2018). Previous studies found that increased N deposition increases the N concentration in green and senesced plant leaf tissue and also changes the stoichiometric ratio of plant leaves (Xia and Wan, 2008; Cory et al., 2011). Nitrogen addition also changes the specific leaf area, plant height, and dry matter content of leaves (Zheng et al., 2017). These results all show that leaf functional traits are closely related to N deposition. The nutritional status of plants and the response of leaf stoichiometric ratios to N enrichment are essential for elucidating how plants adapt to human disturbances (He et al., 2008; Elser et al., 2010).

Atmospheric N deposition involves a complex of N compounds (Galloway, 1995), which is mainly composed of ammonium (NHx) and nitrate (NOy; Yu et al., 2019). Some recent observations have shown differences in the response of soils and plants to different N compound addition type. Across Chinese grasslands, Liu et al. found that declines in soil pH varied with the N compound type (Liu et al., 2021a). In addition, a meta-analysis found that growth of terrestrial plants was enhanced more by $NH4^+$-N than $NO3^-$-N addition (Yan et al., 2019). In addition, the divergent responses of plant to N addition have been ascribed the response of species and ecosystems will be differential to the duration of N addition (Bobbink et al., 2010). Species abundance responded contradictorily to short-term and long-term N addition (Zheng et al., 2019; Yang et al., 2021), and N-induced P limitation also changed with the duration of N addition (Chen et al., 2020). Therefore, the effects of plant functional traits may also depend on the duration of N enrichment. These findings suggest that different N compounds have different effects on soil properties, which may result in different changes in plants. However, the impacts of different N compounds and the duration of N addition on leaf functional traits within the same location is still lacking.

In the present study, three N compounds and six N addition rates were selected to test the effect of N deposition on the leaf functional traits of two typical meadow plants, *Leymus chinensis* and *Thermopsis lanceolata*, based on the length of the fertilization period. We were particularly interested to examine: (1) How do plant leaf functional traits respond to different N addition gradients among different types of N compound? (2) Under the same N addition gradient treatment, are there differences in the response of plant leaf functional traits to short- and long-term N deposition?

MATERIALS AND METHODS

Study Site

The study was conducted at the Erguna Forest-Steppe Ecotone Ecosystem Research Station (N50°10′46.1″, E119°22′56.4″). It is located in HulunBuir City, Inner Mongolia Autonomous Region. The soil is classified as chernozem according to the FAO classification. The region has a cold temperate continental climate with an average annual temperature of −1.59°C and an annual rainfall of 336.5 mm (2000–2020). The rainfall of the sample site was 148.2 mm in 2015 and 139.8 mm in 2020 according to the measurement of the research station. The background value of N deposition in this area is relatively low (Jia et al., 2014), which is conducive to simulation experiments of N deposition. Two dominant species, the C_3 perennial rhizome grass *L. chinensis* and C_3 perennial legume *T. lanceolata*, which together account for more than 50% of the peak community aboveground biomass, were used as our model plant species.

Experimental Design

The grassland of the experiment site has been fenced since 2013 to prevent large animals, such as cattle and sheep, from eating and trampling the vegetation. The N addition experiment was established in 2014. All experimental plots (10 m × 10 m) had similar topography and land-use history. A completely randomized block design was used, and a 1-m buffer area was established between the plots. Three N compounds were selected: ammonium nitrate (NH_4NO_3), ammonium sulfate [$(NH_4)_2SO_4$], and ammonium hydrogen carbonate (NH_4HCO_3). Each N fertilizer was applied at six concentrations: 0, 2, 5, 10, 20, and 50 g N m^{-2} yr^{-1}. There were 18 treatments, with each treatment replicated eight times, resulting in a total of 144 plots. The N compounds were applied annually in late May. In order to ensure that the N compounds were spread evenly in each plot, they were combined with baked fine sand.

Sampling and Leaf Functional Trait Determination

Plant sampling was conducted during the season of vigorous plant growth (August) in 2015 (short term) and 2020 (long term). These sampling dates corresponded to two years in the short-term experiment and seven years in the long-term experiment. Plant height (H) was obtained based on the mean of five healthy individuals at every site. Ten to 15 complete, healthy plants of each species were harvested, placed in sealed bags, and temporarily stored in an insulation bucket in iceboxes. After sampling was completed, the sample was immediately brought to the laboratory to reduce the loss of water dispersion from the plant and to avoid leaf shrinkage.

In the laboratory, all samples were soaked in water for 6 h to ensure full rehydration. Each leaf was then cut from the stem and gently dried with tissue paper before measurement. Water-saturated leaf and stem mass were weighed. The leaves used to

measure the leaf area are scanned with a scanner after being weighed (Canon LiDE120), and then the leaf area was determined by the ImageJ software[1]. All leaves were then dried for 48 h at 65°C and then weighed. The leaf dry matter content (LDMC), specific leaf area (SLA), and stem-leaf ratio (S:L) were calculated. The leaf total N content (LN) was determined by the Kjeldahl apparatus (K9860, Hanon, Dezhou, China) after extraction with sulfuric acid, and the leaf total P content (LP) was determined by a UV-spectrophotometer (UA-5500, METASH, Shanghai, China) with the wavelength set to 700 nm. The N to P ratio (N:P) was calculated as the ratio of the total N content of the leaf to the total P content. All trait measurements were based on previous standardized measurements (Cornelissen et al., 2003; Pérezharguindeguy et al., 2013).

Statistics

The N compound type was used as a categorical variable with three levels: NH_4HCO_3 (AC), NH_4NO_3 (AN), and $(NH_4)_2SO_4$ (AS). The different sampling years were also categorical variables with two levels: 2015 (short term) and 2020 (long term). We used four-way ANOVA to test the effects of N addition rates, N compounds type, period, species, and their interactions on leaf chemical and morphological traits. The effects of different N addition rates on leaf chemical and morphological traits were tested using one-way ANOVA, followed by *post hoc* tests [Tukey's honest significance difference (HSD)] in periods of treatment for each N compound type, and the effect of year was tested by *t*-tests in each N addition rate of each N compound type. When necessary, data were natural log-transformed to meet the normality assumption of ANOVA and *t*-tests (include SLA). The variation in plant leaf functional traits was analyzed based on differences by terms (N addition time), N compound type and N addition rates using PERMANOVA (999 permutations, Adonis function) and visualized by principal component analysis (PCA). In order to determine whether the relationship between the leaf functional traits of the two species in different years had changed, we used the ggpairs function in the Ggally package for plotting and calculated the correlation and significance between each two based on Pearson's correlation analysis (Emerson et al., 2013). All statistical analyses were performed using R software 4.0.3 (R Core Team 2019[2]).

RESULTS

Effects of N Addition Rate, N Compound Type, and Period on the Chemical Traits in the Plant Leaves

The results showed that the N addition rate, species and period had significant effects on the LN, LP and N:P of the two plants ($P < 0.01$), while N compound type had no significant effect (**Table 1**). The N addition rate and period had an interactive effect on the LN, LP and N:P of the two plants ($P < 0.01$, **Table 1**).

Nitrogen addition significantly increased the LN in both species. However, under high N addition (20 and 50 g N m^{-2} yr^{-1}), the LN of the two species showed a slowing down or a slight downward trend with increasing N addition (**Figure 1**). The increasing trend of LN under long-term N addition was significantly higher than that under short-term N addition (**Figure 1**). Under both short- and long-term N addition treatments, the LN of the two species showed significant differences at each N addition rate of the two periods. Similar results were detected for all three N addition types.

[1]http://imagej.nih.gov/ij/

[2]http://www.R-project.org

TABLE 1 | Results (*F* values) of a repeated-measures ANOVA for the effects of N addition rate (R), N compounds type (T), period (Pr), species (S) and their interactions on leaf nitrogen content (LN), leaf phosphorus content (LP), leaf N content and leaf P content ratio (N:P), specific leaf area (SLA), leaf-stem ratio (S:L), leaf dry matter content (LDMC), and plant height (H) of *Leymus chinensis* and *Thermopsis lanceolata*.

	Df	LN	LP	N:P	Log_{10}(SLA)	S:L	LDMC	H
R	5	31.10***	6.69***	10.53***	4.17**	3.26**	2.43*	10.89***
T	2	2.72^{ns}	0.88^{ns}	2.73^{ns}	0.23^{ns}	1.09^{ns}	3.50*	0.62^{ns}
Pr	1	1556.00***	1328.34***	243.87***	686.11***	79.68***	522.70***	2123.17***
S	1	1328.96***	244.22***	70.55***	878.22***	621.18***	1906.96***	4310.95***
R × T	10	0.75^{ns}	0.84^{ns}	0.52^{ns}	1.02^{ns}	0.77^{ns}	0.56^{ns}	0.57^{ns}
R × Pr	5	9.20***	6.82***	0.55^{ns}	6.37***	2.10^{ns}	5.01***	9.65***
T × Pr	2	0.66^{ns}	0.56^{ns}	0.90^{ns}	0.15^{ns}	0.28^{ns}	0.60^{ns}	2.35^{ns}
R × S	5	5.01***	1.82^{ns}	3.08**	3.16**	1.13^{ns}	1.91^{ns}	2.85*
T × S	2	1.66^{ns}	0.07^{ns}	0.13^{ns}	0.66^{ns}	2.66^{ns}	1.43^{ns}	1.69^{ns}
Pr × S	1	13.76***	148.70***	131.81***	205.23***	86.12***	35.91***	515.38***
R × T × Pr	10	0.68^{ns}	1.24^{ns}	1.11^{ns}	1.21^{ns}	0.71^{ns}	1.65^{ns}	0.59^{ns}
R × T × S	10	0.41^{ns}	0.88^{ns}	0.43^{ns}	0.54^{ns}	1.02^{ns}	1.52^{ns}	0.79^{ns}
R × Pr × S	5	1.31^{ns}	2.81*	0.85^{ns}	2.44*	2.17^{ns}	0.56^{ns}	2.26*
T × Pr × S	2	0.45^{ns}	0.58^{ns}	0.89^{ns}	0.31^{ns}	0.91^{ns}	1.40^{ns}	0.31^{ns}
R × T × Pr × S	10	0.70^{ns}	0.59^{ns}	0.76^{ns}	0.93^{ns}	1.20^{ns}	0.93^{ns}	1.53^{ns}

*Asterisks denote significant levels: ns, $P > 0.05$; *, $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.001$, respectively.*

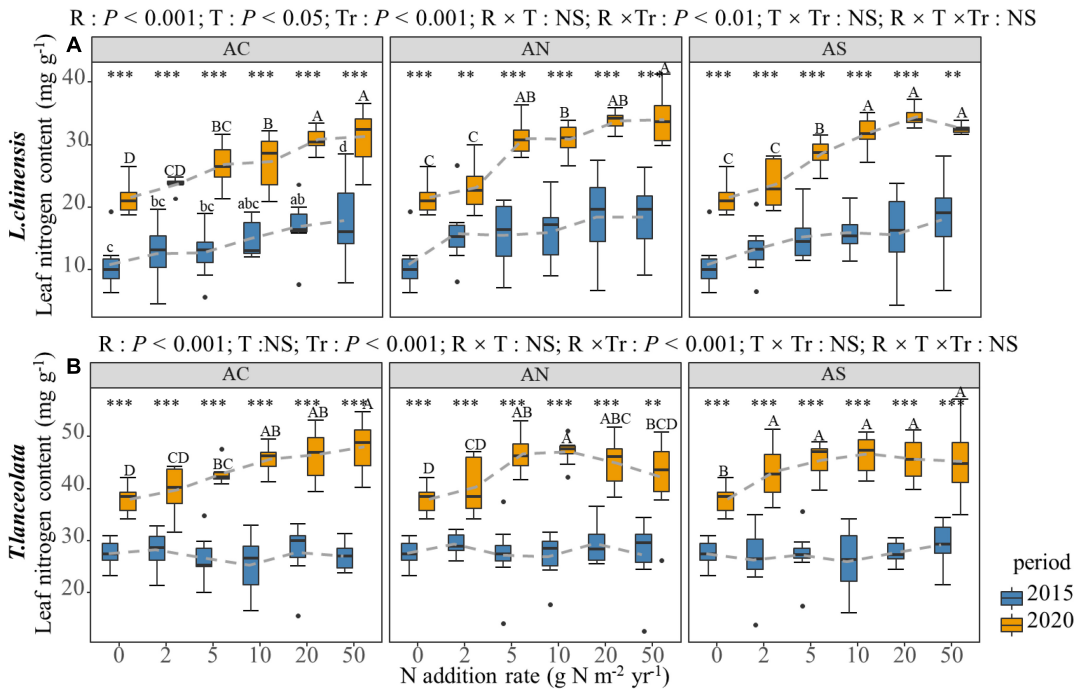

FIGURE 1 | Effects of short- and long-term nitrogen deposition on LN of two species: **(A)** *Leymus chinensis*; **(B)** *Thermopsis lanceolata*. The three-way ANOVA was used to test the effects of Pr, T, and R on LN. The effects of the nitrogen compound type alone and with N addition rate and year on LN are not significant (NS, $P > 0.05$), thus one-way ANOVA (Tukey's HSD) and *t*-tests are used to test the effects of N addition rate and year on LN. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in LN among nitrogen addition rate in the 2015 and 2020 plots, respectively. The asterisk above the picture indicates the significant difference between the two years under each nitrogen addition rate. Note: $^{**}P < 0.01$; $^{***}P < 0.001$. CK: non-fertilizer control = 0, N1 = 2, N2 = 5, N3 = 10, N4 = 20, and N5 = 50 kg N ha^{-1} yr^{-1}; AC (NH_4HCO_3), AN (NH_4NO_3), AS [$(NH_4)_2SO_4$]. The gray dashed line is the connection of the mean value.

The LP of the two species did not show a significant trend in N addition rate with the addition of various N compounds (**Figure 2**). The LP of *L. chinensis* under N addition was only marginally lower than the control. The LP of the two species showed significant differences at each N addition rate of the two periods. The N content under long-term N addition was significantly higher than under short-term N addition (**Figure 2**, $P < 0.05$).

The N:P of the two species differed significantly in response to the N addition rate and period (**Figure 3**). The N:P of the leaf of *L. chinensis* significantly increased under long-term N addition (**Figure 3A**, $P < 0.05$), while that was not significant under short-term N addition. Furthermore, the difference between the two periods was not significant (**Figure 3A**, $P > 0.05$). The change trend of the N:P in the *T. lanceolata* leaves was not obvious with N addition. The N:P under long-term N addition was significantly lower than under short-term N addition (**Figure 3B**).

Effects of N Addition Rate, N Compound Type, and Period on the Morphological Traits in Plant Leaves

According to four-way ANOVA, the N addition rate, species and period had significant effects on the LDMC, S:L, H and SLA of the two plants ($P < 0.05$), while N compound type had no significant effect, except for the LDMC (**Table 1**). The N addition rate and period had an interactive effect on the LDMC, S:L, H and SLA of the two plants ($P < 0.01$, **Table 1**).

With the increase in N addition, the H of *L. chinensis* showed a significant upward trend under long-term N addition ($P < 0.05$), but there was no significant change under short-term N addition (**Figure 4A**). With the exception of the AS treatment under long-term N addition, the response of *T. lanceolata* H to N addition rate was not significant (**Figure 4B**, $P < 0.05$). There was a significant inter-annual difference in the H of the two species. The plant height under long-term N addition was significantly higher than under short-term N addition in both species (**Figure 4**, $P < 0.05$).

The N addition rate significantly affected the LDMC of the two species under long-term N addition (**Figure 5**, $P < 0.05$), but there was no significant difference in short-term N addition. The LDMC showed a gradual decrease with increased N addition. However, under high N treatment (20, 50 g $Nm^{-2}yr^{-1}$), LDMC demonstrated a slowing-down or slight upward trend (**Figure 5**). In period of the response between years, the LDMC of plants under long-term N addition was significantly lower than under short-term N addition (**Figure 5**, $P < 0.05$).

The S:L of the two species demonstrated a significantly different response to the N addition rate and period (**Figure 6**). The S:L of *L. chinensis* showed an upward trend under long-term N addition (**Figure 6A**). The *t*-test showed that the response of *L. chinensis* S:L to period was significant (**Figure 6A**, $P < 0.05$).

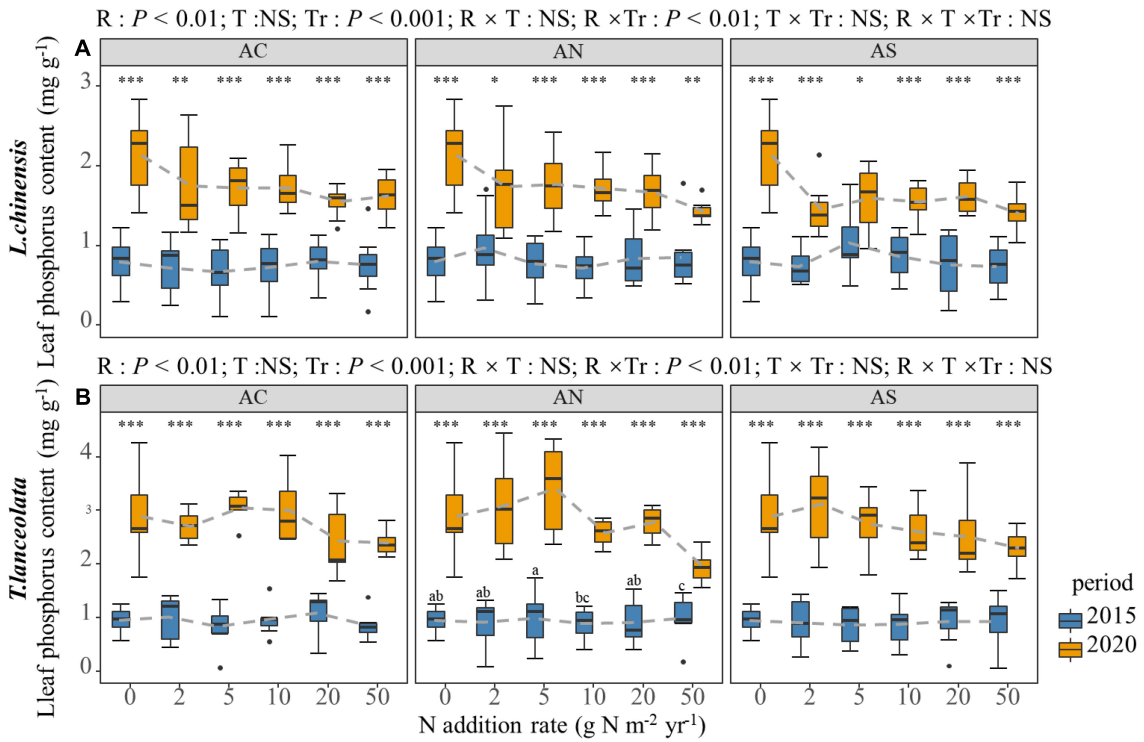

FIGURE 2 | Effects of short- and long-term nitrogen deposition on LP of two species: **(A)** *Leymus chinensis*; **(B)** *Thermopsis lanceolata*. The three-way ANOVA was used to test the effects of Pr, T and R on LP. The effects of the nitrogen compound type alone and with nitrogen addition rate and year on LP are not significant (NS, $P > 0.05$), thus one-way ANOVA (Tukey's HSD) and *t*-tests are used to test the effects of nitrogen addition rate and year on LP. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in LN among nitrogen addition rate in the 2015 and 2020 plots, respectively. The asterisk above the picture indicates the significant difference between the two years under each nitrogen addition rate. Note: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CK: non-fertilizer control = 0, N1 = 2, N2 = 5, N3 = 10, N4 = 20, and N5 = 50 kg N ha^{-1} yr^{-1}; AC (NH_4HCO_3), AN (NH_4NO_3), AS [$(NH_4)_2SO_4$]. The gray dashed line is the connection of the mean value.

The S:L under long-term N addition was significantly higher than that under short-term N addition, while the response of *T. lanceolata* S:L to period was not significant (**Figure 6B**).

Nitrogen addition increased the leaf SLA of the two species, especially under long-term N addition (**Figure 7**). However, under high N addition (20 and 50 g $Nm^{-2}yr^{-1}$), the leaf SLA of the two species slowed down or decreased slightly with the increase in N addition (**Figure 7**). The leaf SLA under long-term N addition was significantly higher than under short-term N addition (**Figure 7**, $P < 0.05$).

Effects of Long- and Short-Term N Deposition on the Correlation of Plant Traits

The PCA of all variables showed that short- and long-term N addition resulted in obvious differences in the spatial aggregation of leaf functional traits, and the response of leaf functional traits to N addition rate and period differed (**Figure 8**). Compared with short-term N addition, *L. chinensis* had higher H, LN, LP, S:L, and SLA and lower LDMC under long-term N addition (**Figure 8A**), and *T. lanceolata* had higher H, LN, LP, and SLA and lower LDMC and N:P. There was a significant correlation between the leaf functional traits of *L. chinensis* and *T. lanceolata* under short- and long-term N addition (**Supplementary Figures 1–4**, $P < 0.05$). Compared with short-term N addition, long-term N addition had more pairs of traits that were significantly related (**Supplementary Figures 1–4**). Under long-term N addition, the H and LP, H and N:P, and LP and LN of both *L. chinensis* and *T. lanceolata* showed a significant change trend that was opposite to that under short-term N addition (**Supplementary Figures 1–4**, $P < 0.05$), which indicated that long-term N addition will alter the relationships of some plant functional traits.

DISCUSSION

Effects of N Addition Rate, N Compound Type, and Period on the Chemical Traits in the Plant Leaves

Leaf N and P concentrations and the N:P stoichiometric ratios of the two species in the meadow steppe showed significant changes in response to the N addition rate and period. N addition led to an increase in the LN of the two species (**Figure 1**), which corroborates the results of previous studies (Huang et al., 2012; Lü et al., 2013). Nitrogen addition caused an increase in soil inorganic N concentrations, which subsequently enhanced the

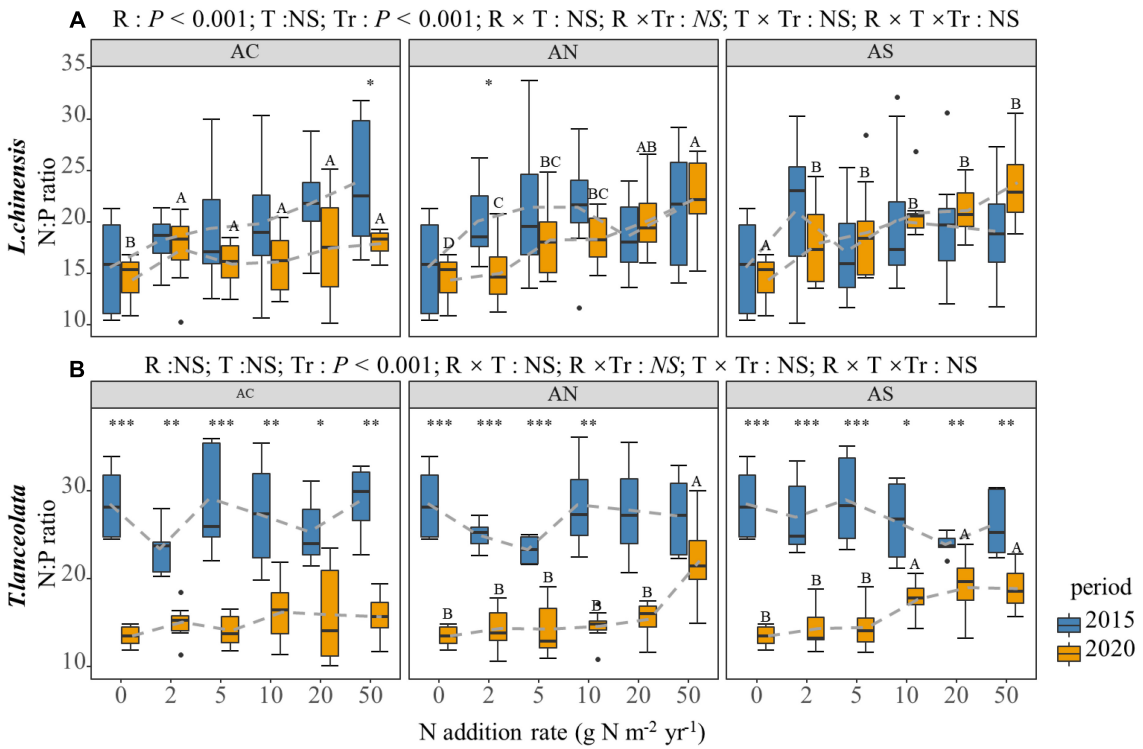

FIGURE 3 | Effects of short- and long-term nitrogen deposition on N:P of two species: **(A)** *Leymus chinensis*; **(B)** *Thermopsis lanceolata*. The three-way ANOVA was used to test the effects of Pr, T and R on N:P. The effects of the nitrogen compound type alone and with nitrogen addition rate and year on N:P are not significant (NS, $P > 0.05$), thus one-way ANOVA (Tukey's HSD) and *t*-tests are used to test the effects of nitrogen addition rate and year on N:P. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in LN among nitrogen addition rate in the 2015 and 2020 plots, respectively. The asterisk above the picture indicates the significant difference between the two years under each nitrogen addition rate. Note: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CK: non-fertilizer control = 0, N1 = 2, N2 = 5, N3 = 10, N4 = 20, and N5 = 50 kg N ha^{-1} yr^{-1}; AC (NH_4HCO_3), AN (NH_4NO_3), AS [$(NH_4)_2SO_4$]. The gray dashed line is the connection of the mean value.

N concentrations in the leaves (Shi et al., 2020). Moreover, N addition increased the re-absorption rate of plant N (Lü et al., 2012; Cao et al., 2021), which in turn increased the plant N content. However, the LN did not always increase with the increase in the N addition rate. At high N addition rate (20 and 50 g $Nm^{-2}yr^{-1}$), the LN decreased slightly, which may be caused by the ammonium toxicity caused by excessive N fertilizer (Van den Berg et al., 2005; Phoenix et al., 2012). Ammonium toxicity effects mainly occur in plants that absorb ammonium ions more easily and exchange these ions with hydrogen ions and potassium plasma, thereby interfering with the cation balance in plants (Van den Berg et al., 2005).

Previous studies on the response of leaf P concentration to N addition obtained different conclusions, including negative (Menge and Field, 2007), neutral (Chen et al., 2015), and positive effects (Lü et al., 2013; Huang et al., 2018). In this work, N addition reduced the LP of *L. chinensis* leaves (**Figure 2**), resulting in a nutrient imbalance. N addition increases the biomass of the plant, and the plant will also grow higher under N addition, which leads to the dilution of the P concentration (Zhang et al., 2017). In order to maintain a stoichiometric balance, the demand for P increases as the N concentration of the leaves increases, and the release of available P from plants through soil formation is usually very slow (Houlton et al., 2008; Zhang et al., 2017). The *T. lanceolata* did not show a similar phenomenon, which may be due to the specific characteristics of the species (Mayor et al., 2014; Wan et al., 2019). The N and P concentrations were less influenced by N addition under short-term N addition than under long-term N addition, probably because of the timing of N application. Because as the time of nitrogen addition increases, the accumulated nitrogen in the soil increases (Sun et al., 2020).

The N-to-P ratio is a measure of plant growth limitation. Güsewell (2004) proposed an N:P > 20 and <10 as the evaluation index of plant N-P limitation. When N-to-P ratio > 20, this indicates that plant growth is limited by P, while N-to-P ratio < 10 indicates that plant growth is limited by N. N-to-P ratio of 10 – 20 indicates that plant growth was not limited or co-limited by N and P (Yan et al., 2017). In this study, the N:P of the *L. chinensis* leaves gradually increased with N addition rate in both periods, which indicated that growth gradually changed to P limitation (**Figure 3A**). The N:P of *T. lanceolata* under long-term N addition was significantly lower than under short-term N addition. According to the criteria proposed by Güsewell, the growth of *T. lanceolata* was mainly limited by P under short-term N addition, while it was limited by both N and P under long-term N addition. The results indicated that N addition could gradually change the elements involved in ecosystem limitations.

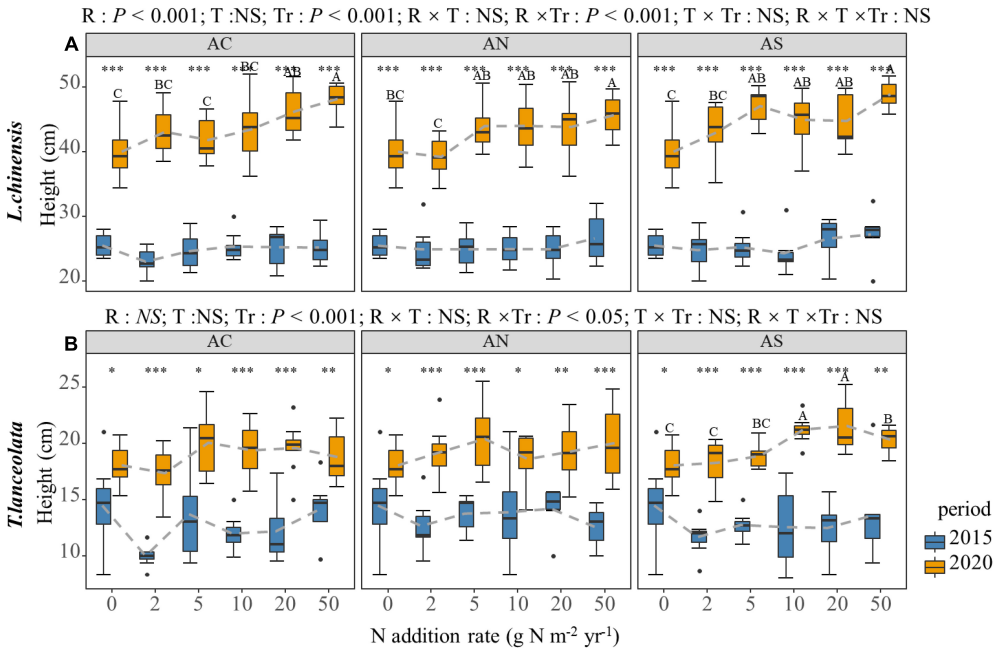

FIGURE 4 | Effects of short- and long-term nitrogen deposition on H of two species: **(A)** *Leymus chinensis*; **(B)** *Thermopsis lanceolata*. The three-way ANOVA was used to test the effects of Pr, T and R on H. The effects of the nitrogen compound type alone and with nitrogen addition rate and year on H are not significant (NS, $P > 0.05$), thus one-way ANOVA (Tukey's HSD) and *t*-tests are used to test the effects of nitrogen addition rate and year on H. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in LN among nitrogen addition rate in the 2015 and 2020 plots, respectively. The asterisk above the picture indicates the significant difference between the two years under each nitrogen addition rate. Note: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CK: non-fertilizer control = 0, N1 = 2, N2 = 5, N3 = 10, N4 = 20, and N5 = 50 kg N ha^{-1} yr^{-1}; AC (NH_4HCO_3), AN (NH_4NO_3), AS [$(NH_4)_2SO_4$]. The gray dashed line is the connection of the mean value.

Effects of N Addition Rate, N Compound Type, and Period on the Morphological Traits of the Plant Leaves

Nitrogen addition had a significant effect on SLA and LDMC in the plant leaves, which aligns with previous studies (Knops and Reinhart, 2000) and demonstrates that N addition influences these leaf functional traits. In this study, N addition increased the H of the plants, indicating that N addition promoted plant growth, the plant growth strongly affected by nitrogen limitation. The results showed that, N addition increased the SLA of the plant leaves and decreased the LDMC. From an inter-year perspective, long-term N application had higher SLA and lower LDMC compared with short-term N application. SLA and LDMC are key indicators of nutrient utilization strategies (Garnier et al., 2004). A high SLA and low LDMC represent rapid nutrient acquisition, which is conducive to the growth of plants in a nutrient-rich environment, while low SLA and high LDMC are suitable for plants in nutrient-poor environments. In the environment, plants adopt a conservative growth strategy (Wilson et al., 2010; Rose et al., 2013). Our results showed that N addition had a beneficial effect on the growth of *L. chinensis* and *T. lanceolata*. Taken together, our results suggest that the plasticity of plant leaf functional traits expressed allowed the plants to adapt to environment changes under increased N deposition (Agrawal, 2001).

Under the background of N addition, changes in plant leaf morphological traits represent a type of phenotypic plasticity. Although our results reflected that the trait plasticity of both plants changed in a favorable direction for plant growth, longer N deposition experiments are encouraged to understand whether N saturation of leaf traits would occur in future.

Our results suggest that the effects of different N compounds on leaf functional traits were not significant. As the N compounds selected in the experiment all have ammonium ions, this shows that different anions (SO_4^-, HCO_3^-, NO_3^-) have no significant effect on leaf functional traits.

Effects of Long- and Short-Term N Deposition on the Correlation of Plant Traits

We found that under long-term and short-term N application, the effect of N addition rates on plant traits was similar. Wright et al. (2004) proposed the "leaf economic spectrum" (LES) and found that the traits of plant leaves are arranged in an orderly manner along a continuously changing combination spectrum of functional traits. The survival strategies of plants are divided into a ""quick investment-return" strategy and "slow investment-return" strategy. In our study, compared with short-term N addition, long-term N addition made plant leaves having higher LN, higher LP, higher plant height, lower LDMC, and higher SLA, indicating that N addition would result in *L. chinensis* and *T. lanceolata* having a tendency toward larger and thinner leaves and a "quick investment-return" strategy. Therefore, meadow grassland plants adapt to the long-term nitrogen deposition

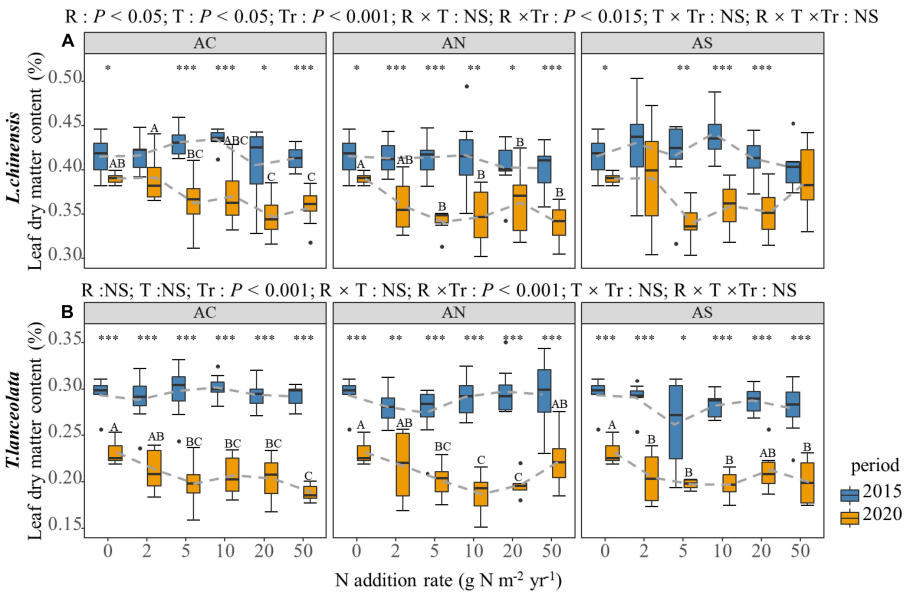

FIGURE 5 | Effects of short- and long-term nitrogen deposition on LDMC of two species: **(A)** *Leymus chinensis*; **(B)** *Thermopsis lanceolata*. The three-way ANOVA was used to test the effects of Pr, T and R on LDMC. The effects of the nitrogen compound type alone and with nitrogen addition rate and year on LDMC are not significant (NS, $P > 0.05$), thus one-way ANOVA (Tukey's HSD) and *t*-tests are used to test the effects of nitrogen addition rate and year on LDMC. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in LN among nitrogen addition rate in the 2015 and 2020 plots, respectively. The asterisk above the picture indicates the significant difference between the two years under each nitrogen addition rate. Note: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CK: non-fertilizer control = 0, N1 = 2, N2 = 5, N3 = 10, N4 = 20, and N5 = 50 kg N ha^{-1} yr^{-1}; AC (NH_4HCO_3), AN (NH_4NO_3), AS [$(NH_4)_2SO_4$]. The gray dashed line is the connection of the mean value.

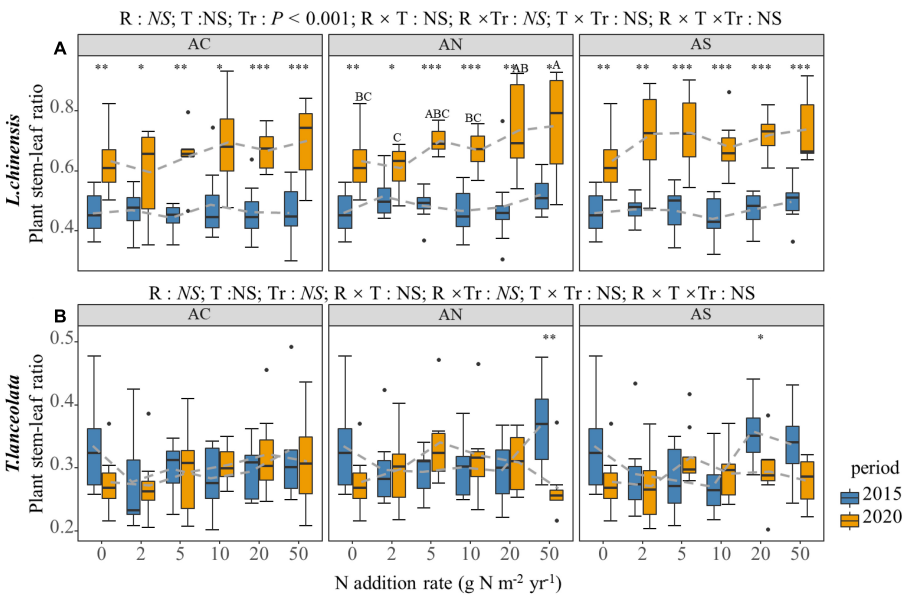

FIGURE 6 | Effects of short- and long-term nitrogen deposition on S:L of two species: **(A)** *Leymus chinensis*; **(B)** *Thermopsis lanceolata*. The three-way ANOVA was used to test the effects of Pr, T and R on S:L. The effects of the nitrogen compound type alone and with nitrogen addition rate and year on S:L are not significant (NS, $P > 0.05$), thus one-way ANOVA (Tukey's HSD) and *t*-tests are used to test the effects of nitrogen addition rate and year on S:L. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in LN among nitrogen addition rate in the 2015 and 2020 plots, respectively. The asterisk above the picture indicates the significant difference between the two years under each nitrogen addition rate. Note: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. CK: non-fertilizer control = 0, N1 = 2, N2 = 5, N3 = 10, N4 = 20, and N5 = 50 kg N ha^{-1} yr^{-1}; AC (NH_4HCO_3), AN (NH_4NO_3), AS [$(NH_4)_2SO_4$]. The gray dashed line is the connection of the mean value.

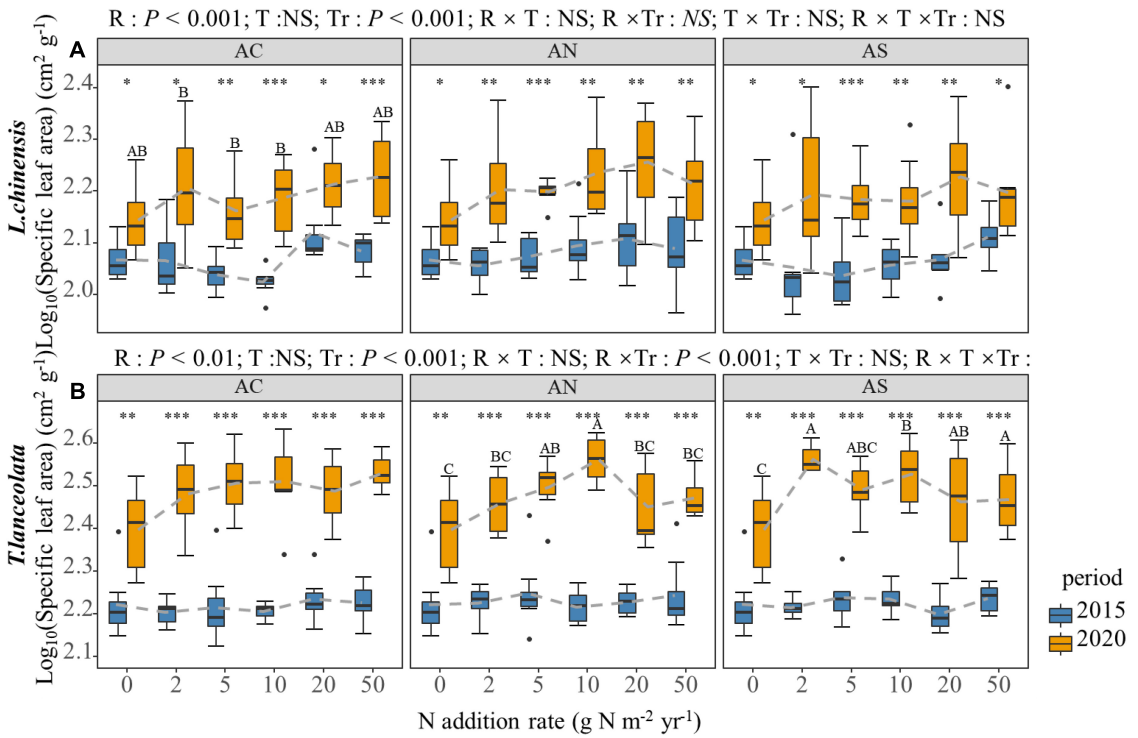

FIGURE 7 | Effects of short- and long-term N deposition on SLA of two species: **(A)** *Leymus chinensis*; **(B)** *Thermopsis lanceolata*. The three-way ANOVA was used to test the effects of Pr, T and R on SLA. In order to make the data more stable, the value of the SLA is the logarithm of the original data. The effects of the nitrogen compound type alone and with nitrogen addition rate and year on SLA are not significant (NS, $P > 0.05$), thus one-way ANOVA (Tukey's HSD) and *t*-tests are used to test the effects of nitrogen addition rate and year on SLA. Different lower-case and upper-case letters indicate significant differences ($P < 0.05$) in LN among nitrogen addition rate in the 2015 and 2020 plots, respectively. The asterisk above the picture indicates the significant difference between the two years under each nitrogen addition rate. Note: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. CK: non-fertilizer control = 0, N1 = 2, N2 = 5, N3 = 10, N4 = 20, and N5 = 50 kg N ha^{-1} yr^{-1}; AC (NH_4HCO_3), AN (NH_4NO_3), AS [$(NH_4)_2SO_4$]. The gray dashed line is the connection of the mean value.

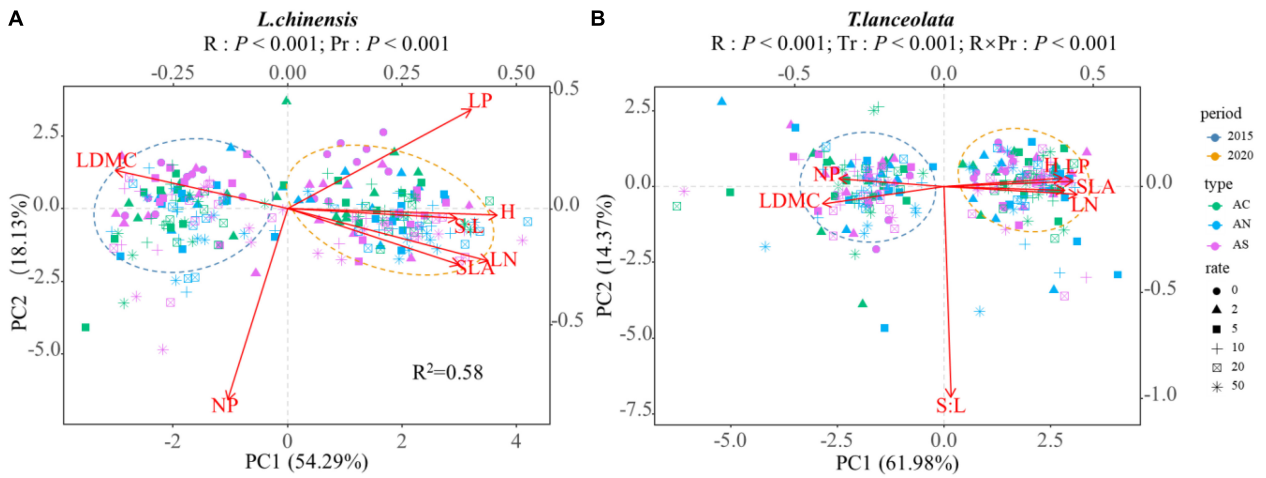

FIGURE 8 | Principal components analysis (PCA) for all leaf functional traits of two species across different N addition rates and different periods: **(A)** *Leymus chinensis*; **(B)** *Thermopsis lanceolata*. The length of a variable vector in the representation space is indicative of the variable's level of contribution. Different shapes represent different nitrogen addition rates, and different colors represent different N compound type. The two ovals are the distribution ranges of plant traits in two periods respectively.

environment mainly through the distribution and regulation of resources. In addition, a fast strategy may be beneficial in N deposition condition because it allows plants to take advantage of high resource, thus increasing their probability of survival. We also found that the correlation between plant leaf functional traits changed under long-term N addition conditions. This is similar to previous studies by Wright and Cannon (2001) and Pensa et al. (2010). Our results suggest that long-term nitrogen application tends to lead to closer relationships between traits. In addition, the correlation of traits involved in the "leaf economic spectrum" tended to prevail under N addition, but the magnitude of the correlation coefficients did not remain constant, and even opposite relationships were observed. This suggests that the relationships between plant traits are not absolutely stable, but vary with the environment. Therefore, when we try to use the general correlation of leaf traits to predict other relatively difficult-to-measure traits from some of the easily measured traits, we need to take into account the environmental changes such as local climate and geographical characteristics.

CONCLUSION

Our results revealed that N deposition will affect leaf functional traits of *L. chinensis* and *T. Lanceolata* in meadow grassland. The N compound type had little effect on the properties, while the N addition rates and the period significantly increased the LN, LP, and SLA, and decreased the LDMC of the plant leaves. The correlation between leaf traits would change with the duration of N addition. The effects of long-term N addition to plant leaf functional traits were significantly higher than short-term treatments, which indicated the cumulative effect of the N deposition. The response of *L. chinensis* and *T. Lanceolata* to the N addition tends to be a "quick investment-return" strategy. These results clearly showed that N deposition can promote plant height, which will increase their light competitiveness and provide advantages for their own growth. And it is necessary for us to use changes in the external environment as one of the factors of reference in the future when predicting traits using the universal relationships related to leaf traits.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

PZ designed the research and secured funding. LS, GY, YZ, SQ, JD, YC, and XL contributed to the field and laboratory measurements. LS and GY analyzed the data. RW provided ideas for writing. LS wrote the manuscript that was intensively edited by all of the authors. All authors contributed to the article and approved the submitted version.

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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.2022.841464/full#supplementary-material

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Nitrogen Deposition Shifts Grassland Communities Through Directly Increasing Dominance of Graminoids: A 3-Year Case Study From the Qinghai-Tibetan Plateau

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Nitrogen (N) deposition has been increasing for decades and has profoundly influenced the structure and function of grassland ecosystems in many regions of the world. However, the impact of N deposition on alpine grasslands is less well documented. We conducted a 3-year field experiment to determine the effects of N deposition on plant species richness, composition, and community productivity in an alpine meadow of the Qinghai-Tibetan Plateau of China. We found that 3 years of N deposition had a profound effect on these plant community parameters. Increasing N rates increased the dominance of graminoids and reduced the presence of non-graminoids. Species richness was inversely associated with aboveground biomass. The shift in plant species and functional group composition was largely responsible for the increase in productivity associated with N deposition. Climatic factors also interacted with N addition to influence productivity. Our findings suggest that short-term N deposition could increase the productivity of alpine meadows through shifts in composition toward a graminoid-dominated community. Longer-term studies are needed to determine if shifts in composition and increased productivity will be maintained. Future work must also evaluate whether decreasing plant diversity will impair the long-term stability and function of sensitive alpine grasslands.

Keywords: alpine grassland, community productivity, N deposition, species composition, species richness, Qinghai-Tibetan Plateau

INTRODUCTION

Atmospheric nitrogen (N) deposition has been increasing globally for decades (Phoenix et al., 2006; Galloway et al., 2008a). N deposition rates in terrestrial ecosystems are projected to increase to 200 Tg N yr^{-1} by 2050 (Galloway et al., 2008b). As N is the principal limiting nutrient for plant growth (Vitousek and Howarth, 1991), N deposition may have positive effects on plant productivity. These effects may be strongest in N-limited ecosystems (Vitousek et al., 1997) such as alpine

grasslands, which are more sensitive to shifts in nitrogen availability (Xu et al., 2018). There is evidence that long-term N deposition can increase the productivity of grassland ecosystems (Bai et al., 2008; Xia and Wan, 2008; Shen et al., 2019), possibly impacting ecosystem function (Gruber and Galloway, 2008; Stevens et al., 2015). High levels of N deposition (72 kg N ha^{-1} $year^{-1}$) in grassland ecosystems have also been shown to alter plant community structure, decreasing plant species richness and altering species composition (Mountford et al., 1993; Bobbink et al., 2010; Pierik et al., 2011; Isbell et al., 2013). As N deposition rates increase, nitrophilous species become more abundant while N-sensitive species become less abundant (Stevens et al., 2004).

Grasslands are important terrestrial ecosystems covering approximately 25% of earth's land surface (Hui and Jackson, 2006). Species richness and composition are two important components of plant diversity in grassland ecosystems (Marini et al., 2007). The effects of N inputs on species richness and composition may be mediated by changes to multiple soil properties (Myklestad, 2004). Different plant functional groups in a grassland ecosystem may respond differently to N deposition because of contrasting N-use efficiencies and adaptation mechanisms (Harpole and Tilman, 2007; Song et al., 2011). Some grassland studies have highlighted the importance of species richness to ecosystem function by demonstrating that species richness is positively correlated with productivity (Tilman et al., 1996; Hector et al., 1999). However, other research has indicated that plant composition represents a stronger influence on productivity than species richness (Hooper and Vitousek, 1997). Therefore, additional research is needed to assess the importance of species richness and composition in determining grassland productivity under N deposition, especially in N-limited habitats such as alpine meadows.

The Qinghai-Tibetan Plateau (QTP), known as the "third pole" on Earth, has experienced increasing N deposition rates in recent decades (Stocker et al., 2013). N deposition rates on the QTP range from 8.7 to 13.8 kg N ha^{-1} $year^{-1}$ and are likely to increase in the coming decades (Lü and Tian, 2007). Alpine grasslands are the largest ecosystem on the QTP (Wang et al., 2012a; Zhao et al., 2017) and provide important life-supporting services for millions of people (Dong et al., 2010). These crucial plant communities are being degraded by numerous stressors, including climate change (Wen et al., 2010), and are likely to be especially sensitive to N deposition.

The objective of our study was to assess the response of plants in an alpine meadow of the QTP to increasing N deposition. We conducted a 3-year field experiment using four levels of N (0, 8, 40, and 72 kg N ha^{-1} yr^{-1}) to simulate atmospheric N deposition. In this experiment, the nitrogen addition level of 8 kg N ha^{-1} $year^{-1}$ was set according to the background value of atmospheric nitrogen deposition in this area (8.7–13.8 kg N ha^{-1} $year^{-1}$) (Lü and Tian, 2007). Galloway et al. (2004) pointed that the N deposition rate on the Qinghai-Tibetan Plateau is predicted to increase to 40 kg N ha^{-1} $year^{-1}$ by 2050, and this rate is estimated to be the N saturation threshold in alpine meadow (Zong et al., 2016). So in our experiment, the setting of 40 and 72 kg N ha^{-1} yr^{-1} was to simulate medium and high nitrogen deposition rate, respectively (Galloway et al., 2004; Bowman et al., 2012; Zong et al., 2016). Moderate nitrogen input can usually increase plant aboveground productivity (Xia and Wan, 2008). However, excessive N input can cause species loss through shifting plant communities to compositions that can tolerate high N levels (Bowman et al., 2008; Clark and Tilman, 2008). Additionally, excessive N input can substantially change soil nutrient balance and decrease soil pH (Phoenix et al., 2012), which may be the main reason that cause species loss (Stevens et al., 2010). Previous studies found that rhizomatous grasses are better adapted to high N levels (Bai et al., 2010). In addition, grasses species are in the upper part of meadow canopy and more competitive for light and soil nutrients (Hautier et al., 2009). Previous study found that grasses species such as *Poa pratensis* and *Stipa aliena* in alpine meadow had a more high N use efficiency with exogenous nitrogen input (Wang et al., 2012b). Based on the above studies, we hypothesized that nitrogen deposition would (1) decrease species richness, (2) increase the abundance and dominance of graminoids (mostly grasses), and (3) increase productivity, primarily through alterations in species composition.

MATERIALS AND METHODS

Study Site

We conducted our study in an alpine meadow near the town of Xihai, Haiyan County (36°56′N, 100°57′E, 3,100 m a.s.l.) in the Qinghai province of China (**Figure 1**). The soil at the study site is comprised primarily of clay. The study site had been fenced (1.2 m high) since 2012 to prevent entry by resident mammalian herbivores such as yak and sheep. The growing season in this region typically begins in early May and ends in late September. During the experimental years of 2015, 2016, and 2017, the annual rainfall totaled 392.8, 396.5 and 556.3 mm, respectively, while the mean annual temperature was 1.8, 3.3, and 1.8°C, respectively. The highest temperatures and precipitation generally occurred from April to October of each year (**Figure 2**).

Experimental Design

We initiated the N addition treatments to simulate N deposition in 2014 on 12 plots, each measuring 2 by 5 m. All plots had similar topography, vegetation, and land-use history. Treatments comprised four N addition rates with three replicates for each rate: 0 kg N ha^{-1} yr^{-1} (control-CK), 8 kg N ha^{-1} yr^{-1} (Low-N), 40 kg N ha^{-1} yr^{-1} (Mid-N), and 72 kg N ha^{-1} yr^{-1} (High-N). We selected these N addition rates based on current and projected N deposition levels for the region (Lü and Tian, 2007). We applied nitrogen fertilizer (powder) to plots as ammonium nitrate (NH_4NO_3) in early May and July from 2014 to 2017 at dusk on a rainy day to avoid needing to water.

Vegetation and Soil Sampling

We carried out vegetation surveys as well as biomass harvests and soil sampling in July and early-August of each year at peak vegetation growth for the region. Within each of the 12 plots, we identified each plant species in a randomly placed 1 by 1 m quadrat and recorded its abundance by point intercept

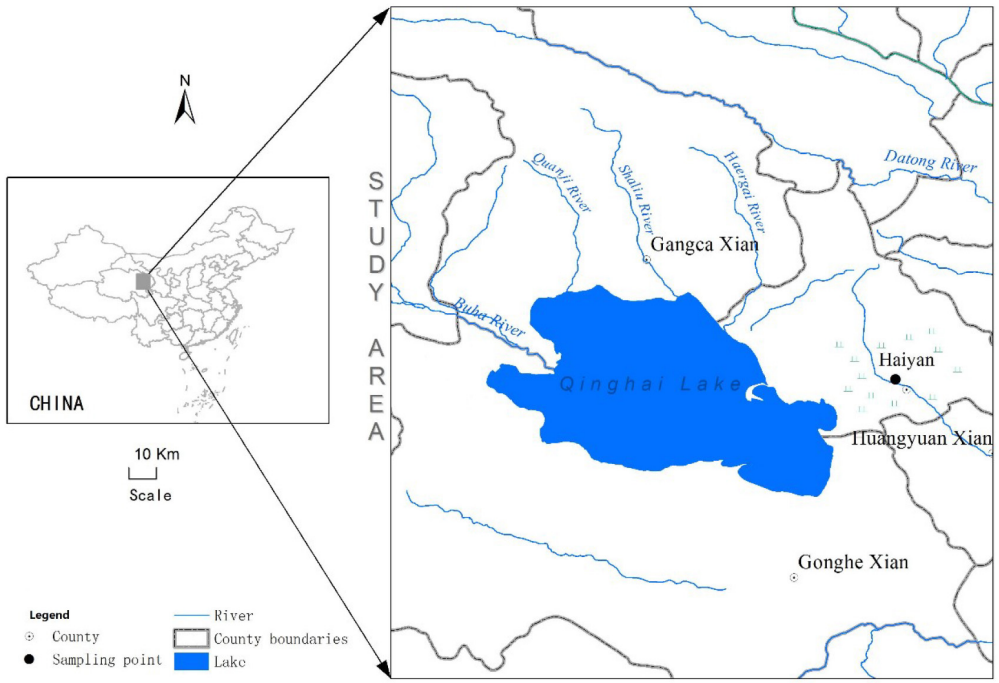

FIGURE 1 | Location of the study site in the Qinghai-Tibetan Plateau of China.

method. In the same quadrats, another survey was carried out to visually estimate the percentage cover (visual estimation) of two functional plant groups: graminoids and non-graminoids. We used these data to calculate the graminoid dominance, the ratio of graminoid percentage cover to forb percentage cover. The graminoid dominance is a measure of functional composition. We also harvested the aboveground biomass of plants of the two functional groups in a 0.5 by 0.5 m sub-quadrat in each plot. Harvested plant samples oven-dried at 65°C (firstly oven-dried at 105°C for 3 h for de-enzyming) to constant weight. Lastly, we collected three soil cores in each plot using a 3.5 cm-diameter soil probe at a depth of 20 cm. Soil samples were mixed, air-dried at 70°C to constant weight, and sieved through a 0.15-mm mesh. We determined total nitrogen (TN) and carbon (TC) content of each composite soil sample using an elemental analyzer (EuroEA 3000, Pavia, Italy).

Statistical Analysis

We performed all statistical analyses using SPSS (SPSS for Windows, version 22.0). We tested the effects of N addition on graminoids dominance (value of graminoids cover/non-graminoids cover), species richness, and productivity using one-way analysis of variance (ANOVA) with treatment means separated using the least significant differences (LSD) test at the $P < 0.05$ level. We used a two-way ANOVA to evaluate interactions between N addition and year. We used least-squares regression to assess relationships between graminoids dominance, species richness, and aboveground biomass. We evaluated associations between these community metrics, soil measurements, and climate factors with principal component analysis (PCA); Non-metric multidimensional scaling (NMDS) to estimate the differences in overall community composition across the treatments, and all these two analyses were carried out in R (version 4.1.2). To test the significance of the observed associations and understand their functional underpinnings that N addition and climatic factors affected plant productivity. The software package AMOS 22.0 was used to develop the SEM model and calculate related path coefficients, squared multiple correlations, direct and indirect effects and model fit. An insignificant χ^2-statistic indicates that the SEM model provides a good fit. A qualified structural equation model (SEM) should match the criterion below (Jonsson and Wardle, 2010; Wei et al., 2013): (1) non-significant χ^2 test, namely $P > 0.05$; (2) Comparative fit index, CFI > 0.95; (3) Root mean square error of approximation, RMSEA < 0.05.

RESULTS

Effects of Nitrogen Deposition on Species and Functional Group Composition

The species composition of our alpine meadow plant community was markedly altered following the 3 years of N addition (**Figures 3**, **4**). In plots subjected to the highest N addition rate, the abundance of grass species such as *Leymus secalinus*, *Agropyron cristatum*, *Poa crymophila,* and *Stipa purpurea* increased substantially during the experimental period while the

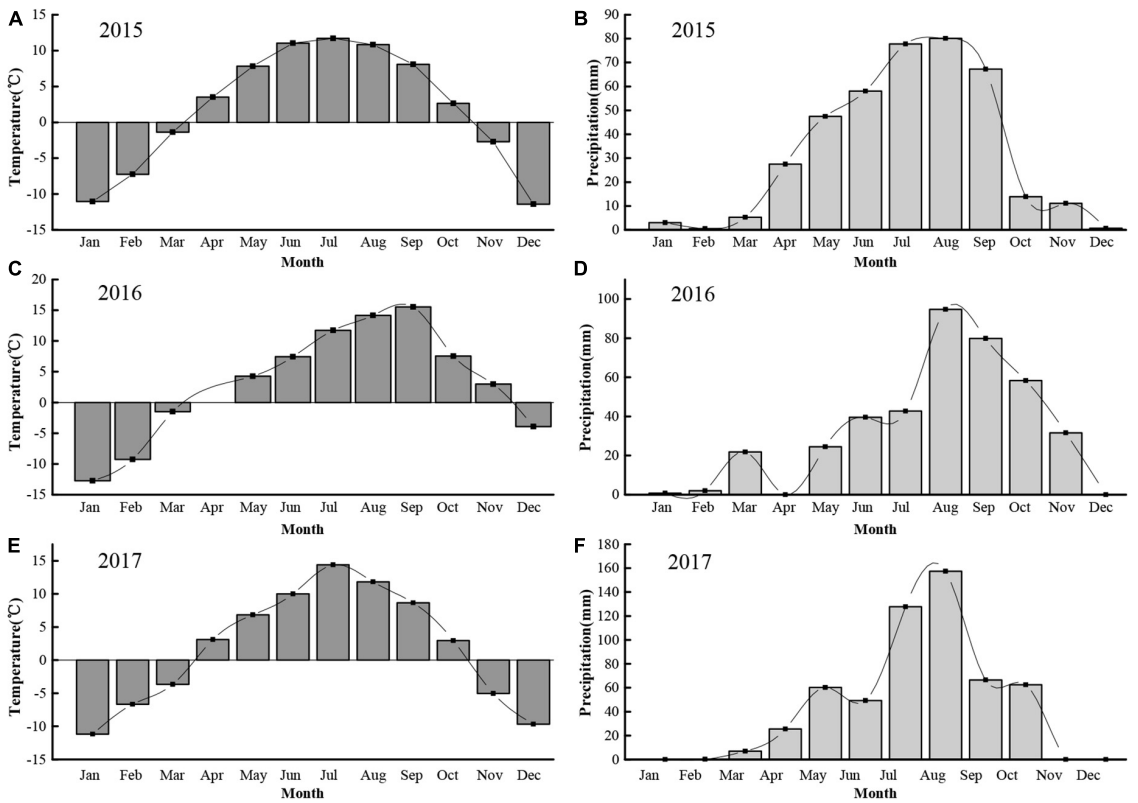

FIGURE 2 | Monthly temperature (°C) and precipitation (mm) during each of 3 years at the study site. **(A,B)** Monthly temperature and precipitation in 2015, respectively; **(C,D)** Monthly temperature and precipitation in 2016, respectively; **(E,F)** Monthly temperature and precipitation in 2017, respectively.

abundance of forb species such as *Aster tataricus* and *Artemisia scoparia* decreased. There was a significant interaction between N treatment and year on the abundance of graminoids (mostly grasses) relative to non-graminoids (F = 12.72, P < 0.05, **Table 1**). In 2015, the first year of sampling after N addition, the abundance of graminoids relative to non-graminoids was greatest at the intermediate and highest N levels. In 2016, the relative abundance of graminoids was greatest at the highest N level. In 2017, following 3 years of N addition, the relative abundance of graminoids increased with increasing N addition rate. The graminoids ratio, the percent cover of graminoids relative to non-graminoids, increased significantly (P < 0.05) from 2015 to 2017 and this trend strengthened with increasing N addition. Species richness was not affected by N addition (**Figure 5A**) rate in our 3-year experiment but varied by year (F = 33.26, P < 0.05, **Table 1**). Species richness decreased significantly in 2016 and 2017 relative to 2015 (**Figure 5B**). Species richness was negatively associated with mean annual temperature (1.8°C in 2015, 3.3°C in 2016, and 1.8°C in 2017).

Effect of Nitrogen Deposition on Plant Aboveground Biomass

There was a significant interaction between N addition level and year (F = 3.76, P < 0.05, **Table 1**) on aboveground biomass. In 2015, after 1 year of N addition, the aboveground biomass of plants subjected to the highest N addition rate was greater than all other treatments (**Figure 5C**). In 2016, plants in the two highest N addition treatments had greater biomass than the control and lowest N addition treatment. In 2017, after 3 years of treatments, aboveground biomass increased with each increase in N addition rate. The total aboveground biomass across N addition levels increased significantly between 2015 and 2017 (**Figure 5C** and **Table 1**).

Relationships Between Species Richness, Functional Group Composition, and Aboveground Biomass

Using data for all 3 years and least-squares regression analysis, we evaluated the relationship between aboveground biomass and functional group composition (i.e., graminoid dominance) (**Figure 6A**) and the relationship between aboveground biomass and species richness (**Figure 6B**). Increasing graminoid dominance was positively correlated with total aboveground biomass (R^2 = 0.729, P = 0.001) while species richness was negatively correlated with the total aboveground biomass (R^2 = 0.249, P < 0.001).

Direct and Indirect Drivers That Affect Plant Productivity

A PCA analysis of plant community performance, soil properties, and climatic factors revealed that PC1 and PC2 explained 70.5% of the variance (**Figure 7**). Data were clearly separated by year.

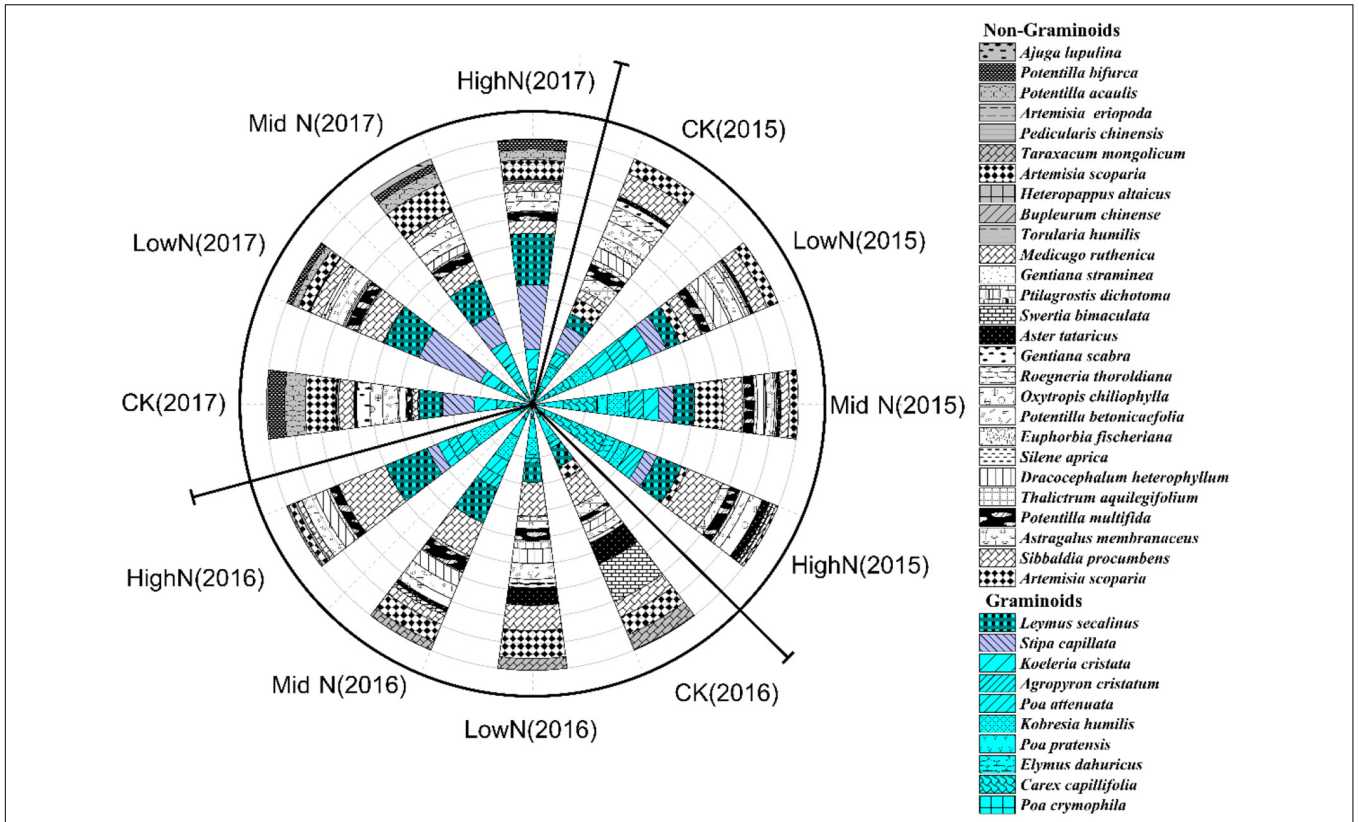

FIGURE 3 | Effect of nitrogen addition rate on species composition based on abundance from 2015 to 2017. Species denoted in color are graminoids (mostly grasses).

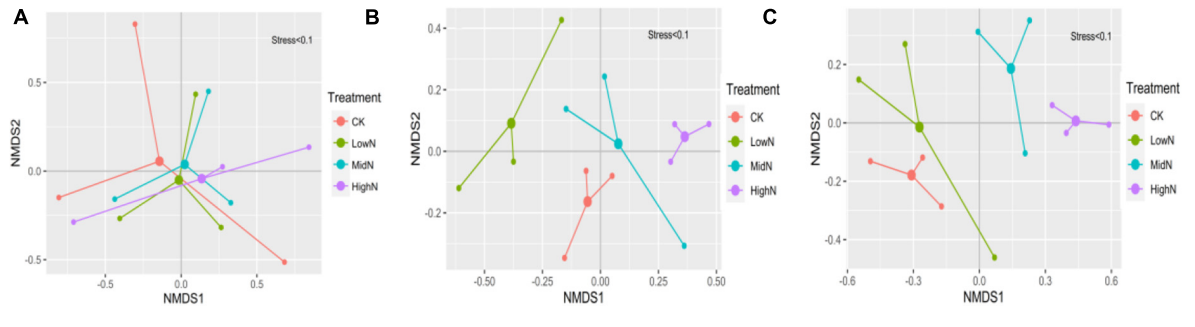

FIGURE 4 | Non-metric multidimensional scaling (NMDS) plot of species composition from 2015 to 2017 under different treatments. **(A)** 2015; **(B)** 2016; **(C)** 2017. 0 kgN ha^{-1} yr^{-1} (CK), 8 kgN ha^{-1} yr^{-1} (LowN), 40 kgN ha^{-1} yr^{-1} (MidN), and 72 kg N ha^{-1} yr^{-1} (HighN).

We found that soil N and soil C/N were the two variables contributing most to the patterns observed. The graminoid dominance and the total aboveground biomass were positively related to N addition rates and mean annual precipitation. We also developed an SEM model (**Figure 8**, χ^2 = 3.066, P = 0.382, GFI = 0.979, NFI = 0.989, RMSEA = 0.025) showing that N addition and climate can influence aboveground community biomass indirectly as well as directly. Nitrogen addition increased aboveground biomass largely by increasing the graminoid dominance. Precipitation influenced aboveground biomass through its effects on both the graminoid dominance and species richness. Lastly, higher mean annual temperatures affected aboveground biomass by decreasing species richness.

DISCUSSION

Inter-Annual Variability in Species Richness

Species richness is a fundamental component of biodiversity. Despite numerous studies, the relationship between productivity and the species richness of a community remains unclear

TABLE 1 | Two-way ANOVA for the effects of year and N-addition rate on species richness, species composition, and total aboveground biomass.

Factors	Species richness		Species composition		Aboveground biomass	
	df	*F*	df	*F*	df	*F*
Year	2	33.26*	2	65.63*	2	85.95*
Nitrogen	3	1.401	3	89.97*	3	57.48*
Nitrogen × Year	6	0.921	6	12.72*	6	3.76*

**Significant according to a LSD test ($P < 0.05$).*

(Mittelbach et al., 2001; Adler et al., 2011). This relationship has sometimes been characterized as a hump-back response in temperate vegetation (Oba et al., 2001). The hump-back model has received some support on the Qinghai-Tibetan Plateau, although productivity-diversity relationships in this region show considerable variation associated with variation in precipitation and species composition (Wu et al., 2014). The downhill slope of the hump-back model may be consistent with previous reports of declines in species richness following N deposition (Bai et al., 2010; Damgaard et al., 2011; Fang et al., 2012). In our study, the impact of N deposition on species richness was not significant in any of the 3 years, although we did observe a decreasing trend with increasing N in 2016 and 2017. We also observed that species richness was negatively correlated with aboveground biomass across the entire dataset.

We found that more variation in species richness was explained by year than by N deposition rates. Average species richness across treatments declined from 17 species in 2015 to 9 species in 2016, and then increased back to 12 species in 2017. These fluctuations in species richness appeared to be inversely associated with mean annual temperatures. Elevated ambient temperatures have been reported to reduce the number of plant species in this region of China (Klein et al., 2004). Additional research and longer-term studies should evaluate this negative relationship between temperature and species richness, which may have significant implications for the stability of sensitive alpine grassland communities in the context of climate change.

Nitrogen Availability and Climate Jointly Influence Productivity

Nitrogen is a limiting soil nutrient in many terrestrial ecosystems (LeBauer and Treseder, 2008). N deposition can improve soil N availability and thereby promote plant productivity (Bai et al., 2010; Liu et al., 2011; Zhang et al., 2015). In the first year of our study, we found that aboveground biomass increased significantly under the high N deposition rate but did not change substantially under the low and intermediate N deposition rates. In the next 2 years, aboveground biomass increased with the rate of N deposition. These results suggest that N may be a critical limiting nutrient for plant productivity in this region, yet there exists hysteresis effect of N accumulation. In this study, soil N and soil C/N were the two variables contributing most to the patterns observed. The graminoid dominance and the total aboveground biomass were positively related to N addition rates and mean annual precipitation. This suggests that soil nutrients which are classically considered to be variable and in this system may be most strongly controlled mechanistically by amount of rainfall, and both soil C and N which classically only change with long-term or very disruptive manipulations ecosystem change are what is driving separation in multivariate space among years. Our findings are consistent with previous studies showing N limitation in grassland ecosystems (Bassin et al., 2007; Dai et al., 2019). Alpine grasslands are usually N-limited, therefore N deposition can release such nutrient restriction to some degree. Our results substantiated previous conclusions that N deposition can enhance alpine grassland productivity (Liu et al., 2011; Zhang et al., 2015), however, hysteresis effect existed as N loads and time length of N deposition increase. Also, climatic factors such as precipitation can obviously interact with N deposition to influence productivity.

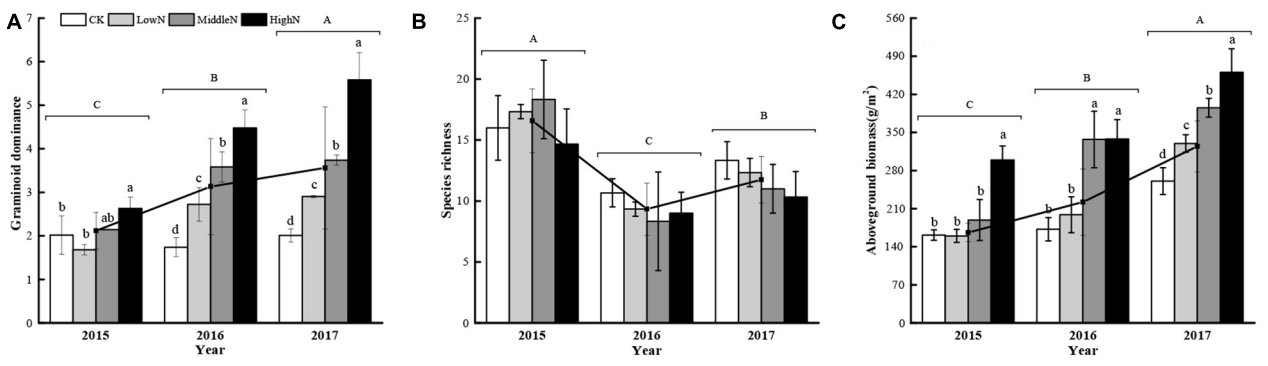

FIGURE 5 | Effects of nitrogen addition rate on **(A)** graminoid ratio, **(B)** species richness, and **(C)** aboveground biomass during the 3-year study. Vertical bars indicate standard deviation of the mean. Lowercase letters indicate significant differences among nitrogen addition rates within years and uppercase letters indicate significant inter-annual differences ($P < 0.05$). Functional composition is expressed as the ratio of graminoid cover relative to the cover of non-graminoids (i.e., graminoid dominance). 0 kg N ha^{-1} yr^{-1} (CK), 8 kg N ha^{-1} yr^{-1} (LowN), 40 kg N ha^{-1} yr^{-1} (MidN), and 72 kg N ha^{-1} yr^{-1} (HighN).

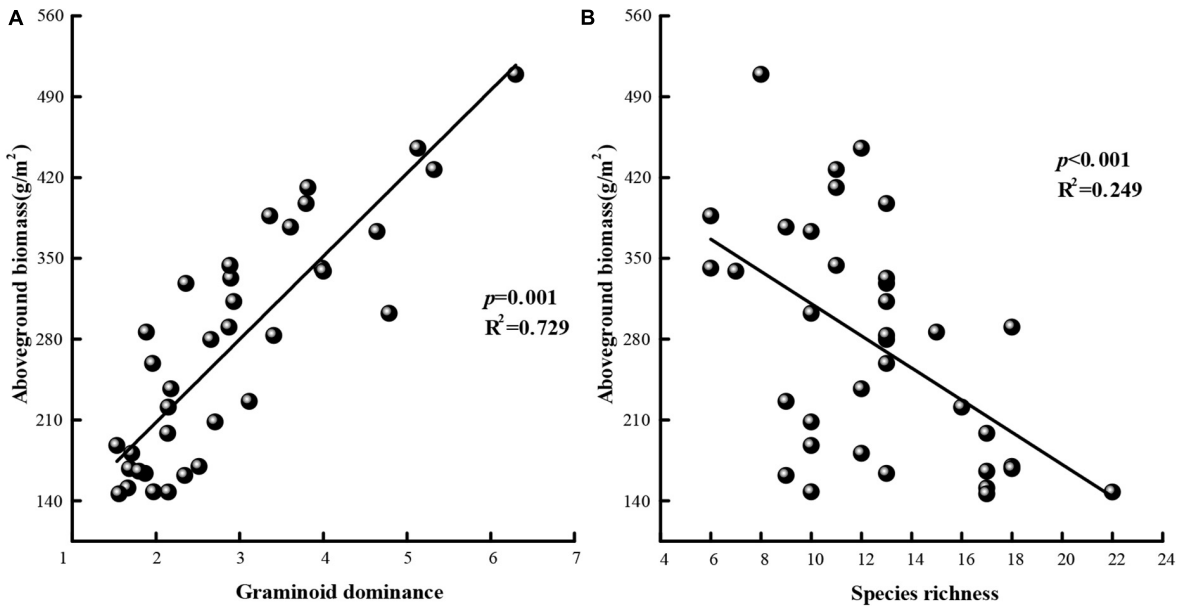

FIGURE 6 | Aboveground biomass in terms of **(A)** graminoid dominance and **(B)** species richness. Fitted lines are based on least-squares regression. Graminoid dominance is expressed as the ratio of graminoid cover relative to the cover of non-graminoids.

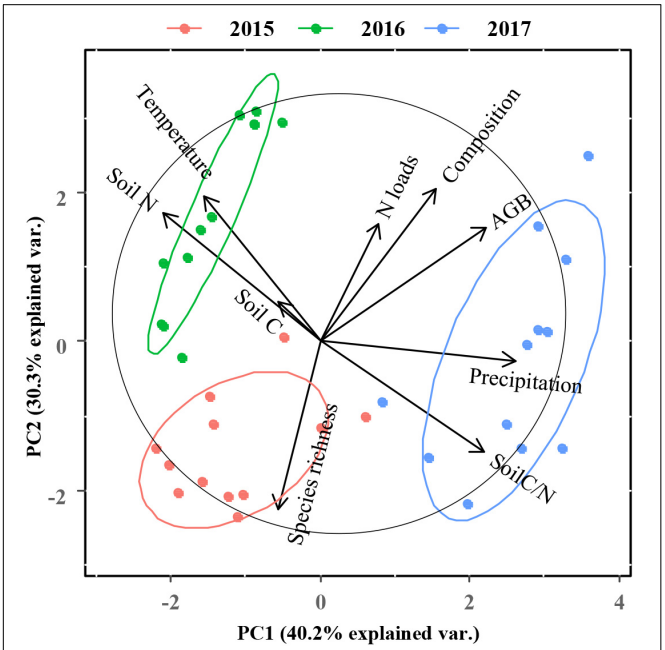

FIGURE 7 | Principal component analysis (PCA) for all plant community parameters, soil properties, temperature, and precipitation across different N addition rates during the 3-year field study. The length of a variable vector in the representation space is indicative of the contribution level of the variable. Composition is expressed as graminoid cover/non-graminoids (i.e., graminoid dominance). AGB, aboveground biomass.

We also observed interannual differences in aboveground plant productivity. These differences are consistent with the idea that N availability interacts with climatic variables, such as temperature and precipitation, to affect plant productivity in grassland ecosystems (Harpole et al., 2007; Sala et al., 2012). Water availability is a particularly important influence on plant responses to N (Hooper and Johnson, 1999; Bai et al., 2008), primarily because increasing productivity increases transpiration, potentially exacerbating water shortages (Harpole et al., 2007). In our study, the effects of N deposition on plant productivity were greatest in the year with the highest precipitation (2017). Our SEM modeling analysis also showed that precipitation may intensify the effects of N on plant productivity. Mean annual precipitation increased from 2015 to 2017, and the mean annual precipitation was highest among 3 years, also the plant community AGB significantly increased over years, suggesting that a higher water availability induced by high precipitation will also favor the grassland productivity in alpine ecosystem. In addition, the high precipitation period was mainly during the growing season (July∼August), indicating that summer precipitation variability plays an important role in determine the increase of grassland productivity. Soil water content is related to temperature and precipitation, higher temperature can cause water evaporation. During the growing season, the temperature increased over years, and precipitation showed the same variation trend, suggesting that the alpine regions is experiencing a warming and more humid environment change. Therefore, soil water content might increase if the increase of soil water availability induced by precipitation can override water loss of evaporation under warmer climate. Our previous study has found that warming could cause water loss in alpine meadow (Shen et al., 2020). In cold alpine regions, temperature and precipitation are usually the limiting factors for plants, though warmer could accelerate water evaporation, some studies reckoned that a higher temperature may favor plant growth

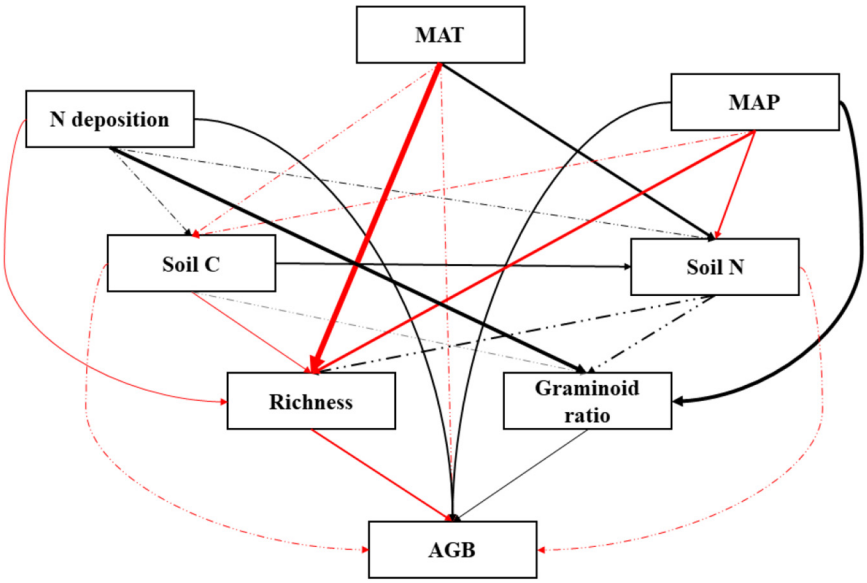

FIGURE 8 | Structural equation model (SEM) testing the relationships between community, soil, and climatic variables. Significant impacts are shown by solid lines whereas non-significant impacts are shown by dashed-dotted lines. Arrow width is proportional to the strength of the relationship. Positive impacts are shown in black and negative impacts are shown in red. MAT, mean annual temperature; MAP, mean annual precipitation; Soil C, soil carbon content; Soil N, soil nitrogen content; AGB, aboveground biomass. $\chi^2 = 3.066$, $P = 0.382$, GFI = 0.979, NFI = 0.989, RMSEA = 0.025.

by releasing cold-limitation in alpine regions (Ganjurjav et al., 2016). However, relation between temperature and soil nutrients is not clear (Kladivko and Keeney, 1987; Sardans et al., 2008; Wang et al., 2016). Higher temperature can cause more soil water evaporation, such water loss is detrimental for plant nutrient uptake as the acquisition of nutrients by plants depends mostly on soil water availability (Querejeta et al., 2021). Yet, at least in our study such negative effects are not obvious than the positive effects from the perspective of community productivity level. Community productivity increases can likely occur in the warmer and wetter alpine regions in the future (Winkler et al., 2016).

Shifts in Community Composition Help Explain Trends in Productivity

Previous nutrient addition studies have reported increases in the aboveground biomass and cover of graminoids with increasing N and concomitant decreases in the dominance of non-graminoids (Stevens et al., 2006; Song et al., 2011; Fang et al., 2012). In agreement with such studies, we found that N deposition increased the abundance and cover of graminoids over non-graminoids. Our data also indicated that N deposition could shift plant species composition in favor of graminoid species within a 3 years period.

We found that the graminoid dominance was positively correlated with community productivity. This finding is consistent with previous research showing that increases in productivity following N deposition were largely due to increases in the prevalence and aboveground growth of grasses (Xu et al., 2015; Fu and Shen, 2016; You et al., 2017). In our study site, grasses grew much taller than non-graminoids and likely outcompeted forb species for light after N deposition. Grasses have also been shown to have higher soil nutrient-use efficiencies, especially for soil N (Huang et al., 2008). Lastly, N deposition can lead to soil acidification and accumulation of Mn^{2+}, which reduces photosynthetic rates more in non-graminoids than grasses (Tian et al., 2016). The differential effects of N on grasses and non-graminoids suggest that N deposition is likely to have significant effects on community function in the long-term (Shaver et al., 2001).

CONCLUSION

Simulated N deposition led to large differences in plant community performance in an alpine meadow of the Qinghai-Tibetan Plateau over 3 years. Nitrogen deposition significantly increased the total aboveground biomass of the plant community and shifted community composition in favor of graminoids. These results suggest that continued N deposition in alpine meadow could significantly alter plant communities diversity, function, and stability. Increased productivity of these generally N-limited habitats could support greater densities of wild or domesticated grazing animals, but the long-term potential effects of N on these sensitive ecosystems are largely unknown. The effects of temperature and precipitation on species richness and plant community responses to N also require further research. Our 3-year field study demonstrates that N deposition and climate variability can have extensive impacts on alpine grassland plant communities, even over short periods. These changes are likely to be magnified if N deposition persists over longer time periods or interacts with the effects of climate change.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SD designed the study. HS analyzed the data and contributed to writing. SD and AD helped to revising the manuscript. HS, JX, YZ, and LW carried out the experiment. All authors contributed to the article and approved the submitted version.

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N-Induced Species Loss Dampened by Clipping Mainly Through Suppressing Dominant Species in an Alpine Meadow

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Nitrogen addition and clipping can exert substantial impact on species diversity but their interactions and the underlying mechanisms still remain unclear. Resource competition theory holds that sufficiently strong competitive ability of dominant species can lead to the losses of subordinate species through competitive exclusion, while niche differentiation theory suggests that the persistence of subordinate species in competitive systems can be promoted by guaranteeing positive growth rates of rare species. Taking advantage of a field experiment with nitrogen addition (10 g N m^{-2} $year^{-1}$) and different clipping intensities (2, 15, and 30 cm) treatments in a Tibetan alpine meadow across 2015–2020, we assessed the relative importance of competitively dominant species and niche differentiation in driving species diversity changes *via* using community weighted mean (CWM) and variation coefficient of nearest neighbor distance (CV_NND) of functional traits including height, specific leaf area (SLA) and leaf dry matter content (LDMC). We show that nitrogen enrichment drove a strong plant diversity loss ($P < 0.001$). Clipping at different intensities had little effect on species diversity, but it can reduce the N-induced diversity loss. Nitrogen addition and clipping caused changes in community diversity were mainly indirectly attributed to their effects on community functional composition, and the competitive ability of dominant species. Nitrogen increased the CWM of functional traits to improve the competitive ability of dominant species. In contrast, clipping influenced species diversity positively by decreasing CWM_{height} ($P < 0.001$), and also negatively by increasing CWM_{SLA} ($P < 0.001$) and decreasing CV_NND_{SLA} ($P < 0.05$). Interacting with N addition, clipping resulted in a neutral effect on species diversity, because clipping could offset the negative effects of nitrogen addition through an opposite effect on CWM_{height}. This study provides new insights into the mechanisms of diversity maintenance with respect to nitrogen addition and clipping. Thus, clipping is recommended as a useful management strategy to alleviate the species loss caused by nutrients enrichment and maintain the diversity of grassland ecosystems.

Keywords: competitive ability, niche differentiation, functional traits, CWM, CV_NND, grassland

INTRODUCTION

Increasing human activities and environmental changes have caused the loss of species in many ecosystems, especially in grasslands (Borer et al., 2014; DeMalach et al., 2017; Harvey et al., 2018; Brandt et al., 2019; Molina et al., 2021). The dramatic decline in grassland diversity is often attributed to increased nutrient enrichment (Stevens et al., 2004; Harpole et al., 2016, 2017; Seabloom et al., 2021) or changed land-use (Golodets et al., 2011; Herrero-Jáuregui and Oesterheld, 2017; Zhang et al., 2018; Rahmanian et al., 2020). One of the potential mechanisms explaining species coexistence is the competitive ability of dominant species (Mortensen et al., 2017; Saiz et al., 2019). Sufficient functional dominance can promote competitive exclusion, leading to the losses of subordinate species (Wilsey and Polley, 2004; Hautier et al., 2009). Niche differentiation is the other possible mechanism maintaining species coexistence (Harpole and Tilman, 2007; Isbell et al., 2009; Doležal et al., 2018), which has been shown to promote the persistence of subordinate species in communities (Palmer, 1994; Gonzalez and Loreau, 2009). However, the relative importance of competitively dominant species and niche differentiation remains unclear.

Nutrient addition is a commonly used management practice to improve grassland production (Schellberg et al., 1999; Conant et al., 2001; Socher et al., 2012). While increasing community productivity, nutrient enrichment is also a major driver of biodiversity loss (Phoenix et al., 2006; Southon et al., 2013; Borer et al., 2014). Since plants have different resources acquisition ability, nutrient enrichment may cause asymmetric resource availability and further result in asymmetric competition amongst competing plants (Rajaniemi, 2002; Niu et al., 2008; Hautier et al., 2009; Goodwillie et al., 2020). For example, Rajaniemi (2002) attributed the loss of diversity to increased underground competition after N-P-K fertilization. Similarly, Hautier et al. (2009) showed that nitrogen addition can reduce plant diversity *via* inducing aboveground light competition. Moreover, nutrient enrichment can alter the competitive intensity *via* shifting plants' resources allocation strategies. For example, Niu et al. (2008) found that fertilization significantly decreased leaf allocation for forbs but increased leaf allocation for grasses, thereby resulting in an increase in competitive ability of grasses. Due to the increased light limitation, nitrogen addition may also favor taller species thereby excluding almost all shorter species.

Land-use management, such as grazing or clipping also plays an important role in maintaining plant diversity in grasslands (Klimek et al., 2008; Speed et al., 2013; Beck et al., 2015; Bakker et al., 2016; Doležal et al., 2018; Kapás et al., 2020). Since high biodiversity can be maintained under moderate grazing intensity (Grime, 1973; Connell, 1978; Li et al., 2021), regular clipping (simulated grazing) also is likely to produce similar effects (Antonsen and Olsson, 2005; White et al., 2014; Nagata et al., 2016; Smith et al., 2018; Yang et al., 2019). For instance, clipping at a low intensity usually imposes moderate disturbance which can significantly raise plant diversity in grasslands, whereas high levels of clipping lead to a decline in species diversity (Socher et al., 2012). *Via* inhibiting the competitive ability of dominant species, clipping was able to decrease the advantage of dominant species and release forbs species that can withstand grazing pressure (such as low-stature and creeping-growth forms) from the competition, thereby contributing to the maintenance of diversity (Isbell et al., 2009; Doležal et al., 2018). Moreover, grazing or clipping may induce niche differentiation of species in plant communities, which is a critical mechanism to promote species co-existence (Pierce et al., 2007; Niu et al., 2015; Wang et al., 2021). For example, Niu et al. (2015) found that grazing promoted species diversity *via* inducing differentiation of leaf phosphorus in an alpine meadow; Wang et al. (2021) also found that clipping promoted the asynchronous response of subordinate species (Doudová and Douda, 2020), and ultimately increased community species diversity. Thus, grazing or clipping may alleviate the negative effects of fertilization on plant community diversity.

To distinguish the relative importance of competitively dominant species and niche differentiation for diversity changes in the context of clipping and nitrogen addition, we used a trait-based approach. Since community weighted means (CWM) were weighted by the relative contribution of species to calculate the mean trait value for a given community (Díaz et al., 2007), the metric can capture the effect of dominant species (Avolio et al., 2019) and represent the competitive ability of dominant species. To indicate niche differentiation effects, the variation coefficient of nearest neighbor distance (CV_NND) of traits was used (Schöb et al., 2012). CV_NND was calculated using the relative distances between traits (Jung et al., 2010) which can quantify trait spacing and indicate the microscale environmental heterogeneity and resource allocation (Schöb et al., 2012).

Here, we conducted a 5-year experiment with nitrogen addition and clipping in an alpine meadow on the eastern Qinghai-Tibet Plateau to assess the independent and interactive effects of nitrogen addition and clipping on plant community diversity. We measured three plant functional traits [i.e., height, specific leaf area (SLA), and leaf dry matter content (LDMC)] for most species (coverage greater than 5%) and calculated indices relating to the competitive ability of dominant species and niche differentiation at the community level (i.e., CWM and CV_NND of each trait, respectively). These analyses allow us to examine the relative importance of the two ecological mechanisms in driving plant diversity. We hypothesized that: (1) the negative effect of nitrogen addition on the diversity of plant communities would be counteracted by clipping; (2) the decline in species diversity due to nitrogen addition is often accompanied by a dramatic increase in the height and competitiveness of dominant species; however, (3) clipping may increase plant diversity or alleviates negative effects of nitrogen addition on plant diversity *via* reducing the competitive ability of dominant species and increasing niche differentiation.

MATERIALS AND METHODS

Study Site and Experimental Design

Our field experiment was carried out at the Research Station of Alpine Meadow and Wetland Ecosystems of Lanzhou University

(Maqu Branch Station), Gansu province, northwestern China, at the elevation of approximately 3,500 m (33° 40′N, 101° 52′E). According to the past 35-year observation (from the Maqu County Meteorological Bureau), the mean annual precipitation in the region is about 620 mm, and mainly distributed in the short summer. The average annual temperature is 1.2°C, ranging from −10°C in January to 11.7°C in July. The vegetation is characterized by typical alpine meadow, and the soil is subalpine meadow soil. The natural habitat is dominated by perennial sedges (e.g., *Kobresia graminifolia*), Gramineae (e.g., *Elymus nutans* and *Poa poophagorum*), and forbs (e.g., *Anemone rivularis*). The study area had received no fertilizer or clipping before this experiment. During the study, fencing was used to prevent large mammal grazing.

Our experiment was established at a flat site in May 2014. Within the experimental area, twenty 2 × 2 m plots were laid out in four columns and five rows with a 2 m-wide buffer zone between the plots (**Figure 1**). Four clipping treatments and five replicates were randomly assigned to plots and applied every week from June to July every year. By removing plant above ground parts at different heights, three clipping intensity levels were implemented including (1) low intensity (stubble height: 30 cm), (2) moderate intensity (stubble height: 15 cm), and (3) high intensity (stubble height: 2 cm). Each plot was divided into two subplots, resulting in a total of 40 subplots. Since the threshold for changes in biomass, species diversity and community composition in response to nitrogen addition in mature Eurasian grasslands is about 10.5 g N m^{-2} $year^{-1}$ (Bai et al., 2010), we randomly assigned each subplot to one of two N treatments: ambient (N_0, control) or N addition (N_{10}, 10 g N m^{-2} $year^{-1}$, in the form of NH_4NO_3). Fertilization was carried out once a year in late May every year.

Vegetation Sampling and Plant Functional Trait Measurements

Species richness and species abundance were recorded annually within a 0.5 × 0.5 m quadrat that was randomly placed within each subplot in late-August from 2015 to 2020. In May 2018, we measured three plant functional traits for each species, including plant height (H, cm), (SLA, cm^2/g) and LDMC, which are easy to measure and closely related to resource acquisition and utilization. Plant height has a direct impact on plant competition, because taller species can gain a competitive advantage through preferential exposure to light (Westoby et al., 2002). SLA is positively related to photosynthetic capacity, leaf longevity, relative growth rate, and competitive ability, therefore increased CWM_{SLA} represents a good indicator of eutrophication (Ordoñez et al., 2009). LDMC is also widely used as an indicator of plant resource-use strategy (Vaieretti et al., 2007).

In each subplot, 10 individuals of each species were randomly selected and their heights were recorded. If the number of individuals was less than 10, all individuals of the species were measured. One mature and complete leaf was selected from each individual per species in all subplots, and the fresh weight of each leaf was weighed with an electronic analytical balance. Leaf areas were calculated with ImageJ software after scanning by an Epson-V300 scanner. After dried at 75°C for 48 h to a constant weight, the biomass of each leaf was weighed. Then the SLA was calculated as the ratio of leaf dry weight to leaf area, and LDMC as the ratio of leaf dry mass to leaf fresh weight.

Indices of Plant Functional Diversity

Two functional metrics, CWM and coefficient of variation of nearest neighbor distance (CV_NND), were calculated.

The CWM value of each trait was calculated in each community according to Lavorel et al. (2008):

$$CWM = \sum_{i=1}^{n} p_i \times trait_i$$

where p_i and $trait_i$ are respectively the relative abundance and trait value of species i in the community. CWM is strongly driven by the trait values of dominant species; a high CWM value indicates a strong role of dominant species in the community.

We also estimated individual trait differentiation by calculating the CV_NND of each trait individually. According to Schöb et al. (2012), the CV_NND values were calculated as the coefficient of variation of differences between successive trait values of neighbors within a plot. With CV_NND, a lower value reflects niche differentiation (i.e., even spacing of traits) (Jung et al., 2010), while a higher value indicates clumping of species in trait space (Schöb et al., 2012).

Statistical Analyses

To meet the assumptions of normal distribution and variance homogeneity, the data were log-transformed when necessary. Two-way ANOVAs were employed to assess the effects of clipping, nitrogen addition, and their interaction, on species diversity and CWM and CV_NND of different functional traits. Two-way ANOVAs with Tukey tests were used to test for differences in each index among treatments.

Structural equation modeling (SEM) was used to explore effects of clipping, nitrogen addition, and their interaction on Shannon diversity through CWM and CV_NND of each trait. Our experimental treatments (nitrogen addition and clipping) could impose certain environmental constraints within the community that limited the range of trait values (Díaz et al., 1998), which can be reflected in functional composition and functional dispersion (denoted by CWM and CV_NND, respectively). Therefore, we conducted SEM according to *a priori* model with the following premises: (1) clipping, nitrogen addition and their interaction could directly affect CWM and CV_NND of different functional traits; (2) Shannon diversity was indirectly mediated by clipping, nitrogen addition and their interaction through CWM and CV_NND of different traits.

To test the goodness of SEMs, we used a combination of χ^2 test, root mean square error of approximation (RMSEA) test and comparative fit index (CFI). A non-significant χ^2 and RMSEA test, and CFI > 0.9 indicate a good fit of the model to the data.

All data were analyzed using R software, version 4.1.0 (R Development Core Team, 2021). Shannon diversity was calculated with the "vegan" package (Oksanen et al., 2020),

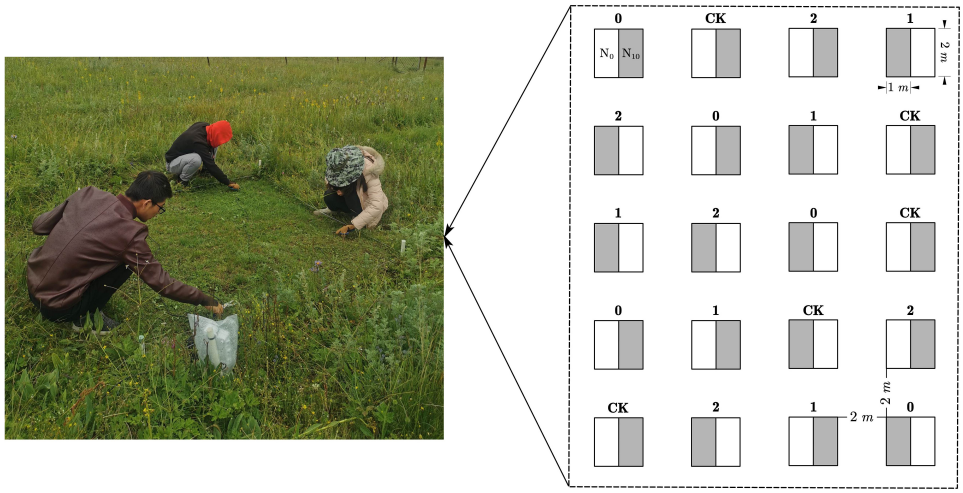

FIGURE 1 | Schematic representation of the experiment design. Twenty 2 × 2 m plots were laid out in four columns and five rows with a 2 m-wide buffer zone between the plots. Labels above the boxes represent the clipping treatments that were randomly assigned to the plots (CK: no clipping; 0: high intensity; 1: moderate intensity; 2: low intensity). Each 2 × 2 m plot was divided into two subplots, one was dedicated to the ambient N (N_0, subplot A white), another to nitrogen addition treatment (N_{10}, subplot B gray).

while the SEMs were conducted using the "lavaan" package (Rosseel, 2012).

RESULTS

Species richness ($P < 0.001$) and Shannon diversity ($P < 0.001$) were significantly lower in the N_{10} plots than in N_0 plots. Clipping at different intensities did not cause significant changes in Shannon diversity compared with the control (**Table 1** and **Figure 2**). However, clipping alleviated the decline of plant diversity under fertilized plots. Since the changes of species richness in each treatment were consistent with Shannon diversity, only Shannon diversity was used to illustrate the results in this paper.

Nitrogen addition, clipping and their interaction had significant effects on the community weighed means for the three functional traits (**Table 1**). Specifically, nitrogen application significantly increased CWM for plant height and SLA (height, $P < 0.001$; SLA, $P < 0.001$; LDMC, $P = 0.138$) (**Table 1**), indicating a positive effect on

TABLE 1 | *P*-values of two-way ANOVA to evaluate the effect of clipping, nitrogen addition and their interaction on community indices.

Community indices	Clipping	Nitrogen	Clipping × N
Richness	*0.085*	**<0.001**	0.304
Shannon diversity	0.952	**<0.001**	0.485
CWM_{height}	**<0.001**	**<0.001**	**<0.001**
CWM_{SLA}	**<0.001**	**<0.001**	**<0.01**
CWM_{LDMC}	**<0.001**	0.138	**<0.001**
CV_NND_{height}	**<0.001**	0.118	**<0.05**
CV_NND_{SLA}	**<0.05**	0.317	0.225
CV_NND_{LDMC}	**<0.001**	0.764	0.264

Probabilities considered statistically significant ($P < 0.05$) and marginally significant ($P < 0.1$) are indicated in bold and italic typeface, respectively.
CWM, community weighted mean; CV_NND, coefficient of variation of nearest neighbor distance; SLA, specific leaf area; LDMC, leaf dry matter content.

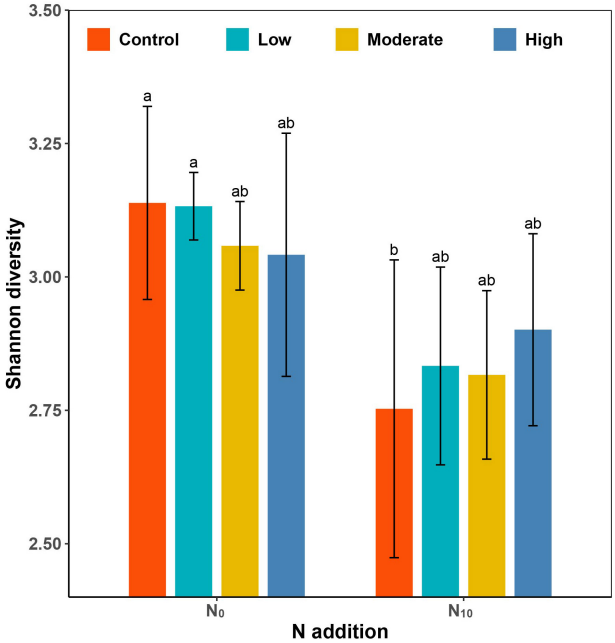

FIGURE 2 | Barplot showing the differences in Shannon diversity among fertilization (N_0: control, N_{10}: nitrogen addition) and clipping (i.e., control, low intensity, moderate intensity, high intensity) treatments. Values (±SE) are means of five replicates of each treatment. Different letters denote significant differences between treatments ($P < 0.05$, Tukey's HSD tests).

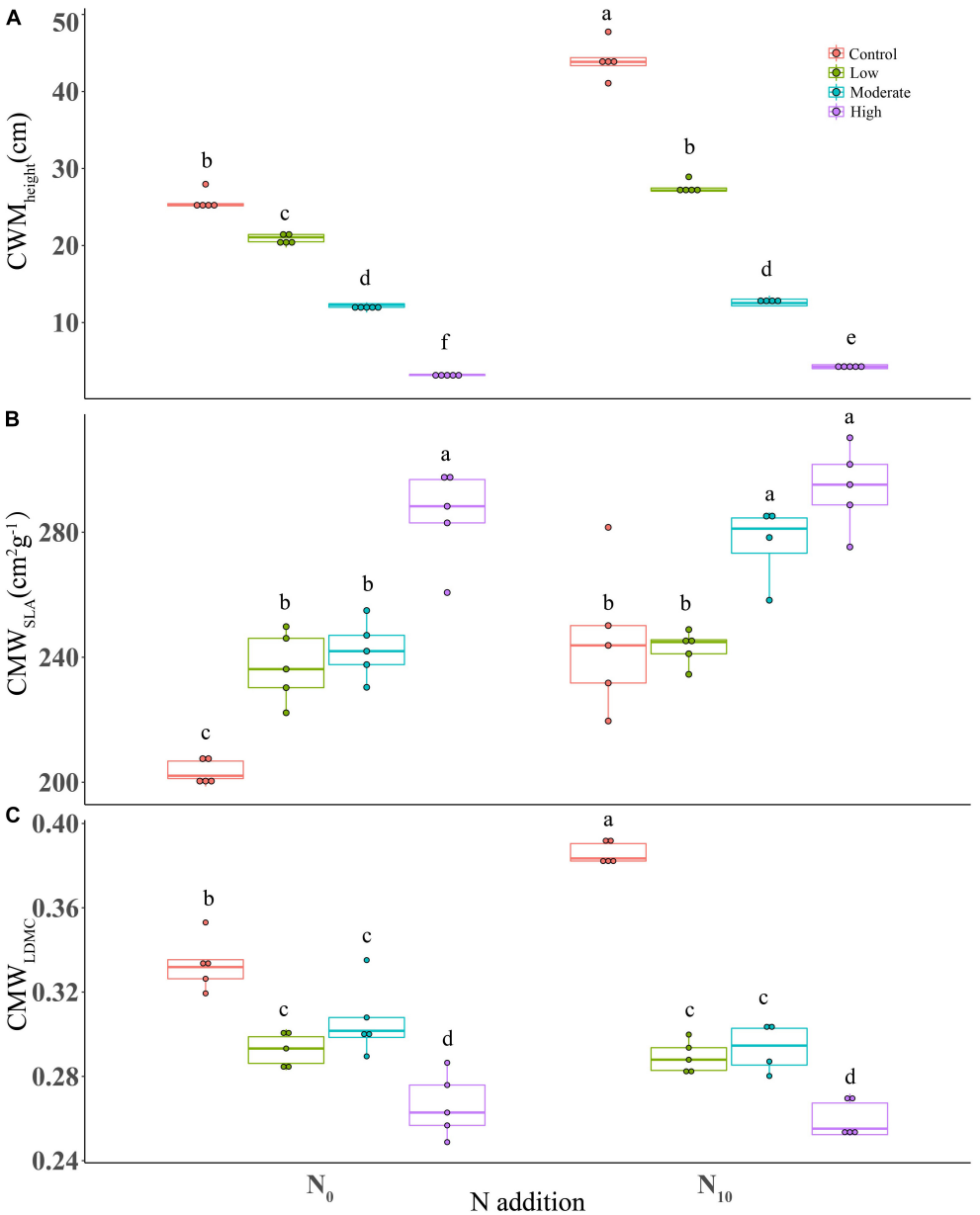

FIGURE 3 | Boxplots showing the differences in CWM of functional traits among fertilization (N_0: control, N_{10}: nitrogen addition) and clipping (i.e., control, low intensity, moderate intensity, high intensity) treatments. **(A)** height, **(B)** specific leaf area, and **(C)** leaf dry matter content. CWM, community weighted mean. The box signifies the upper and lower quartiles, and the whiskers extend up to 1.5 times that intra-quartile range. Median is represented by horizontal line. For a given trait, different letters between treatments donate significant differences ($P < 0.05$, Tukey's HSD tests).

the competitive ability of dominant species. Clipping significantly increased CWM_{SLA} ($P < 0.001$), but decreased CWM_{height} ($P < 0.001$) and CWM_{LDMC} ($P < 0.001$) (**Figure 3**). Increasing clipping intensities could partially offset the changes of CWM traits caused by N addition. At high and moderate intensities, clipping counteracted the differences in CWM_{height} due to nitrogen addition (**Figure 3A**). The increase in CWM_{SLA} under nitrogen addition could be offset by clipping at low and high intensities (**Figure 3B**). Similarly, N-induced increase in CWM_{LDMC} could also be alleviated under different intensities of clipping (**Figure 3C**).

Nitrogen addition had no significant effects on the coefficient of variation of nearest neighbor distance (CV_NND) of all traits (height, $P = 0.118$; SLA, $P = 0.317$; LDMC, $P = 0.764$) (**Table 1**). Clipping at high intensity remarkably increased the CV_NND of plant height and LDMC (**Figures 4A,C**), but significantly decreased CV_NND_{SLA} at moderate intensity (**Figure 4B**). The interaction between clipping and N addition had a significant negative effect on CV_NND_{height} (**Table 1**). High clipping

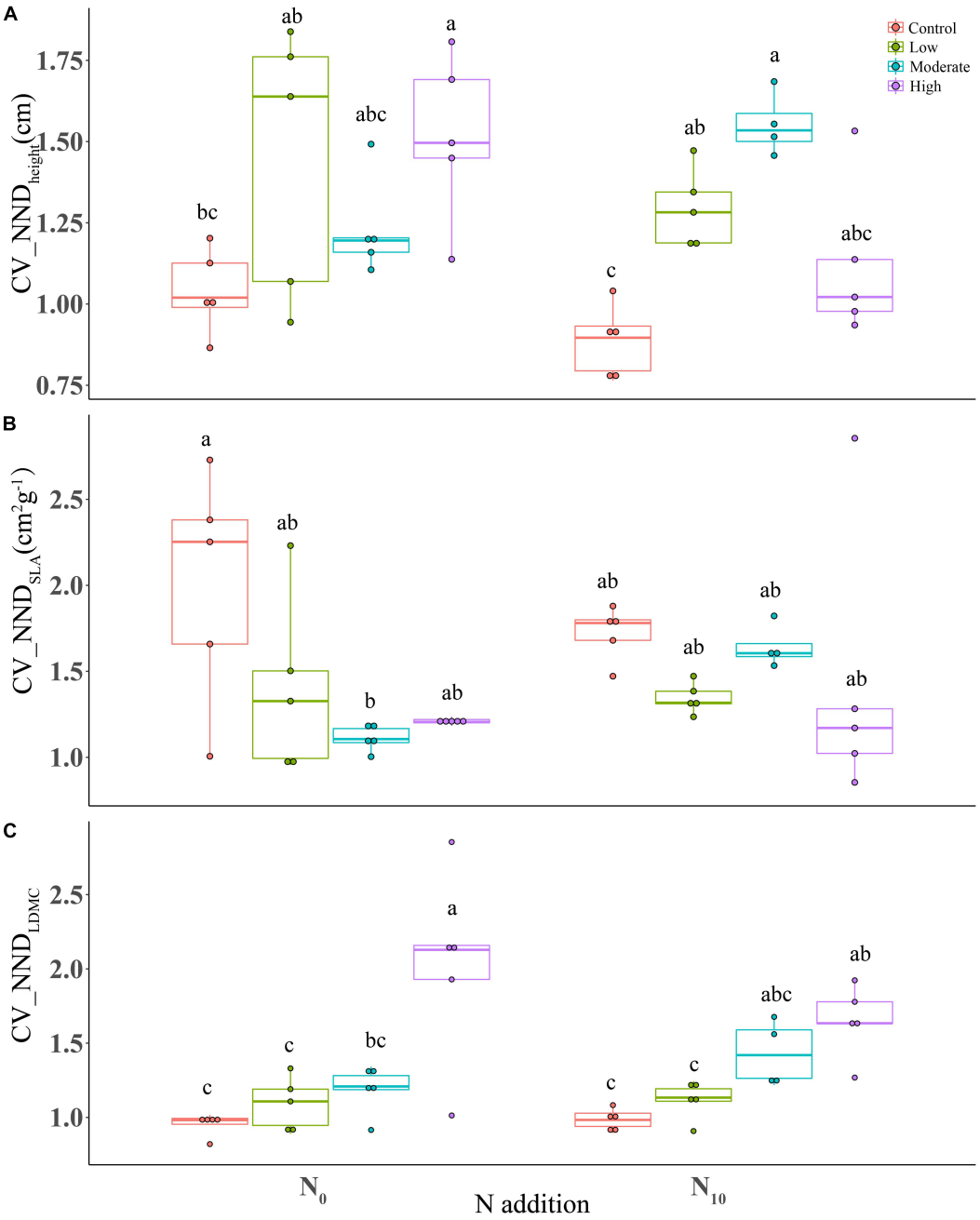

FIGURE 4 | Boxplots showing the differences in CV_NND of functional traits among fertilization (N_0: control, N_{10}: nitrogen addition) and clipping (i.e., control, low intensity, moderate intensity, high intensity) treatments. **(A)** height, **(B)** specific leaf area, and **(C)** leaf dry matter content. CV_NND, coefficient of variation of nearest neighbor distance. The box signifies the upper and lower quartiles, and the whiskers extend up to 1.5 times that intra-quartile range. Median is represented by horizontal line. For a given trait, different letters between treatments donate significant differences ($P < 0.05$, Tukey's HSD tests).

intensity significantly reduced the variation of CV_NND_{height} under N addition (**Figure 4A**).

The SEM model showed that the CWM of functional traits was the most important attribute in influencing community diversity when including both direct and indirect effects (**Figure 5**). Community diversity was mainly altered by treatments and their interaction indirectly rather than their direct effects (**Figure 5**). Specifically, nitrogen addition showed a negative effect on Shannon diversity indirectly through increasing CWM_{height} and CWM_{SLA}, whereas the combination of N addition and clipping positively affected Shannon diversity mainly through decreasing CWM_{height}. Clipping indirectly affected Shannon diversity simultaneously through CWM and CV_NND of different functional traits. In particular, clipping had a positive

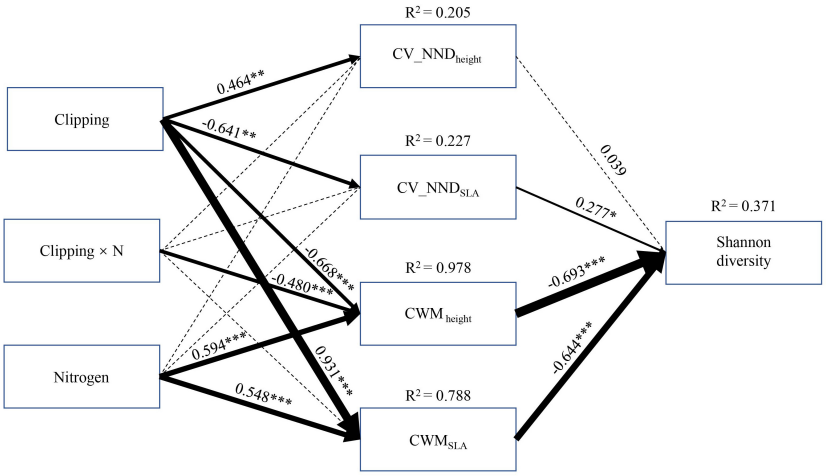

FIGURE 5 | Structural equation modeling of the clipping by nitrogen addition effects on the Shannon diversity of plant community. For abbreviations see **Figures 3**, **4**. df = 19, *P*(Chi-square) = 0.078, CFI = 0.980, *P*(RMSEA) = 0.141. Black solid arrows indicate a significant effect (at the level $P < 0.05$), and black dashed arrows indicate non-significant effect (at the level $P > 0.05$). Values associated with solid arrows represent standardized path coefficients, which are also indicated by arrow width. R^2 values associated with response variables indicate the proportion of explained variation by relationship with other variables. Black solid arrows indicate a significant effect (at the level $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

effect on Shannon diversity by decreasing CWM_{height} and a negative effect on diversity by increasing CWM_{SLA}. At the same time, clipping could negatively affect Shannon diversity through decreasing CV_NND_{SLA}, but the effect of this pathway was weak.

DISCUSSION

Nitrogen enrichment drove a strong plant diversity loss through the increasing competitive ability of dominant species. Clipping could affect both the competitiveness of dominant species and niche differentiation. In particular, clipping reduced the N-induced diversity loss mainly by suppressing competitive dominant species. We also observed significant interaction between nitrogen addition and clipping on dominant species' height, a trait that closely associated with light competition. Together, the results show that competitive ability of dominant species plays a substantial role in diversity maintenance under nitrogen addition and clipping conditions.

Extensive studies have shown that enhanced competition for light is a major mechanism for the diversity loss under fertilization (Hautier et al., 2009; Socher et al., 2012; Yang et al., 2012; Goodwillie et al., 2020). According to the light competition hypothesis (Hautier et al., 2009; Borer et al., 2014; DeMalach et al., 2017), N enrichment releases plant species from symmetrical competition for belowground resources, intensifying asymmetric competition for aboveground light. Taller species generally capture more light resources than shorter ones, which could further expand their competitive advantages, ultimately resulting in the competitive exclusion (Goodwillie et al., 2020). In our study, N-induced negative effects on Shannon diversity were mainly due to the increasing CWM of plant height and SLA, so we inferred that nitrogen addition may decrease species diversity by favoring taller dominant species. Increasing CWM_{height} and CWM_{SLA} under nitrogen addition are expected to increase canopy height and reduce light available for smaller species in the understory, resulting in a decline in species diversity (Hautier et al., 2009). Since rare species are generally of smaller stature and occupy higher diversity (Lavergne et al., 2004; Pfestorf et al., 2013), reduced understory light due to N addition may reduce the number of rare species. Thus, the negative effects nitrogen addition imposed on Shannon diversity were mainly through promoting the competitive advantage of dominant species.

Most previous studies have shown that clipping can increase plant diversity in diverse ways, such as increasing light availability (Borer et al., 2014), reducing species dominance (Lepš and Wan, 2014), promoting rare species regeneration (Wilsey and Martin, 2015) and enhancing seedling germination (Foster and Gross, 1998). A few studies have found that clipping has a weak or even negative effect on plant diversity. For instance, Socher et al. (2012) reported that clipping early in the growing season and clipping at high frequency both decreased biodiversity. Morgan (2015) also found a decrease in diversity when clipping produced excessive litters which suppressed seedling establishment. However, here we found that clipping had little effect on species diversity. With the increasing intensities, clipping promoted dominant species by increasing CWM_{SLA}, and simultaneously exerted suppressive effects by decreasing CWM_{height}, indicating that broadleaf forbs with lower height and higher SLA such as *A. rivularis* were favored under clipping treatments. While clipping could help maintain species diversity by suppressing dominant resource competitors such as tall grasses (Borer et al., 2014), broadleaf species with higher SLA could also reduce light availability to understory and outcompete species that are less effective at light capture (Yang et al., 2015). Besides, clipping could also promote niche differentiation through the decreasing CV_NND_{SLA} under moderate intensity. There may be trade-off

between these pathways, resulting in no significant clipping effect on diversity in our study.

Our results showed that clipping could alleviate the negative effect of nitrogen enrichment on species diversity to some extent. Under fertilized plots where nutrients reinforce the competitive advantage of dominant species (Farrer and Suding, 2016), clipping could inhibit dominant taller species through the decreasing CWM_{height}, which alleviates light competition and promotes random colonization of rare local species (Song et al., 2020), thus maintaining local-scale plant diversity (Hillebrand et al., 2007). We also found a significant negative effect of the interaction between nitrogen addition and clipping on CWM_{height}. However, the effects of nitrogen addition and clipping were not totally counteractive. Nitrogen addition and clipping are both associated with increased CWM_{SLA} thereby negatively affected Shannon diversity, reflecting the increased performance of dominant species under nitrogen addition and clipping conditions (Xu et al., 2018). Besides, clipping could also cause niche differentiation among co-occurring species by altering the spacing of functional traits. CV_NND_{height} increased under high clipping intensity, indicating that overgrazing was related to strong selection effect, and the plant height distribution would be limited to a certain range of grazing tolerance. The decrease in CV_NND_{SLA} under moderate clipping intensity indicated that moderate disturbance facilitated the functional differentiation of SLA, probably due to the exclusion of species with similar traits or the colonization of species with distinct traits (Xu et al., 2018). However, we can only observe a significant positive correlation between species diversity and CV_NND_{SLA}, suggesting that the net effect of clipping on diversity through niche differentiation was negative. Therefore, these results showed that clipping can mitigate N-induced diversity loss mainly through suppressing dominant species.

CONCLUSION

In conclusion, our results show that both the competitive ability of dominant species and niche differentiation (represented by the CWM and CV_NND of different functional traits, respectively) modulated the effects of nitrogen addition and clipping on plant community diversity. Diversity loss due to nitrogen addition were mainly driven by the enhancement of dominant species through increasing CWM. In contrast, clipping influenced species diversity positively by decreasing CWM_{height}, and also negatively by increasing CWM_{SLA} and decreasing CV_NND_{SLA}, resulting in a weak clipping effect in our experiment. However, the negative effects of nitrogen addition on plant diversity can be alleviated mainly through their opposite effects on CWM_{height}. Since nitrogen addition and clipping are two main anthropogenic factors in driving grassland diversity, management strategies could consider incorporating clipping into conservation programs to maintain the diversity of grassland ecosystems.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

WW and XZ conceived and performed the research. WW and ZR mainly contributed to the investigation and data curation. XW analyzed the data. GD, XZ, and XW made suggestions for the revision of the manuscript. WW wrote the manuscript. All authors have read and agreed to the draft manuscript.

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Heterogeneous Nitrogen Supply With High Frequency and Ramet Damage Increases the Benefits of Clonal Integration in Invasive *Hydrocotyle vulgaris*

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Nitrogen (N) deposition significantly affects the growth and the function of invasive clonal plants. However, the effects of heterogeneous N supply with different frequencies on the growth and the potential contribution of clonal integration in invasion plants are still unclear, especially in the complex environment considering ramet damage. To address this question, apical and basal ramets of the clonal invader *Hydrocotyle vulgaris* were connected or disconnected, N was added to the basal ramets with a high frequency, a low frequency, or no supply, and the total N quantity was the same for the different frequency. Furthermore, 8 aphids were placed on the apical ramets, and 30% of each leaf was cut off to cause damage. The connection between ramets significantly increased the biomass, total carbon (C), and total N of the basal and apical ramets. Higher frequency N supply significantly increased the biomass, total C, and total N of the basal ramets and the entire clonal fragment biomass. The damage had no significant effect on the growth of basal and apical ramets. Especially, under the high N frequency and ramet damage condition, the connection between ramets more significantly increased the biomass, total C, and total N of the apical ramets and the entire clonal fragment biomass. In addition, the uptake rates of $^{15}NH_4^+$ and $^{15}NO_3^-$ in *H. vulgaris* had no significant difference, and N supply increased the uptake rates of $^{15}NH_4^+$ and $^{15}NO_3^-$ of the basal ramets. Our results suggest that both higher frequency N supply and clonal integration are beneficial to the growth of *H. vulgaris*. Moreover, the heterogeneous N supply with high frequency and ramet damage increases the benefits of clonal integration in *H. vulgaris*. These findings improve our understanding of the response of clonal invader *H. vulgaris* to nitrogen deposition and ramet damage.

Keywords: herbivory, invasive plant, physiology integration, stable isotope, wetland plant

INTRODUCTION

Alien plants can quickly adapt to new environments, replacing the local plants and seriously damaging the local ecosystem due to some specific traits (Richardson et al., 2000; Kleunen et al., 2010). Studies have shown that clonal integration may be an important trait for alien plants to quickly adapt and successfully invade new environments (Liu et al., 2006; Yu et al., 2009; Song et al., 2013). Besides, due to the influence of fertilization, disturbance, and soil properties, the distribution of soil nutrients needed for plant growth is often heterogeneous in habitats (Zhang et al., 2016; Shen et al., 2019). In clonal plants, heterogeneous resources and colonization of habitats are moderated through clonal integration, where water, nutrients, and carbohydrates are translocated among ramets through a connecting rhizome or stolon, subsequently promoting their growth (Wei et al., 2019; Yu et al., 2019; Zhang et al., 2019; Franklin et al., 2020).

Due to human activities, the total amount of atmospheric nitrogen (N) deposition and the rate keep increasing, which significantly affects the growth and function of plants (Gutiérrez, 2012; Peñuelas et al., 2012; Valliere and Allen, 2016). Previous studies showed that N addition can improve the division of labor of invasive clonal plants and promote their growth (Huang et al., 2018; Lin et al., 2018). In addition, it has been reported that the clonal integration benefits of clonal plants in heterogeneous N environments are more significant (Dong et al., 2015; Liu et al., 2017a; Ying et al., 2018). However, previous studies on the N environment and clonal plants were only based on the supply level of N (Huang et al., 2018; Lin et al., 2018; Dong et al., 2019). In fact, N deposition in these environments is a continuous process, and the frequency of deposition also significantly impacts plant growth (Carreiro et al., 2000; Phoenix et al., 2004). A single high amount of N addition amplifies the ecosystem pulse effect in the short term and weakens the long-term impact of N deposition (Moldan et al., 2018). Thus, we should consider the potential effects of N supply frequency when exploring the responses of plant growth to simulated N deposition (Cao et al., 2020, 2021). This study aims to provide an experimental test for the effects of heterogeneous N supply with different frequencies on the growth and the clonal integration of clonal plants.

Besides heterogeneous N resources, studies have shown that ramet damage also significantly affects the growth and the clonal integration of cloned plants (Hellström et al., 2006; Liu et al., 2007). For clonal plants, under the action of clonal integration, plants can deal with the ramet damage through overall resource allocation (Liu et al., 2009; Tewari et al., 2014). For example, damage can also be transmitted between ramets as a signal so that normal ramets can deal with damage in advance (Hettenhausen et al., 2017; Zhuang et al., 2018). In addition, a recent study shows that the damage of ramets may also induce the negative effects of clonal integration (Gao et al., 2021). However, less is known about the interaction effects between N deposition and ramet damage on the growth of cloned plants and their clonal integration (Dong et al., 2019).

In addition, the translocation of resources among ramets is not equal, so clonal integration has different effects on different ramets (Salzman and Parker, 1985; Gao et al., 2014; Wang et al., 2017). Generally, all ramets will benefit from clonal integration due to the rational division of labor and resource integration among ramets (Roiloa and Retuerto, 2007; Zhang et al., 2009). However, under some conditions, low resource ramets do not always obtain support from high resource ramets (Klimeš and Klimešová, 1999; Hay and Kelly, 2008). Besides, the reallocation of resources from the high- to the low-resource or damaged ramets may also result in neutral or negative effects on the high resource ramets (Yu et al., 2002; Pauliukonis and Gough, 2004; Wang et al., 2009; Chen et al., 2015). Therefore, it is necessary to quantify the effects of heterogeneous N supply and damage on ramets located in different environments.

To explore the response mechanism of the growth and the clonal integration of invasive clonal plants under heterogeneous N supply with different frequencies and ramet damage conditions, the clonal invader *Hydrocotyle vulgaris* was used as the model plant in a control experiment. Apical and basal ramets of *H. vulgaris* were connected or disconnected, N at different frequencies was added to the basal ramets, 8 aphids were placed on the apical ramets, and 30% of each leaf was cutoff to cause damage. We measured the morphological and physiological indexes of *H. vulgaris*, including the isotopic identification of the $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$. Specifically, we addressed the following two questions: (1) How do the clonal integration and heterogeneous N supply with different frequencies and ramet damage affect the growth of *H. vulgaris*? (2) How do the heterogeneous N supply with different frequencies and ramet damage affect the benefits of clonal integration in *H. vulgaris*?

MATERIALS AND METHODS

Experimental Materials

Hydrocotyle vulgaris L. is a native perennial herb in the family Apiaceae, growing in moist to wet habitats across Europe and northwestern Africa (Dong et al., 2015; Wang et al., 2018, 2020). It was introduced to China as a garden plant in artificial wetlands in the 1990s, from which it spread into natural habitats (Liu et al., 2014). *H. vulgaris* is a typical clonal plant with high phenotypic plasticity, strong clonal ability, and wide tolerance to habits. It can occupy a wider ecological amplitude than the native species (Wang et al., 2018, 2020). In our study, *H. vulgaris* was collected from the Xixi wetlands in Hangzhou, Zhejiang Province, China, in May 2015. The collected samples were vegetatively propagated in a greenhouse at the Forest Science Company, Ltd. of Beijing Forest University, Beijing, China.

The green peach aphid, *Myzus persicae* Sulzer, is a small, euryphagic, piercing, and sucking insect of the family Aphididae that infests the majority of agricultural crops and wild plants globally (Goggin, 2007). Its piercing-sucking phenomenon contributes to the rapid spread of plant viruses, such as *Potato virus* and *Turnip yellows virus* (Mondal and Gray, 2017; Congdon et al., 2019), which significantly affect the productivity of agricultural and forestry crops (Guerrieri and Digilio, 2008). A recent study showed that aphids can also cause some damage to *H. vulgaris* (Liu et al., 2017a). In our study, aphids were collected

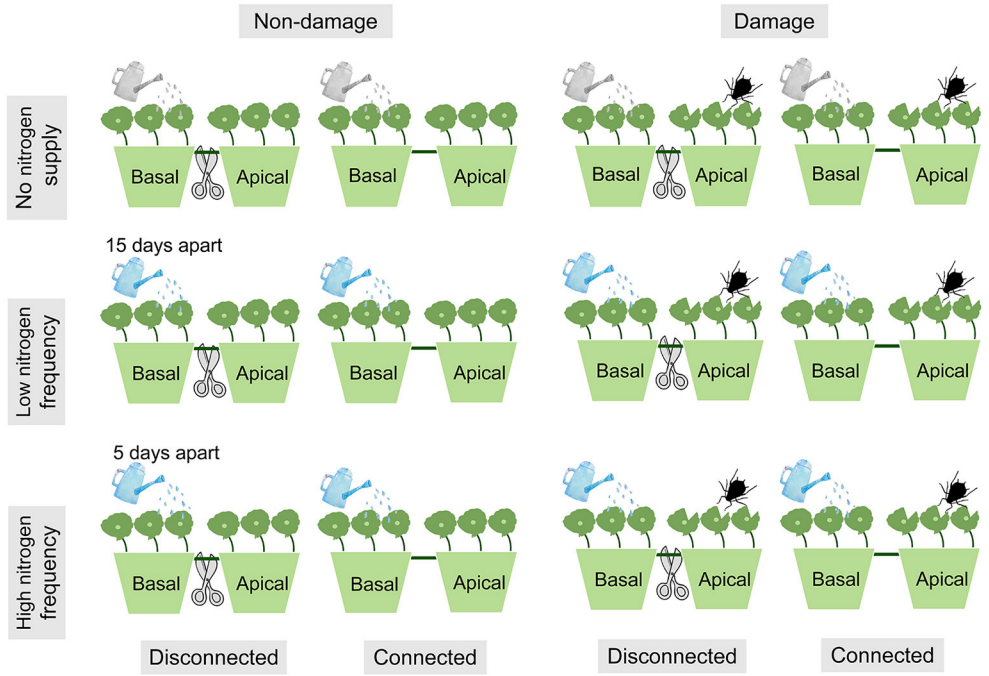

FIGURE 1 | Experimental design. The ramets in each pot were connected. All treatments were sprayed with 100 ml of deionized water every 5 days. In addition, 0.039 g NH_4NO_3 was added to deionized water every 15 days at low nitrogen (N) frequency, and 0.013 g NH_4NO_3 was added to deionized water every 5 days at high N frequency.

from *Rosa chinensis* Jacq in the greenhouse at the Forest Science Company, Ltd. of the Beijing Forest University in May 2015. They were then multiplied using *H. vulgaris* as the host plant for 1 year. Aphids from a single *H. vulgaris* clonal fragment were selected in this study.

Experimental Design

The experiment used a fully factorial design consisting of three N frequency treatments crossed with two damage treatments (ramets damage or non-damage) and two connection treatments (clonal fragment connected or disconnected) (**Figure 1**). Each *H. vulgaris* fragment has 6 nodes, and every 3 nodes were planted in pots that had 17 cm inner diameter, 19 cm outer diameter, and 13 cm height. The older and younger ramets represented the basal and apical ramets, respectively. Since aphids prefer to feed on young ramets, we addeded aphids to the apical ramets (Gao et al., 2021). Corresponding to the damage, N was added to the basal ramets. In addition, connection or disconnected treatments were carried out in the middle parts of the fragments.

The three N supply frequencies were high frequency, low frequency, and no N supply on the basal ramets. All treatments were sprayed with 100 ml of deionized water every 5 days. In addition, 0.013 g NH_4NO_3 was added to deionized water every 5 days at high N frequency, and 0.039 g NH_4NO_3 was added to deionized water every 15 days at low N frequency. The total N deposition for both the low N frequency and the high N frequency treatments was 15 g N m^{-2} a^{-1}. The total amount and frequency of N in the experiment were set according to the atmospheric N wet deposition and the precipitation in the natural distribution area of *H. vulgaris* in China (Li et al., 2011; Zhou et al., 2015).

Ramet damage was carried out by aphids throwing and cutting leaves on the apical ramets. During the experiment, 8 aphids were released on the apical ramets and checked regularly to keep the number stable (Liu et al., 2017a). Meanwhile, the container where apical ramets are growing was covered with gauze cages (length, 25 cm; width, 25 cm; height, 50 cm) to prevent the spread of aphids between containers. In addition, considering that the purpose of our study was to test the response of *H. vulgaris* growth and clonal integration to N deposition and ramet damage, we are not concerned about the effects of aphids on *H. vulgaris*. Therefore, we also simulated the leaf damage of the animal and mechanical injuries to stimulate the *H. vulgaris* response to ramet damage. More specifically, we removed 30% of each leaf of all apical ramets on the 45th day after the start of the experiment (Portela et al., 2019).

We added $^{15}NH_4NO_3$ and $NH_4^{15}NO_3$ isotopes 24 h before harvest. Six replicates were randomly and equally divided into two groups for the addition of $^{15}NH_4NO_3$ and $NH_4^{15}NO_3$, respectively. To detect an appropriate amount of ^{15}N in ramets after 24 h, we checked the ^{15}N abundances in $^{15}NH_4NO_3$ and $NH_4^{15}NO_3$, which were 99.11 and 99.23%, respectively, and the added total amount in each pot was 12.5 $^{15}NH_4NO_3$ or $NH_4^{15}NO_3$ mg·m^{-2} (Gao et al., 2021). The applied isotope was dissolved into 100 ml distilled water and applied on the soil surface evenly using a needle tube.

TABLE 1 | Effects of connected treatment, nitrogen (N) frequency, damage, and their interaction on the biomass, root mass, stem mass, and leaf mass of the entire clonal fragment **(A)**, basal ramets **(B)**, and apical ramets **(C)** of *Hydrocotyle vulgaris*.

	Connection (C)	Nitrogen frequency (NF)	Damage (D)	C × NF	C × D	NF × D	C × NF × D
	$F_{1,60}$	$F_{2,60}$	$F_{1,60}$	$F_{2,60}$	$F_{1,60}$	$F_{2,60}$	$F_{2,60}$
(A) Entire clonal fragment							
Biomass	**8.99****	**10.49*****	1.04^{ns}	0.04^{ns}	1.83^{ns}	0.80^{ns}	0.29^{ns}
Root mass	**9.45****	**10.62*****	0.89^{ns}	0.01^{ns}	0.36^{ns}	2.70^{ns}	1.27^{ns}
Stem mass	**8.38****	**6.40****	0.97^{ns}	0.03^{ns}	1.65^{ns}	0.76^{ns}	0.31^{ns}
Leaf mass	**9.39****	**29.00*****	1.18^{ns}	0.35^{ns}	3.00^{ns}	0.44^{ns}	0.11^{ns}
(B) Basal ramets							
Biomass	**4.52***	**15.62*****	1.49^{ns}	0.91^{ns}	0.30^{ns}	1.50^{ns}	0.56^{ns}
Root mass	3.95^{ns}	**12.80*****	1.29^{ns}	0.41^{ns}	0.23^{ns}	2.30^{ns}	2.60^{ns}
Stem mass	**5.25***	**9.97*****	1.10^{ns}	0.91^{ns}	0.38^{ns}	1.21^{ns}	0.47^{ns}
Leaf mass	2.22^{ns}	**36.87*****	2.66^{ns}	0.94^{ns}	0.48^{ns}	1.85^{ns}	0.59^{ns}
(C) Apical ramets							
Biomass	**5.89***	0.68^{ns}	0.05^{ns}	1.96^{ns}	2.54^{ns}	0.36^{ns}	0.61^{ns}
Root mass	**7.46****	0.84^{ns}	0.01^{ns}	0.99^{ns}	2.94^{ns}	0.99^{ns}	0.78^{ns}
Stem mass	**4.77***	0.64^{ns}	0.16^{ns}	1.46^{ns}	2.17^{ns}	0.50^{ns}	0.46^{ns}
Leaf mass[a]	**11.28****	1.15^{ns}	0.00^{ns}	2.64^{ns}	3.05^{ns}	0.08^{ns}	0.94^{ns}

*Numbers are ANOVA F-values. ns, P > 0.05; *P = 0.01–0.05; **P = 0.001–0.01; and ***P < 0.001. [a]indicaes that the data have undergone square transformation.*

The experiment started on 11 July 2016 in the same greenhouse where *H. vulgaris* was cultivated. There were 12 treatments in total, and there were 6 repetitions for each treatment. There were two pots in line together for each treatment to ensure that the basal and apical ramets are placed separately to facilitate the observation of clonal integration. The pots were filled with a 1:1:1 (v:v:v) mixture of peat:vermiculite:quartz sand, and some ceramsite were placed at the bottom of the pots to prevent soil loss. There were 144 pots in total for the experiment.

During the experiment, the mean temperature was 28.4 ± 0.3°C, and relative humidity was 64.4 ± 0.8%, as measured by I Buttons (DS1923; Maxim Integrated Products, Sunnyvale, CA, USA).

Morphological Measurements

Plants of *H. vulgaris* were harvested on 15 October 2016. The clonal fragments in each combination pot were separated into two portions. The basal and apical ramet portions consisted of the original ramet and any new stems and ramets it had produced.

Within each portion of ramets, the numbers of leaves and nodes were counted, and the total stem length was measured. In addition, the total leaf area was measured using a Win FOLIA Pro 2004a (Regent Instruments, Inc., Canada). Plants were then divided into roots, stems, and leaves, dried at 75°C for 72 h, and weighed.

Physiological Measurements

Recent studies have shown that N deposition and ramet damage not only affect plant morphological traits but also significantly affect plant physiological traits (Cao et al., 2021; Gao et al., 2021). Therefore, we measured the total carbon (C) and N of the basal and apical ramets to improve our understanding of the response of *H. vulgaris* to N deposition and ramet damage.

The total C and total N contents were determined with a total organic carbon (TOC) (multi N/C 3100, Analytik Jena, Germany) analyzer and a continuous flow analyzer (SEAL AA3, SEAL, Germany).

The $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$ isotopes of apical and basal ramets were determined using the DELTAV Advantage Isotope Ratio Mass Spectrometer and the Flash 2000 HT Element Analyzer. Considering that there is no resource transfer between ramets when they are not connected (Gao et al., 2021), we did not measure the isotopes of apical ramets when they are not connected. The samples were burned at high temperatures in an elemental analyzer to generate N_2. Then, the mass spectrometer calculated the $\delta^{15}N$ values of the samples after detecting the ^{15}N to ^{14}N ratio of N_2 and comparing it with the international standard (atmospheric N_2). The determination accuracy was $\delta^{15}N$: ± <0.2‰.

Statistical Analyses

A three-way ANOVA was used to test the effects of N frequency, connection, and damage (all factors were treated as fixed and categorical) on each measure of indexes of the entire clonal fragment and basal and apical ramets of *H. vulgaris* (**Tables 1**–**4**). Linear contrasts based on ANOVA were used to compare whether the effect of connection on various indexes of *H. vulgaris* was significant under each N frequency and damage treatment combination (Wang et al., 2020) (**Figures 2**–**5**). A one-way ANOVA was used to test the differences in the uptake rates of $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$ of *H. vulgaris* (**Supplementary Figure 1**). The leaf mass and area of the entire clonal fragment, stem length, number of leaves, and total C and

TABLE 2 | Effects of connected treatment, N frequency, damage, and their interaction on the stem length, number of nodes, number of leaves, and leaf area of the entire clonal fragment **(A)**, basal ramets **(B)**, and apical ramets **(C)** of *H. vulgaris*.

	Connection (C)	Nitrogen frequency (NF)	Damage (D)	C × NF	C × D	NF × D	C × NF × D
	$F_{1,60}$	$F_{2,60}$	$F_{1,60}$	$F_{2,60}$	$F_{1,60}$	$F_{2,60}$	$F_{2,60}$
(A) Entire clonal fragment							
Stem length	**10.18****	**11.30*****	0.06^{ns}	0.18^{ns}	1.44^{ns}	0.52^{ns}	0.13^{ns}
Number of ramets	**8.34****	**6.40****	0.05^{ns}	0.16^{ns}	0.34^{ns}	1.13^{ns}	0.69^{ns}
Number of leaves	**4.61***	**17.75*****	0.25^{ns}	0.45^{ns}	1.04^{ns}	0.70^{ns}	0.63^{ns}
Leaf area[b]	**16.10*****	**53.91*****	0.69^{ns}	**3.38***	2.23^{ns}	0.29^{ns}	0.12^{ns}
(B) Basal ramets							
Stem length [b]	**6.71***	**21.77*****	1.63^{ns}	1.69^{ns}	1.01^{ns}	0.32^{ns}	1.80^{ns}
Number of nodes	**4.33***	**11.39*****	0.11^{ns}	0.42^{ns}	0.08^{ns}	0.99^{ns}	1.47^{ns}
Number of leaves [a]	0.97^{ns}	**34.79*****	0.71^{ns}	0.96^{ns}	0.10^{ns}	0.97^{ns}	2.34^{ns}
Leaf area	0.52^{ns}	3**9.95*****	1.20^{ns}	0.93^{ns}	0.06^{ns}	2.28^{ns}	0.52^{ns}
(C) Apical ramets							
Stem length	**5.52***	0.51^{ns}	0.15^{ns}	2.04^{ns}	3.00^{ns}	0.33^{ns}	0.61^{ns}
Number of nodes	3.85^{ns}	0.26^{ns}	0.56^{ns}	1.96^{ns}	1.53^{ns}	0.42^{ns}	0.41^{ns}
Number of leaves [a]	**5.94***	0.14^{ns}	3.10^{ns}	2.17^{ns}	3.19^{ns}	0.14^{ns}	0.86^{ns}
Leaf area [b]	**27.85*****	**2.54***	0.05^{ns}	0.22^{ns}	3.19^{ns}	0.05^{ns}	1.57^{ns}

*Numbers are ANOVA F-values. ns, P > 0.05; *P = 0.01–0.05; **P = 0.001–0.01; and ***P < 0.001. [a]indicaes that the data have undergone square transformation, and [b]indicaes that the data have undergone logarithmic transformation.*

TABLE 3 | Effects of connected treatment, N frequency, and damage and their interaction on the total carbon and total N of the basal **(A)** and apical **(B)** ramets of *H. vulgaris*.

	Connection (C)	Nitrogen frequency (NF)	Damage (D)	C × NF	C × D	NF × D	C × NF × D
	$F_{1,60}$	$F_{2,60}$	$F_{1,60}$	$F_{2,60}$	$F_{1,60}$	$F_{2,60}$	$F_{2,60}$
(A) Basal ramets							
Total carbon[b]	**6.13***	**26.76*****	3.78^{ns}	2.54^{ns}	2.10^{ns}	0.615^{ns}	1.24^{ns}
Total nitrogen[a]	3.56^{ns}	**14.85*****	1.33^{ns}	**4.15***	1.26^{ns}	1.33^{ns}	**1.66***
(B) Apical ramets							
Total carbon	0.632^{ns}	**5.91****	0.03^{ns}	2.05^{ns}	2.53^{ns}	0.30^{ns}	0.52^{ns}
Total nitrogen[a]	1.29^{ns}	**3.77***	0.13^{ns}	0.34^{ns}	3.63^{ns}	0.47^{ns}	0.67^{ns}

*Numbers are ANOVA F-values. ns, P > 0.05; *P = 0.01–0.05; **P = 0.001–0.01; and ***P < 0.001. [a]indicaes that the data have undergone square transformation, and [b]indicaes that the data have undergone logarithmic transformation.*

TABLE 4 | Effects of connected treatment, N frequency, and damage and their interaction on the $^{15}N\text{-}NH_4^+$ and $^{15}N\text{-}NO_3^-$ uptake rates of the apical **(A)** and basal **(B)** ramets of *H. vulgaris*.

	Connection (C)	Nitrogen frequency (NF)	Damage (D)	C×NF	C×D	NF×D	C×NF×D
	$F_{1,60}$	$F_{2,60}$	$F_{1,60}$	$F_{2,60}$	$F_{1,60}$	$F_{2,60}$	$F_{2,60}$
(A) Basal ramets							
$^{15}N\text{-}NH_4^+$ uptake rate	3.24^{ns}	**29.26*****	0.14^{ns}	0.12^{ns}	0.27^{ns}	0.60^{ns}	2.20^{ns}
$^{15}N\text{-}NO_3^-$ uptake rate	2.75^{ns}	**8.82****	2.40^{ns}	0.14^{ns}	3.33^{ns}	2.03^{ns}	0.13^{ns}
(B) Apical ramets							
$^{15}N\text{-}NH_4^+$ uptake rate		1.57^{ns}	3.14^{ns}			1.84^{ns}	
$^{15}N\text{-}NO_3^-$ uptake rate		1.84^{ns}	0.62^{ns}			0.13^{ns}	

*Numbers are ANOVA F-values. ns, P > 0.05; **P = 0.001–0.01; and ***P < 0.001.*

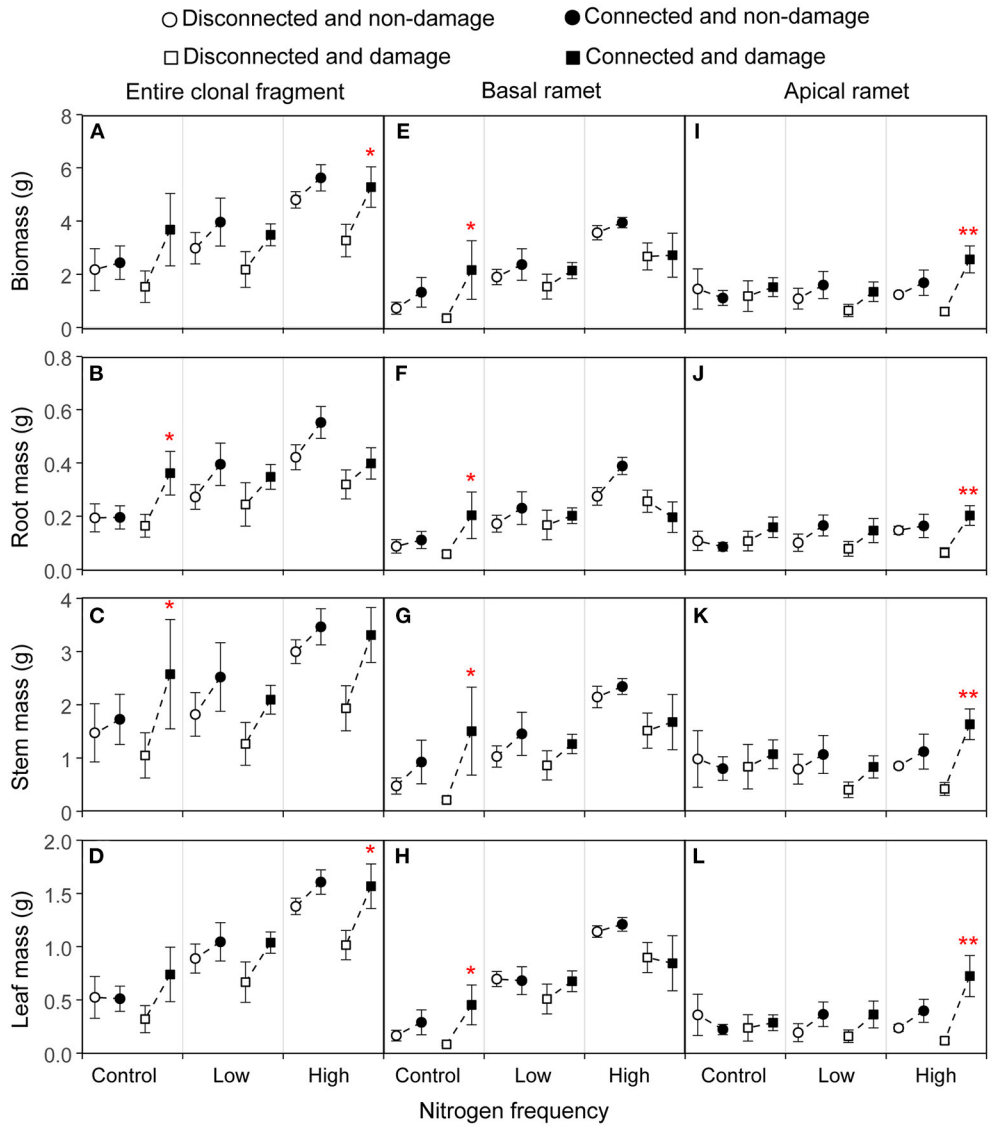

FIGURE 2 | Effects of connection among ramets, N supply frequency, and damage on the biomass, root mass, stem mass, and leaf mass of the entire clonal fragment **(A–D)**, basal ramets **(E–H)**, and apical ramets **(I–L)** of *Hydrocotyle vulgaris*. Bars represent the mean ± SE. The *p*-values with significant difference between disconnected and connected treatments under each N frequency and damage combination (linear contrast based on ANOVA) were marked above the connected treatment. $^{*}P < 0.05$; $^{**}P < 0.01$. Refer to **Table 1** for ANOVAs.

total N of the basal ramets, as well as the number of leaves, leaf area, total N, and the ^{15}N uptake rates of the apical ramets were transformed to the natural logarithm or the square root before analysis as needed to improve homoscedasticity. Figures show untransformed data. All analyses were conducted using SPSS 22.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Morphological Traits of the Entire Clonal Fragment of *H. vulgaris*

The connection between ramets and a higher N frequency had significantly increased the biomass, root mass, stem mass, leaf mass, stem length, number of nodes, number of leaves, and leaf area of the entire clonal fragment ($P < 0.05$; **Figures 2A–D**, **3A–D**; **Tables 1A**, **2A**). However, damage insignificantly decreased all growth traits of the entire clonal fragment ($P > 0.05$; **Figures 2A–D**, **3A–D**; **Tables 1A**, **2A**). Besides, the connection and N frequency interactive effect significantly increased the leaf area of the entire clonal fragment ($P < 0.05$; **Figure 3D**; **Table 2A**).

A linear contrast analysis revealed that, under the treatment of high N frequency at the basal ramet and apical ramet damage, the connection between ramets significantly increased the total biomass, leaf mass, and leaf area of the entire clonal fragment ($P < 0.05$; **Figures 2A,D**, **3D**). Moreover, under the treatment

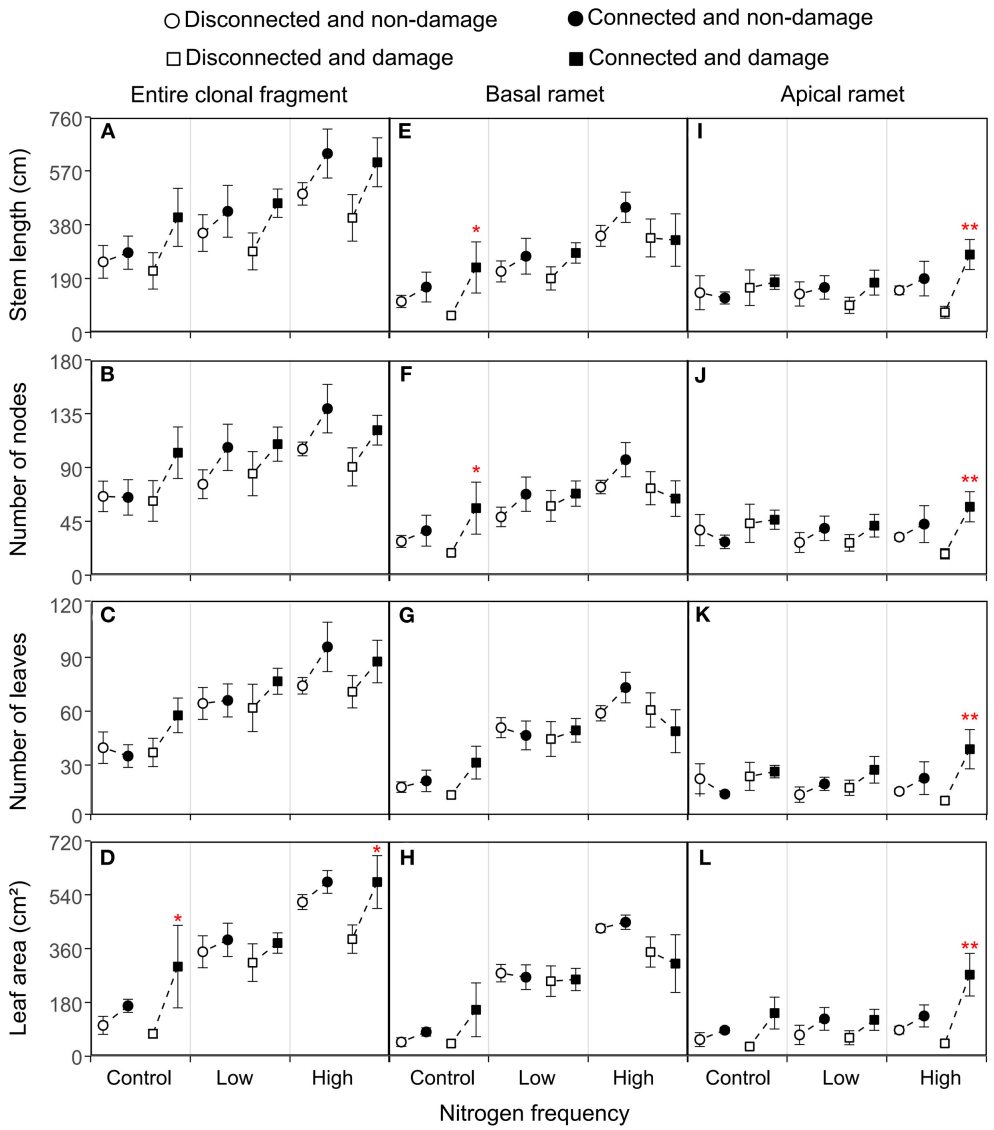

FIGURE 3 | Effects of connection among ramets, N supply frequency, and damage on the stem length, number of nodes, number of leaves, and leaf area of the entire clonal fragment **(A–D)**, basal ramets **(E–H)**, and apical ramets **(I–L)** of *H. vulgaris*. Bars represent the mean ± SE. The *p*-values with significant differences between disconnected and connected treatments under each N frequency and damage combination (linear contrast based on ANOVA) were marked above the connected treatment. $^{*}P < 0.05$; $^{**}P < 0.01$. Refer to **Table 2** for ANOVAs.

of no N supply at the basal ramet and apical ramet damage, the connection between ramets significantly increased the root mass, stem mass, and leaf area of the entire clonal fragment ($P < 0.05$; **Figures 2B,C**, **3D**).

In addition, the basal and apical ramets of *H. vulgaris* had different responses to each treatment (**Figures 2**, **3**; **Tables 1**, **2**).

Morphological Traits of the Basal Ramets of *H. vulgaris*

The connection between ramets significantly increased the total biomass, stem mass, stem length, and number of nodes of the basal ramets ($P < 0.05$; **Figures 2E,G**, **3E,F**; **Tables 1B**, **2B**). At the same time, a higher N frequency significantly increased the biomass, root mass, stem mass, leaf mass, stem length, number of nodes, number of leaves, and leaf area of the basal ramets ($P < 0.05$; **Figures 2E–H**, **3E–H**; **Tables 1B**, **2B**). However, damage to the basal ramets had no significant effect on all growth traits of the basal ramets ($P > 0.05$; **Figures 2E–H**, **3E–H**; **Tables 1B**, **2B**).

A linear contrast analysis revealed that, under the treatment of no N supply at the basal ramet and apical ramet damage, the connection between ramets significantly increased the total biomass, root mass, stem mass, leaf mass, stem length, and number of nodes in the basal ramets (**Figures 2E–H**, **3E,F**).

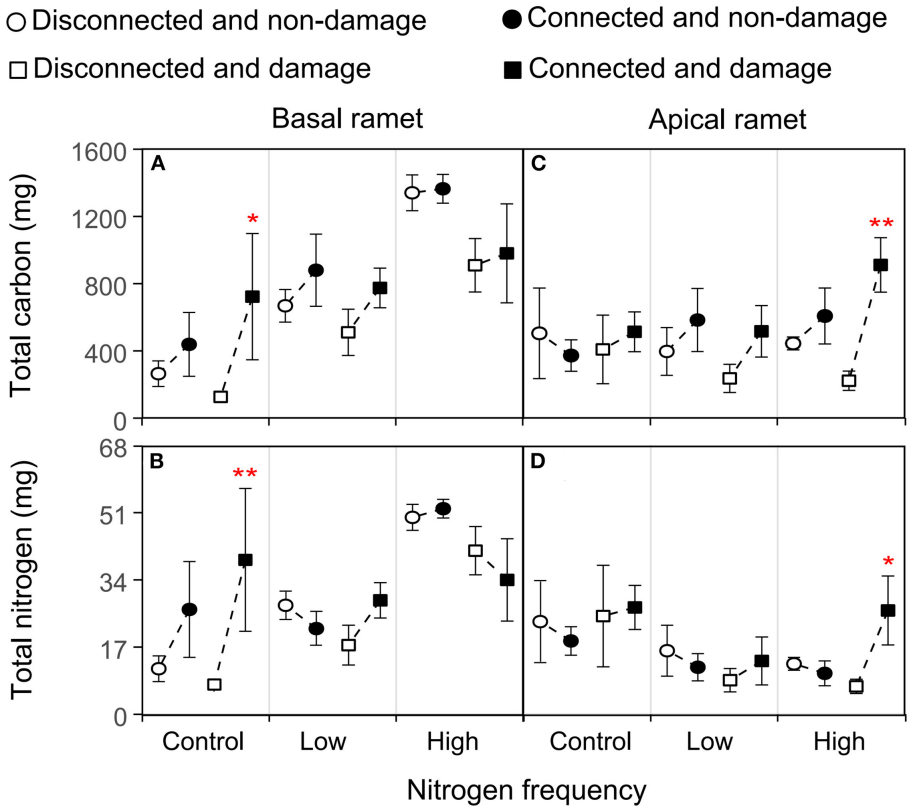

FIGURE 4 | Effects of connection among ramets, N supply frequency, and damage on the total carbon and total N of the basal **(A,B)** and apical **(C,D)** ramets of *H. vulgaris*. Bars represent the mean ± SE. The *p*-values with significant differences between disconnected and connected under each N frequency and damage combination (linear contrast based on ANOVA) were marked above the connected treatment. *$P < 0.05$; **$P < 0.01$. Refer to **Table 3** for ANOVAs.

Morphological Traits of the Apical Ramets of *H. vulgaris*

The connection between ramets significantly increased the total biomass, root mass, stem mass, leaf mass, stem length, number of leaves, and leaf area of the *H. vulgaris* apical ramets ($P < 0.05$; **Figures 2I–L**, **3I,K,L**; **Tables 1C**, **2C**). A higher N frequency significantly increased the leaf area in apical ramets ($P < 0.05$; **Figure 3L**; **Table 2C**). However, damage to the apical ramets had no significant effect on all growth traits ($P > 0.05$; **Figures 2I–L**, **3I–L**; **Tables 1C**, **2C**).

Based on the linear contrast analysis, under the treatment of high N frequency at the basal ramet and apical ramet damage, connected ramets significantly increased all the growth traits of the *H. vulgaris* apical ramets ($P < 0.05$; **Figures 2I–L**, **3I–L**).

Physiological Traits of the Basal Ramets of *H. vulgaris*

The growth and physiological indexes of *H. vulgaris* had a similar trend in all the treatments (**Figures 2–5**). The connection between ramets significantly increased the total C of basal ramets ($P < 0.05$; **Figure 4A**; **Table 3A**), but there was no significant effect on the total N ($P > 0.05$; **Figure 4B**; **Table 3A**). However, the high N frequency significantly increased the total C and N of the basal ramets ($P < 0.05$; **Figures 4A,B**; **Table 3A**). At the same time, damage to the ramets had no significant effect on the total C and N of *H. vulgaris* basal ramets ($P > 0.05$; **Figures 4A,B**; **Table 3A**). In addition, the interactive effect between connection and N frequency and damage, connection, and N frequency significantly affected the total C of the *H. vulgaris* basal ramets ($P < 0.05$; **Figure 4A**; **Table 3A**).

Based on the linear contrast analysis, under the treatment of no N supply at the basal ramet and apical ramet damage, connected ramets significantly increased total C and N of the basal ramets ($P < 0.05$; **Figures 4A,B**).

Physiological Traits of the Apical Ramets of *H. vulgaris*

The connection between ramets slightly increased the total C of apical ramets ($P > 0.05$; **Figures 4C,D**; **Table 3B**). In addition, the N frequency significantly increased the total C ($P < 0.05$; **Figure 4C**; **Table 3B**) and decreased the total N of the *H. vulgaris* apical ramets ($P < 0.05$; **Figure 4D**; **Table 3B**). In contrast, damage to ramets had no significant effect on the total C and N of the apical ramets ($P > 0.05$; **Figures 4C,D**; **Table 3B**).

The linear contrast analysis revealed that, under the treatment of high N frequency at the basal ramets and apical ramet damage,

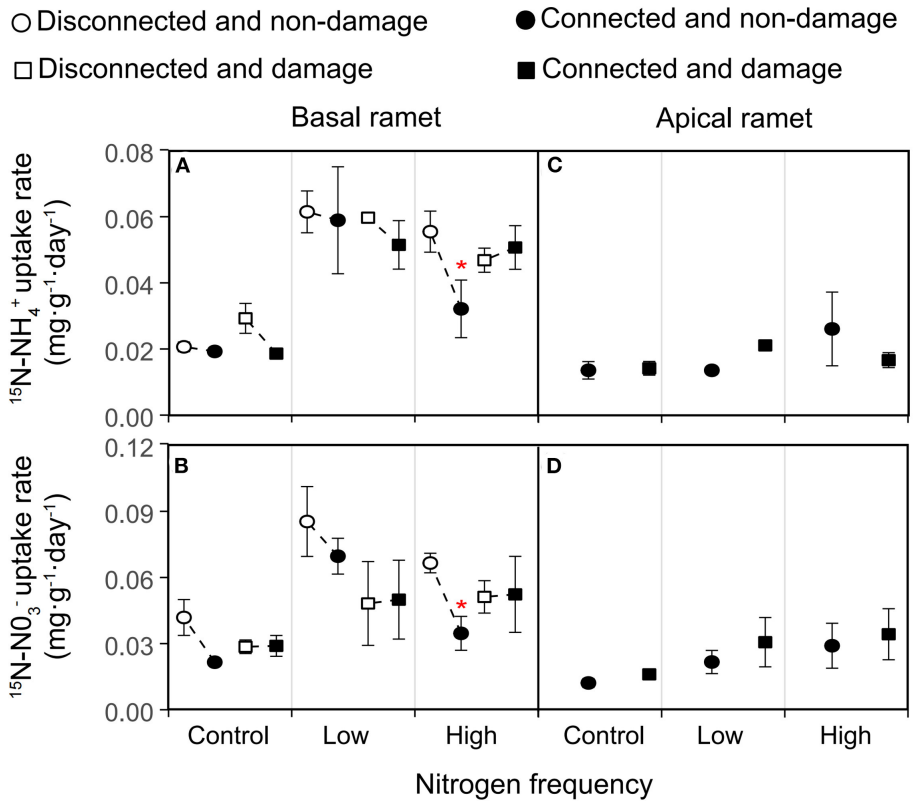

FIGURE 5 | Effects of connection among ramets, N supply frequency, and damage on the ^{15}N-NH_4^+ uptake rate and ^{15}N-NO_3^- uptake rate of the basal **(A,B)** and apical **(C,D)** ramets of *H. vulgaris*. Considering that there is no resource transfer when there is no connection between ramets, the isotope of apical ramets was not measured when there is no connection between ramets. Bars show the mean ± SE. The *p*-values with significant difference between disconnected and connected treatments under each N frequency and damage combination (linear contrast based on ANOVA) were marked above the connected treatment. *P = 0.01–0.05. Refer to **Table 4** for ANOVAs.

connected ramets significantly increased the total C and N of the apical ramets ($P < 0.05$; **Figures 4C,D**).

The ^{15}N-NH_4^+ and ^{15}N-NO_3^- Uptake Rates of the Basal and Apical Ramets of *H. vulgaris*

The ^{15}N-NH_4^+ and ^{15}N-NO_3^- uptake rates of *H. vulgaris* had no significant difference (**Supplementary Figure 1**), and the response trend to each treatment was also consistent (**Figure 5**; **Table 4**). The connection between ramets had no significant effect on the ^{15}N-NH_4^+ and ^{15}N-NO_3^- uptake rates of the basal ramets ($P > 0.05$; **Figures 5A,B**; **Table 4A**). Besides, the high N frequency significantly increased the ^{15}N-NH_4^+ and ^{15}N-NO_3^- uptake rates of the basal ramets, while the low N frequency had no significant effect ($P < 0.05$; **Figures 5A,B**; **Table 4A**). Similarly, the damage had no significant effect on the ^{15}N-NH_4^+ and ^{15}N-NO_3^- uptake rates of the basal ramets ($P > 0.05$; **Figures 5A,B**; **Table 4A**). Moreover, the linear contrast analysis revealed that, under a high N frequency and no damage treatment, the connection between ramets significantly decreased the ^{15}N-NH_4^+ and ^{15}N-NO_3^- uptake rates of the basal ramets ($P < 0.05$; **Figures 5A,B**). In addition, N frequency and damage had no significant effect on ^{15}N-NH_4^+ and ^{15}N-NO_3^- uptake rates of the apical ramets ($P > 0.05$; **Figures 5C,D**; **Table 4B**).

DISCUSSION

Effects of the Clonal Integration on *H. vulgaris* Traits

The connection among ramets significantly increased the biomass, stem mass, and total C of the basal ramets as well as the biomass, root mass, stem mass, leaf mass, stem length, leaf number, leaf area, and total C of the apical ramets (**Figures 2–4**). This implies that clonal integration significantly promoted the growth of *H. vulgaris* basal and apical ramet, with greater growth by the apical ramets. With clonal integration, the basal and apical ramets in clonal plants reallocate resources and reasonable division of labor that promote the growth of apical and basal ramets (Hartnett and Bazzaz, 1983; Roiloa and Retuerto, 2007; Zhang et al., 2009). Moreover, with clonal integration, ramets located in a high-resource habitat act as donor ramets, transferring some resources to those in low-resource habitats (Song et al., 2013;

Wang et al., 2020). Therefore, clonal integration promoted the growth of the entire clonal fragment and basal and apical ramets of *H. vulgaris*, especially promoting the apical ramets more significantly.

Effects of the N Supply With Different Frequencies on *H. vulgaris* Traits

Our results showed that the increase of N frequency promoted the growth of the entire clonal fragment of *H. vulgaris* (**Figures 2**, **3**). N is an important nutrient to maintain plant growth (Gutiérrez, 2012; Song et al., 2012). Moreover, under small amounts of multiple applications of N, plants can absorb and use more N so as to grow better (Chang et al., 2014; Wu et al., 2019). Therefore, a higher N frequency can better promote the growth of *H. vulgaris*. In addition, the increase of N frequency significantly promoted the growth of basal ramets but had no significant effect on the apical ramets (**Figures 2**–**4**). This may be because the increase of N frequency increases the resources of the basal ramet environment. Since the resources captured by clonal ramets can be transferred between ramets (Roiloa and Retuerto, 2007; Zhang et al., 2009), allocating more resources to high resource ramets can make more full use of resources and improve the performance of the whole clonal plants (Ikegami et al., 2008; Huang et al., 2018). For example, under heterogeneous nutrient conditions, clonal integration generally increased biomass allocation to roots in the high resource ramets but decreased it in the low recourse ramets (Wang et al., 2021). Thus, the increase of N frequency only enhanced the growth of the basal ramets and had no significant effect on the apical ramets.

Effects of the Ramet Damage on *H. vulgaris* Traits

The induction of damage on ramets had no significant effect on the *H. vulgaris* growth (**Figures 2**–**4**). Most plants have the ability to resist damage (Nguyen et al., 2016; Hakim et al., 2018; Lu et al., 2020; Qi et al., 2020). Besides, a meta-analysis of 32 invasive species found that invasive plants had stronger tolerance and compensation for damage (Zhang et al., 2018). In addition, more than one study shows that clonal plants can counter the local adverse conditions through reasonable resource allocation and division of labor among ramets, which also supports our results (Schmid et al., 1988; Alpert, 1999; You et al., 2014; Lyu et al., 2016; Liu et al., 2017a; Wang et al., 2017). Therefore, *H. vulgaris*, as an invasive clonal plant, has the ability to resist a certain degree of damage.

Effects of the N Supply With Different Frequencies and Ramet Damage on *H. vulgaris* Clonal Integration

Under the treatment of high N frequency at the basal ramet and apical ramet damage, the connection between ramets more significantly improved the growth of the apical and entire clonal fragment (**Figures 2**–**4**). Most studies suggest that when resources are heterogeneous, clonal integration is beneficial to ramets in low-resource habitats (Friedman and Alpert, 1991; Song et al., 2013; Wang et al., 2017; Lin et al., 2018). Through clonal integration, the increase of available resources in the high resource ramets increases the resource availability in the low resource ramets and also improves the benefits of clonal integration to low resource ramets (Lin et al., 2018). Besides, in clonal plants, damage also stimulates the allocation of resources to damaged ramets to maintain their growth (Schmid et al., 1988; You et al., 2014; Lyu et al., 2016; Wang et al., 2020). Thus, under the treatment of high N frequency at the basal ramet and apical ramet damage, clonal integration significantly promoted the apical ramet growth. Moreover, the surplus resources of basal ramets are more fully utilized, and the ability of apical ramets to resist damage is also improved (Gao et al., 2014; Liu et al., 2017a). Therefore, clonal integration also provides more benefits to the entire clonal fragment.

It is worth noting that the low-frequency N supply and clonal integration had no significant impact on the apical ramet growth (**Figures 2**–**4**). This may be because, although low N frequency provides resources, it is limited. In previous studies, heterogeneous environments were often designed with high contrast, and the resources in high resource environments are often surplus, so the effect on the ramets located in low resource environments is significant (Guo et al., 2011; Wang et al., 2017). However, when resources are limited, clonal plants allocate the limited resources to ramets in the higher resource environment to capture more resources (Ikegami et al., 2008). Therefore, low-frequency N supply has no significant effect on the growth of apical ramets.

In addition, we found no N supply to the basal ramets and damage on the apical ramets, and the connection between ramets significantly increased the basal ramet growth, increasing the benefits of clonal integration on the basal ramets (**Figures 2**–**4**). This may be because the damage to the apical ramets serves as a signal that stimulates resource utilization at the basal ramets and makes an earlier stress response (Lyu et al., 2016; Wang et al., 2017). Another explanation is that clonal plants can enhance the ability to compensate for damage by concentrating ramets in less stressed patches in heterogeneous environments (Wise and Abrahamson, 2007; Sun et al., 2010).

Moreover, as we discussed earlier, when the basal ramet resources are limited, the resources will not be allocated to the apical ramets (Ikegami et al., 2008). However, when the resource availability of the basal ramets is high, some resources will be allocated to the apical ramets, which will reduce the benefits of clonal integration to the basal ramets (Wang et al., 2009; Song et al., 2013; Chen et al., 2015). Therefore, the effect of clonal integration on the basal ramets was significant only when there was no N addition at the base and the apical was damaged. In addition, a recent study showed that apical ramet damage inhibits the growth of basal ramets through clonal integration and reduces overall growth (Gao et al., 2021). Additionally, the treatment in this study is caused by parasitism of *Cuscuta australis* with a length of 15 cm (Gao et al., 2021). It may require more nutrients for growth, which caused greater damage to the clonal plants, exceeding the clonal ramets' resistance to damage, subsequently offsetting the clonal integration benefits.

Effects of the Clonal Integration, N Supply Frequency, and Ramet Damage on the ^{15}N-NH_4^+ and ^{15}N-NO_3^- Uptake Rates of *H. vulgaris*

Compared with no N supply, N supply increases the ^{15}N-NH_4^+ and ^{15}N-NO_3^- uptake rates. N deposition increases the N uptake rate of clonal plant *Leymus chinensis*, and *L. chinensis* shows better advantages than *Stipa grandis* and *Cleistogenes squarrosa* in N deposition, which are similar to the study results by Cao et al. (2021). This suggests that the positive response in N absorption rate following N deposition may be an important factor supporting the diffusion and invasion of *H. vulgaris* (Liu et al., 2017b; Valliere et al., 2017; Wang and Chen, 2019). Besides, this study shows no significant difference in N absorption rate between the low- and high-frequency N supply, implying that the positive response in N absorption rate following N deposition was limited. This explains why frequent applications of small amounts of N significantly improved *H. vulgaris* growth.

In addition, under the conditions of no damage and high-frequency N supply, clonal integration significantly reduced the ^{15}N-NH_4^+ and ^{15}N-NO_3^- uptake rates of the basal ramets and increased the uptake rates of the apical ramets. This is because ^{15}N-NH_4^+ and ^{15}N-NO_3^- were applied 24 h before harvest, and the basal ramets accumulated a large amount of N in treatments with a high-frequency N supply and no damage. Some studies have shown that ramets growing in high resource patches usually transfer resources to low resource ramets to maintain the growth of low resource ramets (Guo et al., 2011; Wang et al., 2017).

A few potential limitations should be considered within the context of this study. First, the experiment is a pot control experiment. The response of *H. vulgaris* to N deposition and ramet damage in the field may be more complex than our research. Second, N was added to the basal ramets, and the damage was in the apical ramets in the experiment. In the field, the heterogeneity of N content and ramet damage is usually random. Whether basal ramet damage and N addition to apical ramets will significantly affect the results is uncertain. Finally, this research mainly focuses on the response of *H. vulgaris* to N deposition, and only one degree of ramet damage treatment is designed. How *H. vulgaris* responds to more severe damage is unclear. Future studies are encouraged to design more damage levels. In addition, the basal and apical ramets should be treated with N addition and damage, respectively, to gain a comprehensive understanding of the response of *H. vulgaris* to N deposition and ramet damage.

CONCLUSION

Both clonal integration and higher frequency N supply promoted the growth of the entire clonal fragment of *H. vulgaris*, and clonal integration more significantly promoted the growth of apical ramets. However, higher frequency N supply more significantly promoted the growth of basal ramets. Ramet damage had no significant effect on the growth of *H. vulgaris*. Besides, the heterogeneous N supply with high frequency and ramet damage increased the clonal integration benefits in ramets in a given environment, subsequently benefiting the entire *H. vulgaris* clonal plant. Moreover, the size of differences in heterogeneous resources affected the resource allocation among ramets. In addition, the uptake rates of NH_4^+ and NO_3^- of *H. vulgaris* had no significant difference, and N supply increased the uptake rates of NH_4^+ and NO_3^- of the basal ramets. Taken together, our study increases the understanding of the growth of invasive clonal plants and their clonal integration in response to N deposition and ramet damage.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

H-LL and Y-NM: conception and design of this study. KS: drafting the manuscript and analysis and interpretation of data. J-FC and YZ: revising the manuscript critically for important intellectual content. S-HA, Y-LS, and L-JY: acquisition of data. KS, J-FC, YZ, Y-NM, S-HA, Y-LS, L-JY, and H-LL: approval of the version of the manuscript to be published. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Transformation of Plant to Resource Acquisition Under High Nitrogen Addition Will Reduce Green Roof Ecosystem Functioning

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Ecosystem engineering, such as green roof, provides numerous key ecosystem functions dependent on both plants and environmental changes. In the recent years, global nitrogen (N) deposition has become a hot topic with the intensification of anthropogenic disturbance. However, the response of green roof ecosystems to N deposition is still not clear. To explore the effects of N addition on plant ecological strategy and ecosystem functioning (biomass), we conducted a 3-month N addition simulation experiment using 12 common green roof species from different growth forms on an extensive green roof in Tianjin, China. The experiment included three different N addition treatments (0, 3.5, and 10.5 gN m^{-2} $year^{-1}$). We found that plants with the resource-acquisitive strategy were more suitable to survive in a high N environment, since both aboveground and belowground traits exhibited synergistic effects. Moreover, N addition indirectly decreased plant biomass, indicating that ecosystem functioning was impaired. We highlight that there is a trade-off between the survival of green roof species and keeping the ecosystem functioning well in the future N deposition. Meanwhile, these findings also provide insights into how green roof species respond to global climate change and offer important information for better managing and protecting similar ecosystem engineering in the background of high N deposition.

Keywords: resource acquisition strategy, green roof, nitrogen deposition, plant functional traits, trait plasticity, ecosystem functioning

INTRODUCTION

Global climate changes and human disturbances are destroying terrestrial ecosystem processes and functioning, especially in urban areas (Grimm et al., 2008; Radhi et al., 2013). Due to the fast development of urbanization, the areas available for urban greening continue to decrease, coupled with the expansion of population in cities, resulting in a growing number of ecological and environmental problems (Davidson, 2009). Ecosystem engineering, such as green roof, can mitigate the adverse effects of urbanization and provide numerous key ecosystem functions (e.g., stormwater management, reduce heat transfer through building roofs, provide habitat for heterotrophs, and improve the air quality, Lundholm, 2015; Lundholm and Williams, 2015;

Nagase and Koyama, 2020). Thus, it has become an effective measure to increase the greening areas and improve the quality of the ecological environment. Considering that global climate changes may affect the survival of species, they will inevitably impact the green roof functions (Du et al., 2018). However, it remains unclear how global climate changes affect these processes in green roofs, which also limits the understanding of the role of green roof ecosystems.

Due to the increase of atmospheric reactive nitrogen (N) caused by anthropogenic disturbance, global N deposition has received considerable attention in the recent years, especially in cities, which have become the hot areas of N deposition (Galloway et al., 2004; Davidson, 2009). Increased N deposition has profound consequences for natural and anthropogenic ecosystems. For example, studies based on natural ecosystems have found that moderate N addition would improve the ability of plants to utilize various forms of N for their own photosynthesis and accumulation of organic compounds, which promoted plant growth and biomass, as well as contributed to productivity and ecosystem functioning (Bauer et al., 2004; Ren et al., 2019). On the other hand, excessive N deposition would lead to "N saturation" of ecosystems, increasing the other nutrients loss, and considerably threatening the normal growth of plants and the balance of ecosystems, especially in urban areas (Liu et al., 2010). However, there are still few studies examined the response of green roof ecosystems to N deposition so far. A better understanding of how plants respond to N deposition in green roof ecosystems will provide important information for selecting suitable species, improving sustainable ecosystem engineering, developing greening policies, and getting multiple social, economic, and environmental benefits (Du et al., 2018; Shafique et al., 2018).

Plant functional traits can reflect the plant resource utilization ability, adaptability to environmental changes, and trade-offs among different function strategies (Cornelissen et al., 2003; Lundholm and Williams, 2015). They are often used to explain the underlying causal direction between environmental change and various ecological processes of plants (Lundholm, 2015). Meanwhile, species can be categorized based on the effects of plant functional traits on ecological processes so as to achieve the purpose of species screening for green roofs (Nagase and Koyama, 2020). To resist harsh roof conditions (high winds, drought, large temperature fluctuations, and low substrate water availability, Speak et al., 2012), the plants with conservative traits, such as small-stature succulent plants with thick leaves, seem to be reliable and provide better ecological functions in green roofs (Zhang et al., 2020). Most experiments based on N addition in natural ecosystems found that plant functional traits could respond quickly to the changes in N (Trocha et al., 2017), and plants shifted toward resource acquisition strategy (acquire and use nutrients quickly) with the increase of N availability. Considering that the aboveground and belowground parts of plants face different selection pressures, resource acquisition strategies of aboveground and belowground traits may be different and will change with local environments (Hu et al., 2019). Therefore, it is still controversial whether the responses of aboveground and belowground traits to N deposition are consistent in the green roof ecosystem, which may be largely related to environmental factors and remains to be further explored (Li et al., 2019; Hu et al., 2021). Moreover, trait plasticity is a vital indicator for plants to adapt to varied environments by modifying plant structure and function in response to stress, disturbance, or inputs from environments (Matesanz et al., 2010). Generally, trait plasticity tends to be higher in favorable environments, promoting species to optimize resource acquisition to improve their fitness, while it is lower in stress habitats due to the environmental filtering (Ryser and Eek, 2000; Portsmuth and Niinemets, 2007). For example, with the increase of N and phosphorus (P) supply, *Lycium Ruthenicum* improved the plasticity index of specific root length, which could better access to nutrients and adapt to these environments (Li et al., 2021). Thus, to get a comprehensive understanding of the mechanisms underlying species' response to environmental changes, it is necessary to explore the response of plant traits and trait plasticity to N deposition in green roofs.

Most studies have suggested that plant biomass is directly related to multiple ecological processes and can be used as a proxy for essential ecosystem functions (Costa et al., 2012; Bongaarts, 2019; Farrell et al., 2021). Likewise, for green roof ecosystems, numerous studies have demonstrated that biomass is positively associated with ecosystem functioning and multifunctionality (Lundholm, 2015; Xie et al., 2018). Previous studies found that excessive and long-term N deposition in urban areas might cause soil acidification, decreasing the availability of soil resources and stability of plant communities (Lu et al., 2014), seriously affecting plant biomass and ecosystem functions. Moreover, green roof ecosystem is vulnerable to the environmental changes because of relatively poor resilience and stability (Lundholm et al., 2015; Zhang et al., 2020). Thus, it is crucial to explore the effects of N deposition on plant biomass and ecosystem functioning in green roof ecosystems to better protect and manage similar ecosystems.

In this study, we attempted to explore the effects of N addition on plant ecological strategy and ecosystem functioning (biomass) in a green roof ecosystem. Here, we selected 12 common green roof plants in Tianjin and assessed the effects of N addition on aboveground and belowground traits, trait plasticity, and biomass of plants. The study focused on three hypotheses: (a) N addition would promote the transformation of plant ecological strategy from resource conservation to resource acquisition, and the aboveground and belowground traits of plants showed similar changes to N addition; (b) N addition would inhibit the trait plasticity of plants and decrease their adaptability to the environment; (c) high N addition would inhibit the biomass of plants, thereby reducing green roof ecosystem functioning.

MATERIALS AND METHODS

The experimental site was on the roof of a four-story building (approximately 10 m aboveground) at the College of Environmental Science and Engineering in Nankai University, Tianjin, China (latitude 38.9878° North, longitude 117.3312° East). The annual average temperature in this region is 13.8 °C,

the annual average precipitation is 704.5 mm, and the annual average relative humidity is 59%. In the recent years, N deposition rate has been increasing continuously in Tianjin, and the current average level of atmospheric N deposition is up to 35 kgN ha^{-1} $year^{-1}$ (Yu et al., 2019).

The Experimental Design

We customized twelve wooden modules (120 cm × 120 cm × 30 cm), and each module was divided into nine small pots (30 cm × 30 cm × 30 cm), composed of a growing medium layer, a filter membrane containing a piece of a non-woven geotextile, a drainage system of HDPE (high-density polyethylene) drainage panels, and a root barrier layer including HDPE membranes, totally 108 pots (**Supplementary Figure 1**). In this study, the substrate employed was made up of 40% pumice, 35% sand, 15% peat, and 10% vermiculite by volume (Zhang et al., 2020; all these materials from Xuebin Horticulture Ltd., in Tianjin), and substrate depth of 15 cm was chosen.

On 23 May 2021, twelve common green roof species from different growth forms in Tianjin (**Supplementary Table 1**) were grown. We randomly assigned twelve wooden modules into three N addition groups (**Supplementary Figure 1**). Within each group, one seedling of twelve species was randomly assigned to be planted into each of the 36 pots, with 3 replicates for each species, respectively. Pots were irrigated every day for the first 2 weeks and every 2 days for the next 2 weeks. Seedlings that died during this period were replanted in time. After the first month, we tried not to water the roof until a week when it did not rain. At the end of the acclimation period, the three groups were randomized to one of the three N treatments, including the control (no N addition), normal N addition (3.5 gN m^{-2} $year^{-1}$), and high N addition (10.5 gN m^{-2} $year^{-1}$). For the two N addition groups, an aqueous solution of NH_4NO_3 was applied one time a month from July to September (18 July, 26 August, and 21 September, respectively). Meanwhile, the control treatment received the same amount of water every time.

Plant Sampling and Trait Measurement

A dataset of aboveground and belowground functional traits including leaf, stem, and root was collected for each species under each N addition treatment by standard methods of Pérez-Harguindeguy et al. (2013). These traits were universally acknowledged as the valid indicators of plant ecological strategies for acquiring, using, and preserving resources such as nutrients, light, and water (Maire et al., 2012; Garnier et al., 2017; **Figure 1** depicted trait names and abbreviations; **Supplementary Table 2** described the link between the measured traits and their associated functions). After the growing season, the plants were harvested on 21 October to measure plant aboveground and belowground functional traits. Before harvesting the whole plant, we measured the height of each plant (PL). Then, three fully expanded, young and healthy, undamaged leaves per plant were selected randomly to scan and measure leaf length (LL), width (LW), and area (LA) immediately using ImageJ software (version 1.51j8; National Institutes of Health, Bethesda, MD, United States). The scanned leaf fresh weight was measured using an analytical balance (ADAM PGL 203). After that, the leaves and the rest of aboveground leaves and stems (culm plus leaf sheath) were oven-dried at 75°C for 48 h to estimate the dry leaf and aboveground weight (She et al., 2016). Then, we calculated specific leaf area (SLA, the ratio of the leaf area to the leaf dry mass) and leaf dry matter content (LDMC, the ratio of leaf dry mass to fresh mass). Leaf thickness (LT) was calculated as 1/(SLA × LDMC). Finally, dry leaves were powdered for the measurement of leaf carbon content (LCC) and leaf nitrogen content (LNC) using an elemental analyzer (Vario MAX C/N-Macro Elemental Analyzer) and then calculated LCC/LNC.

All plant roots were collected from the pots as completely as possible. After cleaning the fresh roots, 108 root samples for the morphological and architectural measurements were scanned at 400 dpi using a scanner (EPSON Perfection V700/V750) and analyzed (WinRhizo root analysis system, Canada) to calculate the total root length (RL), root area (RA), specific root length (SRL), specific root area (SRA), branching intensity (BRI), and average root diameter (ARD). The corresponding root samples were dried at 75°C for 48 h to determine the final belowground biomass (Lundholm, 2015; She et al., 2016) and then calculated root tissue density (RTD). SRL was calculated as the root length per unit dry weight, SRA was calculated as the root area per unit dry weight, BRI was obtained as the ratio of tip counts to the total root length, and RTD was calculated from root dry weight divided by its volume (Comas and Eissenstat, 2009). In a similar way, dry roots were powdered for the measurement of root carbon content (RCC) and root nitrogen content (RNC), using an elemental analyzer and then calculated RCC/RNC. Finally, we calculated the root–shoot ratio (RSR) of each species using the ratio of belowground dry weight to aboveground dry weight.

Statistical Analyses

To fulfill the Kolmogorov–Smirnov test of normality distribution and Levene's test of homogeneity of variances, the data of plant biomass (aboveground, belowground, and total biomass) were log-transformed prior to analysis. Then, one-way analysis of variance (ANOVA) was performed to test the differences in plant biomass (aboveground, belowground, and total biomass) among N addition treatment using the SPSS22.0 (IBM SPSS Inc., Chicago, United States). A p-value < 0.05 was considered significantly different, and the means were compared using Tukey's *post hoc* test.

In addition, to assess the shift in the plant traits along with N addition, separate principal component analyses (PCAs) for aboveground and belowground traits were performed to describe variations in plant traits and visualize the trait space occupied by all 12 plant species. Because the first two axes of PCA explain a high proportion of the plant functional traits (**Supplementary Tables 3, 4**), the scores of these axes can be used as the overall variations in aboveground and belowground traits (PC of traits) and represent the trends of aboveground and belowground traits under N addition gradient. Because RSR did not belong to the aboveground or belowground traits, we conducted a linear regression between RSR and N addition to assess how RSR values change along the N gradient. Furthermore, pairwise relationships between all aboveground and belowground traits were evaluated by Pearson's correlation analysis and linear regressions.

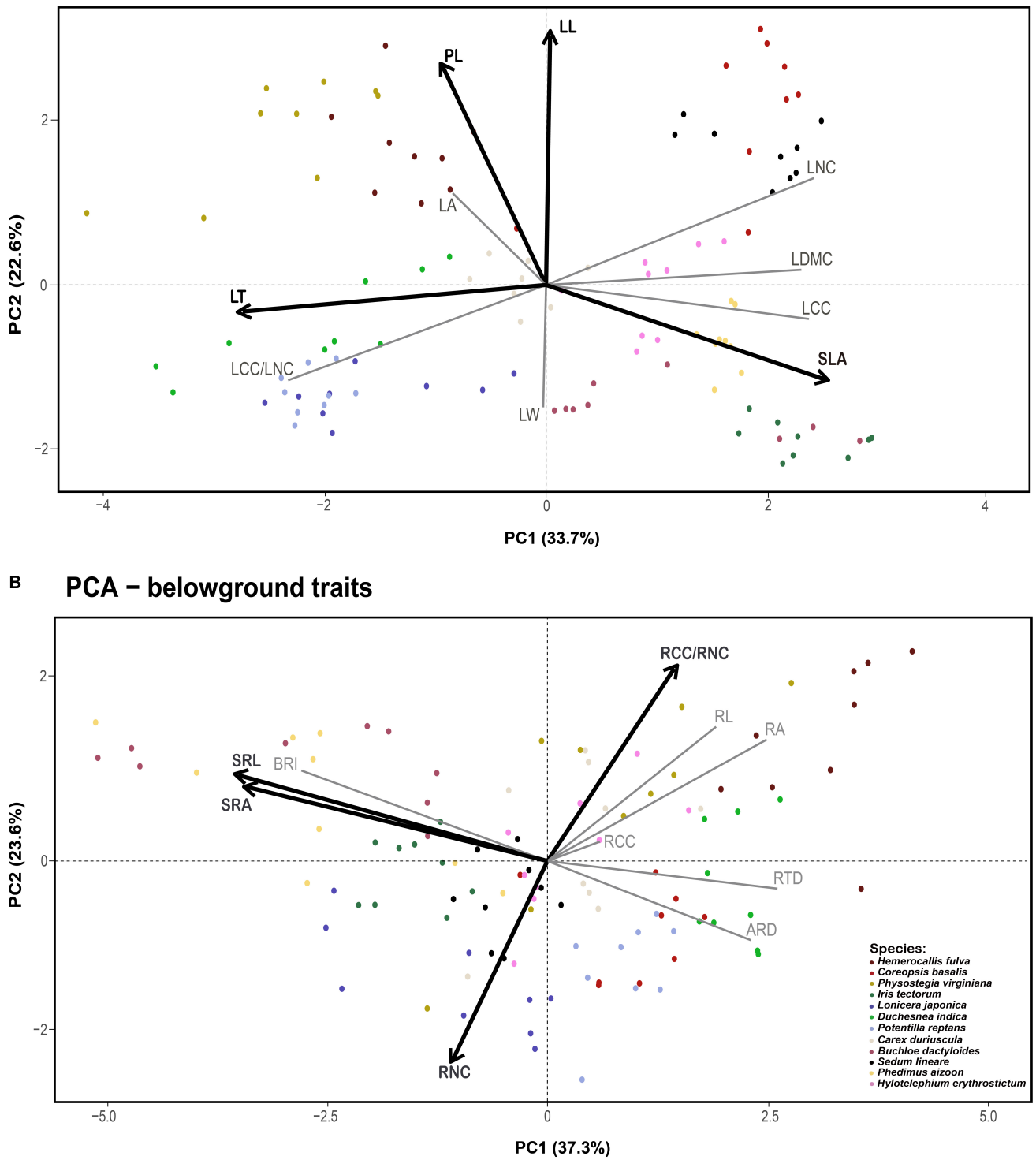

FIGURE 1 | Principal component analysis for aboveground **(A)** and belowground traits **(B)**. Vectors represent the relative contribution of each plant trait to the axis. The two variables that load most strongly on each axis are shown in dark black lines, with the remaining variables shown in light gray. Dots of different colors denote the different species samples. Trait acronyms: LA = leaf area, SLA = specific leaf area, LDMC = leaf dry matter content, LL = leaf length, LW = leaf width, LT = leaf thickness, LNC = leaf nitrogen content, LCC = leaf carbon content, LCC/LNC = the ratio between leaf carbon and nitrogen, PL = plant height, SRL = specific root length, RNC = root nitrogen concentration, RCC = root carbon concentration, RCC/RNC = the ratio between root carbon and nitrogen, RTD = root tissue density, RA = root area, SRA = specific root area, BRI = branching intensity, RL = root length, ARD = average root diameter.

To quantify the response of the plant traits to N addition, plasticity index (PI) was calculated (Valladares et al., 2000).

$$PI = (T_{iMax} - T_{iMin})/T_{iMax}$$

where T_{iMax} and T_{iMin} are the trait maximum and minimum scores of PC1 or PC2 of species i under each N addition treatment, respectively. Thus, we could get trait plasticity (PI_{PC1} and PI_{PC2}) for each species under each N addition treatment.

After this, we used functional traits (PC of traits) and trait plasticity (PI_{PC}) of each species in each N addition treatment to make a linear regression with N gradient to explore their connections. Meanwhile, to evaluate whether plant functional traits were properly matched with plant biomass at different N addition, we also performed linear regressions between plant biomass and PC of traits and PI_{PC}.

Finally, to quantify the relative importance of each predictor (N addition, PC1 and PC2 of aboveground and belowground traits, and PI_{PC1} and PI_{PC2} of aboveground and belowground traits) in influencing the plant biomass (aboveground, belowground, and total biomass), we performed a variance partitioning analysis with full-model predictors.

All these analyses mentioned above were performed using the packages "FactoMineR," "factoextra," "variancePartition," "psych," and "vegan" in R version 4.1.1 (R Core Team, 2021).

RESULTS

Effects of N Addition on Plant Functional Traits and Trait Plasticity

The first two independent principal components together explained 56.3 and 60.9% of the total variation for aboveground and belowground traits, respectively (**Figure 1**). For aboveground traits, the first PCA axis explained 33.7% of the variance and was mainly represented by SLA, LNC, and LT (Leaf Economics Spectrum, LES). We found that PC1 of aboveground traits increased with N addition, which meant the changes in leaf traits reflected a shift from a slow to a fast strategy as N increased (**Figure 2A**). The second dimension explained an additional 22.6% of the variance, indicating a resource utilization strategy at a trade-off for plant elongation or leaf and stem structure construction. However, N addition did not affect PC2 of aboveground traits (**Figure 2B**). As for belowground part, the first PCA axis accounted for 37.3% of the variance and described the conservation-acquisition trade-off in root traits (Root Economics Spectrum, RES). N addition had no significant effects on PC1 of belowground traits (**Figure 2C**), either. The second PCA axis accounted for an extra 23.6% of the variance, indicating N uptake capacity of roots. We observed that PC2 of belowground traits was positively correlated with N addition (**Figure 2D**). In addition, we found that N addition had no significant effects on the plasticity index of aboveground and belowground traits (**Supplementary Figure 2**).

Furthermore, we also found a synergy effect between aboveground and belowground traits in response to N addition. The results of Pearson's correlation analysis showed that LNC was positive with RNC and negative with LCC/LNC and RCC/RNC (**Figure 3** and **Supplementary Figure 3**). SRL and SRA were positively correlated with SLA and both of them were negatively correlated with LT. Likewise, SLA was negatively correlated with RTD and ARD, respectively (**Supplementary Figures 3, 4**). Moreover, N addition did not affect the plant RSR, and aboveground and belowground biomass had an isometric relationship (**Supplementary Table 5** and **Supplementary Figure 5**), which also proved that the aboveground and belowground biomass allocation strategies of plants did not change.

Effects of N Addition on Plant Biomass

N addition did not have a significant effect on the whole plant biomass (aboveground, belowground, and total biomass; **Supplementary Table 5**). The result of variance partitioning analysis also indicated that N addition explained the lowest proportion of variance among all predictors (**Figure 6**). However, we found that the observed responses of plant biomass to N addition were species-specific-dependent. For example, the aboveground biomass of *Sedum lineare* was significantly increased in high N treatment, but *Buchloe dactyloides* showed the opposite results (**Supplementary Figures 6–8**).

Relationships Between Plant Biomass and Functional Traits and Trait Plasticity

Plant biomass (aboveground, belowground, and total biomass) decreased with PC1 of aboveground traits while increased with PC2 of aboveground traits and PC1 of belowground traits. In addition, only belowground biomass increased with PC2 of belowground traits (**Figure 4**). The relationship between plant biomass and trait plasticity (PI_{PC1} and PI_{PC2} of aboveground and belowground traits) was similar to the relationships between plant biomass and PC of aboveground and belowground traits (**Figure 5**).

By variance component analysis, the sum of unique effects of all predictors explained the 30.9, 78.6, and 72.1% of the total variation in aboveground, belowground, and total biomass, respectively (**Figure 6**). PI_{PC1} of aboveground traits was the highest variable on aboveground biomass. Next, PC1 of aboveground and belowground traits and PC2 of belowground traits had the similar effects on aboveground biomass (**Figure 6**). The results obtained for variables from the belowground and total biomass were similar, where the PC1 and PC2 of belowground traits were the first two highest variables and the sum of them could explain 72.7 and 59.1% of the total variation, respectively (**Figure 6**).

DISCUSSION

We investigated the effects of N addition on plant resource strategies and ecosystem functioning (biomass) in green roofs. Our findings indicated that plants shifted to resource acquisition strategies with N addition, and the response of these ecological strategies for aboveground and belowground was synergistic. However, we did not find the significant effect of N addition

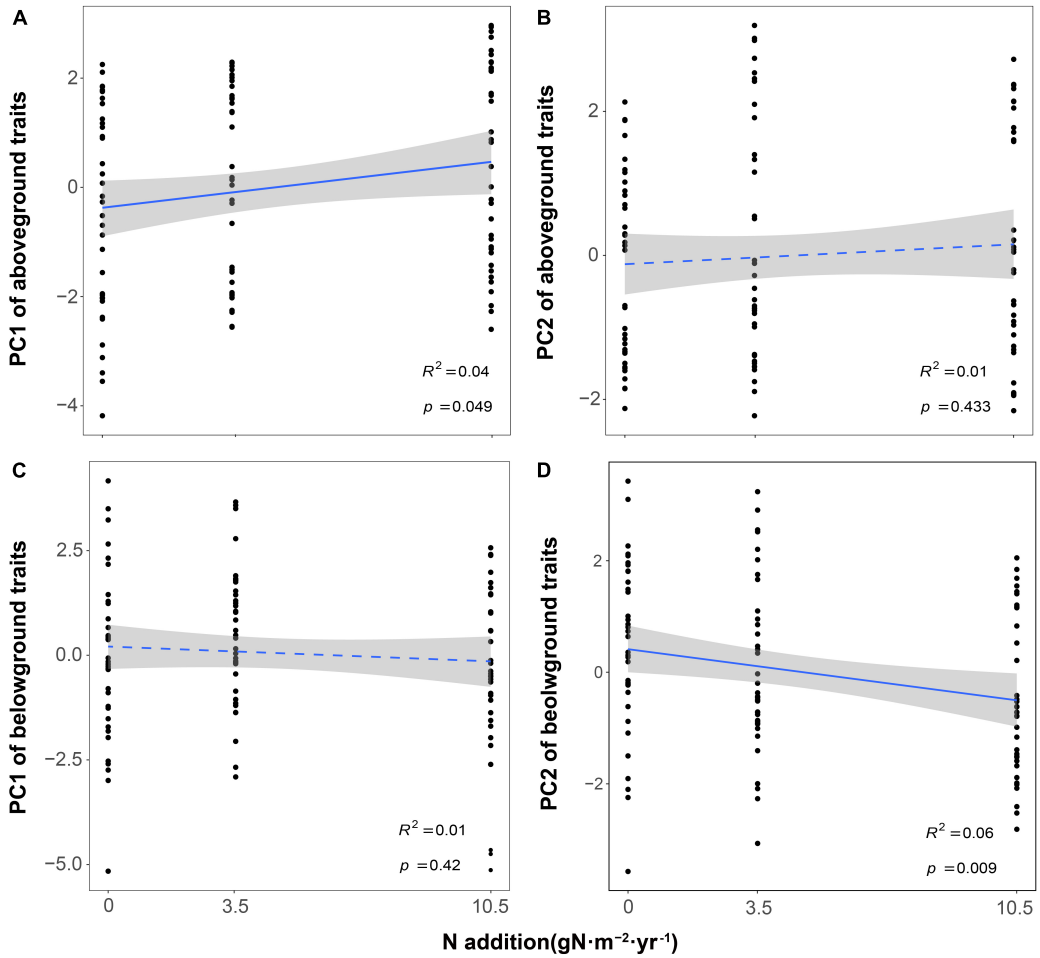

FIGURE 2 | Effects of nitrogen addition on plant functional traits [**(A,B)** for PC of aboveground and **(C,D)** for PC of belowground]. The R^2 (coefficient of determination) and *p*-values are obtained from the linear regression analyses. Shaded areas show 95% confidence interval of the fit test.

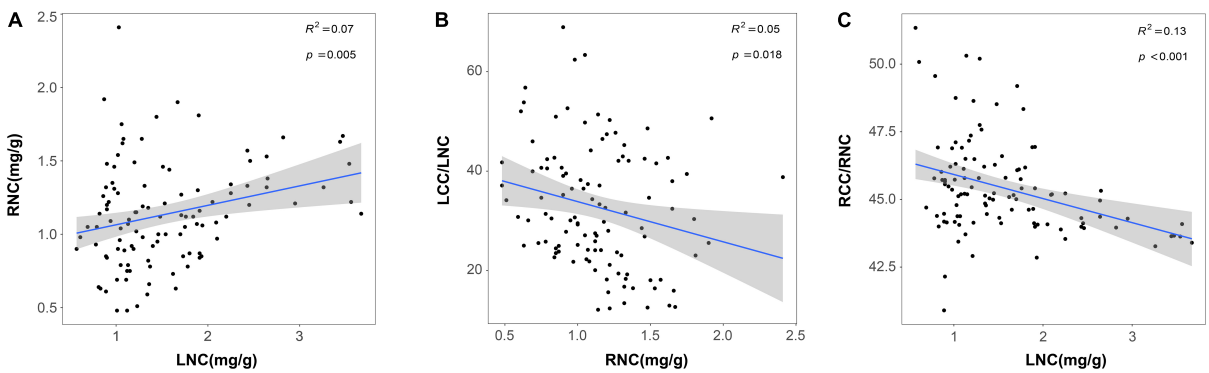

FIGURE 3 | Relationships between LNC and RNC **(A)**; RNC and LCC/LNC **(B)** and LNC and RCC/RNC **(C)**. Trait acronyms: LNC = leaf nitrogen content, LCC/LNC = the ratio between leaf carbon and nitrogen, RNC = root nitrogen concentration, RCC/RNC = the ratio between root carbon and nitrogen. The R^2 (coefficient of determination) and *p*-values are obtained from the linear regression analyses. Shaded areas show 95% confidence interval of the fit test.

on trait plasticity. In addition, despite N addition did not have significant effects on plant biomass, it could inhibit biomass indirectly by increasing plant resource-acquisitive traits, such as SLA, LNC, and RNC. Therefore, in the context of N deposition, there was a trade-off between the survival of green roof species and keeping the ecosystem functioning. Simultaneously,

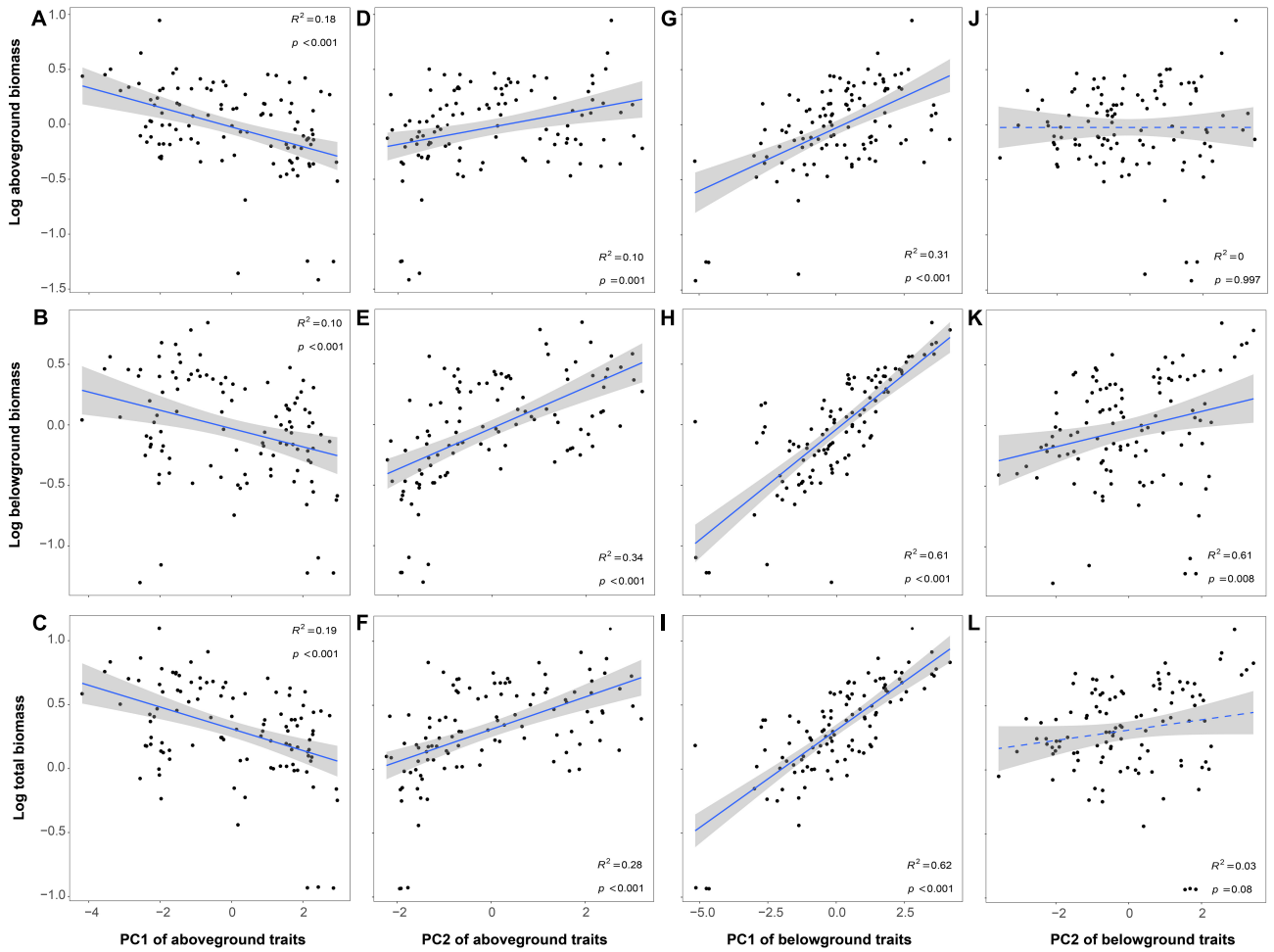

FIGURE 4 | Relationships between plant biomass and plant functional traits [**(A–F)** for PC of aboveground and **(G–L)** for PC of belowground]. The R^2 (coefficient of determination) and *p*-values are obtained from the linear regression analyses. Shaded areas show 95% confidence interval of the fit test.

compared with aboveground traits, root traits played a more important role in the total biomass. In future studies, more attention should be paid to the root growth mechanism of green roof plants under the condition of high N deposition.

Response of Aboveground and Belowground Traits and Trait Plasticity to N Addition

Our result showed PC1 of aboveground traits increased and PC2 of belowground traits decreased with the increase of N addition, suggesting that both aboveground and belowground traits of plants tended to shift from resource conservation to resource acquisition to adapt to the high N deposition environment. This was similar to the majority of studies in natural ecosystems (Cunningham et al., 1999; Kitajima and Poorter, 2010; Kramer-Walter et al., 2016). For example, SLA and LNC increased and LT decreased with increasing resource availability (such as nutrient and water availability) for the aboveground traits (Cunningham et al., 1999), and RNC was positively correlated with N addition on fertilization experiments for the belowground traits (Helmisaari et al., 2009; Wang P. et al., 2016). N addition could increase soil N availability, which improved the foliar N storage and root absorption efficiency (Butler et al., 2016). Plants with higher LNC tended to have a faster photosynthetic rate, larger SLA, and shorter leaf span (LT is positive with leaf span) in high N deposition (Heberling and Fridley, 2016). Therefore, N addition could promote photosynthetic efficiency and resource acquisition capability which made plants more tolerant to the high N environment. Meanwhile, RNC was thought to be an adequate predictor of root respiration, and roots with high N content increased metabolic activity and nutrient absorption rates (Trocha et al., 2017). With the increase of RNC, more N became available for root uptake and stocked in root tissues, thereby accelerating the cycle of C and N in green roof ecosystems (Nadelhoffer, 2000). In addition, we found that LNC was positive with RNC, both of them were negative with LCC/LNC and RCC/RNC (**Figure 3**), and N addition did not significantly affect the plant RSR (**Supplementary Figure 5**). These results also proved that there was a strong coordination

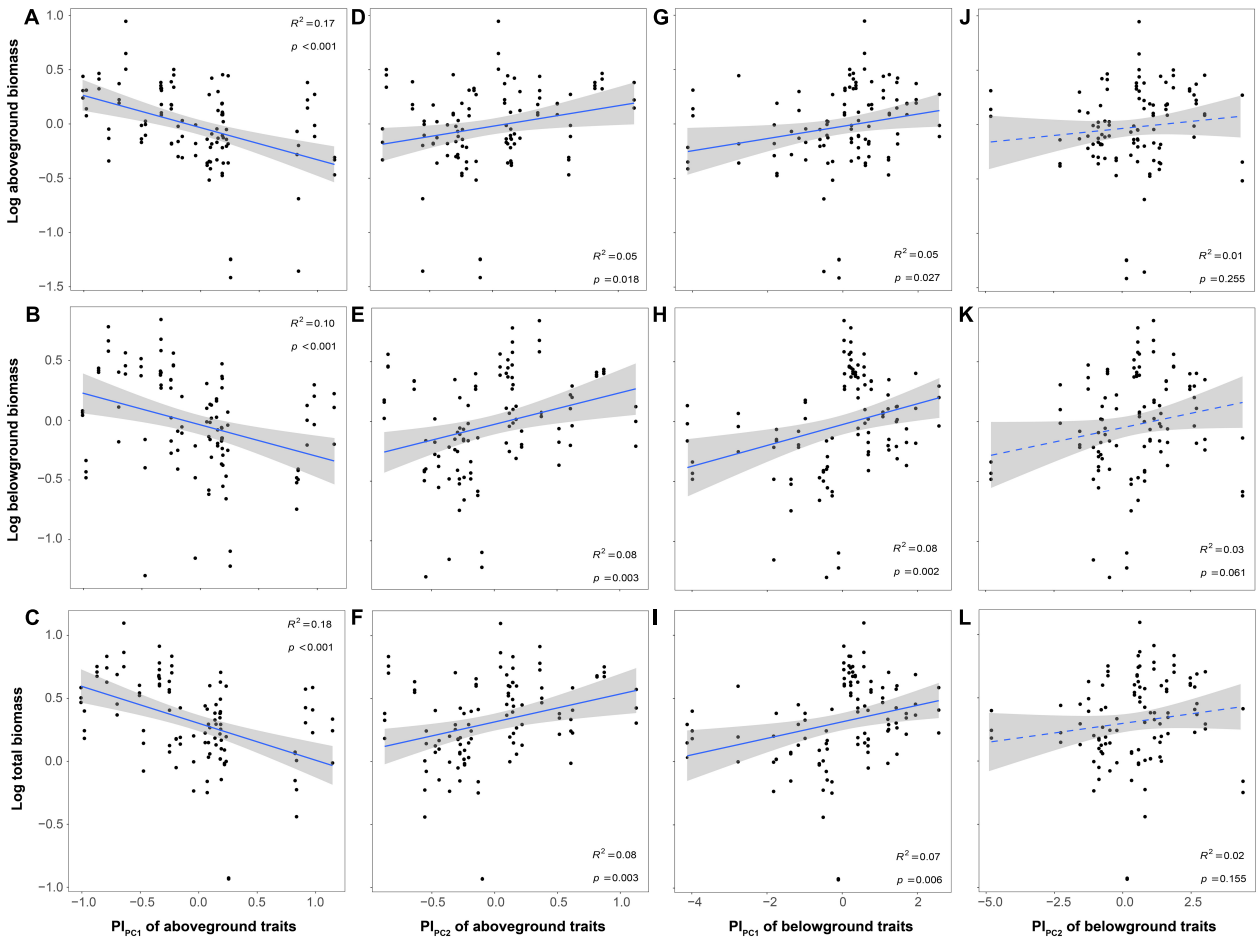

FIGURE 5 | Relationships between plant biomass and trait plasticity [**(A–F)**, for PI_{PC} of aboveground and **(G–L)** for PI_{PC} of belowground]. The R^2 (coefficient of determination) and *p*-values are obtained from the linear regression analyses. Shaded areas show 95% confidence interval of the fit test.

between aboveground and belowground traits of roof plants in the direction of resource acquisition strategy in the high N deposition environment (Freschet et al., 2010; Hu et al., 2019).

However, there were no correlations between N addition and PC2 of aboveground traits and PC1 of belowground traits. PC2 of aboveground traits described a trade-off for plant elongation or leaf and stem structure construction (Weiner and Thomas, 1986), playing an important role in determining light interception. This result was similar to Yin et al. (2011), who reported that corn traits related to light interception (such as plant height) were not correlated with N addition. Because of the high winds in the green roof environment (Speak et al., 2012), investing more resources in height to improve light access would incur more costs in construction and maintenance of the stem, resulting a decrease in individual plant fitness and survival rate. Therefore, plants mainly increased LNC and SLA rather than PL in response to N addition. In addition, PC1 of belowground traits described the conservation-acquisition trade-off in the uptake of root resources. Our results showed that N addition did not affect RES strategy, which was different from other studies (Kramer-Walter et al., 2016; Li et al., 2016; Wang et al., 2017). Previous studies found that SRL and SRA showed significantly positive responses to N addition (Li et al., 2016), which was mainly because N could change soil N availability and improve the root absorption efficiency (Butler et al., 2016). However, we did not reach the same conclusion, and the mechanism of N addition on root resource uptake strategies of plants in roof ecosystems is still unclear. We could only deduce that N was not a limiting factor for root resource uptake in our study. In the follow-up roof experiments, further attention and understanding of root system of roof plants are still needed.

Trait plasticity is an important mechanism for plants to optimize resource access and adapt to environmental changes (Wang P. et al., 2016), so we found a positive association between trait plasticity and plant biomass (**Figure 5**). However, we did not find the significant effect of N addition on trait plasticity, regardless of aboveground or belowground (PI_{PC} of aboveground or belowground). Considering the extreme environmental conditions of the roofs, most of plants we selected were apt to be trait-conservative, and the functional plasticity indexes of these plants were relatively low (Zhang et al., 2020). Therefore, trait plasticity might not be the main strategy for

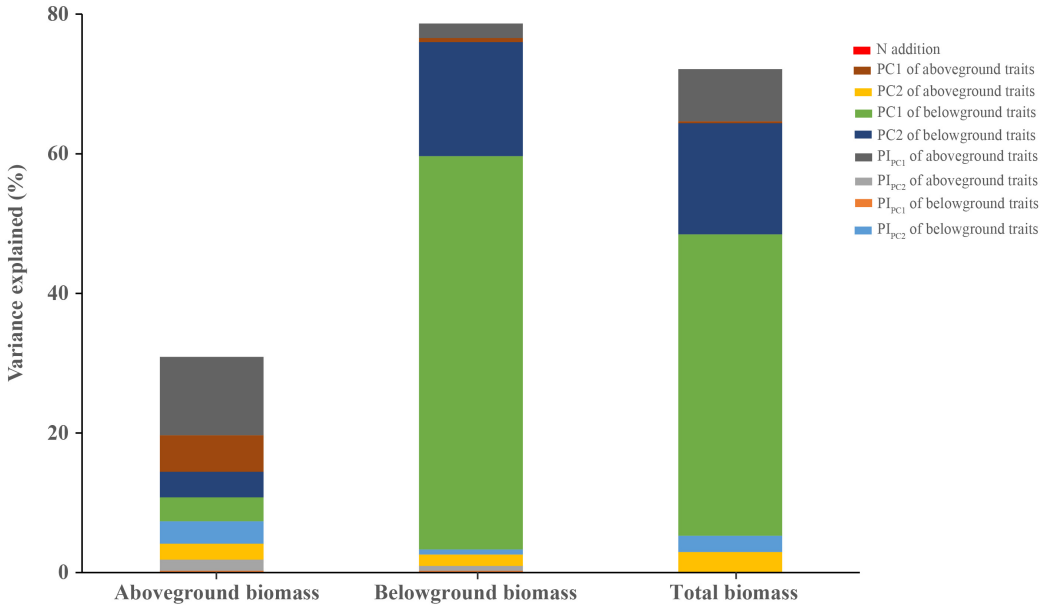

FIGURE 6 | Variance components show the unique portion of variation (percentage of total R^2) explained by each predictor in aboveground, belowground, and total biomass. The predictors used are N addition, plant functional traits (PC1 and PC2 of aboveground and belowground traits), and trait plasticity (PI_{PC1} and PI_{PC2} of aboveground and belowground traits).

the whole species group to respond to N addition in green roofs. Instead, the roof environment might be the main factor limiting the plasticity of plant traits in green roofs. Species with specific acquisitive traits through environmental filtering were more suitable for growth in this environment (Maire et al., 2012).

Response of Plant Biomass to N Addition

Plant biomass can represent multiple key ecosystem functions in green roof ecosystems (Xie et al., 2018; Mayer et al., 2021). For example, plant species with greater root biomass had a greater ability in providing nutrient retention and rainwater capture, which also could indicate a general increase in plant resource demand and water consumption (Nagase and Dunnett, 2012). N is one of the most critical nutrients for managing plant function and ecosystem stability, which can affect plant productivity and biomass for both above and below parts (Hermans et al., 2006). However, our results showed that N addition did not influence plant biomass (**Figure 6** and **Supplementary Table 5**), which was different from other studies (Lee et al., 2017; Mao et al., 2017). There might be owing to three reasons. First, the response of species to N addition was specific, leading to an independent correlation between each species' biomass and N treatment (Ren et al., 2019). Second, N had both positive and negative effects on plant growth (Lu et al., 2014; Li et al., 2016). Therefore, the overall effect was not significant probably because the positive and negative effects were offset each other. Finally, it might be owing to our short experiment period (only one growing season; Lundholm, 2015), which could not reflect sufficiently the relationship between N addition and plant biomass.

Although N addition did not affect biomass directly, it could decrease plant biomass by mediating plant functional traits. Because of the harsh environment on green roofs (Speak et al., 2012), plants adopting resource conservative strategy had higher biomass and ecosystem functioning (**Figure 4**). However, N addition indirectly decreased the plant biomass and ecosystem functioning by the shift to resource-acquisitive traits. This might be because N addition broke the stoichiometric ratio of original elements in plant organs, leading to the imbalance of nutrient element relations, which was not conducive to plant growth (Nadelhoffer et al., 1999; Baptist and Aranjuelo, 2012; Liu et al., 2018). For example, N addition increased LNC and RNC, which caused that the C/N ratio dropped (LCC/LNC and RCC/RNC, **Figure 3**). The changes of C and N circulation indicated the original C-N balance of the plant nutrient system would be broken and eventually caused the decrease in plant biomass (C/N balance hypothesis, Bryant et al., 1983; Wang L. et al., 2016). Moreover, resource-acquisitive species appeared to be more affected by water scarcity than conservative ones, due to their higher water demand to maintain high growth rates (Grime et al., 1997; Fort et al., 2014). In our study, N addition promoted the development of plant traits toward the direction of resource acquisition, which intensified the effects of water shortage on roof plants. Thus, considering the environment of water-deficient in green roofs, this might also be one of the main reasons for limiting the growth and biomass of resource-acquisitive species under high N deposition (Fort et al., 2014; Khan et al., 2020).

In addition, the results of variance partitioning analysis showed that the first two principal components of belowground traits played the most important roles in total biomass (**Figure 6**), which was in line with the understanding that plants would invest more resources for the root growth to survive under stress environment conditions (Wang P. et al., 2016). Therefore, given

that root traits had more influence on the total biomass compared with aboveground traits and N addition, it is important to consider how root traits and environmental changes impact plant selection and ecosystem function in green roofs. Meanwhile, it is also worth noting that our experimental periods are relatively short (only a 3-month N addition simulation experiment). The next important question to explore is whether our results on the response of aboveground and belowground traits and biomass to N deposition can be generalized to a longer time scale.

CONCLUSION

Our findings demonstrate that plant ecological strategy shifted from resource conservation to resource acquisition with N addition and the responses of both aboveground and belowground traits of plants were synergistic in green roofs. Therefore, considering the survival of plants, plants with resource acquisition traits (large specific leaf area, high leaf and root N concentration, and thin leaves) were more suitable for roof planting in high N deposition. However, the transformation of green roof plants to resource acquisition as N addition also resulted in the decline of biomass and ecosystem functioning. In the context of future high N deposition, a trade-off between the survival of species and their ecosystem functioning needs to be considered when planning and managing similar ecosystem engineering. Furthermore, the research on root traits should be strengthened in the future conservation of green roof plants. These findings provide insights into how species respond to global climate changes in green roofs and support the possibility of the development of sustainable green roofs in a background of high N deposition.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

GH, NY, and HL conceived the ideas and devised the methodology. GH, QZ, ML, LL, and BK collected the data. QZ and GH analyzed the data. QZ, GH, and HL led the writing of the manuscript. All authors made significant contributions to the drafts, which were eventually approved 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.2022.894782/full#supplementary-material

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Mowing Did Not Alleviate the Negative Effect of Nitrogen Addition on the Arbuscular Mycorrhizal Fungal Community in a Temperate Meadow Grassland

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As nitrogen deposition intensifies under global climate change, understanding the responses of arbuscular mycorrhizal (AM) fungi to nitrogen deposition and the associated mechanisms are critical for terrestrial ecosystems. In this study, the effects of nitrogen addition and mowing on AM fungal communities in soil and mixed roots were investigated in an Inner Mongolia grassland. The results showed that nitrogen addition reduced the α-diversity of AM fungi in soil rather than that of root. Besides, nitrogen addition altered the composition of AM fungal community in soil. Soil pH and inorganic nitrogen content were the main causes of changes in AM fungal communities affected by nitrogen addition. Mowing and the interaction of nitrogen addition and mowing had no significant effect on AM fungal community diversity. In contrast, while mowing may reduce the negative effects of nitrogen addition on the richness and diversity of plants by alleviating light limitation, it could not do so with the negative effects on AM fungal communities. Furthermore, AM fungal communities clustered phylogenetically in all treatments in both soil and roots, indicating that environmental filtering was the main driving force for AM fungal community assembly. Our results highlight the different responses of AM fungi in the soil and roots of a grassland ecosystem to nitrogen addition and mowing. The study will improve our understanding of the effects of nitrogen deposition on the function of ecosystem.

Keywords: arbuscular mycorrhizal fungi, nitrogen addition, diversity, community composition, environmental filtering

INTRODUCTION

Human activities have caused increasing atmospheric nitrogen deposition that has seriously affected the biodiversity and functioning of terrestrial ecosystems (Smith et al., 1999; Yu et al., 2019). For plants, nitrogen deposition can affect both plant community composition and diversity (Wei et al., 2013; Zhang et al., 2014) due to soil acidification, aluminum toxicity (Wei et al., 2013),

light limitation (Borer et al., 2014), and increased nitrogen availability (Liu et al., 2020). Meanwhile, lower pH and increased nitrogen availability following nitrogen deposition can lead to alteration in the soil microbial community structure and reductions of species diversity (Wang et al., 2018; Liu et al., 2020). Moreover, grasslands are mowed to harvest hay, one of the most important ways of managing grassland ecosystems. Previous studies have shown that mowing can mitigate the negative effects of nitrogen deposition on plant diversity by removing litter and alleviating light limitation (Luo et al., 2019; Yang et al., 2019). However, information on the impact of mowing on belowground microbial communities under the background of nitrogen deposition in grassland ecosystems is limited.

Arbuscular mycorrhizal (AM) fungi can form mutualistic associations with most land plants, thereby providing mineral nutrients to their plant partners in exchange for the photosynthetic products used to maintain growth and function (Lu and Hedin, 2019; Tedersoo et al., 2020). In addition, AM fungi have important ecological functions in the succession, biodiversity, productivity, and material and energy cycling of terrestrial ecosystems (Vályi et al., 2016; Powell, 2018; Tedersoo et al., 2020). A recent meta-analysis showed that nitrogen deposition significantly decreased the AM fungal abundance (Han et al., 2020). However, some studies have shown that fertilization has no significant or positive effect on the AM fungal community (Zheng et al., 2014; Pan et al., 2020). The response of the AM fungal community to nitrogen deposition may be affected by different ecosystem types as well as the rates and duration of nitrogen deposition (Han et al., 2020; Ma et al., 2020). Nitrogen is limited resource in grassland ecosystems, and nitrogen deposition removes plants from nutrient limitation. So that plants are less dependent on AM (Olsson et al., 2010). AM obtain less photosynthetic products from host plants (Pearson and Jakobsen, 1993; Smith et al., 1999), thereby intensifying competition among AM fungi and changing their community composition and structure. Moreover, nitrogen deposition may alter plant community composition, and host plant may also actively select AM fungal taxa that can help the host plants obtain more benefits. (Veresoglou and Rillig, 2014). Researches of the effects of mowing on AM fungal community are also controversial. Mowing altered the community composition of AM fungi through soil properties (Sepp et al., 2018). In addition, there were different views that mowing has no significant effects on the species composition of AM fungi (Zubek et al., 2022) and the colonization of AM fungi in roots (Eom et al., 1999). However, it remains unclear whether mowing alters the effects of nitrogen deposition on AM communities in grassland ecosystems.

In general, deterministic processes are important in regulating ecological communities when environmental filtering and competitive exclusion contribute to community assembly, and stochastic processes are important when dispersal and chance shape community assembly (Vályi et al., 2016). However, nitrogen deposition may affect the relative importance of deterministic and stochastic processes. Nitrogen deposition and mowing can change soil environmental variables, and the assembly process of AM fungal community is influenced by soil pH, soil nutrients, and soil type (Johnson, 2010; Liu et al., 2012). Furthermore, spore dispersal is an important way for AM fungi to disperse (Janos et al., 1995). Spore density of AM fungi is altered after nitrogen deposition (Zheng et al., 2014), which affects the dispersal process of AM fungi. The phylogenetic structure of AM fungi has been used to clarify the dominant ecological processes that drive AM fungal community assembly (Webb et al., 2002). Environmental filtering, stochastic processes, and competitive exclusion may lead to phylogenetic clustering, random distributions, and over-dispersion of the AM fungal community, respectively (Webb et al., 2002). An eight-year fertilization experiment showed that the phylogenetic pattern of AM fungal communities shifted from clustering under no fertilization to a random distribution under low fertilization treatments and over-dispersion under high fertilization treatments (Liu et al., 2015). Chen et al. (2017) found that AM fungal communities were phylogenetically clustered according to nitrogen deposition, suggesting that environmental filtering was the main driver of AM fungal community assembly. At present, evidence is inconclusive concerning whether the ecological processes driving AM fungal community assembly under nitrogen deposition are consistent.

In this study, long-term (seven years) nitrogen addition and mowing experiments in the temperate grassland of Inner Mongolia were conducted. We investigated the composition of the plant community and used high-throughput sequencing to study how plant and AM fungal communities responded to nitrogen addition and mowing. We addressed three questions: (1) How do plant and AM fungal communities respond to nitrogen addition? We assumed that the diversity of the plant community and the AM fungal community would decrease with increasing nitrogen addition. (2) Will mowing change the responses of plant and AM fungal communities to nitrogen addition? We hypothesized that mowing would alleviate the negative effects of nitrogen addition on the composition and diversity of the plant and AM fungal communities. (3) What are the main ecological processes responsible for the AM fungal community assembly under nitrogen addition and mowing treatments? We hypothesized that environmental filtering was the primary driver of AM fungal community assembly.

MATERIALS AND METHODS

Study Site and Experimental Design

The study site was located in a temperate meadow grassland in Inner Mongolia of northern China (50°10′N, 119°22′E). Mean annual temperature and precipitation at the site are −1.59°C and 336.5 mm (2000–2020), respectively. The study area has been fenced since 2013 to avoid disturbance by large animals. Vegetation is dominated by *Leymus chinensis*, *Stipa baicalensis*, *Cleistogenes squarrosa*, *Thermopsis lanceolate*, *Cymbaria daurica*, and *Carex duriuscula*.

A nitrogen addition experiment was initiated in 2014 and has been maintained since then. Nitrogen fertilization were added when grasslands turned green. The nitrogen fertilization was mixed with the roasted fine sand and uniformly spread

into the corresponding experimental plots. A series of 10 m × 10 m plots were laid out and separated by 1 m walkways. We selected five nitrogen rates (0, 2, 5, 10, and 20 g m^{-2} $yr.^{-1}$) of urea for the nitrogen addition experiment. Mowing treatments (non-mown and mown) were set up based on nitrogen addition, with a total of 10 treatments (nitrogen addition without mowing treatments: N0, N2, N5, N10, N20; Nitrogen addition with mowing treatments: MN0, MN2, MN5, MN10, MN20), each replicated five times. The mowing treatment was carried out at the end of August every year, and plants were mown with a height of about 10 cm remaining.

Plant and Soil Sampling

Samples of plant species were collected in August by clipping all plants at the soil surface using a 1 m × 1 m quadrat randomly placed in each plot. After bringing the plant sample back to the laboratory, plants were dried (105°C for 1 h, and 65°C for 48 h) and weighed for dry mass. Soil samples were collected from the top 15 cm of the soil profile using a soil core sampler (6 cm internal diameter). Six soil cores were collected from each plot after the plant biomass harvest and mixed to give one composite sample. Thus, a total of 50 mixed stratified soil samples were collected. The soil samples were passed through a 2.0 mm sieve and homogenized. Plant roots were carefully picked out with tweezers. Roots and a portion of fresh soil were stored at −80°C for DNA extraction and −20°C for inorganic N analysis, and the remaining soil was air-dried and stored at room temperature for determination of soil chemical properties.

Soil Properties Analyses

Soil moisture was determined as the mass loss after drying the soil at 105°C for 24 h. The soil pH was measured with a soil-water ratio of 1:5 using a pH meter. The ammonium (NH_4^+ 以N) and nitrate nitrogen (NO_3^- 以N) were leached with 2 mol L^{-1} KCl and determined by MgO-Devarda alloy distillation. The total nitrogen (TN) and phosphorus (TP) contents were digested with concentrated sulfuric acid, then determined by the Kjeldahl nitrogen and molybdenum antimony colorimetric methods, respectively. The total carbon content (TC) of the soil was measured using potassium dichromate heating. The available phosphorus content (AP) of the soil was leached with $NaHCO_3$ and determined by the molybdenum antimony colorimetric method.

DNA Extraction and Sequencing

Mycorrhizal fungal DNA was extracted from the soil and mixed root samples using a Fast DNA SPIN Kit (MP Biomedicals LIC, Santa, Ana, CA). The quality and quantity of the extracted DNA were determined by electrophoresis on a 2.0% agarose gel. All DNA samples were amplified by nested PCR. The first step was performed with the primer pair AML1F (5′-ATCAACTTTCGATGGTAGGATAGA-3′) and AML2R (5′-GAACCCAAACACTTTGGTTTCC-3′; Lee et al., 2008). The PCR mixture consisted of 4 μl of 5 × FastPfu Buffer, 2 μl of dNTPs mixture (each 2.5 mM), 0.8 μl of each primer, 0.4 μl of FastPfu polymerase, 0.2 μl of BSA, and 10 ng of template DNA combined with sterile deionized H_2O to a total volume of 20 μl. The thermal cycling conditions were an initial denaturation at 95°C for 3 min, 32 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s followed by a final extension at 72°C for 10 min. The PCR products were further amplified in the second step with the primer pair AMV4-5NF (5′-AAGCTCGTAGTTGAATTTCG-3′) and AMDGR (5′-CCCAACTATCCCTATTAATCAT-3′; Sato et al., 2005). Except for the number of cycles being set at 30, the PCR conditions for the second PCR were similar to the first PCR.

PCR products were sent to Majorbio Bio-pharm Technology Co., Ltd., (Shanghai 201203, China) for Illumina MiSeq sequencing. The raw high-throughput sequencing data were first processed using the Quantitative Insights into Microbial Ecology (QIIME)[1] toolkit. 2365781 valid readings were obtained after quality control filtering. We clustered our sequences into operational taxonomic units (OTUs) with the criterion of sequence identity ≥97% and used the representative sequence of each OTU to Blast against the MaarjAM database[2] for taxonomic assignment (Öpik et al., 2010). After normalization, 924 OTUs were obtained, of which 573 OTUs were identified at the family level with known AM fungal families. The raw reads were submitted to the NCBI Sequence Read Archive (SRA) database under the accession numbers PRJNA790751.

Statistical Analyses

In this study, the structural equation modeling (SEM) was performed using the AMOS software (IBM SPSS AMOS 24.0.0), other statistical analysis and graphing were performed in R (R version 4.0.5). Richness, Shannon-Wiener index (H), Simpson index (D), and Pielou index (E) were used to measure the α-diversity of the community.

$$H = -\sum_{i=1}^{s} p_i \log_2 p_i$$

$$D = 1 - \sum_{i=1}^{s} p_i^2$$

$$E = \frac{H}{\log_2 N}$$

Where p_i is relative biomass of plant species *i* or OTU abundance of AM fungi *i*. *N* is plant species richness or OTU abundance of AM fungi. *H* is Shannon-Wiener index; *D* is Simpson index. *E* is Pielou index.

Two-way ANOVA was used to examine the effects of N addition, mowing and their interaction on soil properties, plant and AM fungal communities. The effects of different nitrogen addition rates on soil properties and plant and AM fungal

[1]http://qiime.org/install/index.html
[2]http://maarjam.botany.ut.ee

α-diversity were tested using one-way ANOVA followed by Tukey HSD test for each mown or unmown treatment. The differences between mown and unmown treatments were tested by independent *t* tests. Before the ANOVA, we performed a normality test and a test for the homogeneity of variance on all data. For the data that did not satisfy the assumptions of normality of the distribution or the homogeneity of variance, the logarithmic, reciprocal, or square root transformations were performed, and the nonparametric Kruskal-Wallis test was used for the data that still did not satisfy the conditions after the transformation.

Principal component analysis (PCA) was conducted to reduce the number of variables for plant and AM fungal community composition using the R package 'vegan'. The AM fungal community structure was visualized by non-metric multidimensional scaling (NMDS) ranking based on the Bray-Curtis distance matrices using the 'metaMDS' function of the R package 'vegan' (Faith et al., 1987). Permutational multivariate analysis of variance (PERMANOVA, 999 permutations) was used to test the effects of nitrogen addition and mowing on the AM fungal community structure by using the 'adonis' function of the R package 'vegan' (Warton et al., 2012).

Structural equation modeling was used to examine the causal pathways by which nitrogen addition and mowing affected the AM fungal community. Based on our knowledge of the effects of nitrogen deposition and mowing on the AM fungal community, we constructed *a priori* model (**Supplementary Figure S1**). The generalized least squares method was used to fit the data into the model. Chi square, root mean square error of approximation (RMSEA), and goodness of fit index (GFI) were used to assess model fit (Grace, 2006). We calculated NRI and NTI using the 'ses.mpd' and 'ses.mntd' functions (NRI and NTI were equivalent to −1 times output of 'ses.mpd' and 'ses.mntd') of the R package 'picante', respectively (Webb et al., 2008; Chen et al., 2017).

RESULTS

Responses of Soil Parameters and Plant to Nitrogen Addition and Mowing

Nitrogen addition significantly reduced the soil pH from 6.86 to 5.72 ($F=23.65$, $p<0.001$; **Table 1**). TN, NH_4^+ N, NO_3^- N, and inorganic nitrogen in the soil increased with the increase of nitrogen addition, but only the N10 or N20 treatments were significantly different from the controls ($F=5.356$, $p<0.01$; $F=6.956$, $p<0.01$; $F=3.588$, $p<0.05$; $F=7.947$, $p<0.001$; **Table 1**). Nitrogen addition had no significant effect on soil TC, TP, or AP. When nitrogen addition and mowing were carried out simultaneously, the response of soil parameters to nitrogen addition was consistent with that of only nitrogen addition (**Table 1**). Mowing did not significantly change soil properties ($p>0.05$, results not shown).

Plant richness and the Shannon-Wiener, Simpson, and Pielou indices were significantly reduced under the nitrogen addition ($p<0.001$; **Figure 1**), where plant richness was reduced to about two species under N20. After mowing, the plant richness and Shannon-Wiener index were significantly improved at N5 and higher treatments compared with only nitrogen addition (*t*-test, $p<0.001$; **Figure 1**). At the same time, the Simpson and Pielou indices were significantly increased under N2 and higher treatments (*t*-test, $p<0.001$; **Figure 1**).

Responses of AM Fungal Communities in Soil and Roots to Nitrogen Addition and Mowing

There was no significant difference in AM fungal operational taxonomic unit (OTU) richness in the soil under all treatments (**Figure 2A**). The Shannon-Wiener and Pielou indices of AM fungal communities in the soil under mowing conditions were significantly different only under MN20 compared with the control treatment, but Shannon-Wiener, Simpson, and Pielou indices generally decreased with the increase of nitrogen addition (Shannon-Wiener: $p<0.05$, Simpson: $p<0.05$, Pielou: $p<0.05$; **Figures 2B–D**). Nitrogen addition only exerted an influence on the OTU richness of AM fungal community in roots ($p<0.05$, **Figure 2A**), while no other significant difference was found in the Shannon-Wiener, Simpson, and Pielou indices of AM fungal communities in the roots in other treatments ($p>0.05$, **Figures 2B–D**). Mowing did not change the richness or α-diversity indices of AM fungal communities in the soil or roots (*t*-test, **Figure 2**).

The OTU richness of Glomeraceae was more diverse and had higher ratio reads than other families in all treatments (**Figure 3A**). Regardless of mown or unmown treatment, the relative abundances of Glomeraceae and Diversisporaceae families decreased with increasing nitrogen addition in the soil; the Paraglomeraceae family showed an opposite trend to the Glomeraceae and Diversisporaceae families (**Figure 3B**). However, Glomeraceae assumed a supermajority of the percentage in the roots (**Figure 3B**). Regardless of soil or roots, both AM fungal community composition and beta diversity were significantly affected by nitrogen addition (PERMANOVA, $R^2=22.18\%$, $p=0.001$; $R^2=11.31\%$, $p=0.041$; **Table 2**). There was no detectable effect of mowing on AM fungal community structure in the soil or roots (**Table 2; Figure 4**).

The SEM model adequately fitted data describing the interaction pathways between plants, AM fungal communities, and soil parameters in response to nitrogen addition and mowing (Soil: Chi square = 11.477, Df = 10, $p=0.322$, GFI = 0.932, RMSEA = 0.055; Root: Chi square = 6.307, Df = 7, $p=0.504$, GFI = 0.962, RMSEA = 0; **Figure 5**). The final model explained 22.9, and 34% of the variation in AM fungal community composition and richness in roots (**Figure 5A**), and explained 58.3, and 28.5% of the variation in AM fungal community composition and Shannon-Wiener index in soil, respectively (**Figure 5B**). Regardless of in soil or roots, soil pH showed significant correlations with AM fungal composition, whereas inorganic N showed significant correlations with AM fungal richness of root or AM fungal Shannon-Wiener index of soil (**Figure 5**).

Ecological Process of AM Fungal Community Assembly

The NRI and NTI of the AM fungal community were all significantly greater than zero (**Figure 6**), indicating that AM fungal species

were more closely related than expected by chance in all treatments. In other words, the phylogenetic structure of AM fungal communities was always clustered in all treatments. Except that the treatment of mowing under nitrogen addition had a significant effect on the NTI of the AM fungal community in soil ($p < 0.05$, **Figure 6D**), there was no significant difference in the NRI and NTI of AM fungal communities in other treatments ($p > 0.05$, **Figure 6**). However, the NRI and NTI of the AM fungal community had a downward trend across the nitrogen addition rates (but were always greater than zero), which only occurred in the soil (**Figure 6**).

DISCUSSION

The Effects of Nitrogen Addition and Mowing on Plant Community

Consistent with previous research (Clark and Tilman, 2008; Tian et al., 2016), nitrogen addition reduced the diversity of plant community (**Figure 1**). We found that the soil pH decreased and the inorganic nitrogen content increased significantly after nitrogen addition (**Table 1**). In addition, resource competition and soil acidification were considered to be the main causes of species loss (Stevens et al., 2004; Farrer and Suding, 2016; Greaver et al., 2016). On the one hand, nitrogen addition led to competition for limiting resources among different species or functional groups, including competition for light from aboveground resources (Grime, 1973; Newman, 1973) and competition for nutrients from belowground resources (**Supplementary Figure S2A**; Wei et al., 2013). On the other hand, soil acidification increased concentrations of harmful Al^{3+}, Mn^{2+}, and Fe^{3+} (Maskell et al., 2010), thereby reducing plant diversity (**Supplementary Figure S2B**).

Previous studies have shown that mowing can reduce the negative impact of nitrogen on plant species diversity by removing the nitrogen accumulated in the soil (Storkey et al., 2015; Tilman and Isbell, 2015). However, mowing did not change inorganic N concentrations in the present study (**Table 1**) but did mitigate the negative effects of nitrogen addition on plant diversity (**Figure 1**). Nitrogen addition promoted the rapid growth of some nitrogen-loving plants, which caused shading to other plants in the community, resulting in the loss of these shaded plant species from the community due to light limitation (Grime, 1973; Newman, 1973). Mowing may have slowed the

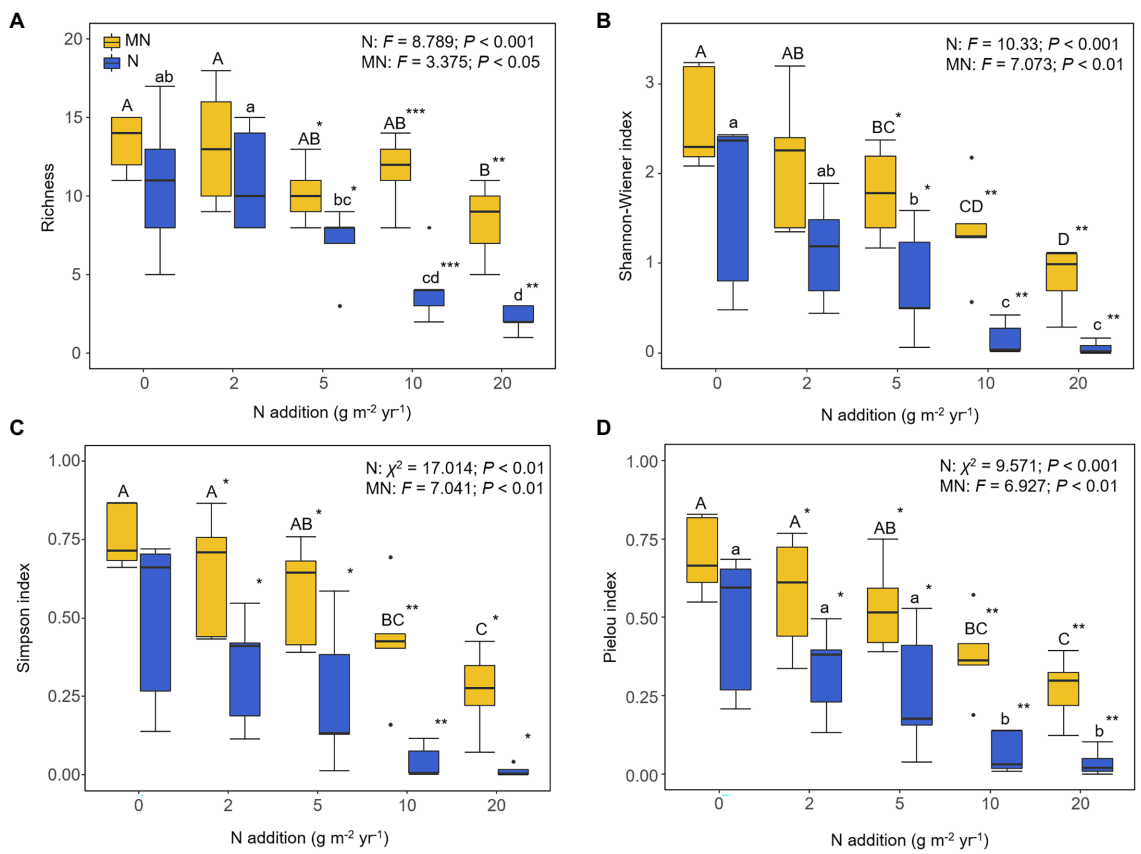

FIGURE 1 | Effects of nitrogen addition (N) and mowing (M) on plant α-diversity: **(A)** plant richness, **(B)** plant Shannon-Wiener index, **(C)** plant Simpson index, and **(D)** plant Pielou index. One-way ANOVA was used to test the response of plant α-diversity in each nitrogen addition and mowing treatment. Different lower- and upper-case letters denote significant difference ($p < 0.05$) among nitrogen addition rates in the mown and unmown treatments. The symbol "*" at the right corner of lower- and upper-case letters indicates a significant difference between mown and unmown treatments at each nitrogen addition rate (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). Treatments 0, 2, 5, 10 and 20 denote nitrogen addition with 0, 2, 5, 10 and 20 g m^{-2} $yr.^{-1}$, respectively.

TABLE 1 | Soil properties tested by one-way ANOVA in N and MN treatments [results are means ± SE, $n = 5$, except NH_4^+ N, NO_3^- N, Inorganic N of MN5 ($n = 4$)] and summary of results of ANOVA (*F*-values and significance levels).

	Soil moisture %	pH	Total C (mg/g)	Total N (mg/g)	Total P (mg/g)	Available P (mg/kg)	NH_4^+ N (mg/kg)	NO_3^- N (mg/kg)	Inorganic N (mg/kg)
N0	15.81 ± 0.465	6.86 ± 0.102a	26.10 ± 1.533	2.58 ± 0.071b	0.43 ± 0.011	2.58 ± 0.429	7.87 ± 0.990b	6.44 ± 1.396b	14.31 ± 1.222c
N2	14.63 ± 0.565	6.57 ± 0.041b	23.22 ± 1.404	2.55 ± 0.148b	0.41 ± 0.011	2.96 ± 0.499	8.73 ± 1.522b	7.21 ± 1.289b	15.94 ± 2.350bc
N5	14.41 ± 0.905	6.46 ± 0.107b	22.26 ± 0.752	2.71 ± 0.084b	0.41 ± 0.012	2.47 ± 0.142	7.62 ± 1.018b	5.31 ± 1.151b	12.92 ± 1.676c
N10	14.78 ± 0.540	5.94 ± 0.058c	24.82 ± 2.295	2.82 ± 0.093b	0.41 ± 0.016	3.00 ± 0.265	19.10 ± 5.766a	11.02 ± 2.948ab	30.12 ± 8.624b
N20	15.20 ± 0.652	5.72 ± 0.140c	24.70 ± 2.787	3.21 ± 0.154a	0.42 ± 0.026	3.30 ± 0.490	39.93 ± 9.995a	16.79 ± 3.552a	56.71 ± 12.609a
Significance of									
N	0.737	23.65***	0.625	5.356**	0.313	0.76	6.956**	3.588*	7.947***
MN0	14.02 ± 0.757	6.90 ± 0.063a	24.96 ± 2.611	2.60 ± 0.052bc	0.43 ± 0.014	2.24 ± 0.347	7.85 ± 0.756cd	5.97 ± 1.826bc	13.82 ± 2.262c
MN2	13.84 ± 0.536	6.80 ± 0.095a	22.72 ± 1.442	2.32 ± 0.112c	0.38 ± 0.015	2.28 ± 0.490	7.00 ± 0.507d	4.41 ± 0.424c	11.41 ± 0.800c
MN5	13.69 ± 0.527	6.26 ± 0.104b	24.98 ± 1.193	2.77 ± 0.086ab	0.41 ± 0.014	3.07 ± 0.427	10.79 ± 0.62bc	8.83 ± 1.848abc	19.62 ± 2.256bc
MN10	13.38 ± 0.837	6.05 ± 0.074b	24.00 ± 0.968	2.94 ± 0.164ab	0.45 ± 0.015	2.77 ± 0.233	17.70 ± 4.603ab	9.85 ± 1.324ab	27.55 ± 4.495b
MN20	12.54 ± 0.524	5.42 ± 0.109c	24.02 ± 1.628	3.06 ± 0.166a	0.40 ± 0.021	3.49 ± 0.352	45.38 ± 12.708a	12.11 ± 1.814a	57.49 ± 13.519a
Significance of									
MN	0.805	43.84***	0.336	5.596**	2.689	1.975	10.93***	4.22*	12.14***

*N0, N2, N5, N10 and N20, nitrogen addition with 0, 2, 5, 10, and 20 g m^{-2} yr^{-1} respectively; MN0, MN2, MN5, MN10, and MN20, mowing and nitrogen addition with 0, 2, 5, 10 and 20 g m^{-2} yr^{-1}, respectively. Different letters denote significant ($p < 0.05$) differences among treatments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. N, the effect of nitrogen addition; MN, the effect of mowing and nitrogen addition.*

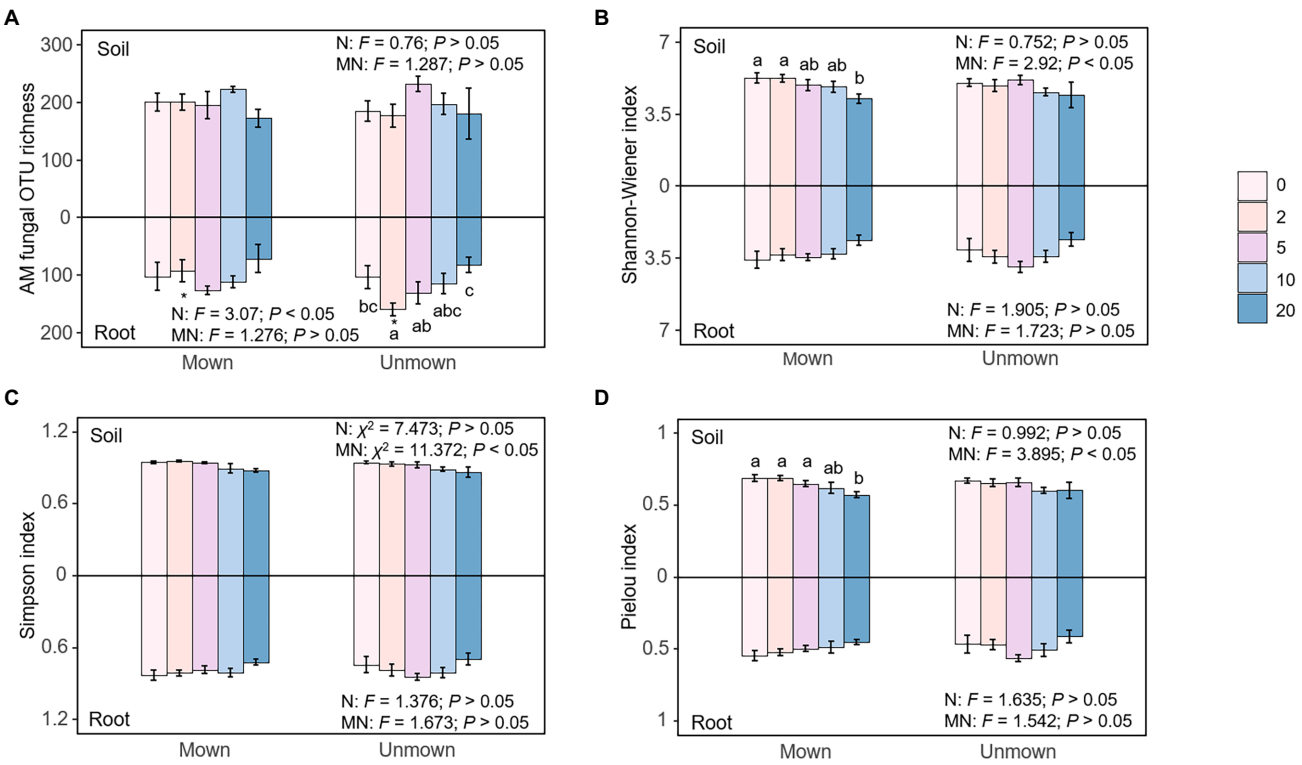

FIGURE 2 | Effects of nitrogen addition (N) and mowing (M) on AM fungal α-diversity in soil (upper) and roots (lower): **(A)** AM fungal operational taxonomic unit (OTU) richness, **(B)** AM fungal Shannon-Wiener index, **(C)** AM fungal Simpson index, **(D)** AM fungal Pielou index. All data are expressed as mean ± SE ($n = 5$). One-way ANOVA, Tukey's HSD, and *t* tests were used to test the effects of nitrogen addition and mowing on AM fungal α-diversity in the soil and roots in each nitrogen addition and mowing treatment. Different letters denote significant differences ($p < 0.05$) among nitrogen addition rates in the mown and unmown treatments at soil or root. The symbol "*" at the top or bottom of the column indicates a significant difference ($p < 0.05$) between mown and unmown at each nitrogen addition rate in soil or root. The numbers 0, 2, 5, 10, and 20 denote nitrogen addition with 0, 2, 5, 10, and 20 g m^{-2} yr.$^{-1}$, respectively.

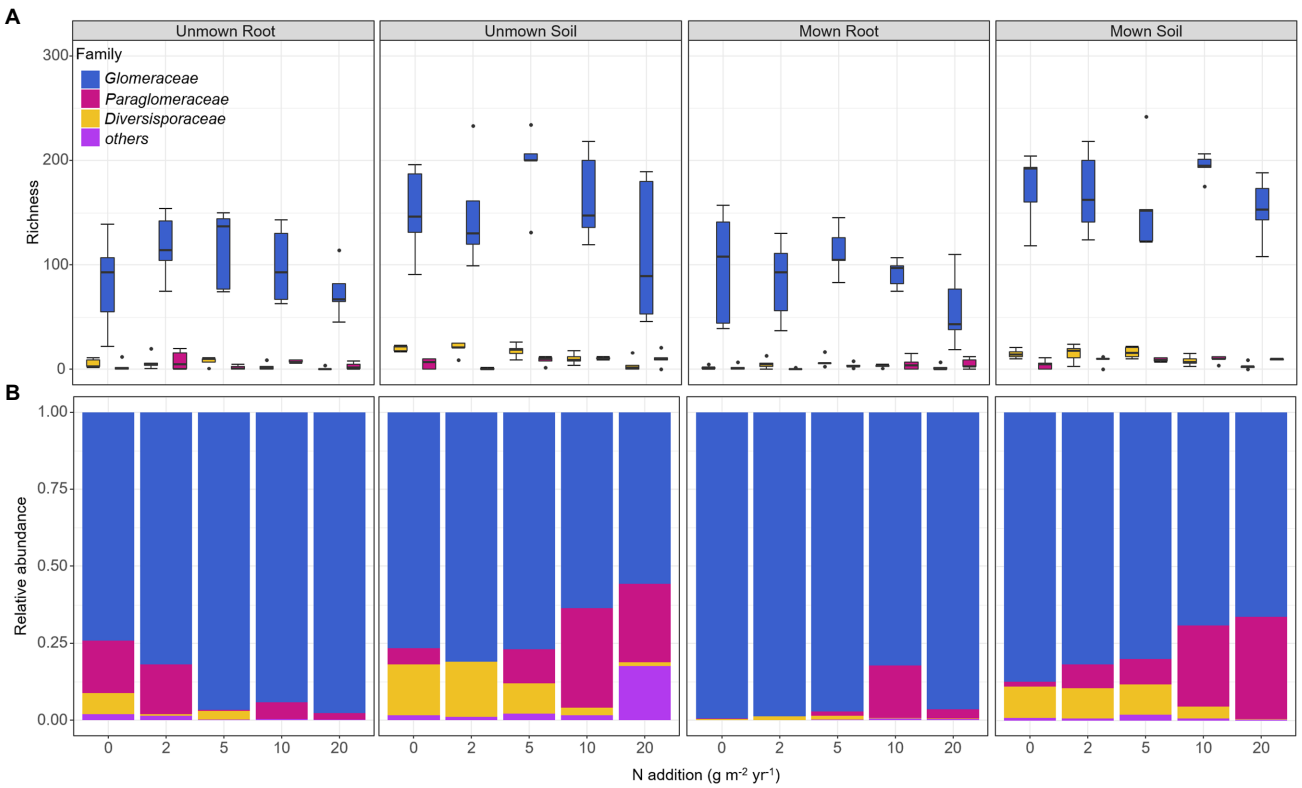

FIGURE 3 | Effects of nitrogen addition and mowing on the richness and relative abundance of AM fungal dominant families. **(A)** Richness of AM fungal dominant families and **(B)** relative abundance of AM fungal dominant families. The mid-horizontal lines within boxplots represent the median. 0, 2, 5, 10, and 20 denote nitrogen addition with 0, 2, 5, 10, and 20 g m^{-2} $yr.^{-1}$, respectively.

TABLE 2 | Results from a permutational multivariate analysis of variance (PERMANOVA) testing the effects of nitrogen addition, mowing, and the interaction on Bray–Curtis dissimilarity of AM fungal community in soil and roots, respectively.

	Treatment	R^2	*P*
Root	*M*	1.69%	0.551
	N	11.31%	**0.041**
	M × N	9.25%	0.204
Soil	*M*	2.63%	0.072
	N	22.18%	**0.001**
	M × N	6.79%	0.461

N, the effect of nitrogen addition; M, the effect of mowing; N × M, the interaction of nitrogen addition with mowing. Bold means a significant difference ($p < 0.05$).

light competition by changing plant height. Unfortunately, we did not quantify the availability of light in this experiment; this prevented us from directly assessing the role of light restriction in explaining the mechanisms generating plant diversity.

The Effects of Nitrogen Addition and Mowing on the AM Fungal Community

As expected, N addition significantly caused negative effects on AM fungal diversity in soil (**Figure 2**), supporting our first hypothesis. On the one hand, nitrogen addition can increase the inorganic nitrogen content and lower the soil pH, toxic ions released from soil acidification and increased ammonium toxicity both affected AM fungal community composition (Da Silva et al., 2014; De Beenhouwer et al., 2015). On the other hand, increased soil nitrogen availability makes host plant less dependent on AM (Olsson et al., 2010). The benefits that plants provide to AM were reduced, intensifying competition among AM fungi. Moreover, nitrogen addition may indirectly affect AM fungal communities through changing plant community (Chen et al., 2014). However, we did not find a significant relationship between plant richness and AM fungal community in the SEM (**Figure 5**). This may be due to the correlation between the plant and AM fungi being strong after short-term nitrogen addition, but the correlation weakens after long-term treatment (Li et al., 2015). The study site had been adding nitrogen for 7 years at the time of sampling. Long-term nitrogen addition may have weakened the relationship between plants and AM fungal communities. Therefore, it is necessary to carry out long-term monitoring of the changes of plant and AM fungal communities. Contrary to our second hypothesis, mowing did not alleviate the negative impact of nitrogen addition on AM fungal diversity (**Figures 2, 3**), possibly because nitrogen addition reduced AM fungal diversity by modulating soil inorganic nitrogen, but mowing did not change soil properties, especially soil pH and inorganic nitrogen content (**Table 1**), in our experiments.

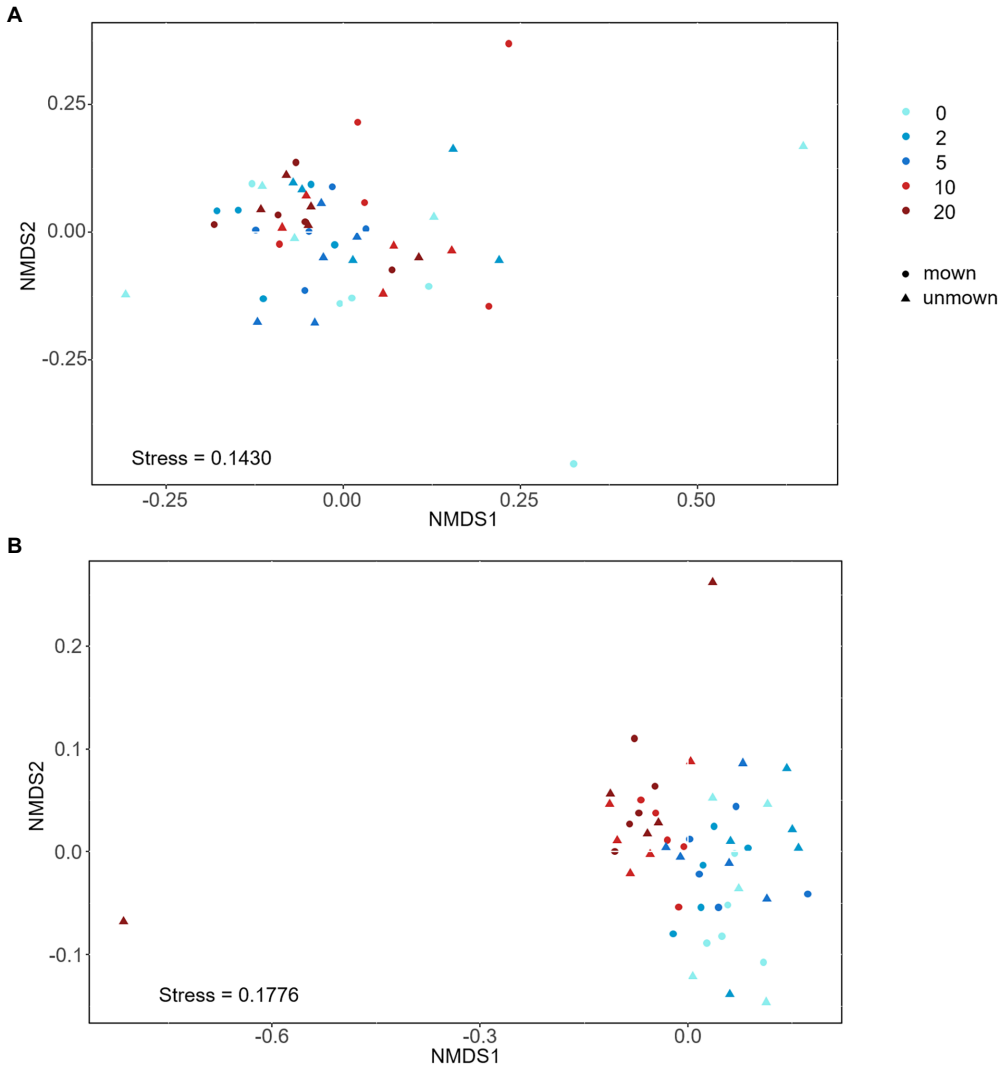

FIGURE 4 | Nonmetric multidimensional scaling (NMDS) analysis of AM fungal community structures under nitrogen addition: **(A)** AM fungal community composition in roots, **(B)** AM fungal community composition in soil. 0, 2, 5, 10, and 20 denote nitrogen addition with 0, 2, 5, 10, and 20 g m^{-2} $yr.^{-1}$, respectively.

We found significant differences between soil and root AM fungal community composition (**Figure 3**). Some studies have also shown that Paraglomeraceae and Glomeraceae are dominant in soil, but Glomeraceae are dominant in roots (Powell et al., 2009; Varela-Cervero et al., 2015). AM fungi occupy different ecological niches in time and space. In addition, the evolutionary dynamics that drive the formation of AM fungal communities in soil and root systems are different, resulting in different AM fungal community compositions in roots and soil (Pringle and Bever, 2002; Liu et al., 2012). The present results indicated that nitrogen addition affected the AM fungal community composition in soil but not in roots (**Figure 3B**), consistent with the study of Lü et al. (2020). The effect of nitrogen addition on the soil AM fungal community composition can be attributed to changes in soil nutrients (Zheng et al., 2014). As such, AM fungi in soil may be more sensitive to changes in soil nutrients, while AM fungi in roots are more closely associated with the roots of the host plant. The decrease in the richness and relative abundance of Glomeraceae in the soil with nitrogen addition (**Figure 3**) suggested that some members of the family have greater carbon requirements, nitrogen addition can promote down-regulation by the host or competitive exclusion among members of the family (Kiers et al., 2011; Duenas et al., 2020).

Ecological Process of AM Fungal Community Assembly

Consistent with the third hypothesis, environmental filtering was a major process of AM fungal community assembly under mowing and nitrogen deposition. The role of environmental filtration has been demonstrated in many previous experiments with nitrogen addition (Chen et al., 2014; De Beenhouwer et al., 2015; Li et al., 2015). When nutrients were limited, host plants would rely more on AM fungi to obtain nutrients such as soil nitrogen and phosphorus. Nitrogen addition had no significant effect on NRI or NTI of AM fungal communities, and AM fungal species always

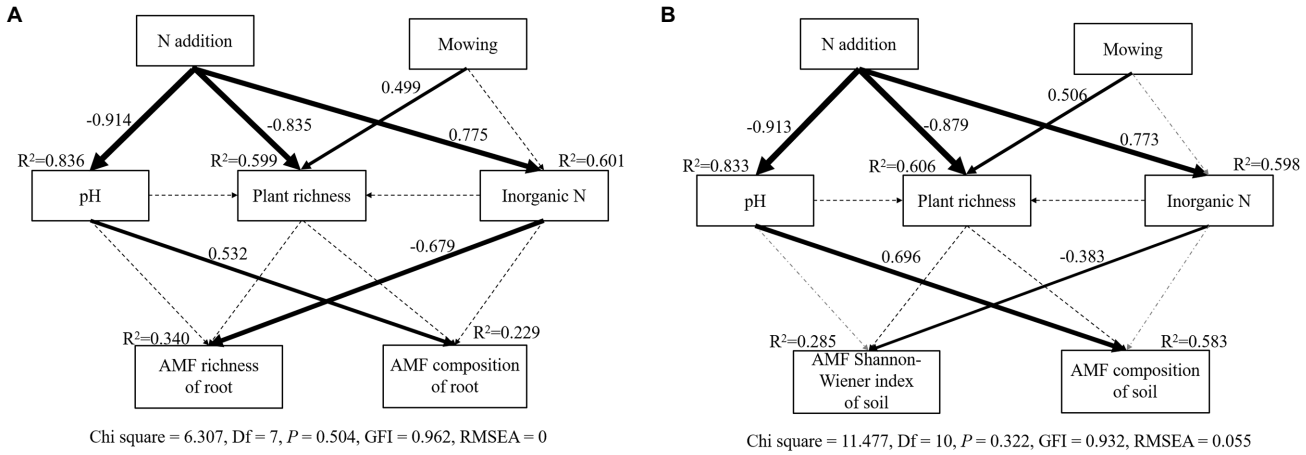

FIGURE 5 | The structural equation modeling (SEM) analysis of the effects of nitrogen addition and mowing on AM fungal richness, community composition in roots **(A)** and AM fungal community Shannon-Wiener index, community composition in soil **(B)** *via* the pathways of nitrogen addition, mowing, soil pH, soil inorganic N, plant richness. Numbers adjacent to arrows are path coefficients, and width of the arrows is proportional to the strength of path coefficients. Black dashed arrows indicate non-significant relationships ($p > 0.05$). Gray dashed arrows indicate paths removed to improve model fits. Percentages close to endogenous variables indicate the variance explained by the model (R^2).

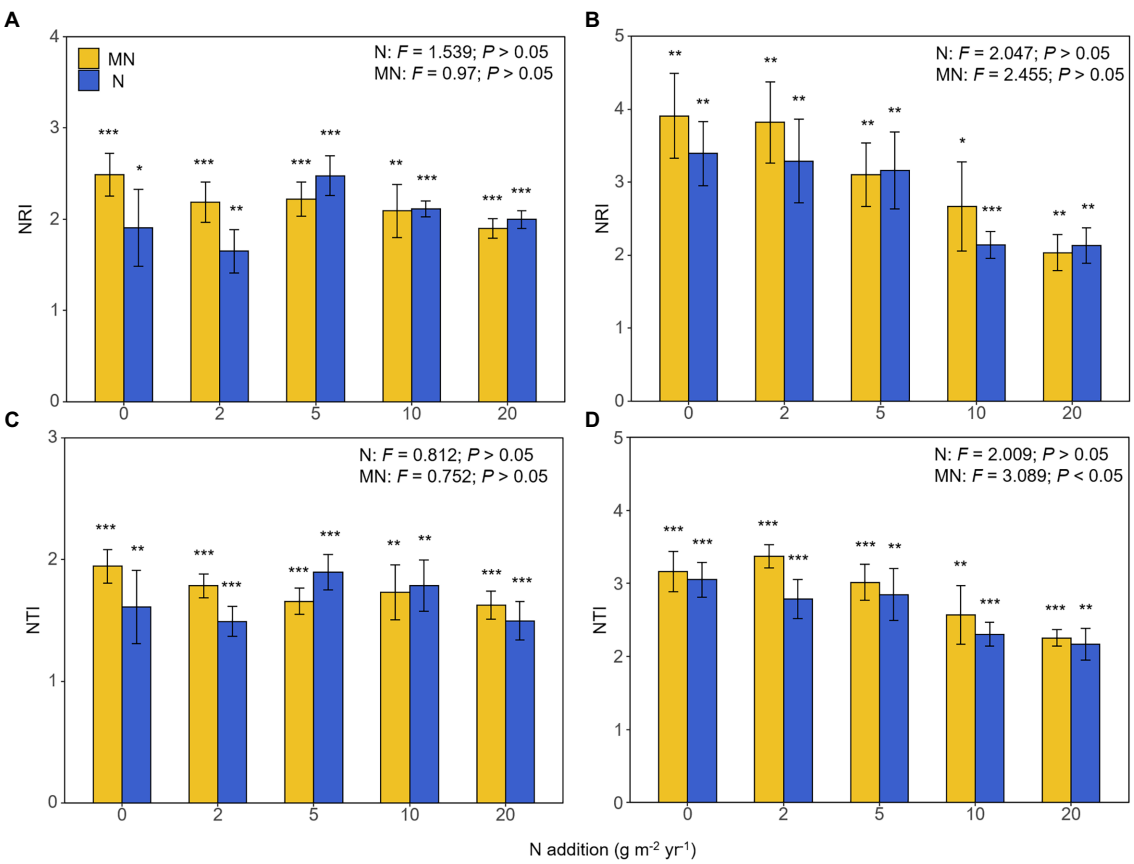

FIGURE 6 | The responses of the nearest relative index (NRI) of the arbuscular mycorrhizal fungal community in roots **(A)** and soil **(B)** to nitrogen addition and mowing treatments, and nearest taxa index (NTI) of the arbuscular mycorrhizal fungal community in roots **(C)** and soil **(D)** to nitrogen addition and mowing treatments. N, the effect of nitrogen addition; MN, the effect of mowing and nitrogen addition. *$p < 0.05$ of *t*-test, **$p < 0.01$ of *t*-test, ***$p < 0.001$ of *t*-test.

remained clustered in roots (**Figures 6A,C**). AM fungal communities that colonized roots were often clustered in natural ecosystems (Saks et al., 2014; Shi et al., 2014). NRI and NTI had a downward trend with increasing nitrogen addition in soil (**Figures 6B,D**). This also corroborated the finding that Glomeraceae occupied the majority of the AM fungal composition within the roots, but the relative abundance of Glomeraceae in the soil gradually decreased with increasing N fertilization, and new species emerged (**Figure 3B**). AM fungi occupied different ecological niches in time and space. Some AM fungi existed only in certain soil nutrient conditions.

CONCLUSION

In the present study, we found that nitrogen addition reduced the diversity of plant and AM fungal communities in soil by lowering pH and increasing inorganic nitrogen concentration. We suggested that mowing mitigated light limitation by changing the height of plants to alleviate the negative effects of nitrogen addition on the diversity of plant communities, but it could not alleviate the negative effects on AM fungal community. Mowing had important practical significance in grassland management. Whether in soil or roots, AM fungal communities clustered phylogenetically in all treatments, indicating that environmental filtering is the major driving force for AM fungal community assembly. Therefore, our research in future needs to pay more attention to long-term monitoring to explore the feedback between plant and AM fungi, and the response mechanism of AM fungal community to nitrogen deposition.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA790751.

AUTHOR CONTRIBUTIONS

PZ, GY, and RW conceived the research. SQ, GY, LS, YC, and YZ collected the samples. SQ, LS, YZ, JD, and MS performed the lab analyses. SQ, XL, and NW analyzed the data. PZ, GY, and SQ wrote the manuscript. All authors contributed to the article and approved the submitted version.

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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.2022.917645/full#supplementary-material

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Nitrogen Addition Affects Ecosystem Carbon Exchange by Regulating Plant Community Assembly and Altering Soil Properties in an Alpine Meadow on the Qinghai–Tibetan Plateau

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Nitrogen (N) deposition can affect the global ecosystem carbon balance. However, how plant community assembly regulates the ecosystem carbon exchange in response to the N deposition remains largely unclear, especially in alpine meadows. In this study, we conducted a manipulative experiment to examine the impacts of N (ammonium nitrate) addition on ecosystem carbon dioxide (CO_2) exchange by changing the plant community assembly and soil properties at an alpine meadow site on the Qinghai–Tibetan Plateau from 2014 to 2018. The N-addition treatments were N0, N7, N20, and N40 (0, 7, 20, and 40 kg N $ha^{-1}year^{-1}$) during the plant growing season. The net ecosystem CO_2 exchange (NEE), gross ecosystem productivity (GEP), and ecosystem respiration (ER) were measured by a static chamber method. Our results showed that the growing-season NEE, ER and GEP increased gradually over time with increasing N-addition rates. On average, the NEE increased significantly by 55.6 and 65.2% in N20 and N40, respectively ($p < 0.05$). Nitrogen addition also increased forage grass biomass (GB, including sedge and Gramineae) by 74.3 and 122.9% and forb biomass (FB) by 73.4 and 51.4% in N20 and N40, respectively ($p < 0.05$). There were positive correlations between CO_2 fluxes (NEE and GEP) and GB ($p < 0.01$), and the ER was positively correlated with functional group biomass (GB and FB) and soil available N content (NO_3^-–N and NH_4^+–N) ($p < 0.01$). The N-induced shift in the plant community assembly was primarily responsible for the increase in NEE. The increase in GB mainly contributed to the N stimulation of NEE, and FB and the soil available N content had positive effects on ER in response to N addition. Our results highlight that the plant community assembly is critical in regulating the ecosystem carbon exchange response to the N deposition in alpine ecosystems.

Keywords: alpine meadow, nitrogen deposition, net ecosystem carbon exchange, soil available nitrogen, aboveground biomass, plant community

INTRODUCTION

Accelerating industrialization and increasing nitrogen (N) fertilizer application have substantially increased the N deposition in most areas of the earth (Canfield et al., 2010), which will make reducing greenhouse gas emissions harder in the coming decades (IPCC, 2018). The release and deposition of global atmospheric reactive N into the Earth's surface is predicted to total 200 Tg N $year^{-1}$ by 2050 (Galloway et al., 2008). Increasing the N deposition alters plant-community compositions and affects the biogeochemical cycling of terrestrial ecosystems (Simkin et al., 2016), particularly in N-limited grasslands (Canfield et al., 2010). Consequently, the N deposition can have many effects on the plant productivity and ecosystem carbon fluxes in grasslands (Niu et al., 2010; Zhang J. et al., 2020).

Net ecosystem carbon exchange (NEE), which is defined as the difference between gross ecosystem productivity (GEP) and ecosystem respiration (ER), is widely used to describe the changes in ecosystem carbon sinks and sources and is a vital function in the global carbon cycle (Mahecha et al., 2010; You et al., 2020). However, the effect of N addition on grassland NEE has been shown to be either positive (Niu et al., 2010) or non-significant (Kim and Henry, 2013) in different grassland ecosystems. Such inconsistent findings are likely due to different frequencies and concentrations of N addition (Cao et al., 2019) and the changes in climate factors (temperature and precipitation) (Niu et al., 2010; Yan et al., 2011). Additionally, the soil temperature, water availability, soil nutrient availability (Leff et al., 2015; Wang et al., 2020), and community composition (Xu et al., 2016) affect ecosystem carbon dioxide (CO_2) fluxes under N addition.

The dynamic response of ecosystem carbon exchange to N addition is derived from shifts in species composition and colimitation with other abiotic resources (Jiang et al., 2012). A long-term N addition affects the plant biomass and community composition in grassland ecosystems by increasing soil N availability (Bai et al., 2008; Yang et al., 2012; Xu et al., 2014; Shen R. N. et al., 2022). For example, a long-term N addition could promote the functional groups of grass by negatively impacting forb in alpine grasslands (Zhang L. et al., 2020), which is an effect related to the different mycorrhizal distributions, N absorption amounts and utilization mechanisms between grasses and forbs (Wang et al., 2015; Kang et al., 2019). Chronic or excessive N addition can also reduce the species richness and diversity when soil N exceeds the saturation threshold (Xia and Wan, 2008; Li S. et al., 2019). Furthermore, many studies have shown that N addition can affect ER and NEE by stimulating plant growth and altering the plant community composition. For example, N addition stimulated NEE mainly by increasing the cover of dominant species in alpine meadows (Shen H. et al., 2022). Nitrogen-induced shifts in plant functional groups are considered to be an essential factor affecting carbon exchange in ecosystems (Niu et al., 2010; Fang et al., 2011; Chen et al., 2020), generally favoring a few nitrophilic plant species while suppressing the growth of many other species (Duprè et al., 2010; Jiang et al., 2012). In addition, studies showed that an increase in soil respiration was positively related to soil nutrient availability under N addition (Leff et al., 2015; Peng et al., 2015; Riggs et al., 2015).

Nitrogen deposition is a prominent global change driver on the Qinghai–Tibetan Plateau (QTP) (Liu et al., 2015) because the rapid development of the regional economy and the long-distance monsoon transport of atmospheric N deposition from South Asia have been related to the conspicuously increasing trend of the N deposition rate (Lü and Tian, 2007; Liu et al., 2015). Alpine meadows are one of the typical or dominant grassland ecosystem types on the QTP and are vital carbon pools in the "third pole" (Wang et al., 2014; Gao et al., 2016). Alpine meadows have long been affected by N and water deficiencies (Qiu, 2008; Liu et al., 2018; Dong et al., 2020), and their vegetation compositions and ecological processes are highly sensitive to the N deposition (Xu et al., 2018; Ma et al., 2019). The effects of N deposition on ecosystem CO_2 fluxes and plant community assembly have been widely studied in this region. A study from the QTP found that the N deposition could shift plant species composition in favor of graminoids (Shen H. et al., 2022). The responses of the functional traits of alpine plants to the N deposition showed cascading effects from dominant species to functional groups and plant communities (Li et al., 2022). A long-term N addition can greatly reduce species richness (Li S. et al., 2019), and NEE may increase under N addition (Niu et al., 2009, 2010). Multigradient N-addition experiments have shown that the GEP revealed a non-linear increasing trend with increasing the N deposition rates (Tian et al., 2015). In addition, some researchers have highlighted that the ecosystem CO_2 fluxes were affected by plant productivity (Bai et al., 2008; Tian et al., 2015). However, how to achieve the combination of functional groups and promote ecosystem carbon fluxes under the N deposition in alpine meadows remains unclear. Based on the above studies, we hypothesized that N addition could stimulate ecosystem CO_2 fluxes *via* increasing sedge and forb biomass, thereby affecting the responses of the carbon sink function in alpine meadows.

A 5-year (2014–2018) field-manipulation experiment with four N deposition rates (i.e., N0, N7, N20, and N40) was conducted in an alpine meadow on the QTP. Our study aimed to (1) determine how plant functional groups and ecosystem CO_2 fluxes respond to increasing N deposition and (2) clarify how the associated changes in the plant community assembly affect the responses of ecosystem CO_2 fluxes to N addition.

MATERIALS AND METHODS

Study Site

The research area is located at the Nagqu National Agricultural Experimental Station for the Agricultural Environment, Tibet Autonomous Region, China (31.441°N, 92.017°E) at an elevation of 4,500 m. The mean annual temperature and precipitation are −1.2°C and 431.7 mm, respectively. The main functional groups are sedge, *Kobresia pygmaea*, *Carex moorcroftii*, Gramineae, *Poa pratensis* and forbs (mainly including *Potentilla acaulis* and *Oxytropis ochrocephala*) (Zhou et al., 2021). The soil is clay silt and is classified as alpine meadow soil. The soil bulk density is 1.01 g cm^{-3}. The experimental field was grazed by yak (*Bos grunniens*) every summer before 2010, after which the study area was fenced off, and grazing and mowing were prohibited throughout the experiment (Ganjurjav et al., 2020). The growing

TABLE 1 | Results of repeated measures analysis of variance (RMANOVA) on the individual and interactive effects of N addition (N), year on soil properties.

Factors		NO_3^-–N (mg kg^{-1})		NO_3^-–N (mg kg^{-1})			MBC (mg C kg^{-1})		MBN (mg N kg^{-1})	
	df	*F*	*p*	*F*	*p*	*df*	*F*	*p*	*F*	*p*
Year	4	20.61	**<0.001**	4.33	**0.009**	3	15.34	**<0.001**	6.29	**0.001**
Nitrogen	3	4.65	**0.007**	8.22	**<0.001**	3	10.91	**0.001**	4.70	**0.006**
Year × Nitrogen	12	1.05	0.42	0.43	0.91	9	4.14	**0.001**	0.81	0.61

The numbers in bold express significant effects ($p < 0.05$). NH_4^+–N, soil ammonium nitrogen; NO_3^-–N, soil nitrate-nitrogen; MBC, soil microbial biomass carbon; MBN, soil microbial biomass nitrogen.

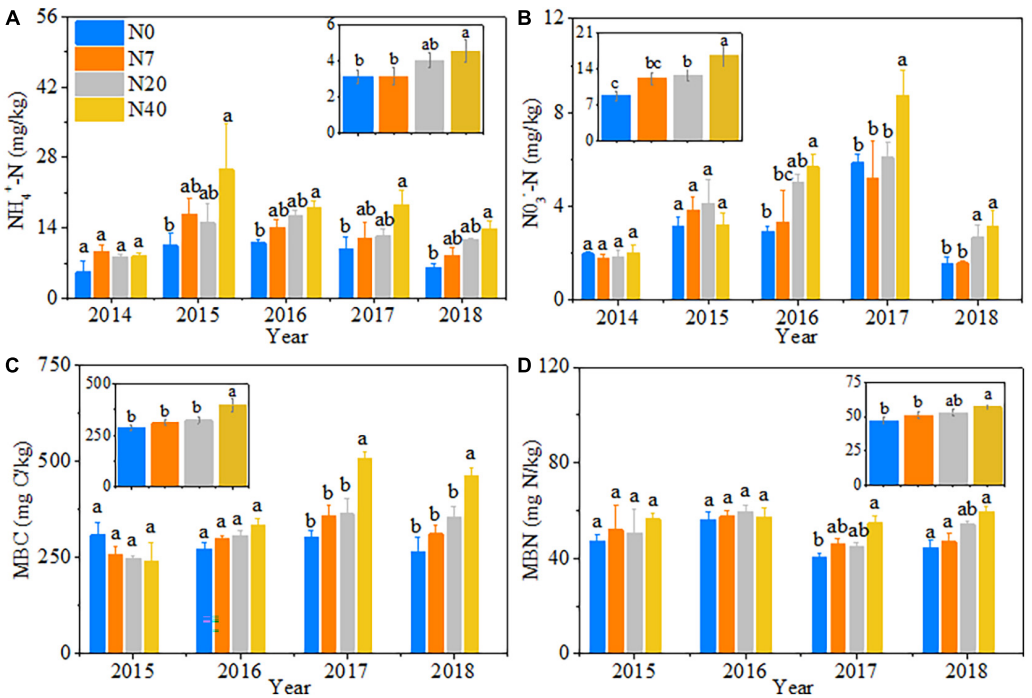

FIGURE 1 | Patterns in soil properties with increasing N addition from 2014 to 2018. N0–N40: expressed as control, 0, 7, 20, and 40 kg N $ha^{-1}year^{-1}$. **(A)** NH_4^+–N, soil ammonium nitrogen; **(B)** NO_3^-–N, soil nitrate–nitrogen; **(C)** MBC, soil microbial biomass carbon; **(D)** MBN, soil microbial biomass nitrogen. The error bars represent the standard error (SE). Different lowercase letters indicate significant differences ($p < 0.05$) among different N-addition levels (hereinafter inclusive). Some results are cited from Yan (2019).

season is from May to September when the average temperature is above 0°C, and the precipitation during this time accounts for more than 90% of the total precipitation. The precipitation was lower during the middle of the growing season in 2015 and 2017, and the temperature showed an undulating increasing trend during the growing season in the time period 2014-2018 (**Supplementary Figure 1**).

Experimental Design

The total dry and wet N deposition rate was approximately 7 kg N $ha^{-1}.year^{-1}$ (6.96–7.55 kg N $ha^{-1}.year^{-1}$) in the Tibet Autonomous Region (Lü and Tian, 2007; Liu et al., 2013). Therefore, we set N-addition rates of approximately 1, 3, and 6 times 7 kg N $ha^{-1}.year^{-1}$ to simulate the N deposition effects on ecosystem carbon exchange in the study area. We initiated a 5-year (from 2014 to 2018) N deposition experiment in an alpine meadow using a randomized block design with 16 experimental plots (each plot of size 3 m × 3 m) separated by a 2-m buffer. One control (unfertilized, N0) and three N addition treatments termed N7, N20, and N40 (0, 7, 20, and 40 kg N ha^{-1} $year^{-1}$, respectively) were randomly assigned to plots. Four replicate plots were selected for use in our study. Ammonium nitrate (NH_4NO_3) was applied to simulate atmospheric N deposition, and each plot was sprayed with a solution of NH_4NO_3 dissolved in 5 L water 4 times per year (early in each month from May to August). The same volume of water was sprayed on the control plots to avoid an impact from added water. The total annual volume of water applied to each plot was 40 L, which was equivalent to 1% of the local annual precipitation (Yan et al., 2018).

Plant Community Assembly

The community characteristics were surveyed twice a month during the growing season from 2014 to 2018. The community

TABLE 2 | Results of repeated measures analysis of variance (RMANOVA) on the individual and interactive effects of N addition (N), and year on CO_2 fluxes (GEP, ER and NEE), biomass (AGB, GB and FB) and diversity indices.

Factors		NEE (μ mol $m^{-2}s^{-1}$)		ER (μ mol $m^{-2}s^{-1}$)		GEP (μ mol $m^{-2}s^{-1}$)		AGB ($g.m^{-2}$)		GB ($g.m^{-2}$)		FB ($g.m^{-2}$)		Species richness		Shannon–Wiener index	
	df	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*p*
Year	4	20.85	**<0.001**	27.07	**<0.001**	17.45	**<0.001**	15.57	**<0.001**	6.29	**<0.001**	5.77	**0.001**	4.18	**0.005**	8.01	**<0.001**
Nitrogen	3	7.50	**<0.001**	4.81	**0.005**	8.18	**<0.001**	32.31	**<0.001**	16.61	**<0.001**	13.45	**<0.001**	12.74	**<0.001**	12.70	**<0.001**
Year × Nitrogen	12	1.93	**0.048**	0.69	0.76	1.37	0.21	2.75	**0.005**	2.62	**0.007**	1.34	0.22	2.17	**0.03**	1.18	0.32

The numbers in bold express significant effects ($p < 0.05$). GEP, gross ecosystem productivity; ER, ecosystem respiration; NEE, net ecosystem carbon exchange; AGB, aboveground biomass; GB, forage grass biomass; FB, forb biomass.

characteristic data during the peak growing season (late July or early August) were used in this study. A small quadrat (of size 0.5 m × 0.5 m) in each plot was selected for the determination of vegetation characteristics using sampling and visual methods. First, we recorded and measured the numbers and heights of all plant species. The heights of five individuals of each species were measured, and the mean value was used to represent the species height. Second, we estimated the total community coverage (total cover) and the coverage of each species (percentage coverage) using a visual method. Third, the aboveground biomass of different functional groups was determined by non-destructive measurement (Ganjurjav et al., 2020). The non-destructive measurement method included establishing a regression equation of plant height, coverage, and biomass in the vicinity of the sample plot and estimating the plant biomass in the plot using the developed equation (see **Supplementary Table 1**). The species richness index was equal to the number of species. Species dominance was evaluated based on the importance value index (*P*), which was computed as follows:

$$P_{\mathrm{i}} = (FC_i + RF_i)/2 \quad (1)$$

where RC_i and RF_i are the relative cover (equal to the ratio of the species coverage to the total coverage of all species) and relative height (equal to the ratio of the species height to the total height of all species), respectively (Sun et al., 2021).

The plant community diversity was computed using the Shannon–Wiener index as follows:

$$H' = -\Sigma P_i \ln P_i \quad (2)$$

where P_i represents the importance value index of the *i*th species (Magurran, 1988).

Ecosystem Carbon Dioxide Fluxes

A portable photosynthesis system (Li-6400; LI-COR Inc., Lincoln, NE, United States) and the transparent chamber method were used to measure the carbon exchange 1–3 times a month during the growing season from 2014 to 2018. We carried out these field measurements at 10:00–12:00 local time on sunny days. First, on the inner side of the top of each transparent polyethylene chamber (of size 0.3 m × 0.3 m × 0.4 m), a fan was installed to mix the gas inside the chamber during the measurement. Second, a transparent polyethylene chamber was placed in each quadrat to measure NEE for 90 s. Then, we removed the chamber to ensure that the air humidity and CO_2 reached at ambient levels. Finally, we placed the chamber back in each quadrat, covered it with an opaque shade cloth, and measured ER for 90 s. Gross ecosystem productivity was calculated using the NEE and ER values. For more information on this methodology, see Ganjurjav et al. (2015).

Soil Properties

The soil samples were collected from 0 to 10 cm soil layer in each plot using an auger in mid-August every year. The soil available N content (NH_4^+–N and NO_3^-–N) was extracted with a 2 mol L^{-1} KCl solution and determined with a continuous flow spectrophotometer (FIAstar 5000 Analyzer; Foss

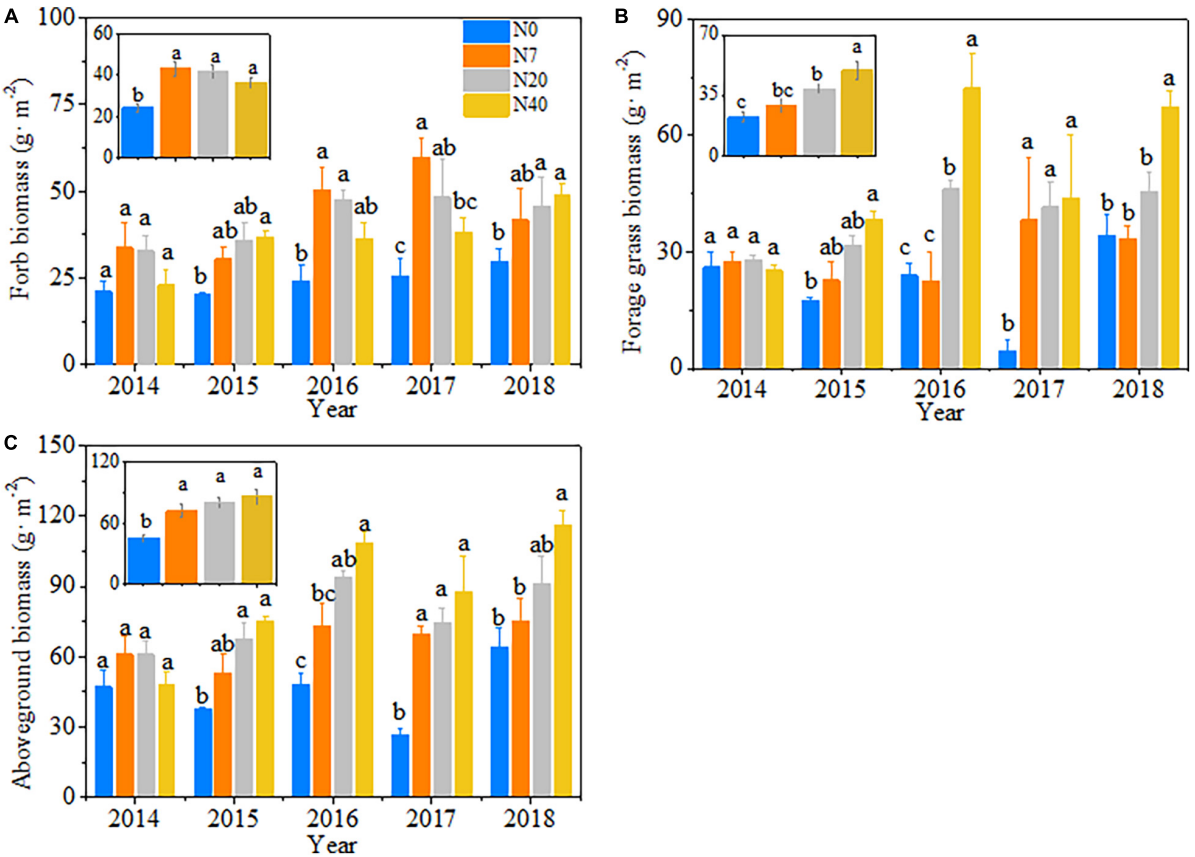

FIGURE 2 | Patterns in **(A)** forb biomass, **(B)** forage grass biomass, and **(C)** aboveground biomass with increasing N addition from 2014 to 2018. The error bars represent the standard error (SE). N0–N40: expressed as control, 0, 7, 20, and 40 kg N $ha^{-1}year^{-1}$. Different lowercase letters indicate the significant differences ($p < 0.05$) among different N-addition levels.

Tecator, Hillerød, Denmark). The soil microbial biomass C (MBC) and N (MBN) were measured using the chloroform fumigation extraction method (Brookes et al., 1985; Fu et al., 2012). Fumigated and unfumigated soil samples were extracted with 100 ml 0.5 mol L^{-1} K_2SO_4 and filtered with a 0.45-μm membrane. The extractable organic carbon and total N were determined by an automatic carbon analyzer (Phoenix 8000) and flow injection nitrogen analyzer (FIAStar5000, FOSS Inc), respectively. Extractable carbon and N were converted to MBC and MBN using a conversion coefficient of 0.45 for both measurements (Fu et al., 2012).

Data Analysis

The annual maximum ecosystem carbon exchange value (obtained between late July and early August in each year) was used to present the annual variation in NEE, GEP, and ER during the growing season. We log_{10}-transformed all variables before performing the data analysis to stabilize the residual variances. One-way ANOVA and *post hoc* tests were used to analyze significant differences in ecosystem CO_2 fluxes, soil properties, community diversity, and functional group biomass among the N0, N7, N20, and N40 plots over 5 years. The functional group biomass included forage grass and forb biomass (GB and FB, respectively), and forage grass was defined as the combination of sedge and Gramineae (Huang et al., 2020). Differences were considered significant when $p < 0.05$. We also applied repeated-measures analysis of variance (RMANOVA) to analyze the individual and interactive influences of year and N addition on CO_2 fluxes (i.e., NEE, ER, and GEP), soil properties, community diversity, and functional group biomass. Pearson correlation analysis was employed to analyze the relationships between CO_2 fluxes (NEE, GEP and ER) and soil properties, community diversity, and functional group biomass over 5 years. Meanwhile, to determine the long-term effect of N addition, we calculated the response ratios under the treatments (N7, N20, and N40) relative to the control (N0) to examine the changes in biomass, and the change in carbon flux was defined as the difference between the value under each N addition treatment and that under N0 treatment. All analyses were carried out using SPSS (SPSS for Windows, version 22.0).

We established *a priori* structural equation modeling (SEM; IBM SPSS Amos 24) based on correlation analyses between the CO_2 fluxes (NEE and ER) and GB, FB and soil properties (**Supplementary Figure 4** and **Supplementary Table 4**). Next, we explored how N addition affected NEE and ER by changing

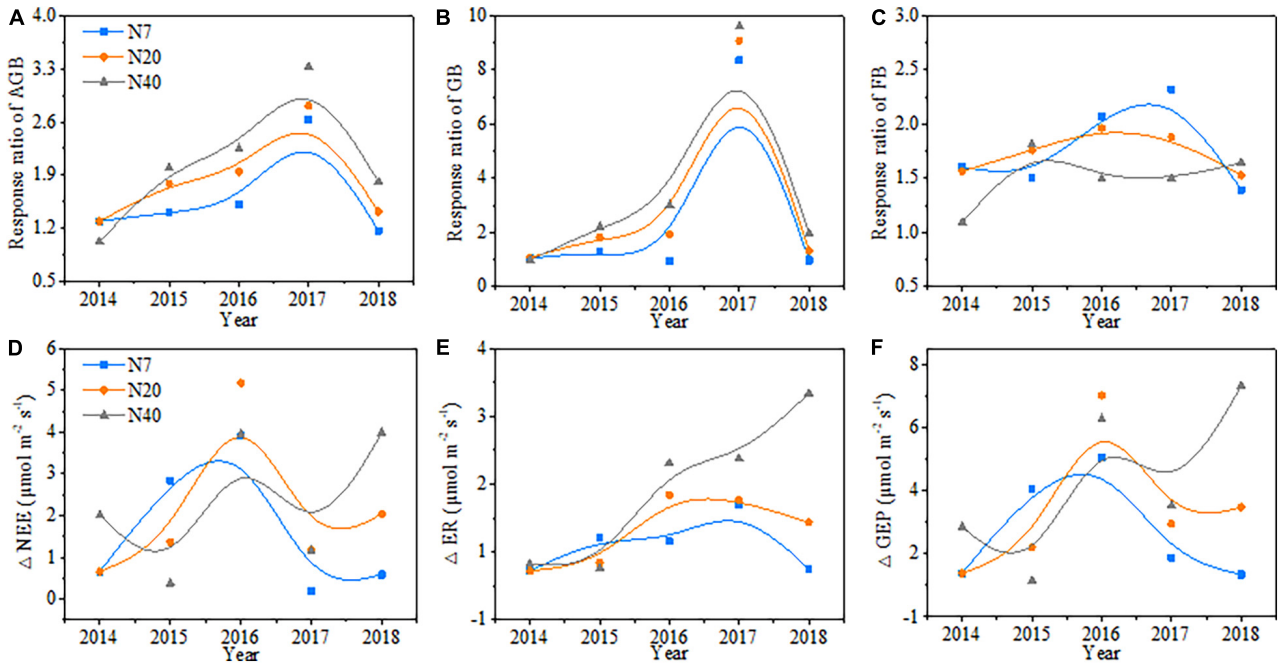

FIGURE 3 | Response ratio of **(A)** aboveground biomass (AGB), **(B)** forage grass biomass (GB) and **(C)** forb biomass (FB) and changes in **(D)** net ecosystem CO_2 exchange (NEE), **(E)** ecosystem respiration (ER), and **(F)** gross ecosystem productivity (GEP) from 2014 to 2018.

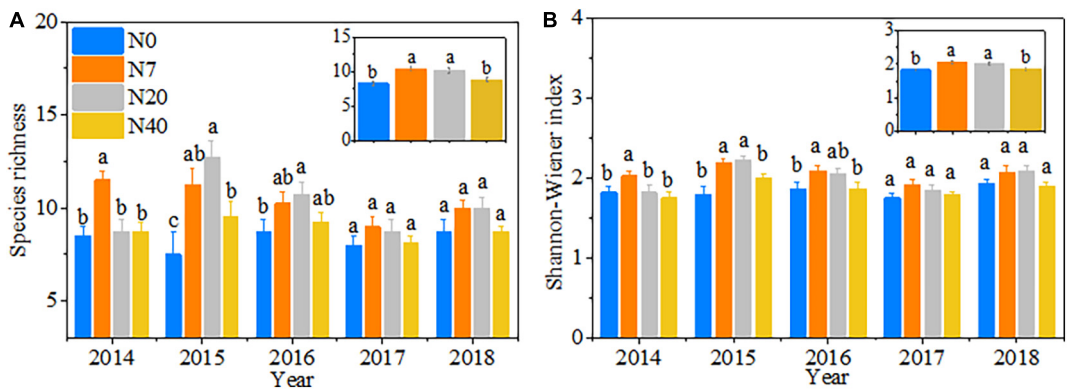

FIGURE 4 | Patterns in **(A)** species richness and **(B)** Shannon–Wiener index from 2014 to 2018. The error bars represent the standard error (SE). N0–N40: expressed as control, 0, 7, 20, and 40 N $ha^{-1}year^{-1}$. Different lowercase letters indicate significant differences ($p < 0.05$) among different N-addition levels.

soil properties and functional group biomass. A multivariate index representing the soil available N content was created by principal component analysis (PCA) of the soil NO_3^-–N and NH_4^+–N contents. The first principal component explained 66.5% of the total variance and was used in the SEM analysis. First, we hypothesized that N addition would increase the soil properties, and alter the plant community assembly by increasing soil nutrient availability (Bai et al., 2008; Yang et al., 2012; Xu et al., 2014), which would then affect the ER and NEE responses to N addition (Duprè et al., 2010; Jiang et al., 2012). We optimized the prior model based on the Chi-squared (χ^2) statistic ($p > 0.05$), root mean square error of approximation (RMSEA) < 0.05, comparative fit index (CFI) and Tucker–Lewis index (TLI) > 0.95, and the lowest Akaike information criterion (AIC) value (Wei et al., 2013).

RESULTS

Changes in Soil Chemical Properties

Nitrogen addition had significant effects on soil properties (**Table 1**, $p < 0.05$). Compared to the N0 plot, the soil NO_3^-–N, NH_4^+–N, MBC, and MBN contents increased almost exclusively in the N40 treatment (**Figure 1**). On average, the soil NH_4^+-N, MBC and MBN contents increased by 44.3, 38.5, and 21.0% in N40, respectively, and the soil NO_3^-–N content

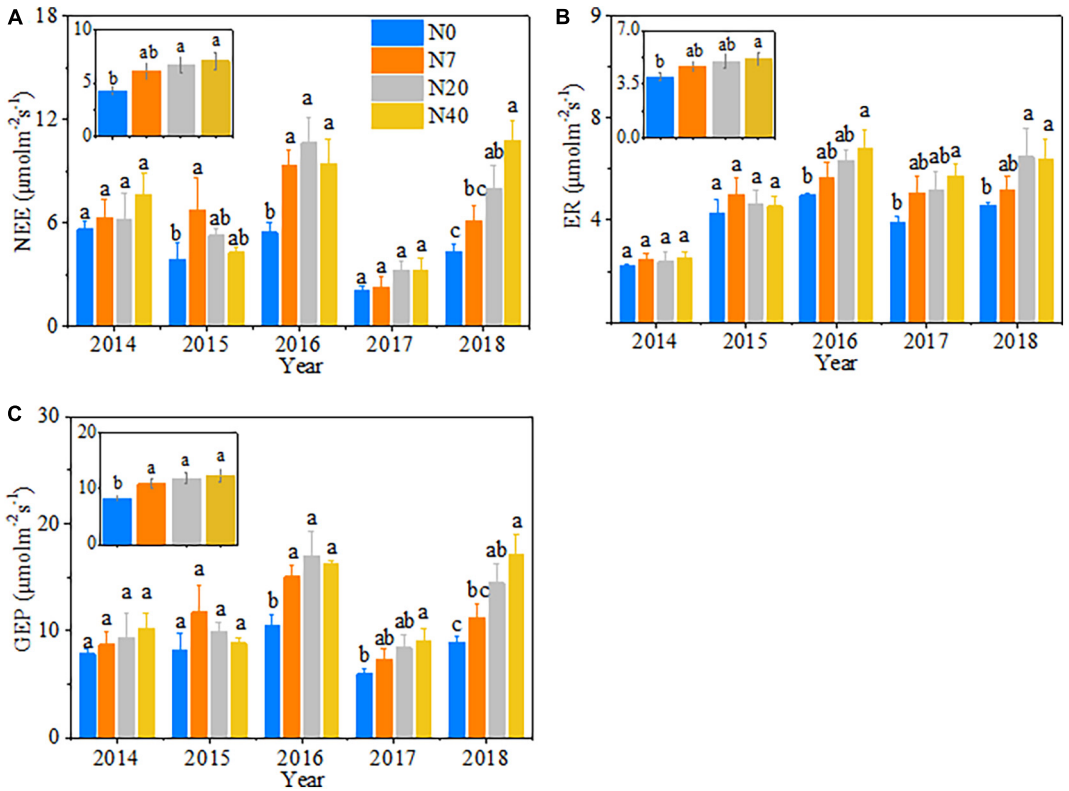

FIGURE 5 | The changes in **(A)** net ecosystem carbon exchange (NEE), **(B)** ecosystem respiration (ER), and **(C)** gross ecosystem productivity (GEP) with increasing N addition from 2014 to 2018. N0–N40: expressed as control, 0, 7, 20, and 40 kg N $ha^{-1}year^{-1}$. The error bars represent the standard error (SE). Different lowercase letters indicate significant differences ($p < 0.05$) among different N-addition levels.

increased by 45.7 and 88.0% in N20 and N40, respectively ($p < 0.05$). The soil properties changed significantly during the 5 studied years (**Table 1**, $p < 0.05$). Specifically, in N40, the soil NH_4^+–N content was 153.6, 63.4, 86.9, and 124.9% higher than that in N0 in 2015–2018 (**Figure 1A**). Additionally, the soil MBC content was 67.7 and 74.4% while MBN content was 35.7 and 33.5% higher than that in N0 in 2017–2018, respectively (**Figures 1C,D**). The soil NO_3^-–N content was also 93.5, 49.8, and 99.4% higher than that in N0 in 2016–2018, respectively (**Figure 1B**). In N20, the soil NO_3^-–N content was 71.1 and 69.6% higher than that in N0 in 2016 and 2018, respectively (**Figure 1B**).

Changes in Forb and Forage Grass Biomass and Aboveground Biomass

The variations in aboveground biomass and forage grass and forb biomass mainly depended on the N-addition level, and differences were also observed among the 5 studied years (**Table 2**, $p < 0.05$). The aboveground biomass and forage grass biomass mostly showed gradual increasing trends (**Figures 2B,C**), and forb biomass mostly showed a unimodal trend with N addition across the 5 studied years (**Figure 2A**). On average, the aboveground biomass increased by 59.1, 78.1, and 90.1% in N7, N20, and N40, respectively; forage grass biomass increased by 74.3 and 122.9% in N20 and N40, respectively; and forb biomass increased by 78.1, 73.4, and 51.4% in N7, N20, and N40, respectively ($p < 0.05$). The effects of N addition on AGB and GB followed a unimodal temporal trend, with the maximum response peak in 2017 (**Figures 3A,B**). The response ratios of forb biomass to N addition showed a different temporal trend, with N addition increasing FB in most years (**Figure 3C**).

Changes in Plant Community Diversity

Species richness was prominently affected by the year, N addition, and their interactions (**Table 2**, $p < 0.05$). The variations in the Shannon–Wiener index mainly depended on the N-addition level ($p < 0.05$). The species richness and the Shannon–Wiener index first increased and then decreased over time with increasing N-addition rates, peaking between the N7 and N20 plots (**Figures 4A,B**). The differences were also observed among the 5 studied years (**Table 2**, $p < 0.05$). In 2014, the species richness and Shannon–Wiener index in N7 were significantly higher than those in the other three plots (**Figures 4A,B**, $p < 0.05$). In 2015, the species richness and Shannon–Wiener index in N7 and N20 were significantly higher than those in N0 and N40 (**Figures 4A,B**, $p < 0.05$). In 2016, the maximum species richness and Shannon–Wiener index

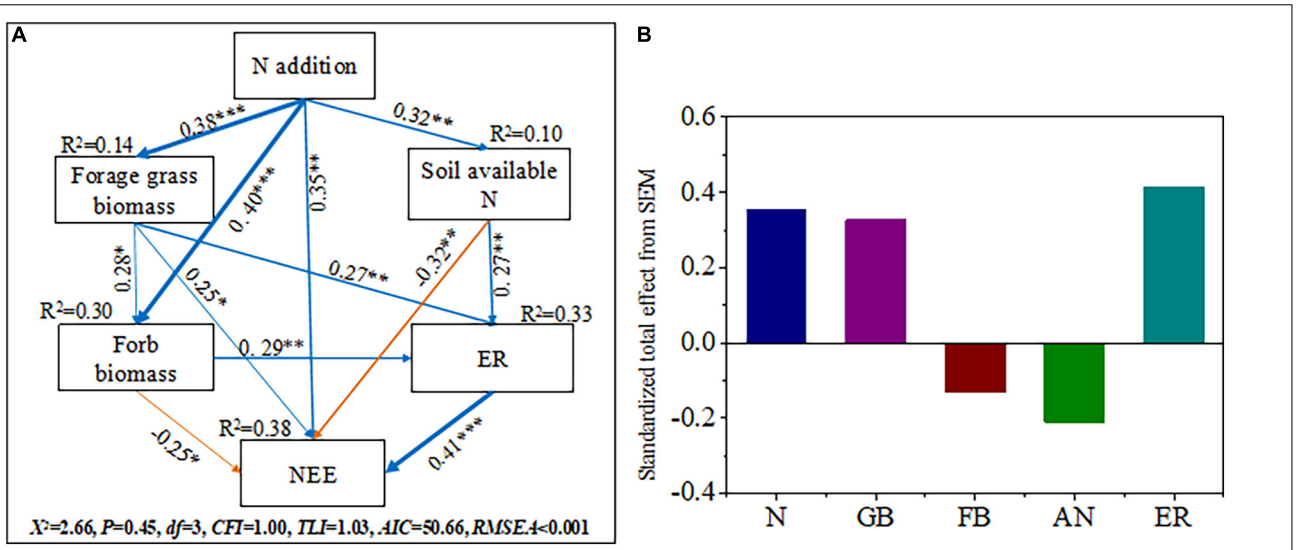

FIGURE 6 | Structural equation models reveal direct and indirect influences of soil available N, forage grass biomass and forb biomass on the CO_2 fluxes (NEE: net ecosystem CO_2 exchange, ER: ecosystem respiration) to N addition **(A)** and **(B)**. N, N addition; GB, forage grass biomass; FB, forb biomass; AN, soil available N. Single-arrowed pathways indicate the directional effect between variables. The values associated with pathways are the standardized path coefficients. The R^2-values are given for soil available N, forage grass biomass, forb biomass, ER, and NEE, indicating the variance explained by the model (R^2). The fitness statistics, Chi-squared (χ^2), degrees of freedom (*df*), P-value and root mean square error of approximation (RMSER), Tucker–Lewis index (TLI), comparative fit index (CFI) are given for the fitness of the model. The χ^2-test with $p > 0.05$ and the RMSER ≤ 0.05 indicates that the model is acceptable. The width of the arrows indicates the strength of the relationships. Blue arrows indicate significant positive relationships and orange arrows indicate significant negative relationships. The numbers on the line indicate standardized path coefficients. Stars indicate significant correlations. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}P < 0.001$. The data shown here include all data collected across the treatments and years. See **Supplementary Figure 4** and **Supplementary Table 4** for more details of the *a priori* model.

values were found in N20 and N7, respectively (**Figures 4A,B**, $p < 0.05$).

Changes in Ecosystem Carbon Dioxide Exchange With Increasing Nitrogen Addition

The variations in ecosystem CO_2 fluxes (i.e., NEE, ER, and GEP) mainly depended on N-addition level ($p < 0.05$), and significant differences in NEE were observed among the 5 studied years (**Table 2**, $p < 0.05$). Generally, NEE, ER, and GEP increased gradually over time with increasing N addition rates and showed an interannual fluctuating trend across the 5 years (**Figures 3D–F**). On average, NEE increased by 55.6 and 65.2% in N20 and N40, respectively, ER increased by 30.3% in N40, and GEP increased by 30.7, 42.8, and 48.4% in N7, N20, and N40, respectively ($p < 0.05$). In 2015, NEE, ER, and GEP showed a unimodal trend with the N-addition level, and in N7, NEE was 72.8% higher than that in N0 (**Figure 5A**, $P < 0.05$). In 2014 and 2017, the response of NEE to N addition showed a gradual increase but did not significantly differ from that of N0 (**Figure 5A**, $P > 0.05$). In 2016, N addition (N7, N20, and N40) significantly increased NEE, which reached 71.6, 94.9, and 72.5%, respectively ($p < 0.05$). In 2018, NEE significantly increased by 83.7 and 152.2% in N20 and N40, respectively ($p < 0.05$). Meanwhile, ER gradually increased in 2016–2018 with increasing N-addition levels. Specifically, ER significantly increased by 36.5 and 48.2% at N40 in 2016 and 2017, respectively ($p < 0.05$), and in 2018, ER increased significantly by 42.0 and 39.2% in N20 and N40, respectively (**Figure 5B**, $P < 0.05$). In addition, the response of GEP to N addition showed a change pattern similar to that of NEE (**Figure 5C**). In 2016, the GEP increased significantly by 43.6, 62.3, and 55.1% in N7, N20, and N40, respectively ($p < 0.05$). In 2017, the GEP increased by 51.0% in N40 ($p < 0.05$). In 2018, the GEP increased by 62.3 and 92.1% in N20 and N40, respectively ($p < 0.05$). The seasonal variations in CO_2 fluxes showed that the maximum values all appeared between late July and early August in each year (**Supplementary Figure 5**).

Plant Community Assembly and Soil Property Effects on Carbon Dioxide Fluxes

There were significant positive correlations between AGB and GB and NEE, GEP, and ER ($p < 0.01$). Both ER and GEP were positively affected by FB ($p < 0.05$). In addition, NEE was positively correlated with the soil MBN content ($p < 0.01$); ER was positively correlated with the soil properties (NO_3^-–N, NH_4^+–N, MBC, and MBN) and Shannon–Wiener index ($p < 0.05$); GEP was positively correlated with the soil MBN content and Shannon–Wiener index ($p < 0.01$).

Structural equation models adequately fitted the data describing the interaction pathways among the soil available N (AN), forage grass biomass (GB), forb biomass (FB), and ecosystem CO_2 fluxes (NEE and ER) in response to N addition (**Figures 6A,B**). The models explained 10.0, 14.4, 30.2, 32.7,

TABLE 3 | Pearson's correlations between soil properties and plant community characteristics and CO_2 fluxes during the growing season from 2014 to 2018.

	NEE (μ mol $m^{-2}s^{-1}$)	ER (μ mol $m^{-2}s^{-1}$)	GEP (μ mol $m^{-2}s^{-1}$)	AGB ($g.m^{-2}$)	GB ($g.m^{-2}$)	FB ($g.m^{-2}$)	NO_3^--N (mg kg^{-1})	NH_4^+–N (mg kg^{-1})	MBC (mg C kg^{-1})	MBN (mg N kg^{-1})	Shannon–Wiener index
NEE (μmol $m^{-2}s^{-1}$)	1										
ER (μmol $m^{-2}s^{-1}$)	0.39**	1									
GEP (μmol $m^{-2}s^{-1}$)	0.93**	0.69**	1								
AGB ($g.m^{-2}$)	0.35**	0.53**	0.48**	1							
GB ($g.m^{-2}$)	0.39**	0.45**	0.48**	0.89**	1						
FB ($g.m^{-2}$)	0.15	0.45**	0.30**	0.78**	0.41**	1					
NO_3^--N (mg kg^{-1})	−0.20	0.32**	−0.03	0.17	0.14	0.20	1				
NH_4^+-N (mg kg^{-1})	0.12	0.32**	0.21	0.29**	0.28*	0.17	0.35**	1			
MBC (mg C kg^{-1})	0.09	0.30*	0.16	0.48**	0.47**	0.28*	0.33*	0.05	1		
MBN (mg N kg^{-1})	0.48**	0.32*	0.48**	0.37**	0.39**	0.13	−0.11	0.26*	0.15	1	
Shannon–Wiener index	0.17	0.25*	0.24*	0.22	0.01	0.39**	−0.12	0.16	−0.42**	0.14	1
Species richness	0.18	0.10	0.18	0.16	0.04	0.26*	−0.06	0.22*	−0.39**	0.14	0.89**

*NEE, net ecosystem exchange; ER, ecosystem respiration; GEP, gross ecosystem productivity; AGB, aboveground biomass; GB, forage grass biomass; FB, forb biomass; MBC, soil microbial carbon; MBN, soil microbial nitrogen. *Correlation is significant at the 0.05 level. **Correlation is significant at the 0.01 level.*

and 38.2% of the variations in soil available N, forage grass biomass, forb biomass, ER and NEE, respectively (**Figure 6A**). The results of SEM analysis illustrated that N addition increased NEE indirectly through soil available N, forage grass biomass, and forb biomass and ER (0.35). Forage grass biomass (0.341) and ER (0.41) were positively correlated with NEE, while soil available N (-0.32) and forb biomass (-0.25) were negatively correlated with NEE ($p < 0.05$). Nitrogen addition indirectly increased ER *via* soil available N, forage grass biomass and forb biomass (0.33); ER was positively correlated with soil available N (0.27), forage grass biomass (0.27), and forb biomass (0.29). In addition, N addition significantly increased soil available N (0.32, $p < 0.01$), forage grass biomass (0.38, $p < 0.001$), forb biomass (0.40, $p < 0.001$), and NEE ($p < 0.01$, 0.35). Forage grass biomass was positively correlated with forb biomass ($p < 0.05$).

DISCUSSION

Effects of Nitrogen Addition on Functional Group Biomass and Plant Community Assembly

The soils of the most grassland ecosystems are characterized as N-poor, which affects the plant community composition and biomass accumulation (Wang et al., 2015; Shen R. N. et al., 2022). Our results found that AGB, GB, and FB increased significantly with N-addition levels under seasonal N application during the growing season (**Figure 2**). Our results confirm the previous studies indicating that N addition significantly enhances aboveground biomass by providing a large amount of N required for plant growth (Bai et al., 2010; Tian et al., 2015). Simulated N deposition has many positive impacts on plants when N application occurs more evenly throughout the year instead of at a single time (Cao et al., 2019). We also found that soil available N (NO_3^-–N and NH_4^+–N) increased significantly at higher N levels (**Figures 1A,B**), providing sufficient nutrients for the plant community growth (Han et al., 2019). There were significant correlations between AGB and GB and the soil NH_4^+–N content (**Table 3**) which also supported the above results. Meanwhile, consistent with previous studies (Xu et al., 2014; Armstrong et al., 2015), we found that AGB and functional group biomass largely depended on precipitation and temperature during the growing season (**Supplementary Figure 2**). In particular, AGB and GB increased significantly in 2017 (**Figures 3A,B**), possibly because the higher precipitation (114.05 mm) in the early growing season (**Supplementary Figure 2**) improved the plant biomass production by increasing the uptake and utilization of N. Moreover, these results indicated that the synergistic effects of climate and soil factors promoted grassland productivity (Shen R. N. et al., 2022).

There were differences in the sensitivity of different functional groups to N addition because of the difference in the N utilization capacity of plant roots and leaves (Kang et al., 2019). Some previous studies in alpine (Bassin et al., 2007) and subalpine

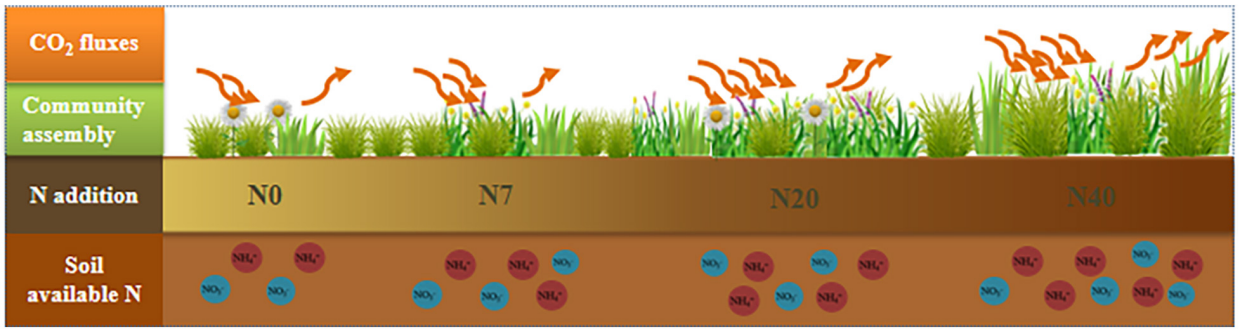

FIGURE 7 | Conceptual diagram of the response of ecosystem CO_2 fluxes (NEE, ER, and GEP) to regulation by the plant community assembly and soil properties under N addition (N0, N7, N20, and N40). Blue and red circles represent the soil available N content (NO_3^-–N and NH_4^+–N), respectively. The downward arrow is gross ecosystem productivity (GEP), and the upward arrow is ecosystem respiration (ER). Net ecosystem carbon exchange is defined as the difference between GEP and ER.

grasslands (Soudzilovskaia and Onipchenko, 2005) reported that sedges were characterized by high respiration efficiency and growth rates (Aerts, 1999; Shane et al., 2006; Mcclean et al., 2011), which will make sedges have a higher resource competitiveness than the other functional groups in alpine meadow ecosystems. We can partially support our hypothesis that a higher N addition could change the plant community assembly through shifts in composition toward forage grass-dominated communities. Sedge and Gramineae dominance over forb increased at high N (N40) level (**Supplementary Figure 3**), especially during a dry year (in 2015) (**Supplementary Figure 1**), which is consistent with the results of a previous study (Shen et al., 2019). This result may be because the whisker root system of forage grass (sedge and Gramineae) mainly distributed in the surface soil in alpine meadows and can access soil available N and water resources more easily than the root system of forb. Gramineae species such as *P. pratensis* are in the upper part of meadow canopy and more competitive for light and soil nutrients (Hautier et al., 2009). Significant increases in sedge coverage and Gramineae height also limited the competitiveness of forb for light (**Supplementary Tables 2,3**). Thus, N deposition is likely to enhance the dominant position of forage grass over forb in alpine meadows. Moreover, similar to the previous studies (Niu et al., 2010; Li S. et al., 2019), we found that plant diversity (species richness and Shannon–Wiener index) had a non-linear response trend, and this facilitating effect decreased with increasing N-addition rates (**Figure 4**). Nitrogen deposition is an ongoing process, and high N input may lead to a saturation response. The limiting resource for plants may be the availability of water, light, and other resources instead of N (Niu et al., 2010). The rapid growth of N-loving plants (forage grass) reduces the light transmittance of vegetation and causes the loss of plant diversity due to plant light competition (Hautier et al., 2009).

Effects of Nitrogen Addition on Ecosystem Carbon Dioxide Fluxes

A long-term N addition will influence carbon cycling (Simkin et al., 2016). Inner Mongolian grassland experiments found that the responses of CO_2 fluxes all exhibited non-linear patterns with increasing N-addition rates (Niu et al., 2010; Tian et al., 2015). In contrast to the previous results, our results showed that the response of ecosystem CO_2 fluxes (NEE, GEP, and ER) showed an increasing pattern with increasing N addition over the 5 years (**Figures 5A–C**). This pattern may illustrate that N and water availability are relatively lower in alpine meadows than in alpine steppes (Li S. et al., 2019), and alpine meadow ecological processes are highly sensitive to the N deposition during the growing season (Liu et al., 2015; Ma et al., 2019). Meanwhile, the relative contributions of GEP and ER significantly increased NEE because the response of GEP to cumulative N addition was more sensitive than that of ER (**Figure 5**). This result is because GEP is related only to plant photosynthesis, whereas ER is also affected by microorganisms and animals in the soil (Niu et al., 2009). There were significant correlations between ER and soil factors (MBC and MBN) (**Table 3**), possibly as a result of the increased availability of soil microbial respiration. A global meta-analysis by Zhou et al. (2017) also suggested that N rates less than 100 kg N $ha^{-1}year^{-1}$ stimulate microbial growth. Similar to a previous study (Tian et al., 2015), we demonstrated that the precipitation pattern affects the peak ecosystem carbon fluxes (**Supplementary Figure 2**); thus, NEE exhibited interannual differences (**Figures 3D–F** and **Table 2**). For example, the response of NEE to N addition was lower in 2017 than in other years, which may have been because lower precipitation amounts during the growing season (**Supplementary Figure 1**) lead to low plant photosynthetic rates (Niu et al., 2010). This phenomenon may also be because the increase in productivity was offset by boosting respiration consumption, which was also detected in temperate steppe (Niu et al., 2010) and Irish pasture systems (Peichl et al., 2011).

Plant Community Assembly and Soil Properties Regulate Ecosystem Carbon Dioxide Fluxes Under Nitrogen Addition

Our study provided evidence that the effects of N addition on ecosystem CO_2 exchange are regulated through soil available N and the plant community assembly (**Figures 6**, **7**). Kim and

Henry (2013) found that the net influx was observed during periods of peak aboveground biomass. Our findings confirmed the previous conclusion that plant productivity plays a major role in adjusting the responses of ecosystem CO_2 fluxes to N addition (Niu et al., 2009). Nitrogen addition alleviates soil nutrient constraints by increasing the soil available N at higher N-addition level (**Figure 1** and **Table 1**); thus, promoting a rapid vegetation growth and biomass accumulation (Bai et al., 2008; Yang et al., 2012; Xu et al., 2014; Shen R. N. et al., 2022). Higher productivity provides more substrates and increases the leaf area index and photosynthetic units associated with photosynthesis production (Bassin et al., 2007; Wang et al., 2015).

According to our hypothesis (**Supplementary Figure 4**), the plant community assembly would positively regulate the responses of ecosystem CO_2 fluxes to N addition (**Figure 7**). Nitrogen-stimulated forage grass could effectively promote NEE and ER because forage grass is construction species in the study area, and GB is significantly positive correlated with AGB and GEP (**Figure 3**, $p < 0.05$). Litter decomposition from forage grass roots and leaves continuously provides many carbon and N sources to the soil microbial community, which boosts soil respiration and NEE (Mcclean et al., 2011). Additionally, the positive response of ER to N addition depended on the increase in FB (**Figure 6**). The increase in forb coverage contributed to the higher levels of CO_2 uptake (**Supplementary Table 3**, $p < 0.05$) because the larger leaf spread area of forb was accompanied by the higher leaf breathing capacity (Ammann et al., 2007). Therefore, the N-induced shift in the plant community assembly toward forage grass-dominated community affected the formation of carbon sinks in alpine meadows. In addition, the changes in soil available N also affected ER because N addition may increase soil available resources and in turn increase ER by increasing heterotrophic respiration in the soil (Leff et al., 2015; Riggs et al., 2015), and this finding was similar to that of a previous study on the QTP (Peng et al., 2015). Nitrogen addition will increase the chlorophyll concentration in the different functional groups, which might be another reason for the increases in NEE and ER (Yan et al., 2014). However, we found negative effects of soil available N on NEE possibly because the soil acidification induced by excessive N addition resulted in a decrease in community diversity; thus, reducing the soil carbon sink capacity (Tian et al., 2015; Li J. et al., 2019).

Implications and Limitations

Consequently, our study showed that the plant community assembly significantly affected the response of the ecosystem CO_2 fluxes to N deposition, thus promoting the grassland function as a carbon sink. Our results emphasized that the functional group (forage grass) was a more important regulator in promoting NEE and ER responses to the N deposition than soil available N in the alpine meadow (**Figure 6B**). However, we did not measure the changes in soil microbial activity in this experiment and could not explore the relationships between the plant community composition and microbial community composition (Hodge and Storer, 2015) or their responses to N addition. Future studies need to explore how these factors regulate the response of NEE to the N deposition and the soil carbon stability maintenance mechanism in alpine meadows.

CONCLUSION

In an alpine meadow on the QTP, the ecosystem CO_2 fluxes responded positively to N addition, with a higher increase in GEP than in ER, and N addition ultimately increased the net carbon uptake (measured as NEE) during the 5-year experiment. The forage grass and forb biomass all showed positive feedback to N addition because soil available N significantly increased with N addition. The plant community assembly changed in favor of forage grass under higher N addition. Nitrogen addition stimulated the ecosystem CO_2 fluxes by regulating all functional group biomass and soil available N in the alpine meadow. Specifically, forage grass and forb had different regulatory effects on the response of CO_2 fluxes to N deposition, and the increase in forage grass biomass mainly contributed to the N stimulation of NEE. Our study highlights the significant role of the plant community assembly in regulating NEE and ER under N addition. However, we found that excessive N addition reduced species diversity. Therefore, more attention should be given to the impacts of N addition on alpine meadows. Future works must evaluate whether changing the community composition and decreasing plant diversity will impair the long-term carbon sink functions of sensitive alpine meadows.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

QG designed the research. YY, HG, GH, LD, SH, WX, and JY performed the experiments. LH analyzed the data and wrote the manuscript. LH, HG, GH, and JW interpreted the results and proofread the text. All authors contributed to this work and approved the final manuscript before submission.

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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.2022.900722/full#supplementary-material

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High N Storage but Low N Recovery After Long-Term N-Fertilization in a Subtropical *Cunninghamia lanceolata* Plantation Ecosystem: A 14-Year Case Study

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Forests are among the most important N pools of all terrestrial ecosystems. Elevated atmospheric N deposition in recent decades has led to increased interest in the influences of N application on forest N cycles. However, accurate assessments of N storage in forest ecosystems remain elusive. We used a 14-year experiment of a Chinese fir [*Cunninghamia lanceolata* (Lamb.) Hook] plantation to explore how long-term N fertilization affected N storage and recovery rates. Our study plots were located in a field that had been continuously fertilized over 14 years (2004–2017) with urea at rates of 0 (N0, control), 60 (N60, low-N), 120 (N120, medium-N), and 240 (N240, high-N) kg N $hm^{-2}a^{-1}$. Data were collected that included N content and biomass in the understory, litter, and various plant organs (i.e., leaves, branches, stems, roots, and bark), as well as soil N content and density at different depths. Results showed that the total ecosystem N storage in the N-fertilized plots was 1.1–1.4 times higher than that in the control plots. About 12.36% of the total ecosystem N was stored in vegetation (plant organs, litter, and understory) and 87.64% was stored in soil (0–60 cm). Plant organs, litter, and soil had higher N storage than the understory layer. Significantly higher plant N uptake was found in the medium-N (1.2 times) and high-N (1.4 times) treatments relative to the control. The N recovery rate of the understory layer in the N-fertilized treatments was negative and less than that in the control. Application of long-term N fertilizer to this stand led to a low N recovery rate (average 11.39%) and high loss of N (average 91.86%), which indicate low N use efficiency in the Chinese fir plantation ecosystem. Our findings further clarify the distribution of N in an important terrestrial ecosystem and improve our understanding of regional N cycles.

Keywords: long-term N fertilization, N storage, plant organs, ecosystem components, *Cunninghamia lanceolata* plantation

INTRODUCTION

Nitrogen (N) is a fundamental constituent of many biomolecules, including proteins and nucleic acids, and therefore one of the most limiting factors on plant growth and productivity (Nadelhoffer, 2008; Xu et al., 2020; Qubain et al., 2021). N concentration in terrestrial ecosystems is particularly sensitive to anthropogenic disturbance, including N fertilization (Bouwman et al., 2002; Tian et al., 2006; Etzold et al., 2020; Verma and Sagar, 2020). Unfortunately, human activity has led to ubiquitous excessive and inappropriate application of N fertilizers that may influence the global N cycle (Lu et al., 2011), upsetting the balance of N uptake among forest plants (Liu et al., 2020 and references therein), alter forest ecosystem structure and function, and inhibit ecosystem productivity by tipping nutrient balances (Nadelhoffer, 2008; Tian et al., 2018; Li et al., 2019). N fertilization also reduces ecosystem biodiversity through soil acidification and ammonium toxification (Talhelm et al., 2013; Zhang et al., 2014; Midolo et al., 2019; Wu et al., 2021), and can decrease ecosystem stability *via* altered dominant species abundance and plant functional group composition (Wu et al., 2020).

Forests are the largest N pools of terrestrial ecosystems and account for 35.86% of the total N storage in China (Xu et al., 2020). Changes in forest N pools caused by N application can alter global N cycle processes (Fenn et al., 2020; Xu and He, 2020; Zhang et al., 2020). "Closed" N cycles are formed in the soil-litter-plant continuum found in forest ecosystems, which are sensitive to N deposition (Nadelhoffer, 2008; Liu and Wang, 2021; Qubain et al., 2021). In this closed N cycle, annual plant N uptake per unit area is balanced by annual N return to litter and soil (Nadelhoffer, 2008). Several studies have demonstrated that the mass balance of N in a forest ecosystem is generally characterized by the difference between N inputs, vegetation and soil N sinks, N leaching, and gaseous loss (Lovett and Goodale, 2011; Gurmesa et al., 2016). In forest ecosystems, external N inputs, especially soil fertilizers, are first applied to the soil and then absorbed and used by plants (Qubain et al., 2021). Most N is retained by litter and soil, and only a small fraction is taken up and retained by plants (Nordin et al., 2001; Gurmesa et al., 2016; Wang et al., 2018; Li et al., 2019). The N uptake capacity of soils and vegetation may be a major factor determining tree growth and mortality in response to N addition (Wallace et al., 2007; Lovett and Goodale, 2011). The N recovery rate is often used as an indicator of N use efficiency (Ju and Zhang, 2003; Tian et al., 2011; Congreves et al., 2021). Even small fluctuations in the forest N pool can dramatically influence global N cycles (Xu and He, 2020). For example, external fluctuations of N inputs decreased main ecosystem compartments recovery to different degree in "N-rich" tropical forest (Gurmesa et al., 2016). Mapping the distribution of fertilized N in forest ecosystem components therefore represents the importance of N storage and N recovery to forest ecosystems N cycles.

Although numerous studies have focused on how N fertilization affects forest ecosystems, it is widely concerned that the response of ecosystem N cycle to N fertilization various in short-term and long-term N application, because the responses of forest ecosystems to N fertilization cannot completely reveal in a short time (Zhu et al., 2015). Previous studies have been reported that N application tended to have positive effect on litter N pool and soil N cycle processes (i.e., accelerating soil N mineralization rate) in short-term fertilization experiment (Lu et al., 2011; Zhu et al., 2015), but had negative effects when forest reached N saturation in long-term N fertilization experiments, such as reduced soil microbial biomass and respiration (Zhu et al., 2015; Zhang T. A. et al., 2018). However, N-induced changes in the aboveground plant N pool did not show any significant correlations with experimental duration (Lu et al., 2011). The effects of fertilized N depend greatly on the fate of fertilized N (Gurmesa et al., 2016); thus, empirical studies that follow N over long time scales are critically needed to better understand how N fertilization influences ecosystem N cycle.

It is widely accepted that excessive N fertilization enhances the leaching of ecosystem nutrients (Wilson and Tiley, 1998), especially in N-saturated forests (Gurmesa et al., 2016). In a review of studies across China, Tian et al. (2018) found that an N application rate exceeding 80 kg N $hm^{-2}a^{-1}$ can be regarded as an extremely high level of N in forests. Further, rates higher than 90 kg N $hm^{-2}a^{-1}$ can lead to negative feedbacks between soil N availability and transformation (Verma and Sagar, 2020). High levels of N input can cause forest decline (Wilson and Tiley, 1998). For example, one study found that N fertilization at 150 kg N $hm^{-2}a^{-1}$ over 15 years induced high tree mortality (Högberg et al., 2006). Two common symptoms of N-induced damage are nutrient deficiencies caused by accelerated plant growth and a loss of soil base cations and species by soil acidification (Högberg et al., 2006; Zhang Y. Q. et al., 2018). N leaching loss can range from 2% to 20%, with higher N dosages leading to higher losses (Tian et al., 2018). While several studies have addressed the effects of N fertilization on plant growth and nutrient cycles in forest ecosystems over short time spans, they provided limited information on the long-term effects of high N fertilization on forest N uptake and N storage, particularly with respect to N recovery rate.

The average rate of N deposition in China has increased by approximately 60% over the past three decades (Yu et al., 2019), up to 150 kg N $hm^{-2}a^{-1}$ in some areas (Zhang et al., 2014). Higher leaf, aboveground plant and litter N pools were observed when N application rate from 0–50 to 50–100 kg N $hm^{-2}a^{-1}$, but no more increase from 50–100 to 100–150 kg N $hm^{-2}a^{-1}$ (Lu et al., 2011). While rapid plant species loss in long-term low frequency of N application showed the same results with the high rates (Zhang et al., 2014). The rate of N application in this study is much higher than the atmospheric N deposition rate (33.2 kg N $hm^{-2}a^{-1}$ on average) across this region (Xu et al., 2018). In previous work we showed that our current study site is N-saturated (Wu et al., 2017, 2021); thus, an extremely high dose of N fertilizers (i.e., 120 and 240 kg N $hm^{-2}a^{-1}$) provides a unique opportunity for studying the effects of high N inputs on forest ecosystems.

Forests cover about 220 million ha in China, which is 22.96% of the global terrestrial ecosystem (FAO, 2015; FRA, 2020).

China's plantation forests are the largest in the world, accounting for 27% of the total global area of planted forests (FAO, 2015). Chinese fir [*Cunninghamia lanceolata* (Lamb.) Hook] is an economically important species in China (Zhang et al., 2020), covers an area of 8.93 million ha (Nation Forestry and Grassland Adminstration, 2019). *Cunninghamia lanceolata* does not fix N_2 and therefore depends upon combined or fixed forms of mineral N (Tian et al., 2018). The objective of our study was to accurately estimate the N storage of *C. lanceolata* plantation ecosystem after 14 years N fertilization. Since 2004, we carried out an N deposition experiment in subtropical region with four urea-N fertilization rates (0, 60, 120, and 240 kg N $hm^{-2}a^{-1}$), with the aim to assess the allocation of N amongst plant organs (i.e., leaves, branches, bark, stems, and roots) and ecosystem components (plants, understory, litter, and soil), as well as N recovery rate. We hypothesized that: (1) N storage of both *C. lanceolata* and the total ecosystem would increase with fertilization rate under long-term N fertilization and (2) N recovery rate would decrease with N fertilization rate.

MATERIALS AND METHODS

Study Site

Our experiments site is located in Guanzhuang National Forest Farm (117°43′E, 26°30'N), Sanming City, Fujian Province in southeastern China, at an altitude of approximately 200 m. This area has a mid-subtropical monsoon climate with abundant rainfall, characterized by an average annual temperature of 20.1 ± 1.96°C, precipitation of 2,777 ± 40.2 mm a^{-1} (>80% of which falls from May–October, **Supplementary Figure 1**), and a frost-free period of 271 days (climatology based on measurements from 2004 to 2017).

The Chinese fir plantation was planted in 1992 over an area of 6 hm^2 at a density of 1,660 individual trees per hectare. There were no legumes in this area, and it had received no fertilizer prior to this experiment. In December 2003, 12 plots (20 m × 20 m each and with a 15 m × 15 m central area) were randomly selected within the forest with a minimum distance between plots of 10 m. The average tree height of the entire plantation was 12 m, and the mean diameter at breast height (DBH, 1.3 m from the ground) was 16.1 cm. A background investigation conducted in 2003 revealed that the understory was sparse, with coverage between 3% and 5%; dominant understory species included *Miscanthus floridulus*, *Dicranopteris olichotoma*, and *Pteridium aquilinum* var. *Latiusculum* (Wu et al., 2013; Shen et al., 2019a). We also measured soil physical and chemical properties; the soil was acidic (pH = 4.67) with an organic carbon content of 18.39 g kg^{-1}, a total N content of 0.79 g kg^{-1}, and was classified as an Acrisol (Fan et al., 2014; Shen et al., 2019a; Wu et al., 2021).

Experimental Design

This N fertilization experiment started on January 1, 2004. There were four rates of ureas-N fertilization, 0, 60, 120, and 240 kg N $hm^{-2}a^{-1}$, referred to as N0 (control), N60 (low-N), N120 (medium-N), and N240 (high-N), respectively. Each treatment was applied to three replicate plots on the same mountain slope. The N-plots were fertilized monthly and continued to 2017 [14 years prior to Shen et al. (2019a)]. According to the N concentration of each treatment, urea fertilizer was weighed and then dissolved in 20 L of water; and the solution was sprinkled evenly with a backpack sprayer to the forest floor of each plot. The control plots received the equivalent 20 L of water alone.

Field Sampling

Sampling was conducted on 15 December 2017. Following a per-wood inspection, DBH statistical analysis over the treatment period (2004–2017), and the map of all Chinese firs in plots, we selected a standard tree (normal growth, no pests or diseases, and few scars) in each plot for wood tests, for a total of 12 standard trees subjected to biomass measurements. We determined the orientation of the selected trees with a compass. Before felling, we marked the orientation of the stem at breast height. The central cross-sectional differentiation quadrature method was used to intercept the stem disc (5 cm thick) at the midpoint of each zone segment (every 2 m of the whole tree beginning at the coarse roots). After felling, plant organs (i.e., leaves, branches, stems, bark, and roots) samples were collected from various points and locations in the field per standard tree per plot and weighed. Briefly, leaves were taken from various direction and composite to one sample; branch, bark, and stem samples were taken from various points and locations. The root system biomass was measured following total root excavation (Niiyama et al., 2010). Root samples were divided into coarse roots (>10 mm) and fine roots (<2, 2–5, and 5–10 mm) and were cleaned with deionized water and weighed. Then all subsamples (5 kg of each fresh mass) were transported to the laboratory.

Forest litter was sampled using a 1 m × 1 m frame (three random samples per plot); the samples were separated into an undecomposed layer (L layer) and a semidecomposed layer (F layer), and the layers were then evenly mixed into composite samples, in total, 12 plots × 2 layers × 3 replicate = 72 composite litter samples. The L layer was distinguished by litter with leaves still maintaining their original shape, color, and without superficial evidence of decomposition; by contrast, the leaves of the F layer were crushed with degraded outlines and mostly decomposed mesophyll. All litter samples were brought back to the laboratory to measure water content, total N content.

Six soil cores, 2.5 cm in diameter, down to 60 cm and divided the core into 0–20, 20–40, and 40–60 cm soil sample, and thoroughly composited the six cores from each plot to end up with three replicate soil samples from each depth, in total, 12 plots × 3 depths × 3 replicate = 108 composite soil samples, for soil water content and total N content analysis. The soil samples were transported to the laboratory, sieved through a 2-mm mesh to remove nonsoil materials, and then divided into two sub-samples. One sub-sample was oven-dried at 105°C until the weight remains constant for soil water content determination. The other sub-sample was air-dried then powdered to sieve through a 0.15-mm mesh for analysis of soil total N content. We took an additional 5 cm inner diameter core at

each of the three replicate plots from the 0–20, 20–40, and 40–60 cm depths, taking care to collect the exact volume the core, for soil bulk density determination.

To capture the understory species diversity when more seasonal vegetation was visible, we investigated the diversity of the understory on 16 September 2017. Three 5 m × 5 m survey subplots were randomly set in each plot, and we recorded the identity, height, and crown width of species, as well as the number of small trees, lianas, herbs, and shrubs taller than 5 cm in each plot. The biomass of the understory layer was determined by harvesting; small trees and shrubs were collected from 2 m × 2 m sampling areas, and herbs were collected from 1 m × 1 m sampling areas. Whole understory plants were excavated and divided into above- and belowground components. The aboveground components consisted of leaves, branches, and stems of small trees, and leaves and branches of herbs, while the belowground components consisted of roots.

Stem disc samples were taken at breast height and air-dried in ventilated conditions. They were sanded with sandpaper until the annual rings were visible. The discs were then cross-dated to the tree core samples at each sample point and tree-ring width was determined using LinTab 6 (TSAP-Win, Germany).

Laboratory Analysis

After being returned to the laboratory, all fresh mass of each sample was measured, followed by determine water content, dry biomass, and N content. All fresh leaves were first washed with 10% dilute hydrochloric acid and then repeatedly washed with deionized water to completely remove dust and particulates adsorbed on the surface, then heat-killed at 105°C for 15 min and oven-dried at for 48 h (Liu and Wang, 2021). The branch and fine root samples were washed with clean water, heat-killed at 105°C for 15 min, and then dried at 75°C for 48 h to a constant weight (Liu and Wang, 2021). The bark and litter samples were dried at 85°C to a constant mass. The dry biomass of plant organs was calculated per unit area based on the water content. All vegetation subsamples (i.e., plant organs, understory, and litter samples) were crushed with a microplant crusher, passed through a 100-mesh sieve, and kept in a dry environment prior to analysis. The total N content of plant organs, litter, understory samples was determined through initial digestion with H_2SO_4–H_2O_2 followed by measurement with the micro-Kjeldahl method (Liu and Wang, 2021). The soil N content was determined by the Kjeldahl method after digestion with H_2SO_4 (Shen et al., 2019a). Soil bulk density cores were sieved to 4 mm and oven-dried (105°C for 48 h; Soong et al., 2020), and bulk density was estimated as the mass of the oven-dried soil divided by its volume.

Calculation

Basal Area Increment

We calculated the basal area increment (BAI, cm^2) using the following equation:

$$BAI = \pi\left(R_n{}^2 - R_{n-1}{}^2\right) \quad (1)$$

where n is the number of tree rings and R_n is the radius of the n^{th} ring (cm).

Soil N Storage

Soil N density (N_d, kg m^{-2}) refers to the storage of N in the soil layer at a specific depth per unit area and was calculated using the following equation:

$$N_{di} = 0.1 \times T_{Ni} \times \gamma \times H_i \times \left(1 - \frac{\delta}{100}\right) \quad (2)$$

where 0.1 is the conversion factor, i represents the soil layer (cm), T_{Ni} represents the total soil N content in soil layer i (%), γ represents the soil bulk density (g cm^{-3}), H represents the soil depth (cm), and δ indicates the percentage of gravel in soil with a diameter>2 mm (%).

Soil N storage (N_S, t hm^{-2}) was calculated as:

$$N_s = \sum\left(N_{\mathrm{di}} \times A\right) \quad (3)$$

where A represents area (hm^{-2}).

Plant N Uptake and Recovery Rate

We used the definition of fertilizer N recovery efficiency (RE_N) as the percentage of fertilizer N that is taken up by the plant, accounting for background soil N levels (Congreves et al., 2021). The definition is also sometimes referred to as the apparent recovery (Tian et al., 2011; Congreves et al., 2021), calculated as:

$$PlantN_f = Plant_{Biomass} \times N\% \quad (4)$$

$$RE_N = \frac{PlantN_f - PlantN_0}{FertilizerN} \times 100\% \quad (5)$$

where N (%) is the N content of major ecosystem components, $Plant_{\mathrm{Biomass}}$ (t hm^{-2}) is the plant biomass of a plot in this study, $PlantN_f$ (kg N hm^{-2}) is the total plant N uptake measured in above- plus belowground biomass in a plot that received N dose (*Fertilizer N*; i.e., 0, 60, 120, or 240 kg N $hm^{-2}a^{-1}$), and $PlantN_0$ is the total plant N uptake in unfertilized N plots (N0).

Statistical Analysis

We used two-way ANOVA with a *post hoc* Tukey test to detect the effects of N treatments and components (litter components, understory layers, and ecosystem components) on biomass, N content, and N uptake; the effect of N treatments and soil layers on density, N content, and N storage; and the effect of N treatments and DBH class on number of trees. One-way ANOVA with Dunnett's *post hoc* LSD test was used to determine the effects of the N treatments on plant biomass, N content, N uptake, understory species, soil N content, and N storage. All values were given as means ± standard error (*SE*). Results

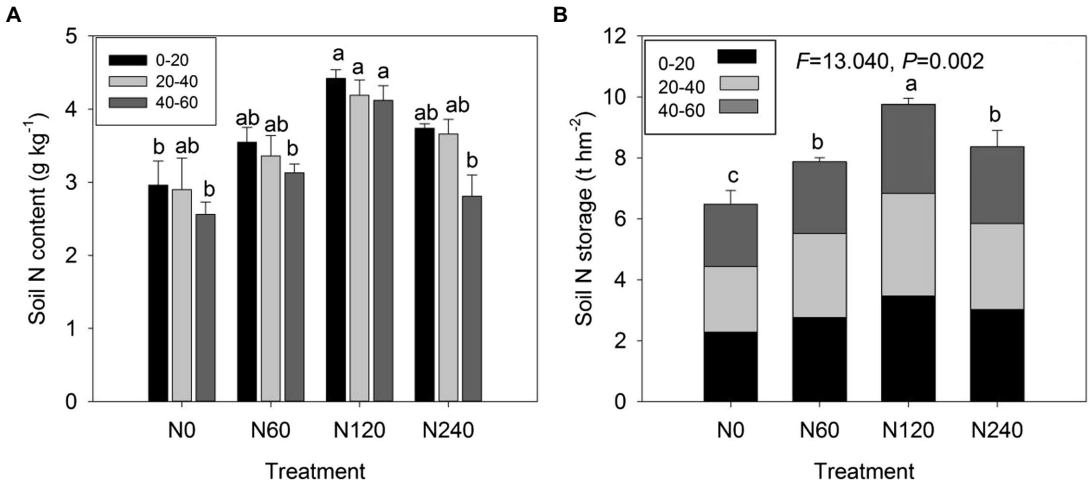

FIGURE 1 | Soil N content **(A)** and N storage in total **(B)** at three soil depths (0–20, 20–40, and 40–60 cm) under different N treatments. N0, control; N60, low-N; N120, medium-N; and N240, high-N. The values show means ± SE ($n = 3$). Lowercase letters indicate significant differences at $p < 0.05$ between different treatments.

TABLE 1 | Two-way ANOVA of soil density, N content, and N storage in three soil layers under different N treatments.

Variables	*F* (*P*) value					
	Density (kg m^{-2})		N content (g kg^{-1})		N storage (t hm^{-2})	
Treatment	2.898	0.056	18.711	**<0.001**	5.392	**0.027**
Layer	11.948	**<0.001**	4.99	**0.015**	12.00	**<0.001**
Treatment × layer	0.877	0.526	0.603	0.725	0.877	0.527

Values in bold are statistically significant at p < 0.05.

were considered significant at $p < 0.05$. All analyses were conducted using SPSS 19.0 (SPSS, Inc., Chicago, IL, United States). Figures were created with SigmaPlot 13.0 (Sysat software Inc., San Jose, CA, United States).

RESULTS

Variation in Soil N Storage

N fertilization significantly increased soil N content and storage ($F = 13.040$, $p = 0.002$), which was maximal under the N120 treatment (**Figure 1**). There were significant effects of N fertilization on the N content of all three soil layers: 0–20 cm ($p = 0.018$), 20–40 cm ($p = 0.048$), and 40–60 cm ($p = 0.003$; **Table 1**; **Figure 1A**). Soil N storage showed significant differences in all three layers among the four N treatments [$p = 0.024$ (0–20 cm), $p = 0.044$ (20–40 cm), $p = 0.014$ (40–60 cm); **Table 1**; **Figure 1B**]. The N120 treatment increased soil N storage in the 0–20 cm layer by 32.53% compared to that in the N treatment but did not have an effect in the 20–40 or 40–60 cm layers.

Variation in DBH and BAI

The tree diameters were normally distributed (**Figure 2**), but there was no significant difference in average DBH among N treatments (**Supplementary Figure 2**). High-N fertilizer significantly decreased the number of trees with a DBH between 10 and 14 cm, but increased the number of trees with a DBH > 30 cm (**Figure 2**). Tree mortality was significantly lower in the N0 (2.98%) and N60 (2.55%) treatments compared to the N120 (8.76%) and N240 (7.32%) treatments, with medium-N significantly increased mortality. During the N treatment period (2004–2017), significant differences in BAI and annual BAI between N treatments occurred only in 2013 ($p = 0.032$) and 2014 ($p = 0.031$; **Supplementary Figure 3**).

Variation in Vegetation N Storage

The dry mass and N content of each plant organ were very similar but nonsignificant differences across N treatments (**Table 2**). N content of leaves and fine roots showed approximately two to four times higher than other organs. Total N uptake of plant organs in the N120 and N240 treatments increased by 27.61% and 39.16%, respectively, relative to that in N0. Significant differences were detected in total plant N uptake in 2017 ($F = 7.906$, $p = 0.009$) and over the study period from 2003 to 2017 ($F = 60.257$, $p < 0.001$) between N treatments (**Figure 3**; **Supplementary Table 1**).

The understory diversity decreased with increasing N fertilization rates, with species counts of 50, 33, 28, and 16 in N0, N60, N120, and N240, respectively (**Supplementary Figure 4A**). The

abundance of small trees and lianas decreased sharply (approximately 4-fold) in N120 and N240 ($P<0.05$) compared to that in N0. Similarly, the total, above- and belowground understory biomass in the N-fertilized treatments were significantly lower than those in control ($p<0.05$; **Supplementary Figure 4B**). Understory layer N content showed no difference (**Figure 4A**), but N uptake significantly decreased with increasing N fertilization rates ($p<0.05$) and aboveground N uptake contributed an average of 64.69% of the total understory N uptake (**Figure 4B**).

Significant differences were detected in total litter biomass among N treatments (**Figure 5**; **Table 3**). The total litter biomass in N60 (5.23 t hm^{-2}) was higher than that in N0 (2.55 t hm^{-2}; 104.81% increase; $p<0.05$; **Supplementary Figure 5A**). The biomass of F-leaf and F-branch in N60 was 1.5 and 4.5 times higher, respectively, than that in N0 (**Supplementary Figure 5B**). N fertilization had significant effects on the N content on both leaf litter ($F=15.577$, $p<0.001$) and branch litter ($F=25.026$, $p<0.001$), especially in the N120 treatment (**Figure 5A**; **Table 3**). The greatest increase in N uptake was observed in N120 for the total litter and litter components (**Figure 5B**; **Table 3**). Leaf litter contributed more to total litter N uptake (up to 79.41%) than branch litter. The branch N uptake in N120 was more than 4.5 times higher than that in N0.

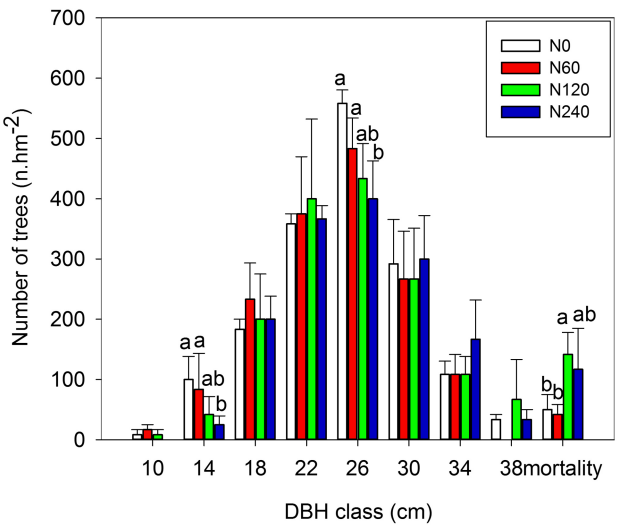

FIGURE 2 | Distribution of trees among DBH classes after 14-year N treatments. DBH classes were defined in 4 cm intervals from 10 cm to 38 cm (in total nine classes); dead trees are all placed in a tenth class as "mortality." There were no trees with diameters less than 10 cm in the N240 treatment. Within each class, the values of *F* and *p* are shown based on one-way ANOVA. N0, control; N60, low-N; N120, medium-N; and N240, high-N. The values show means ± SE ($n=3$). Lowercase letters indicate significant differences at $p<0.05$ between different treatments.

Variation in Ecosystem N Recovery Rate

Among the major components of *C. lanceolata* ecosystem, only the N content of litter and soil differed significantly between N treatments (**Figure 6**). N fertilization increased total ecosystem

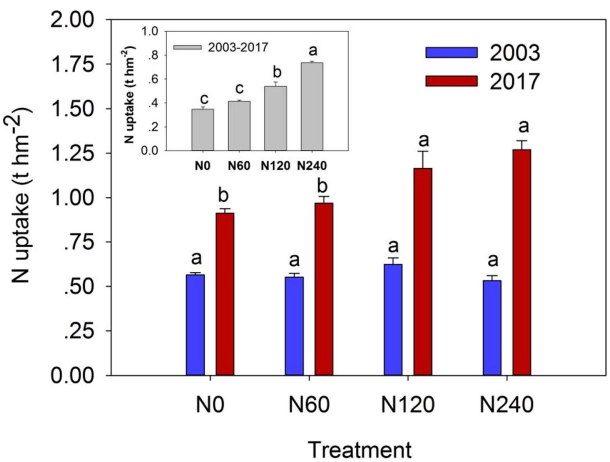

FIGURE 3 | Plant N uptake under different N fertilization rates in 2003 (blue bars), 2017 (red bars), and their differences from 2003 to 2017 (gray bars). Plant N uptake means the sum of N uptake in plant organs (i.e., leaves, branches, bark, stems, and roots). N0, control; N60, low-N; N120, medium-N; and N240, high-N. The values show means ± SE ($n=3$). Lowercase letters indicate significant differences at $p<0.05$.

TABLE 2 | Dry mass and N content of Chinese fir plant organs under different N fertilizer treatments in 2017.

Component	Dry mass (t hm^{-2})				N content (g kg^{-1})			
	N0	N60	N120	N240	N0	N60	N120	N240
Leaves	7.55(0.14)	7.36(0.13)	7.67(0.14)	7.82(0.14)	12.81(1.30)	13.31(1.03)	14.23(1.65)	14.99(1.07)
Branches	19.13(0.50)	18.46(0.47)	19.58(0.52)	20.10(0.51)	6.94(0.31)	7.11(0.43)	7.73(0.54)	7.97(1.18)
Stems	115.25(4.01)	109.71(3.67)	118.90(4.23)	122.79(4.12)	3.00(0.35)	3.48(0.40)	3.78(0.46)	4.86(0.49)
Bark	16.87(0.45)	19.48(0.24)	22.91(0.78)	18.55(0.36)	7.62(0.12)	7.59(0.23)	8.01(0.18)	8.18(0.07)
Coarse roots	35.77(4.82)	34.17(2.41)	42.62(11.61)	33.60(3.37)	4.97(0.15)	5.29(0.21)	5.76(0.27)	6.41(0.59)
Fine roots								
<2 mm	0.57(0.07)	0.53(0.22)	0.32(0.10)	0.37(0.05)	13.87(0.48)	13.82(0.86)	14.55(1.54)	15.36(1.28)
2–5 mm	0.97(0.23)	0.86(0.28)	0.65(0.13)	0.62(0.22)	12.88(0.37)	12.65(0.48)	12.47(1.42)	13.48(0.73)
5–10 mm	0.79(0.16)	0.67(0.14)	0.66(0.35)	0.86(0.18)	11.98(0.43)	12.87(0.49)	13.66(1.24)	14.44(1.47)
Subtotal	196.87	191.24	213.31	204.71				

Values are means ± SEs (n = 3).

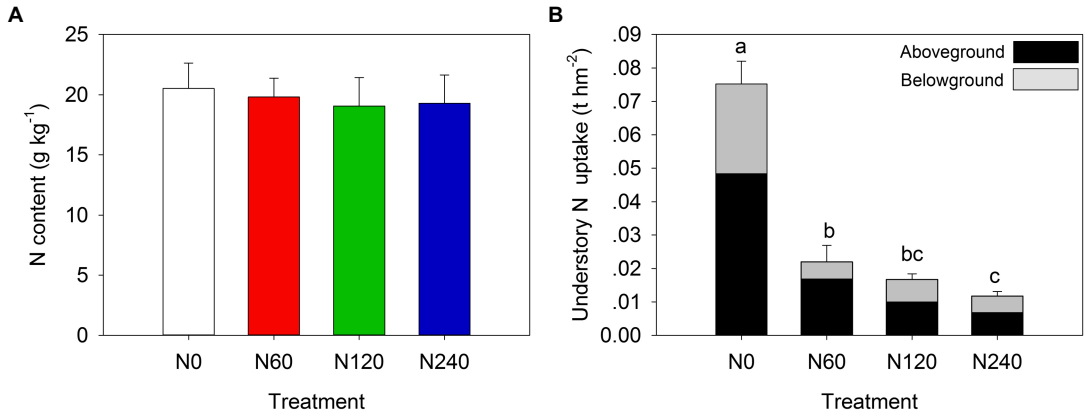

FIGURE 4 | Changes in understory layer N content **(A)** and N uptake **(B)** under different N treatments. N0, control; N60, low-N; N120, medium-N; N240, high-N. The values show means ± SE ($n = 3$). Lowercase letters indicate significant differences at $p < 0.05$ between different treatments.

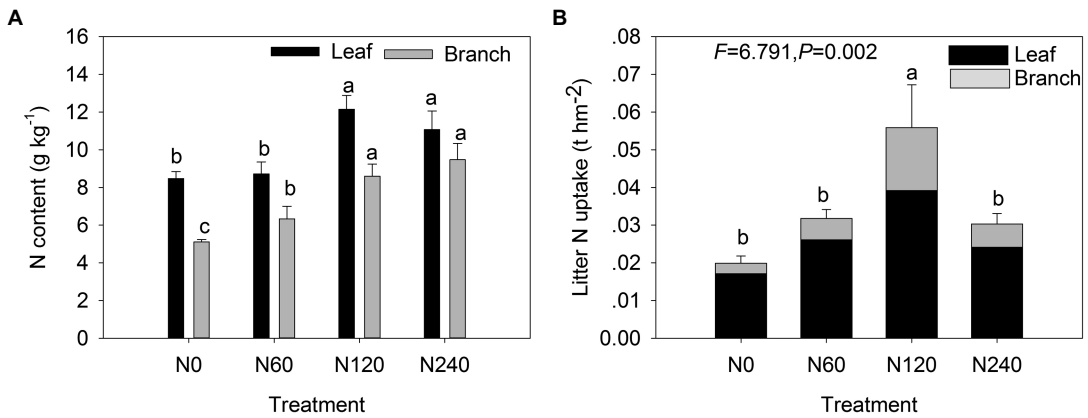

FIGURE 5 | Total litter N content **(A)** and N uptake in total, leaves, and branches **(B)** under different N fertilization treatments. N0, control; N60, low-N; N120, medium-N; and N240, high-N. The values show means ± SE ($n = 3$). Lowercase letters indicate significant differences at $p < 0.05$ between different treatments.

TABLE 3 | Two-way ANOVA of biomass, N content, and N uptake under different N treatments and in different litter components.

Variables	*F* (*P*) value					
	Biomass (t m^{-2})		N content (g kg^{-1})		N uptake (t hm^{-2})	
Treatment	5.367	**0.003**	14.865	**<0.001**	10.928	**<0.001**
Component	39.727	**<0.001**	32.267	**<0.001**	66.213	**<0.001**
Treatment × component	0.505	0.681	0.903	0.461	0.590	0.625

Component: leaves and branches in litter. Treatment: N fertilizer treatments, i.e., N0, N60, N120, and N240.
Values in bold are statistically significant at $p < 0.05$.

N storage (**Table 4**), with average N storage of 12.10% in vegetation and 87.9% in soil in the N treatments and 13.46% in vegetation and 86.54% in soil in the control.

The plant N recovery rate in N120 was much higher than that in N60 and N240; the N recovery rates in vegetation were 2.41%, 19.11%, and 12.67% in N60, N120, and N240, respectively, relative to the control; the N recovery rates of the understory layers in the N fertilizer treatments were lower than in the control (**Table 5**). The N recovery rates of the plant and litter were all positive among N fertilizer treatments, and the N recovery rate was highest in N120 (20.98% in plants and 3.00% in litter). The N storage of vegetation was 14.44, 229.27, and 304.07 kg N hm^{-2} higher in N60, N120, and N240, respectively, relative to N0 (**Supplementary Table 2**).

When N storage in vegetation was compared with the total amount of N fertilizer from 2004 to 2017, the loss of N was estimated to be 825.56 (98.28%), 1450.73 (86.25%), and 3055.9 (90.95%) kg hm^{-2} in N60, N120, and N240, respectively (**Supplementary Table 2**). Together, our results suggest that long-term N fertilization might induce low ecosystem N use efficiency.

DISCUSSION

Tree Growth and Mortality

Although tree mortality rate in the studied forest (2004–2017) was generally low (average 6.21%), it is noteworthy that tree mortality was higher in the treatments with N fertilization. This is consistent with findings of significantly increased mortality after 18 years of N deposition (30 kg N $hm^{-2}a^{-1}$; Talhelm et al., 2013). Tree mortality was sensitive to N deposition, especially at high levels of nitrate deposition, which led to increased tree mortality due to N saturation (Midolo et al., 2019) and soil acidification (Dietze and Moorcroft, 2011; Lovett and Goodale, 2011). Some researchers have attributed this phenomenon to a decrease in the Ca:Al and Mg:N ratios in the foliage and organic soil layer (Aber et al., 1998; Wallace et al., 2007; Lovett and Goodale, 2011). In addition, Fenn et al. (2020) identified a small but significant effect whereby N deposition increased mortality due to crown damage, and Tian et al. (2018) proposed that this increase in tree mortality may indirectly relate to increases in plant disease, changes in soil microbial communities, and greater susceptibility to insect and fungal pathogens. Our finding of increased tree mortality suggests that high N fertilization rates negatively affect trees.

Uniquely, we observed increases in BAI and annual BAI after 10–11 years N treatment, which confirms that N fertilization leads to an increase in relative tree growth (Wallace et al., 2007). The co-occurrence of increased mortality and growth in surviving trees is considered a "threshold" phenomenon in N-saturation forest. The inherent susceptibility of the study site to nitrate leaching and soil acidification in response to N addition, which is mainly regulated by soil and vegetation N uptake capacity, may be the main factor determining tree growth and mortality (Wallace et al., 2007). Enhanced tree growth by N fertilization in this study is inconsistent with results reported by Tian et al. (2018) and Midolo et al. (2019). It has been reported that elevated N deposition had minor effect on forest growth in N-rich forest ecosystems through enhancing transpiration and maintaining nutrient balance (Lu et al., 2018).

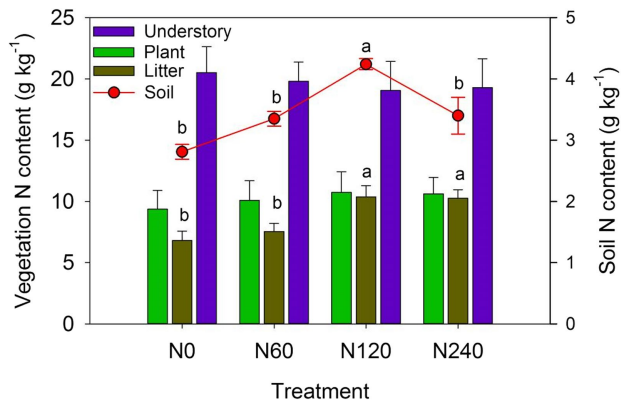

FIGURE 6 | N content of major components of the *Cunninghamia lanceolata* plantation ecosystem under different N fertilizer treatments. Plant: the sum of leaf, branch, stem, bark, fine root, and coarse roots of *C. lanceolata*. N0, control; N60, low-N; N120, medium-N; and N240, high-N. The values show means ± SE ($n = 3$). Lowercase letters indicate significant differences at $p < 0.05$ between different treatments.

N Storage in the Ecosystem

The N contents differed significantly in the soil and litter between N treatments, but did not vary in plant organs or understory. The neutral response to N fertilization on plant organs might be *C. lanceolata* is a non-N_2 fixing species (Tian et al., 2018 and references therein). We observed significant increases in soil N content with N fertilization up to a threshold (N120, 120 kg N $hm^{-2}a^{-1}$), above which no further increase was observed. The soil N content in N120 was increased by 19.24%–51.49% compared with the control after N fertilization, while results of a meta-analysis by Tian et al. (2018) suggested an increase of soil N content in subtropical (5.4%) and up to 26.9% in tropical forests.

As expected, we found consistently negative effects of high N fertilization rates on understory layer species diversity, biomass, N uptake, and N recovery rate. This supports findings that plant total N uptake controls species dominance (Tian et al.,

TABLE 4 | N storage distribution in the Chinese fir plantation ecosystem under different fertilization treatments (t N hm^{-2}).

N fertilizer rate (kg N $hm^{-2}a^{-1}$)	Chinese fir plant	Litter	Understory layer	Soil	Total in ecosystem
N0	0.91 ± 0.03	0.0199 ± 0.0019	0.0752 ± 0.0068	6.47 ± 0.46	7.48 ± 0.26
N60	0.97 ± 0.04	0.0318 ± 0.0024	0.0219 ± 0.0050	7.87 ± 0.15	8.90 ± 0.10
N120	1.17 ± 0.10	0.0559 ± 0.0113	0.0167 ± 0.0017	9.75 ± 0.20	10.99 ± 0.15
N240	1.27 ± 0.06	0.0303 ± 0.0028	0.0117 ± 0.0014	8.36 ± 0.54	9.67 ± 0.35

Chinese fir plant: leaves, branches, stems, bark, fine roots, and coarse roots of Chinese fir. Litter: litter leaves and branches in both L and F layers. Understory layer: small trees, lianas, shrubs, and herbs. Soil: total N storage of three soil layers (0–20 cm, 20–40 cm, and 40–60 cm). Total in ecosystem: the sum of Chinese fir plant, litter, understory layer, and soil. N0, control; N60, low-N; N120, medium-N; N240, high-N.

TABLE 5 | Recovery rate of N (%) by vegetation from N fertilizer of the 60, 120, and 240 kg $hm^{-2}a^{-1}$ treatments relative to the control.

N fertilizer rate (kg N $hm^{-2}a^{-1}$)	Chinese fir plant	Litter	Understory layer	Vegetation
N60	9.31	1.98	−8.88	2.41
N120	20.98	3.00	−4.88	19.11
N240	14.88	0.43	−2.65	12.67

Vegetation: the sum of all plant organs, litter, and understory layer.

2018) and that high levels of N fertilization decrease understory layer diversity (Gilliam et al., 2019). Results of Wu et al. (2021) at the same study site found that, over 13 years N addition, understory plant communities significantly decreased. N addition has previously been reported to reduce understory layer biomass and result in the replacement of dominant understory species, which Talhelm et al. (2013) and Midolo et al. (2019) attributed primarily to different N sensitivities and tolerances.

The control plots in our study provide us with clear insights into the growth and N storage of *C. lanceolata* under natural N deposition. Under no added N fertilizer, growth of *C. lanceolata* over 14 years resulted in substantial N storage (7.48 t N hm^{-2}), although N fertilization greatly promoted plant N storage. This effect was greatest in the N120 and N240 treatments, which had about 1.47x and 1.30x the N storage of the control treatment, respectively (**Figure 3**; **Table 3**). However, the highest N application was not associated with the highest ecosystem N storage, which only partially supports our first hypothesis. The positive effects of N fertilization on the forest ecosystem could be largely driven by the comprehensive effects of N cycles. However, the negative effects on N storage can result from extremely high N fertilization rates. High soil N storage in forest ecosystems and changes in labile soil N factors in response to extremely high N application rates are undeniable (Tian et al., 2011, 2018).

Higher N Storage After 14 Years of N Fertilization

N fertilizer applied to ecosystem can follow three major routes: absorption by plants, remain in the soil, or loss through several pathways (Ju and Zhang, 2003; Xu and He, 2020). Here, *C. lanceolata* accounted for over 90.55% of the total vegetation N storage and the soil (0–60 cm) stored an average of 87.64% of the total ecosystem stored N. When compared with the N stored in vegetation and the total amount of N fertilizer for 14 years, we found N loss was estimated to be over 86%. The N120 treatment showed the highest N recovery rate of all treatments, which did not support our second hypothesis. Although N recovery rate at extremely high doses of N fertilizer (240 kg N $hm^{-2}a^{-1}$) did not result in high N uptake, it is important to note the low N recovery rate (average 11.39%) and high loss of N (91.86% on average) after N fertilization, which indicate low plant N use efficiency. This is consistent with the hypothesis that increased atmospheric N deposition rates have led to lower plant N use efficiency (Liao et al., 2021).

Empirical evidence and meta-analysis indicate that N availability is a major factor determining plant growth (Kiba and Krapp, 2016; Liao et al., 2021). Plants can acquire N through their roots from soil inorganic (i.e., DON, NH_4^+-N and NO_3^--N) and organic (e.g., urea and amino acids) compounds (Kiba and Krapp, 2016; Congreves et al., 2021). The balance of soil N nutrients affects the utilization of N (Aber et al., 1998; Nordin et al., 2001; Kiba and Krapp, 2016). For example, plant community-level N uptake rates can be affected by nitrogen availability (Dybzinski et al., 2021) and low N availability can increase plant N acquisition efficiency (Liao et al., 2021). Zhou et al. (2021) suggested that conifers use different soil N resources (i.e., nitrate and ammonium), and NO_3^--N is a relatively large proportion (42%–52%) of total plant N uptake. Previous research from the same experimental site studied here showed that NH_4^+-N and NO_3^--N concentration in *C. lanceolata* increase with N deposition rate after 12 years N fertilization, with stronger responses from NO_3^--N (Shen et al., 2018), while medium-N and high-N significantly inhibited soil microbial biomass nitrogen with decreased percentage of 27.94%–29.50% (Shen et al., 2019b). However, there is not necessarily a significant association between N use efficiency and soil N availability, and soil P and plant P content can have strong effects on plant N use efficiency (Liao et al., 2021). We have an ongoing study to determine how changes in soil P and plant P content over time affect ecosystem N storage and N use efficiency after long-term N fertilization. The N recovery rate in this study is primarily based on natural N forms. The ^{15}N tracing method provides a good approach to study the fate and retention of N inputs in forest ecosystems over long time scales (Brumme et al., 2009; Gurmesa et al., 2016; Congreves et al., 2021). For example, in an N saturated old-growth tropical forest, Gurmesa et al. (2016) used ^{15}N tracer to estimate plant N recovery and total N retention in N-treatment plots.

Although we report several novel findings, there were limitations in our study design that could be addressed in future research. For example, calculating the N use efficiency without considering an increase or decrease in the soil N pool may lead to biased results (Tian et al., 2011). Vegetation as an important source of active N and changes in N storage in different vegetation types have high uncertainties (Xu et al., 2020). We focused only on *C. lanceolata* plantations, and studies of different tree species are needed to understand the generality of our findings, because plant community composition plays a key role in the use of different N sources (Nordin et al., 2001; Torre and Ávila, 2021). In addition, seasonal N cycling is important to forest N use (Li and Coleman, 2019); more vegetation samples should be collected during different growth seasons and parts (current-year, previous-year, or 2-year-old growth) to increase representation and generalization (Congreves et al., 2021). What's more, detrital biomass, mainly from coarse woody debris, is a large N sink in forests (Wu et al., 2020), and it is necessary to determine how the mass loss from detritus biomass influences ecosystem N pools. Therefore, to assess the applicability of our findings beyond subtropical conifer plantation management, additional studies are needed

covering a broad range of species, their N storage at different rates, and duration of N fertilization.

CONCLUSION

Our study highlights the importance of considering various plant organs and ecosystem components in accurately estimating ecosystem N storage. Our results partially support our first hypothesis—that N storage would increase with N fertilization—but do not support our second hypothesis, that N recovery rate would decrease with N fertilization. With increasing N application rate, *C. lanceolata* N storage increased but ecosystem N storage did not increase beyond a threshold of medium-N fertilization after 14 years. The total ecosystem N storage in fertilized plots was 1.1–1.4 times higher than that of control plots. Taken together, our results suggest that long-term N fertilization lowered N recovery rate and N use efficiency. These findings underscore the progress that has been made towards assessing N storage and regional N cycling.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

FS contributed to data curation, formal analysis, writing—original draft, visualization, conceptualization, and investigation. WL contributed to supervision, conceptualization, methodology, and resources. HD contributed to conceptualization, methodology, and writing—review and editing. JW contributed to supervision, methodology, project administration, software, and validation. CW contributed to writing—review and editing and investigation. YL contributed to writing—review and editing, validation, and funding acquisition. YY contributed to methodology, software, writing—review and editing, and funding acquisition. HF contributed to supervision, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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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.2022.914176/full#supplementary-material

Supplementary Figure 1 | Mean annual precipitation and mean annual temperature of the study site (climatology based on measurements over 14 years from 2004 to 2017).

Supplementary Figure 2 | Average DBH per treatment after 14 years N fertilization. N0, control; N60, low-N; N120, medium-N; N240, high-N. The values show means ± SE ($n = 3$).

Supplementary Figure 3 | Basal area increment **(A)** and annual basal area increment **(B)** from 1992-2017 under different N treatments. N0, control; N60, low-N; N120, medium-N; N240, high-N. The values show means ± SE ($n = 3$). "*" indicated differences at P<0.05 between different treatments.

Supplementary Figure 4 | Changes of understory layer species diversity **(A)** and biomass **(B)** under different N fertilization. N0, control; N60, low-N; N120, medium-N; N240, high-N. The values show means ± SE ($n = 3$). Lowercase letters indicate significant differences at $p < 0.05$ between different treatments.

Supplementary Figure 5 | Total litter input biomass **(A)** and leaf and branch biomass in L and F layers **(B)** under different N treatments. L-leaf: litter leaf of undecomposed layer; L-branch: litter branch of undecomposed layer; F-leaf: litter leaf of semi-decomposed layer; F-branch: branch of semi-decomposed layer. N0, control; N60, low-N; N120, medium-N; N240, high-N. The values show means ± SE ($n = 3$). Lowercase letters indicate significant differences at $p < 0.05$ between different treatments.

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Effects of Nitrogen Addition on Plant Properties and Microbiomes Under High Phosphorus Addition Level in the Alpine Steppe

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Nitrogen (N) addition can increase the vegetative growth, improve the plant production, and restore the degraded terrestrial ecosystems. But, it simultaneously aggravates the soil phosphorus (P) limitation for plant growth, thus affecting its positive effects on ecosystems. However, how plants and soil microorganisms will change under conditions of high P content in soil is still unknown. In this study, we explored the effects of three levels of N addition (0, 7.5, and 15 $g.N.m^{-2}.year^{-1}$) on plants and microorganisms at the high P addition level (13.09 $g.P.m^{-2}.year^{-1}$) in the alpine steppe. We found that the soil microbial community composition had no significant difference between different N addition levels, and the soil AN and AP had a significant effect on the phospholipid fatty acid (PLFA) composition. The abundance of the core PLFAs (i.e., 16:1ω7c, 16:0, a17:1, i17:0, 18:1ω9c, and 18:1ω7c) also remained unchanged after N addition, and microbes at individual, population, and community levels were all correlated with SOM, AK, AN, and pH. Conversely, plant biomass and nutrient content showed linear trends with increasing N addition, especially the dominant functional groups. Specifically, the biomass and plant tissue N content of *Gramineae*, and the total N content of aboveground biomass were all improved by N addition. They were correlated with soil ammonium and AP. The structural equation modeling (SEM) demonstrated that N addition had a direct negative effect on soil microbial biomass, but an indirect positive effect on aboveground biomass *via* soil ammonium. These findings clarify the importance of N-amendment in regulating plants and microorganisms under high P conditions and provide a better understanding of the N-added effects in the alpine steppe.

Keywords: the core species, the Qinghai-Tibetan plateau, nutrient uptake, plant–microbe interaction, nitrogen application

INTRODUCTION

Nitrogen (N) is an essential macro-element for plant growth and development (Mu and Chen, 2021), and they were usually transported to terrestrial ecosystems by anthropogenic N input and natural N deposition (Han et al., 2020). Most previous studies were mainly focused on the responses of plant biomass (Fu and Shen, 2016; Chen et al., 2018) and plant diversity (Foster and Gross, 1998; Bird and Choi, 2017; Soons et al., 2017) to N addition in different terrestrial ecosystems. With the increasing N addition, some studies revealed that the plant diversity was reduced (Bobbink et al., 1998; Stevens et al., 2004; Bird and Choi, 2017; Luo et al., 2019), while others were increased below 8.7 and 13.4 kg N $ha^{-1}.year^{-1}$ in open and closed-canopy vegetation across the continental United States (Simkin et al., 2016). Moreover, some results indicated that the plant diversity and biomass had no response after N addition into the tropical forest and alpine steppe, respectively (Lu et al., 2010; Dong et al., 2016). In addition, by collecting six local plant species (including *Erythronium Americanum*, *Dryopteris intermedia*, *Oxalis acetosella*, *Acer saccharum*, *Viola macloskeyi* F. Lloyd, and *Viola macloskeyi*) in a second-growth northern hardwood forest within the Catskill State Park in New York, Tessier and Raynal (2003) found that the concentrations of plant N were significantly different between plant species with *Oxalis* and *Viola* having the highest and Acer having the lowest, but not for plant populations at varied N-addition levels. Some studies contributed these controversies to the competitiveness of specific plant species that prefer higher N conditions or to eutrophication and soil acidification (Stevens et al., 2018) and the rate and period of N addition (Lu et al., 2010; Dong et al., 2016). Therefore, a better understanding of how plants respond to N addition is critical for maintaining biodiversity and improving plant production.

The impacts of N addition on plants were usually not only by affecting N element but also by interacting with phosphorus (P) to influence the N-induced impacts by creating a N:P imbalance in terrestrial ecosystems (Vitousek et al., 2010; Peñuelas et al., 2013). Before industrial revolution, plants mainly absorb P from soil parent materials, thereafter fertilizers become an essential source (Cordell et al., 2009; Vitousek et al., 2010; Elser and Bennett, 2011), resulting in substantial transfers of P in different ecosystems (Peñuelas et al., 2013). In some terrestrial ecosystems, such as forest, steppe, and meadow, combined application of N and P can enhance plant N and P uptake (Lü et al., 2013, 2016), while the sole application could cause N:P imbalance for plants (Peñuelas et al., 2013) and the aboveground biomass showed an asymptotic relationship with changes of the tissue N:P ratio (Peng et al., 2019). In addition, the impacts of N addition on soil microorganisms were mitigated by P addition in a P-limited paddy soil (Su et al., 2015), which changed their interaction with plants. These findings not only confirmed the positive effects of N addition on plant production but also highlighted that the un-continued positive effects were always related to the soil P conditions (Bobbink et al., 2010; Chen et al., 2019). However, it is still unknown whether P limitation is the main factor for the continued positive effects of N addition on terrestrial ecosystems.

Soil microbes play critical roles in global biogeochemical cycling and form strong bonds with plants in ecosystems (Van Der Heijden et al., 2008). Previous findings indicated that soil microbes can promote the plant growth by enhancing their nutrient acquisition (Adesemoye et al., 2009; Richardson et al., 2009). Under some scenarios in terrestrial ecosystems, soil microbes even act as drivers to plant community structures (Van Der Heijden et al., 2008). Moreover, soil microbes mediated the bioavailability of soil nutrients and aggregation formation (Rashid et al., 2016); conversely, soil microbes were also affected by N addition (Treseder, 2008; Leff et al., 2015; Zeng et al., 2016; Luo et al., 2019). However, some studies revealed that the microbial biomass could remain stable after N addition in the hardwood and pine stands (Frey et al., 2004). After N and P addition, the balance of soil N:P was disturbed, and soil archaea and bacteria responded differently to N, P, and NP additions due to their various urgent needs for N, P, or other resources (Adomako et al., 2022). Furthermore, soil microbes can alter the effects of N:P balance on plant performance, which also depends on nutrient conditions (Ma B. et al., 2019). Based on their key roles in the ecosystem, soil microbes had strong correlations with plants, anyway. Recent studies found that N addition may mediate edaphic properties firstly (Hu et al., 2010; Kang et al., 2018), and then changed the microbial community (Sarathchandra et al., 2001; Eghball, 2002). As the sensitive indicators of surrounding disturbances (Ma X. et al., 2019; Xiao et al., 2019), the soil microbial biomass and community structure, plants biomass, and stoichiometry can respond immediately to N addition which will help us to evaluate the N:P balance and manage the ecosystems, which need further exploration.

Given these problems, we conducted N-added field experiments in the alpine steppe, and previous studies proved that the alpine steppe is sensitive to climate change (Liu et al., 2013), especially in the Tibetan Plateau, which is more vulnerable and promptly responds to climate changes compared to most other regions on Earth due to its ecological fragility (Zhong et al., 2019). It was also reported that the annual N deposition rate reached 15.2 kg N ha^{-1} from 2010 to 2014 in this region (Xu et al., 2015), and prediction showed that the rate will be twice higher than that in the early 1990s by 2050 (Galloway et al., 2004; Basto et al., 2015). In addition, our previous studies found that P is a limited factor for plant and soil microbes at the same field station (Dong et al., 2016, 2020b). These phenomena would be an enormous disaster for the ecosystem, while we still do not know (1) how the plants and soil microbes will change, and (2) whether the positive effects of N addition on them will be continued at high P addition levels in the alpine steppe. Combined with N addition, we also added P fertilization to create a higher soil P condition, and we infer that the P is the main limited factor for plants and microbes if their biomass or nutrient properties would increase linearly with increasing N fertilization, otherwise the soil P is not the main limited factor for the ecosystem. These explorations will be helpful for humans to understand the impacts of increasing N content under conditions of high P

levels, and this might help us to better understand the N-added effects on terrestrial ecosystems.

MATERIALS AND METHODS

Introduction of the Field and Experimental Design

The field experiment (N31°26′, E90°02′, 4678 m a.s.l.) was performed in Baingoin County, Tibet Autonomous Region in southwest China (**Supplementary Figure 1**). This area is a semiarid cold alpine steppe and the soil is Gelic Cambisols according to the Food and Agriculture Organization of the United Nations (FAO) (Baumann et al., 2009; Dong et al., 2020a). As mentioned in our previous articles (Dong et al., 2016, 2020a), *Stipa purpurea* is the dominant plant species, and the accessory plant species are *Leontopodium leontopodioide* and *Heteropappus bowerii* in this place. The average annual precipitation is 301.2 mm, of which 80% falls in the growing season from June to September. The mean annual temperature is −1.2°C, and the maximum mean monthly temperature is 14.7°C in July. The background information of soil properties is presented in **Table 1**, which was also described in our former publication (Dong et al., 2016). To introduce it briefly, the soil TC, TN, and TP were 32.53, 1.65, and 0.62 g/kg, respectively; the soil AN and AP were about 128 and 5 mg/kg period for the establishment of treatments, and the soil pH was nearly 7. In addition, our experimental plots were grazed daily by yaks and sheep before fencing, and no fertilizing history was found. Due to overgrazing, they have been moderately or severely degraded.

The experimental plots were conducted by completely randomized block design in July 2013 on this field station. In each of the five blocks, three subplots were randomly assigned to three N additions (0, 7.5, and 15 g N m^{-2} $year^{-1}$, applied as urea), and each subplot was simultaneously fertilized with high P addition (13.09 g P m^{-2} $year^{-1}$, applied as monocalcium phosphate) to create a higher P condition. Each subplot was 5 × 5 m with five duplications and a 2-m buffer zone of any adjacent plots (**Supplementary Figure 1**). The dry powder of fertilizers was eventually applied over the respective plot at dusk twice each year at the time of the beginning and the vigorous period of plant growth.

Sampling and Analyses

At the vigorous period after 30 days of the second fertilization in September 2014, we surveyed the plant community (i.e., the height, coverage, and plant species) in a 1 × 1 m quadrate of each subplot. Briefly, the quadrates were randomly established in each subplot of four blocks, and each quadrat was divided into equal 100 small subquadrates (1 × 1 cm). We measured the plant height and recorded plant species at the same vertex of each subquadrate. After surveying the plant community, the aboveground biomass was clipped at the ground level and sorted by plant species at the same quadrate. Then, we collected the topsoil layer (0–10 cm) samples by mixing seven soil cores (3.5 cm diameter) in the same clipped subplots. Then, the plant samples were dried at 65°C until constant weight; soil samples were preprocessed to pick out the visible roots and stones and sieved through a 2-mm mesh, and finally separated into two subsamples. A subsample was stored in the room after being air-dried at room temperature for the analysis of some soil physicochemical properties, and the other subsample was stored in a refrigerator at −80°C for soil microbial analysis. The contents of soil organic matter (SOM), soil total N (TN), soil total P (TP), soil available N (AN), soil available P (AP), available potassium (AK), and soil pH were determined using the air-dried soil; the content of soil NH_4^+-N and moisture content (SMC) were determined using the fresh soil (Dong et al., 2016, 2020a).

Soil organic matter was measured using potassium dichromate oxidation and back titration with ferrous sulfate. SMC was determined by a gravimetric method after drying at 105°C for 24 h. AN was determined by the alkaline hydrolysis method. AP was determined using the molybdenum blue method after being extracted with sodium bicarbonate from soil samples. AK was determined using a flame photometric method after being extracted with ammonium acetate (Bao, 2000). Soil pH was measured by using a pH meter (OAKTON® pH, Oakton Instruments, Vernon Hills, IL, United States) at a ratio of 1:5 (weight/volume) for soil vs. distilled water. The content of soil NH_4^+-N was measured using an autoanalyzer (SmartChem140, AMS Alliance, Guidonia, Italy) in 2 M KCl extracts (1:4, soil: extractant). The dried soil samples were ground to a fine powder (through 0.15 mm sieve) to measure the TN and TP using the Kjeldahl method (Liao, 1981) and the molybdenum blue method with an ultraviolet-visible spectrophotometer (UV-2700, Shimadzu, Kyoto, Japan), respectively. The plant samples of each functional group (*Gramineae: S. purpurea, Poaannual*, and *Festuca coelestis; Compositae: L. leontopodioide* and *Heteropappus Puppyflower; Cyperaceae: C. oxyleuca V. Krecz, Carex moorcroftii*, and *Kobresia pygmaea*; and forb for other plants) were ground to a fine powder (using a 0.15 mm sieve) by mixing plant aboveground biomass according to their relative biomass occupied by the whole functional group, and then the total N and total P of each plant functional group were determined by using indophenol blue colorimetry and the Mo-Sb colorimetric method after being digested with H_2O_2-H_2SO_4, respectively (Dong et al., 2020b).

Phospholipid fatty acid (PLFA) profiling has a confidential ability to quantify the responses of soil microbes (Orwin et al., 2018). We used the standard procedure to extract PLFAs from 10 g of fresh soil, as described in detail by

TABLE 1 | The background information of soil properties before the experiment (Dong et al., 2016).

	SOM (g/Kg)	TN (g/Kg)	TP (g/Kg)	AN (mg/Kg)	AP (mg/Kg)	pH
0–10 cm	32.53	1.65	0.62	128.17	4.96	6.97
10–20 cm	18.8	1.09	0.74	77.3	3.04	7.04

SOM indicates total organic matter content in soil, TN indicates total nitrogen content in soil, TP indicates total phosphorus content in soil, AN indicates available nitrogen content in soil, and AP indicates available phosphorus content in soil.

Frostegård and Bååth (1996). Briefly, soil samples were extracted using an extraction mixture of chloroform:methanol:phosphate buffer (1:2:0.8, v/v/v). The extracted fatty acids were then fractionated using solid-phase extraction columns with chloroform, acetone, and methanol, respectively. Phospholipids were trans-esterified to fatty acid methyl esters (FAMEs) with 1:1 methanol:toluene and 0.2 M potassium hydroxide. Methyl nonadecanoate (19:0) was used as an internal standard to calculate each individual fatty acid concentration. The FAMEs were identified by using the MIDI Sherlock Microbial Identification System 6.0 (Microbial ID, Inc., Newark, DE 19713.) The abundance of individual PLFAs was expressed as nmol PLFA g^{-1} dry soil. We found that 22 biomarkers appeared in almost all of samples in this study. The gram-positive bacteria (G+) were presented by i15:0, a15:0, i16:0, i17:0, and a17:0; 16:1ω7c, cy17:0, 18:1ω7c, and cy19:0 were used to present the gram-negative bacteria (G−); the total soil bacteria were presented by combining G+ and G−. The saprotrophic fungus was presented by 18:1ω9c and 18:2ω6,9c; 16:1ω5c was used to present the arbuscular mycorrhizal fungus (AMF), and the total biomarkers of saprotrophic fungus and AMF were used to present the fungus. Notably, 16:0 10-methyl and 18:0 10-methyl were used to present the actinomycetes. Except bacteria, fungus, and actinomycetes, the total microbes were also present by the combination of i15:1, i16:1, 16:0 N alcohol, 16:0, a17:1, 17:1ω8c, 18:1ω5c, and 18:0 (Dong et al., 2020b).

Statistical Method

The responses of soil microbial community and plant properties to N addition at a higher P level were revealed using non-metric multidimensional scaling (NMDS) and permutation multivariate analysis of variance (PERMANOVA) using the adonis function in R package vegan. These analyses were performed by using individual PLFAs to reveal soil microbial community and by using plant traits (plant biomass and nutrient properties of all functional groups) to reveal plant community, respectively. The principal component analysis (PCA) was used to reduce the dimension and find the core factors based on their explained contribution to the first two dimensions (Zosso and Wiesenberg, 2021). The main effects of N addition on edaphic properties, plant properties, and soil microbes were analyzed by one-way analyses of variance (ANOVA) followed by a *post-hoc* mean test (LSD). Redundancy analysis (RDA) was applied to explore a combination of soil physicochemical properties that could explain the divergence in soil microbes and the plant community structure. The Pearson's correlations between soil physicochemical properties and plant properties or soil microbes were also calculated. The structural equation modeling (SEM) was used to explore the relationships between soil microbes, plant biomass, plant-nutrient traits, and edaphic properties by using the AMOS software (IBM SPSS AMOS 25, Chicago, IL, United States). All analyses were conducted using the R software v3.4.4.[1] The histograms and scatterplots were created using OriginPro 2017 (OriginLab Corporation, Northampton, MA, United States).

[1]www.r-project.org

RESULTS

Community Responses of Soil Microbes and Plants

To identify the principal PLFAs that caused the changes of microbial community, we employed PCAs based on all identified PLFAs. Principal components (PC) 1 (explained 68.9%) and PC2 (explained 27.4%) explained 96.3% of the variances (**Supplementary Figure 2**), and they were illustrated by six individual PLFAs (16:1ω7c, 16:0, a17:1, i17:0, 18:1ω9c, and 18:1ω7c), which were named core microbes (**Supplementary Figure 2**). Furthermore, the NMDS results showed that soil microbial community had no significant responses at different N application rates, and there was no linear trend with increasing N addition (**Figure 1A**).

Similar to soil microbial community, PC1 (explained 85.4%) and PC2 (explained 12.2%) together explained most variances (**Supplementary Figure 2**), and six plant variables (i.e., biomass of *Gramineae*, biomass of *Compositae*, and the total aboveground biomass, and they were termed as Plant_biomass; plant tissue N content of *Gramineae*, plant tissue content N of *Compositae*, and total plant tissue N contents, and they were termed as Plant_nutrient) were selected as the core plants traits (**Supplementary Figure 2**). Results of NMDS and PERMANOVA of plants showed that there were significant differences between varied N application rates, and there was a linearly changed trend, especially along the NMDS1 orientation (**Figure 1B**).

Responses of Core Microbes and Plant Traits

To further explore the responses of individual PLFAs upon increasing N addition, we employed one-way ANOVA for core microbes. Results showed that the core microbes had no significant difference between N application rates, and these individual PLFAs also had no linear trend with increasing N fertilization (**Figure 2**). These results confirmed that soil microbes had no responses to N addition, both at individual and community levels.

For Plant_biomass, results of one-way ANOVA showed that the biomass of *Gramineae* was increased by N addition, and each 1 g N m^{-2}.$year^{-1}$ shift was associated with a 14.851 g.m^{-2} aboveground biomass change. Moreover, there was a linear trend for *Gramineae* biomass with increasing N fertilization, while there was no significant linear trend for the biomass of *Compositea* and the total aboveground plant (**Figure 3A**). For Plant-nutrient, our results indicated that the TN of *Gramineae* and total plant community were all increased by N addition, and they all showed a linear trend with increasing N fertilization. In addition, each 1 g N m^{-2}.$year^{-1}$ shift was associated with 512.14 and 842.9 mg.m^{-2} TN for *Gramineae* and total plant community, respectively (**Figure 3B**).

Edaphic Factors Controlling Plants and Microbes

The RDA on soil microbial community constrained by soil properties was conducted to quantify the effects of soil variables

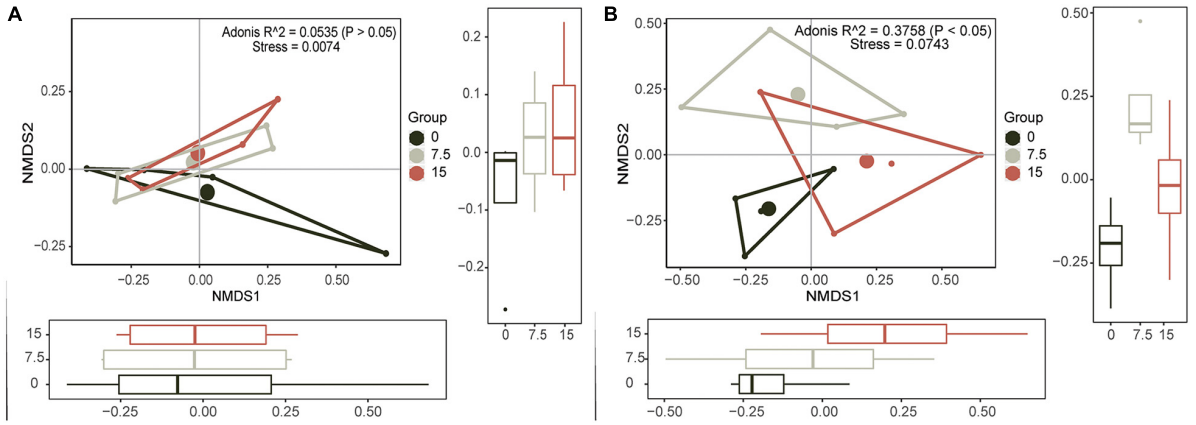

FIGURE 1 | Nonmetric multidimensional scaling (NMDS) plots show the relative differences in community composition of soil microbes **(A)** and plant **(B)** along the increasing N gradient.

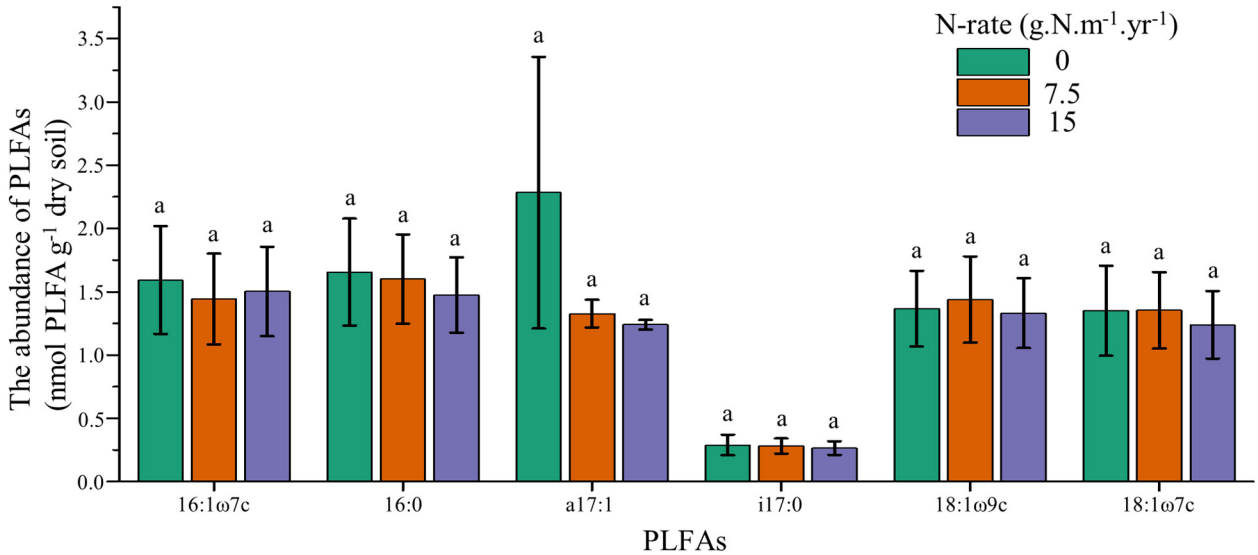

FIGURE 2 | Changes of core microbes under different N application rates. Treatments are expressed by data with means ± SE (n = 4). Different letters above boxes indicate significant differences between N application rates at the $P < 0.05$ level.

on the variation in soil microbial composition (**Figure 4A**). The first two axes explained 67.30% of the variation in the soil microbial community composition. The concentrations of soil AN, AP, and pH were correlated to RDA1, especially AN and AP statistically significantly explained most variations. We then used Pearson's correlations to decipher drivers for these significant decay relationships between microbes and soil properties. From individual PLFAs to functional populations, and then to microbial community levels, the soil microbes were consistently positively correlated with the concentration of SOM, AK, and AN, and negatively correlated with soil pH (**Figure 4D**).

The RDA was also used for plant community that was constrained by soil properties (**Figure 4B**). The first two axes explained 50.07% of the variation in plant community, and the concentration of SMC, NH_4^+-N, and AP could explain most variation. We further conducted the Pearson's correlation analysis between soil properties and plants, and the results showed that the concentration of soil NH_4^+-N was significantly correlated with *Gramineae* biomass, and the total N and P contents of *Gramineae* (**Figure 4C**). In addition, the concentration of soil AP had a significant correlation with aboveground biomass (**Figure 4C**).

The SEM was used to reveal the possible pathways through which soil and microbial attributes structure the aboveground biomass along the gradient of N application (χ^2 = 14.714; Df = 12; P = 0.257; **Figure 5**). This model could explain 37% of the variance in aboveground biomass, and 62% of the variance in microbial biomass. N addition had direct negative effects on soil microbial biomass, and positive indirect effects *via* environmental variables (i.e., NH_4^+-N and AN). For the

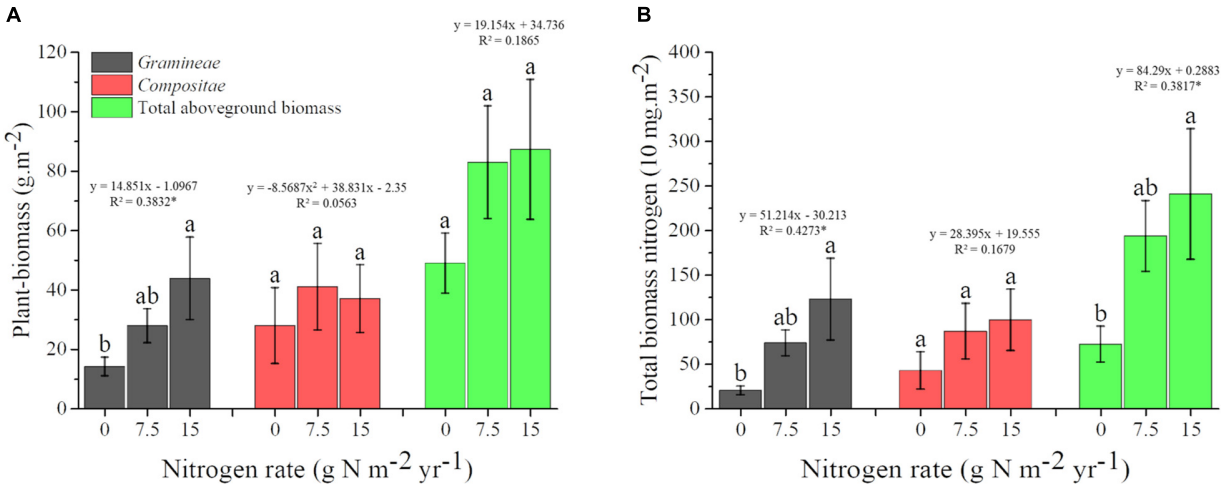

FIGURE 3 | Changes of plant-biomass **(A)** and total biomass nitrogen **(B)** under different N application rate. Treatments are expressed by data with means ± SE (n = 4). Different letters above boxes indicate significant differences between N application rates at the $P < 0.05$. Results of regression analysis were shown above boxes using its function and R2. The * indicates there were significant correlations between plant traits and N application rates at the $P < 0.05$ level.

variation of aboveground biomass, *Gramineae* biomass explained the largest proportion (58.3%), and N addition and soil NH_4^+-N *via Gramineae* biomass explained 30.5 and 31.5% of the variation in the aboveground biomass, respectively (**Figure 5**).

DISCUSSION

Our results reveal that plants have limited responses to N addition in the Tibetan alpine steppe, while soil microbes remain unchanged. Under higher P conditions, the total biomass N content of plant community and *Gramineae* population, and the biomass of *Gramineae* showed linear trends with the increasing gradient of N addition. Conversely, soil microbes had no significant changes facing N addition from individual PLFAs to microbial community levels. These results confirm that plants, especially dominant population, have more responses to N addition compared to soil microbes in the Tibetan alpine steppe, which is consistent with recent findings in the Songnen grassland of China (Gao et al., 2019); and soil N is the limited element for plant growth, but not for soil microbes. These findings improve our understanding of the plant and microbes as indicators of soil quality (Schloter et al., 2003) and ecosystem services (Pommier et al., 2018).

These varied responses of soil microbes and plants to N addition are likely due to their different correlations with environmental variables. The microbial community was usually constructed by their surroundings (Delgado-Baquerizo et al., 2018; Wu et al., 2019). After applying fertilizers, urea was firstly hydrolyzed to NH_4^+, and then denitrified to NO_3^- by ammonia-oxidizing bacteria (Dong et al., 2020a), resulting in more AN (ammonium + nitrite + nitrate) in the soil (Ma et al., 1999). However, only soil NH_4^+ was increased after N and P addition in this study, and no changes of soil AN was found, which may be due to the higher N loss in this area (Che et al., 2017), especially the higher preference of NO_3^--N by the local dominant plants (*S. purpurea* and *L. leontopodioide*) than NH_4^+-N (Hong et al., 2017; Dong et al., 2020a), causing the nitrification product (i.e., NO_3^-) to be immediately absorbed by plants (Caffrey et al., 2007; Shen et al., 2008; Dong et al., 2020a). Importantly, soil AN, SOM, pH, and AK were the main factors for soil microbes, while these parameters remained unchanged. As a result, the soil microbes showed no responses to N addition.

The N demand of plants and their preference for different N forms structured their responses to N addition in this study. Plants have evolved many sophisticated strategies to support their nutrient acquisition and growth (Biemelt and Sonnewald, 2006). According to our results, some plant traits (e.g., the *Gramineae* biomass, the TN content of *Gramineae,* and the aboveground biomass) showed linear trends with increasing N rate, which highlighted the N limitation for plant growth and N acquisition in this study. Furthermore, the monocotyledonous species *S. purpurea* has higher N absorption rates than the dicotyledonous species *L. pusillum* (Liu et al., 2013; Hong et al., 2018), and thus most proportion of N was absorbed by *S. purpurea*, resulting in higher plant biomass production of *Gramineae*. Interestingly, the total P content of *Gramineae* also showed strong correlations with soil NH_4^+ and was co-enhanced by N addition, indicating the N:P balance for plant productivity and growth (Chen and Chen, 2021). In addition, the soil ammonium, nitrite, and nitrate make up the soil AN, and we found that plants and soil NH_4^+ had positive correlations, while AN remained unchanged, which implied the negative correlations between plants and AN except soil NH_4^+, highlighting that these plants prefer nitrate in the Tibetan alpine steppe.

Our findings showed that plants had more sensitive responses to N addition than soil microbes, highlighting the dominant roles of plant in plant–microbe interactions. Plants usually play central roles in complex food webs, with numerous organisms relying

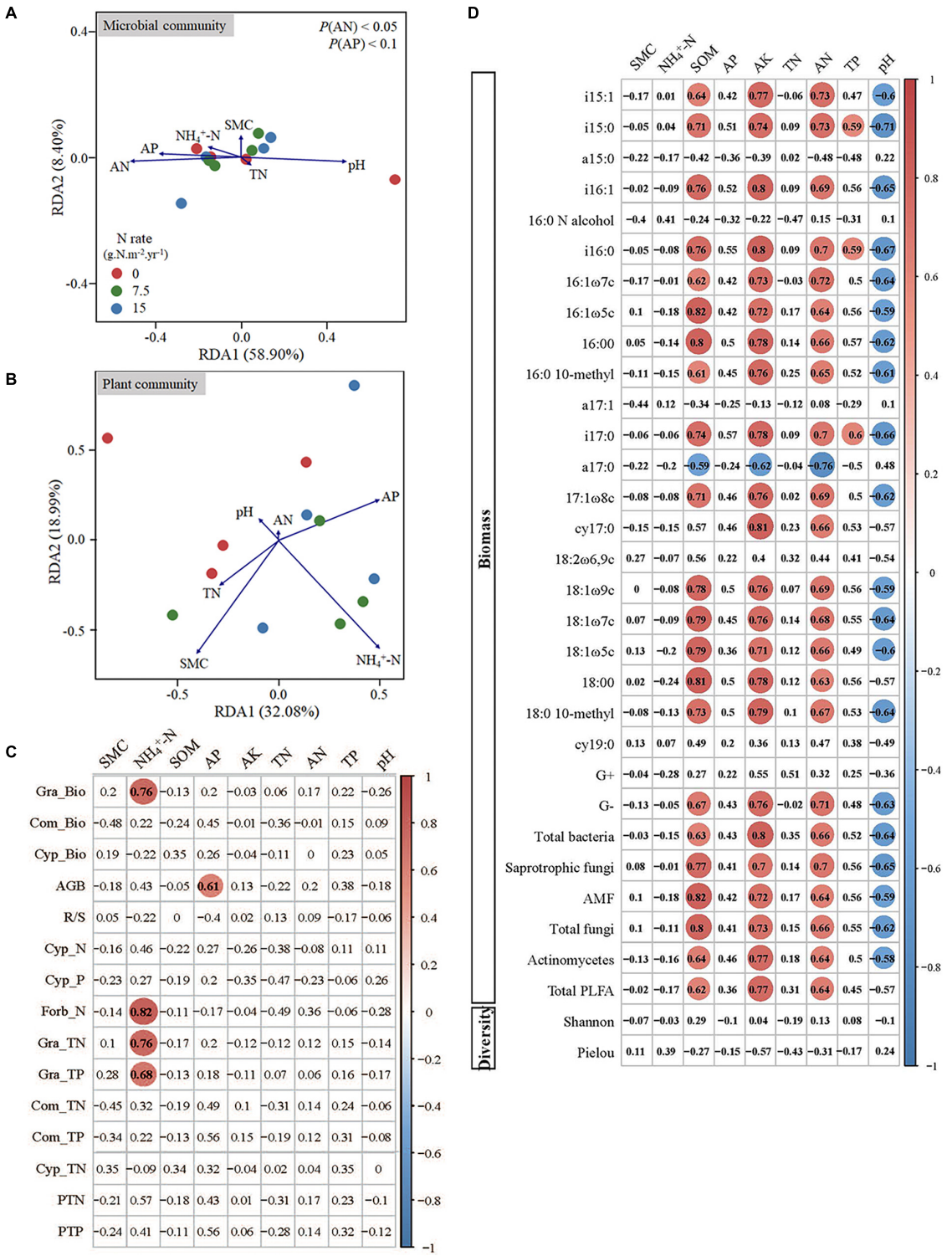

FIGURE 4 | Redundancy analysis biplot for the correlation of plant **(A)** and microbial community **(B)** with soil properties, and the correlations between soil properties and plant traits **(C)** and microbes **(D)**. Red circles in **(C)** and **(D)** indicate significant positive correlations, and blue circles indicate significant negative correlations. SMC indicates the soil moisture content; SOM indicates the soil organic matter content; AP indicates the soil available P; AK indicates the soil available potassium; TN indicates the soil total N content; AN indicates the soil available N; TP indicates the soil total P content; Gra indicates Gramineae; Com indicates Compositae; Cyp indicates Cypositae; AGB indicates the total aboveground biomass; -Bio indicates the aboveground biomass; R/S indicates the ratio of root to shoot biomass; -N indicates the concentration of total nitrogen; -P indicates the concentration of total phosphorus; -TN indicates the total nitrogen content; -TP indicates the total phosphorus content; PTN indicates the total nitrogen content of all aboveground biomass; PTP indicates the total phosphorus content of all aboveground biomass.

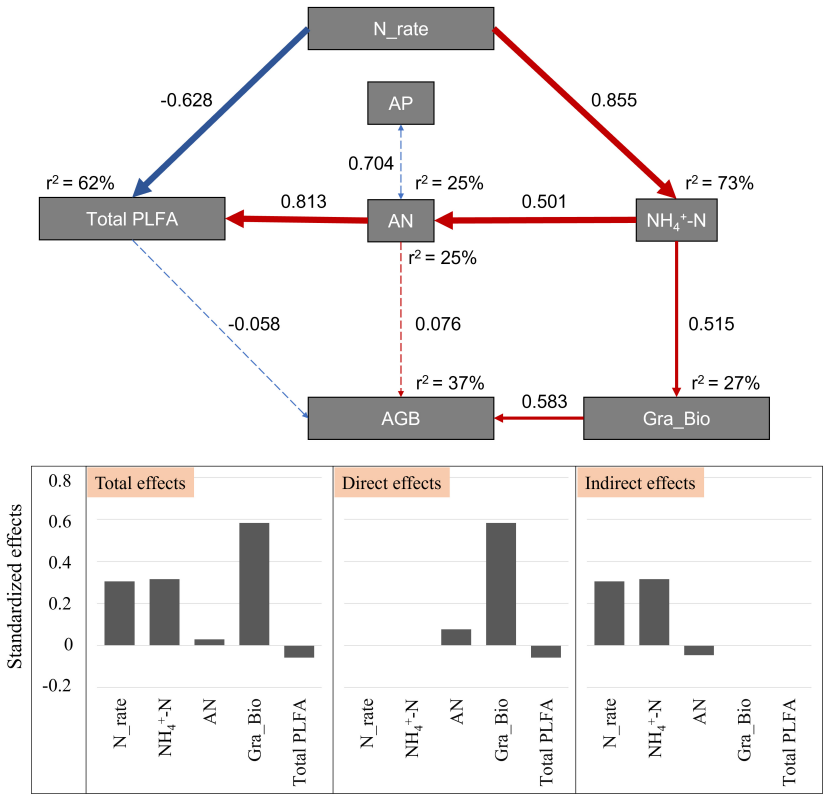

FIGURE 5 | Effects of environmental variables and soil microbes on aboveground biomass after N addition by structural equation model. Blue solid arrows indicate significant positive relationships, and red solid arrows indicate significant negative relationships. Blue dotted arrows indicate negative relationships, and red dotted arrows indicate positive relationships. The thickness of the arrow represents the strength of the relationship. Numbers next to the pathway represent the standardized path coefficients. r2 represents the amount of interpretation. Bar graphs are the standardized effects from SEM on the aboveground biomass. AP indicates the soil available P; AN indicates the soil available N; AGB indicates the total aboveground biomass; Gra_Bio indicates the aboveground biomass of Gramineae.

on their products of photosynthesis (Gruden et al., 2020). One of the most important ways was using root exudates to shape soil microbial community, but it was usually varied between different plant species or soil types (Kourtev et al., 2002; Haichar et al., 2008; Berg and Smalla, 2009). These findings must be based on their tight relationships. In this study, we found that N addition affected plants and soil microbes in different ways, with indirectly shifting plants *via* soil NH_4^+ and directly altering soil microbes. Compared to soil microbes, plants are a superior competitor for N uptake, and the fertilized N was immediately absorbed by plants to lessen their N limitation (Dong et al., 2020b). However, N addition reduced the correlations between plants and soil microbes (Wei et al., 2013), and simultaneously resulted in less photosynthate transport to soil surroundings from roots (Currey et al., 2011). In addition, under the ample P scenarios, we cannot figure out the P limitation for plants and soil microbes in this study, but our former study revealed that P was the limiting factor for soil microbes (Dong et al., 2020b). Taken together, plants absorbed the added-N immediately and the soil surroundings remained constant, resulting in the positive sensitive responses of plants while no changes for soil microbes.

We must point out that this study was conducted at a higher P level at the beginning of fertilization (after 2 years). These findings were limited, but we figured out the varied responses of plants and soil microbes to N addition, and the mechanisms of their different responses. We believe that our results can improve the prediction of responses of plants and microorganisms to N addition in the Tibetan alpine steppe, which might help us to find solutions to global climate changes we face. In addition, the responses of plants and soil microbes at different N rates and different P levels, and the long-term observation will be needed in the future to fully understand the stability of plants and soil microbes to nutrient addition in the Tibetan alpine steppe.

CONCLUSION

In the Tibetan alpine steppe, N was the limiting factor for plants, especially for the dominant functional groups that were indicated by their biomass and N content. These positive responses were related to the soil AN except ammonium, including soil nitrite and nitrate. Soil microbes remained unchanged, which was due to the lessen relationships with plants and their lower competitiveness for N uptake than plants after N addition. We can conclude that N addition was first

beneficial to the dominant plants, by increasing their production and nutrient acquisition and loosening their correlations with soil microbes. These findings would help us to select proper indicators to evaluate the soil quality and ecosystem services at the beginning after fertilization and to understand the plant–microbe interaction in the alpine steppe.

DATA AVAILABILITY STATEMENT

The original contributions presented in this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XC, HN, and SW designed the experiment. JD, JZ, CZ, and ZP conducted the experiment. JD and LL analyzed the data. All authors prepared and approved 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.2022.894365/full#supplementary-material

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Effects of Nitrogen Addition on Soil Carbon-Fixing Microbial Diversity on Different Slopes in a Degraded Alpine Meadow

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Autotrophic carbon-fixing bacteria are a major driver of carbon sequestration and elemental cycling in grassland ecosystems. The characteristics of the response of carbon-fixing bacterial communities to nitrogen (N) addition in degraded alpine meadows are unclear. In this study, it was investigated that the effects of N addition in three levels [they are low (LN), middle (MN), and high (HN) with N supplement of 2, 5, and 10 g $N{\cdot}m^{-2}{\cdot}a^{-1}$, respectively] on soil carbon-fixing bacteria on different slopes in a degraded alpine meadow in the Yellow River on the Qinghai–Tibet Plateau. The results showed that there were significant differences in the abundance of some low abundance genera of carbon-fixing bacteria on the same slope ($P < 0.05$), but the differences in the abundance of various phyla and dominant genera were not significant. MN on gentle slopes significantly reduced the Chao1 index and observed species ($P < 0.05$), whereas N addition on steep slopes had no significant effect on the diversity. The abundance of the Cyanobacteria phylum and 28 genera of identified carbon-fixing bacteria differed significantly between slopes ($P < 0.05$), and observed species of carbon-fixing bacteria were significantly higher on steep slopes than on gentle slopes ($P < 0.05$). Factors affecting the carbon-fixing bacteria community structure include slope, N addition, ammoniacal nitrogen ($N\text{-}NH_4^+$), microbial biomass carbon (MBC), soil water content (SWC), pH, soil C:N ratio, and microbial C:N ratio. Slope, N addition, soil physicochemical properties, microbial biomass, and stoichiometric ratio did not significantly affect the carbon-fixing bacteria diversity. Thus, the effect of exogenous N addition on carbon-fixing bacteria in degraded alpine meadows was dependent on slope conditions, and the response of carbon-fixing bacteria abundance and species number to N addition on gently slope sites was threshold-limited.

Keywords: Qinghai–Tibet plateau, nitrogen addition, carbon-fixing bacteria, *cbbL* gene, alpine meadow, stoichiometric ratio

INTRODUCTION

The global soil carbon pool is about 2.2–3.0 × 10^3 Pg (Cao et al., 2017), and it is the most important carbon sink for terrestrial ecosystems. The carbon sequestration is three times higher than the terrestrial vegetation carbon pool and four times higher than the atmospheric carbon pool (McCarl et al., 2007; Lacis et al., 2010; Vicente-Vicente et al., 2016). Soil carbon pools occupy an important position in the terrestrial carbon cycle, and small changes in them can lead to an increase in CO_2 emissions to the atmosphere, altering the global carbon balance and affecting global climate change (Yousuf et al., 2012). Biological carbon fixation is the most direct and effective way to sequester CO_2 in terrestrial ecosystems, and the main organisms that can fix CO_2 are plants and autotrophic microorganisms (Liu et al., 2018). Among them, autotrophic microorganisms are widely distributed and have strong environmental adaptability in converting CO_2 into soil organic carbon to regulate atmospheric CO_2 concentration and increase soil carbon sequestration (Ge et al., 2013). Some studies have shown that the carbon assimilation by microorganisms in wetlands, grasslands, and forests ranges from 10.3 to 40.1 mg kg^{-1} in 15 days (Lynn et al., 2016), and from the perspective of the material cycle and energy flow of the entire biosphere, CO_2 fixation by microorganisms is a biological carbon fixation force that cannot be ignored (Elsaied and Naganuma, 2001; Nanba et al., 2004). Therefore, it is of great scientific importance to study the ecological and environmental effects of CO_2 fixation by autotrophic microorganisms. The Calvin cycle is one of the key pathways for CO_2 fixation by autotrophic soil bacteria, with the highest carbon sequestration efficiency. This enzyme-catalyzed cycle is catalyzed by Ribulose-1,5-bisphosphate carboxylase or oxygenase (RubisCO), which is encoded by the *cbbL* gene (Tabita, 1999). Therefore, the *cbbL* gene has been widely used by many scholars as a biomarker for the ecological characterization of autotrophic bacteria in soil environments of different ecosystems, including agricultural fields and lakes (Elsaied and Naganuma, 2001; Ge et al., 2013).

With an average altitude over 4,000 m, the Qinghai–Tibet Plateau (QTP) is known as the "roof of the world" and the "third pole." Due to its unique geographical location and climatic conditions, it has formed a typical alpine grassland ecosystem. The alpine meadows cover an area of 1.28 × 10^6 km^2, which is the largest alpine meadows in China (Zhang et al., 2002). The unique biogeochemical processes and fragile ecological environment of alpine meadows make them more sensitive to global climate change and anthropogenic disturbances (Lan, 2004; Wischnewski et al., 2011) and have been ideal sites for ecological studies of diversity distribution patterns. In recent years, the alpine meadow ecosystem has been severely damaged by natural and anthropogenic factors, with large areas of good grassland degraded to bare ground or "heitutan," and the phenomenon of grassland desertification and salinization intensified (Wang et al., 2005), especially in the Sanjiangyuan area in the center of QTP (Chong and Zhang, 2015; Shao et al., 2017). Heitutan is characterized by increased bare land proportion, reduced edible herbage, and commensurate increases in the dominance of unpalatable species (Li et al., 2014). Nearly 4,908,900 hectare of alpine meadows have been degraded to heitutan, with 3,122,400 hectare of degraded heitutan (slopes $< 7°$) on flat areas, 1,533,800 hectare on gentle slopes ($7° \leq$ slopes $< 25°$), and 252,800 hectare on steep slopes ($25° \leq$ slopes) (Dong et al., 2015). In contrast, alpine meadow soils store large amounts of root and organic carbon, which is an important global carbon reservoir and profoundly affects the global terrestrial ecosystem carbon cycle (Fang et al., 1996; Wang L. et al., 2019). The degradation of alpine meadows will undoubtedly have a negative impact on the ecological security of the QTP and even the world (Wang et al., 2021). Therefore, effective measures must be taken to control degraded alpine meadows.

Nitrogen (N) is the most important nutrient limiting plant growth in many ecosystems, and N addition is the most common management to promote productivity in degraded grasslands and maintain nutrient balance in grassland ecosystems (Wang et al., 2017). Exogenous N inputs can increase the amount of available N in the soil, alleviate or eliminate N limitation, change the chemical elemental composition of the soil, and promote plant growth (Verburg et al., 2010; Zhang et al., 2014; Chen et al., 2017). Soil microorganisms have rapid reproduction rates and respond rapidly to soil physicochemical properties changes (Wang et al., 2022), and N addition can affect the composition, structure, and function of soil microbial communities (Wang et al., 2014; Liu H. M. et al., 2017). For example, Yuan et al. (2012) found that long-term fertilization had significant effects on the structure, diversity, and abundance of soil carbon-fixing bacteria revealing the effects of different fertilization regimes (NPK and NPK with straw return) on soil carbon-fixing bacteria. Wang R. et al. (2019) studied changes in the abundance and structure of carbon-fixing bacterial communities in white pulp soils after the conversion of forest land to cropland in the hilly areas of Northeast China and showed that fertilizer application (NPK compound fertilizer) caused changes in the abundance and diversity of carbon-fixing bacterial communities. Zhou et al. (2019) studied the effects of long-term chemical fertilizer regimes (NP, NK, PK, and NPK) on the activity and community composition of soil autotrophic bacteria and showed that the application of chemical fertilizers altered the ecological characteristics of carbon-fixing bacteria and had a significant effect on the carbon-fixing bacteria diversity. Selesi et al. (2005) found significant changes in carbon-fixing bacterial communities' composition and diversity after the application of mineral fertilizers and mixed compost in wheat field soils. Current studies have focused on changes in soil carbon-fixing bacterial communities after fertilizer application in soils such as paddy fields and arable land, and experimental results have also shown that the structure and diversity of carbon-fixing bacterial communities are sensitive to fertilizers. But the report of N addition effecting on carbon-fixing microorganisms in degraded alpine meadows is rare. In addition, due to the complex terrain characterized by the vertical and horizontal ravines in the Sanjiangyuan region, and the large distribution span of different types of terrain such as slope, the composition and diversity of carbon-fixing microbial community may vary in different slopes of the nitrogenous grassland. Rare studies compare carbon-fixing microbial community on different slopes. Therefore, it is of great scientific significance to study the effects

of N addition on soil carbon-fixing microorganisms on different slopes in degraded meadow ecosystems.

Thus, this study aims to answer the following two scientific questions to understand the alpine meadow soil carbon-fixing bacterial communities change rule and provide reference for scientific fertilization system of the degraded alpine meadow in the Sanjiangyuan region: (1) Is there any difference in the characteristics of carbon-fixing microorganisms between N addition levels on different hill slopes in the degraded alpine meadow? (2) What is the relationship between carbon-fixing microorganisms and slope, N addition, microbial biomass, and physicochemical properties?

MATERIALS AND METHODS

Study Area

The sampling site is located in Dawu Town, Maqin County, Guoluo Tibetan Autonomous Prefecture, Qinghai Province, west China (34°25′20.41″ N, 100°19′55.72″ E, average altitude 3,768 m) (**Figure 1**), with a continental cold climate. The average annual temperature is −3.9 to −3.5°C, and the average annual precipitation is 423–565 mm. The surface soil is about 4–10 cm of turf layer, and it belongs to a type of alpine meadow soil. Due to the disturbance of overgrazing, rodent activity, and climate change, this place has become a moderate degraded alpine grassland with vegetation coverage less than 80%. The uncontrolled grazing all year round. The plant community composition of the test sites on the gentle slope and steep slope is basically the same, consisting of *Kobresia humilis*, *Kobresia pygmaea*, *Poa pratensis*, *Elymus nutans*, *Ajania tenuifolia*, *Ligularia virgaurea*, *Aconitum flavum*, *Oplopanax elatus*, *Euphorbia fischeriana*, etceteras.

Experimental Design

In early May 2019, a square of 50 m × 50 m was selected as the sampling sites in steep slope (S) and gentle slope (G) with the slope angle of 27° and 8°, respectively (**Figure 2A**). The aspect of the sampling slope was 12.3° from west to north. A randomized block experimental design was adopted, and 16 quadrats (5 m × 5 m) were set up in two sampling areas, a total of 32 quadrats were set up to conduct N addition experiments with four levels including low equal nitrogen supplemental level (LN): 2 g $N{\cdot}m^{-2}{\cdot}a^{-1}$, middle nitrogen supplemental level (MN): 5 g $N{\cdot}m^{-2}{\cdot}a^{-1}$, high equal amount of nitrogen (HN): 10 g $N{\cdot}m^{-2}{\cdot}a^{-1}$, and 0 g $N{\cdot}m^{-2}{\cdot}a^{-1}$ served as the control (CK). Buffer zone between quadrats was 2.5 m (**Figure 2B**). After the plot was established, nitrogen supplement (NH_4NO_3, grain loading, nitrogen content 35%) was added when the weather was not rainy to reduce fertilizer loss.

Sample Analysis

Soil Sample Collection

In May 2019, the first fertilization was applied. In May 2020, fertilization was applied again in the second year. In August 2020, samples were collected in five plots (10 cm × 10 cm) when herbage was growing vigorously (**Figure 2C**). Sampling tools were disinfected with alcohol, soil samples with the same weight were collected from 0 to 10 cm soil layer using a soil corer (0–10 cm deep with a 3 cm inner diameter), and then they were mixed to obtain one representative composite sample (totally 32 samples). After removing impurities such as roots, a certain amount of soil was put into an aseptic tube. Approximately 10 g of each sample was collected and quickly frozen in a tank of liquid N. After being brought back to the laboratory, it was immediately sealed and transported for sample testing with a large volume of dry ice. We entrusted the determination of soil samples *cbbL* gene to Beijing Allwegene Technology Co., Ltd. The remaining soil samples were brought back to the laboratory. The natural air drying and grinding and screening were conducted for soil physicochemical properties and microbial biomass analysis (**Supplementary Tables 1**, **2** for the determination methods and values).

Soil Microbial Community Structure and Diversity Analysis

The gene primers were *cbbL*-F: 5′-GACTTCACCAAAGA CGACGA-3′ and *cbbL*-R: 5′-TCGAACTTGATTTCTTTCCAC-3′; *cbbL*-F: 5′-CATCATGTTCGACCAGGACT-3′ and *cbbL*-R: 5′-TCGAACTTGATTTCTTTCCA-3′ (Ji et al., 2016). The test steps are: genomic DNA extraction—genomic DNA quality inspection—PCR amplification—PCR product electrophoresis detection—PCR product purification—Miseq library construction—Miseq library quality inspection—Illumina Miseq machine sequencing platform.

DNA Extraction

Soil microbiome DNA was extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., CA) [Omega E.Z.N.A. Stool DNA Kit (Omega Bio-tek, Inc., Beijing, China)] kit instructions. The extracted DNA was assayed for DNA quality and concentration using a Nanodrop 2000 (Thermo Fisher Scientific, Inc., MA, United States). The samples were stored at -20°C for subsequent experiments.

PCR Amplification

PCR reaction system (total system is 25 μL): 12.5 μL 2xTaq Plus Master Mix II (Vazyme Biotech Co., Ltd., China), 3 μL BSA (2 ng/μL), 1 μL Forward Primer (5 μM), 1 μL Reverse Reaction parameters: 95°C pre-denaturation for 5 min; denaturation at 95°C for 45 s, annealing at 55°C for 50 s, extension at 72°C for 45 s, 28 cycles; extension at 72°C for 10 min. The PCR products were amplified using 1% agarose gel electrophoresis to detect the size of the amplified target bands and purified using the Agencourt AMPure XP (Beckman Coulter, Inc., FL, United States) nucleic acid purification kit.

MiSeq Sequencing

PCR products were used to construct microbial diversity sequencing libraries using the NEB Next Ultra II DNA Library Prep Kit (New England Biolabs, Inc., MA, United States) library builder kit. Paired-end sequencing was performed at Beijing Allwegene Technology Co., Ltd., Beijing, China using the

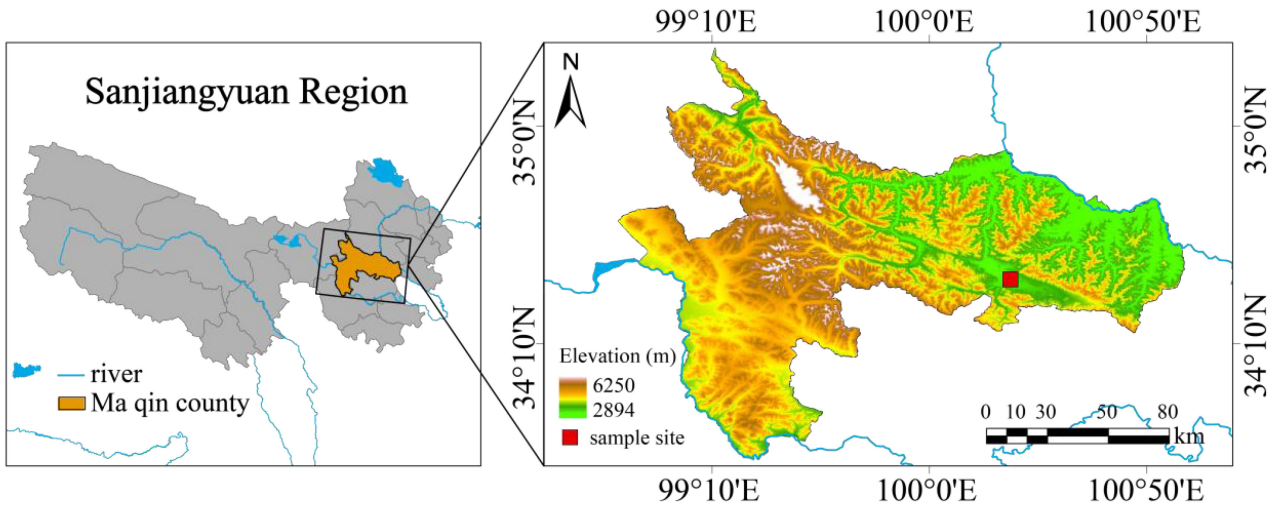

FIGURE 1 | Location of the study site.

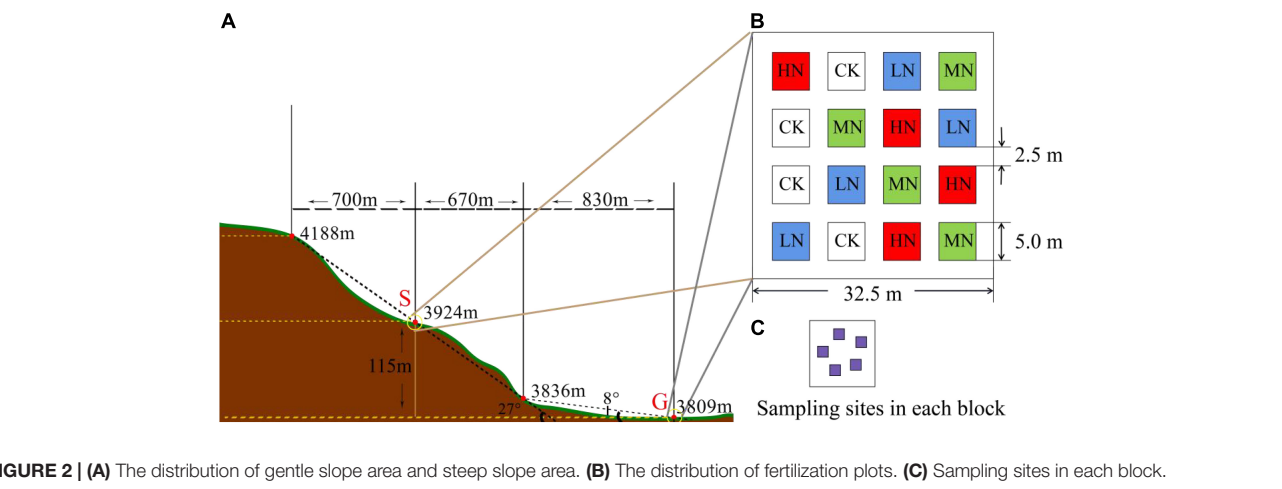

FIGURE 2 | (A) The distribution of gentle slope area and steep slope area. **(B)** The distribution of fertilization plots. **(C)** Sampling sites in each block.

Illumina Miseq PE300 (Illumina, Inc., CA, United States) high-throughput sequencing platform. The sequenced raw sequences were uploaded to NCBI's SRA database.

In order to make the analysis results more accurate and reliable, after the sequencing raw data were launched, data quality control was first performed to obtain optimized sequences through sequence splicing, filtering, and chimera removal, and then OTUs (operational taxonomic units) clustering and annotation were performed. Bioinformatic statistical analysis of OTUs at 97% similarity level was performed using the UPARSE method (Edgar, 2013). Based on the clustering results, diversity analysis could be performed; the OTUs were annotated using the Silva (Release 128/132)[1] database (Quast et al., 2012), and based on the annotation results, species information for each taxon could be obtained, allowing correlation analysis of sample composition and differences in community outcomes between samples.

Alpha diversity analysis includes Chao1, Observed_species, and Shannon, to estimate the species abundance and diversity of environmental communities.

Beta diversity analysis (non-metric multidimensional scaling method, NMDS) focuses on differences in microbial community composition between samples (Rivas et al., 2013).

Data Statistics

The analysis platform of this study is Qiime platform[2], and Kruskal–Wallis test is carried out in R programming (qvalue package) to analyze the N addition treatments for the same slope as well as OTUs of soil carbon-fixing microorganisms and the abundance differences of carbon-fixing bacteria phyla and genus in different slopes.

One-way ANOVA and Duncan are conducted by SPSS Statistics 20.0 statistical analysis software to analyze the effects of different N addition treatments on soil physicochemical

[1]https://www.arb-silva.de/

[2]http://qiime.org/scripts/assign_taxonomy.html

properties, stoichiometric proportion, microbial biomass, Chao1 index, observed species number, and Shannon index for the same slope. Independent sample *t*-test is used to analyze the differences of soil physicochemical properties, stoichiometric proportion, microbial biomass, Chao1 index, observed species number, and Shannon index between different slopes ($\alpha = 0.05$).

Venn diagram and species composition histogram was calculated and plotted by R language tool. The dilution curve was analyzed by mothur based on OTUs clustering results. For NMDS analysis, vegan and ggplot2 packages in R were used for data calculation and mapping. Network analysis was accomplished by R language package and psych package. Mantel test and Pearson correlation analysis were performed with R language ggplot2, ggcor, and dplyr software packages.

RESULTS

Abundance of Soil Carbon-Fixing Bacteria Species

On gentle slope, the number of OTUs common to all treatment soil samples was 246, accounting for 57.3%, while the number of OTUs specific to CK, LN, MN, and HN was 8, 1, 11, and 26, accounting for 1.9, 0.2, 2.6, and 6.1%, respectively (**Figure 3A**). On steep slope, the number of OTUs common to all treatment soil samples was 295, accounting for 41.3%, while the number of OTUs specific to CK, LN, MN, and HN was 71, 21, 19, and 79, accounting for 9.9, 2.9, 2.7, and 11.0%, respectively (**Figure 3B**). The number of OTUs shared between the gentle slope and steep slope soil samples was 559, a proportion of 56.1%, with 101 OTUs specific to the gentle slope and 337 OTUs specific to the steep slope (**Figure 3C**). The Kruskal-Wallis test for OTUs showed that 27 OTUs differed significantly ($P < 0.05$) between N application treatments on gentle slope, 22 OTUs differed ($P < 0.05$) between N application treatments on steep slope, and 450 OTUs differed significantly ($P < 0.05$) between gentle slope and steep slope (**Supplementary Table 3**). A total of 7 Phylum, 11 Classes, 22 Orders, 42 Families, 76 Genus, and 109 Species of carbon-fixing microbial OTUs were identified in the taxonomic lineage.

Community Composition and Relative Abundance of Soil Carbon-Fixing Bacteria

Most of these microorganisms could only be classified in the bacterial groups of the higher taxonomic levels, Proteobacteria and Actinobacteria. Using the phylum as the taxonomic level (**Supplementary Figure 1**), Proteobacteria was the dominant phylum, with the relative abundance of Proteobacteria above 93.0% in all but one sample, SCK_4. At the genus level (**Supplementary Figure 2**), *Sulfurifustis* was the dominant genus with relative abundances ranging from 16.5 to 90.9%, respectively, with an average abundance of 56.5%. The Kruskal–Wallis test showed that at the phylum level, the variation in abundance of each phylum of N-added carbon-fixing bacteria on the same slope was not significant. Only Cyanobacteria with mean abundance <1% differed between gentle slope and steep slope ($P < 0.05$) (**Supplementary Table 4**). At the genus level, the dominant genera were not significantly different between treatments on the same slope and between slopes, but some low abundance genera of carbon-fixing bacteria (<0.5% relative abundance) were significantly different ($P < 0.05$). For example, N addition differed in *Thiorhodococcus*, *Rhodovulum*, *Serpentinomonas* on gentle slope and in *Ectothiorhodospira*, *Sulfuricaulis*, *Cupriavidus* on steep slope ($P < 0.05$). The results of the Kruskal–Wallis one-way AVOVA multiple comparison test for differences between treatments are shown in **Supplementary Tables 5**, **6**. On gentle slope, HN significantly increased the relative abundance of *Rhodovulum* compared to the control ($P < 0.05$). On steep slope, the relative abundance of Ectothiorhodospira was significantly increased ($P < 0.05$) under MN and HN and *Cupriavidus* was significantly increased ($P < 0.05$) under HN compared to the control. There were significant differences ($P < 0.05$) between slopes in the 28 genera of identified carbon-fixing bacteria. The relative abundance of *Cupriavidus* and *Alkalispirillum* was significantly higher on steep slopes than on gentle slope, while the relative abundance of *Rhodovulum*. was significantly lower than on gentle slope (**Supplementary Table 7**). This indicates that the effects of N addition and slope on the dominant phylum and genus of carbon-fixing bacteria in degraded alpine meadows were not significant, but only on some low abundance phylum and genus of carbon-fixing bacteria.

Microbial Network Correlation

The microbial network interactions map shows the complex relationships consisting of highly linked genera, which on gentle slope (**Figure 4A**) form four modules, each belonging to the Proteobacteria. The first module consisted of *Thiobacillus* and *Halorhodospira*, the second, third and fourth modules consisted of *Thiomonas* and *Cupriavidus*, *Bradyrhizobium* and *Alkalispirillum*, *Marichromatium*, and *Endothiovibrio*, respectively, with positive correlations between them ($P < 0.05$). On steep slope (**Figure 4B**), *Diploblechnum* belonged to Streptophyta, *Synechococcus* to Cyanobacteria and the other 10 genera to Proteobacteria. Of these, *Cupriavidus*, which differed significantly between the different N addition treatments, was negatively correlated with the dominant genus *Sulfurifustis* ($P < 0.05$). All other genera were positively correlated with each other. The network interactions between gentle slope and steep slope showed that *Alkalispirillum* and *Bradyrhizobium*, which differed significantly, were positively correlated ($P < 0.05$) (**Figure 4C**).

Carbon-Fixing Community Diversity

The results of the one-way ANOVA showed that MN significantly reduced the Chao1 richness and observed species values of carbon-fixing bacteria on gentle slope ($P < 0.05$). Whereas N addition had no significant effect on Chao1 index, observed species number and Shannon diversity on steep slope (**Figure 5**). Independent sample *t*-tests showed that the observed species number was significantly higher on

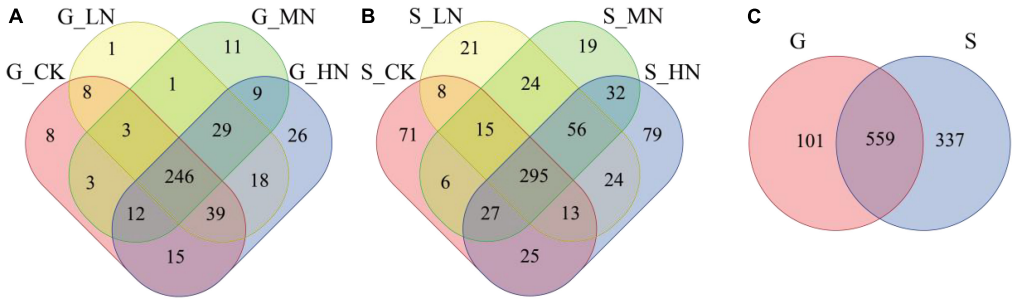

FIGURE 3 | Venn diagrams of different slopes and different nitrogen addition treatments (**A**, the number OTU on gentle slope; **B**, the number of OTU on steep slope; **C**, total OTU number for both gentle slope and steep slope).

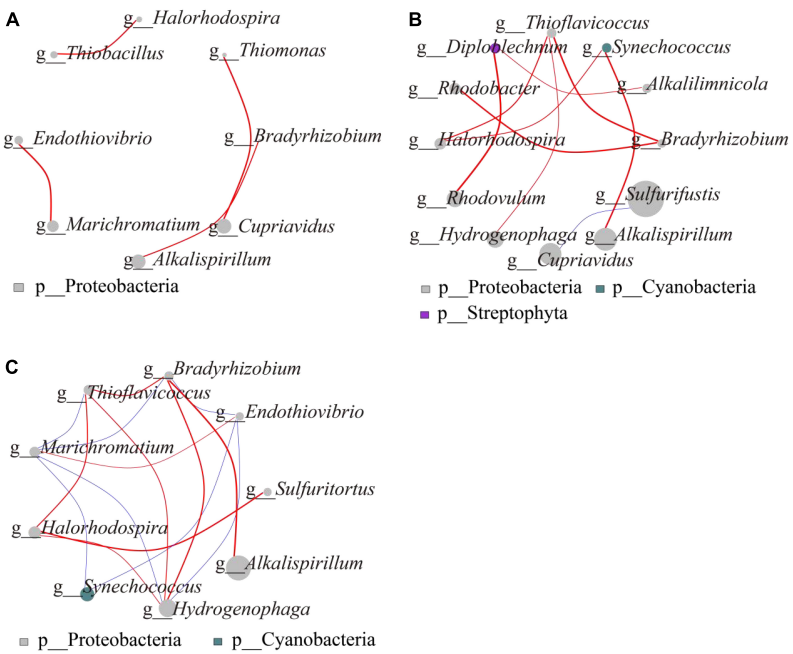

FIGURE 4 | Microbial network interaction of carbon-fixing microorganisms (**A**, microbial network interaction on gentle slope; **B**, microbial network interaction on steep slopes; **C**, network interaction between gentle slope and steep slope). The size of the dot in the graph represents the abundance and the thickness of the line represents the correlation; the color of the dot represents the door to which it belongs, the red line represents a positive correlation, and the blue line represents a negative correlation.

steep slope than on gentle slope ($P < 0.05$). The results of NMDS showed that different treatments on the same slope and different treatment groups on different slopes intersected with each other, indicating that none of the differences in soil carbon-fixing microorganisms between groups were significant (**Figure 6**).

Comparison of Gentle Slope/Steep Slope Soil in CK

Under natural conditions (CK), There were significant differences in TK, MBC and soil C:N between gentle slope and steep slope. Other soil nutrients, stoichiometric ratio, community composition, and diversity were similar (**Supplementary Table 8**).

Carbon-Fixing Bacterial Communities in Relation to Microbial Biomass and Soil Physicochemical Properties

Pearson correlation analysis showed significant positive correlations between slope and SOM ($r = 0.454$, $P = 0.009$), AN ($r = 0.369$, $P = 0.038$), MBC ($r = 0.630$, $P = 0.000$), and microbial C:N ($r = 0.701$, $P = 0.000$), and significant negative correlations with TK ($r = -0.542$, $P = 0.001$), pH ($r = -0.867$, $P = 0.000$), and microbial N:P ($r = -0.417$, $P = 0.018$) (**Figure 7**). There was no significant correlation between the level of N addition and the physicochemical properties and microbial biomass of the soil. The results of Mantel test analysis showed that slope, N level, N-NH_4^+, MBC, SWC, pH, soil C:N, microbial C:N, and carbon-fixing bacteria community abundance were

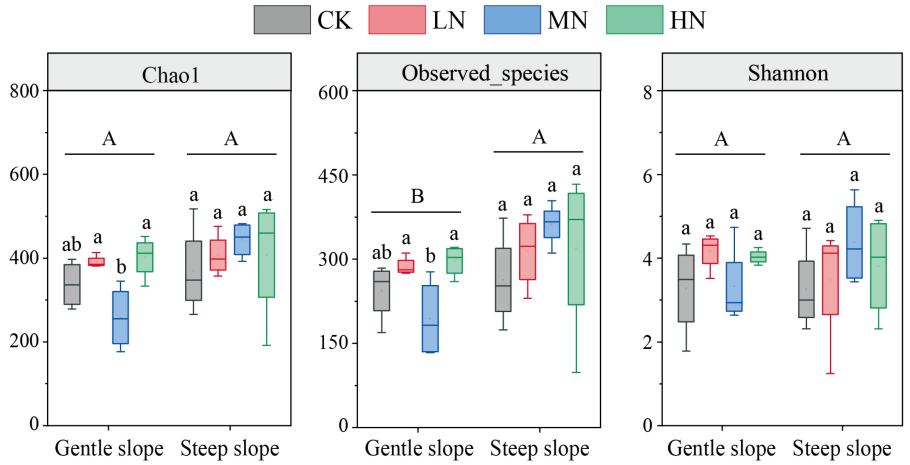

FIGURE 5 | Abundance and diversity index of carbon-fixing microbial community in soil samples. Different lowercase letters represent significant differences among treatments in the same slope, and different uppercase letters represent significant differences among slopes.

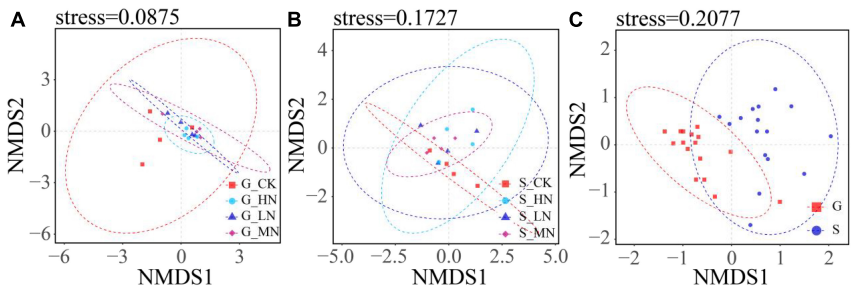

FIGURE 6 | NMDS analysis of OTU level (**A**, groups of nitrogen addition on gentle slope; **B**, groups of nitrogen addition on steep slope; **C**, groups of gentle slope and steep slope). If the soils in the same group are in a circle, it means that the difference between groups is not obvious, and the non-intersection of circles between groups means that there is a certain difference between the groups.

significantly positively correlated ($P < 0.05$). N-NO_3^- and carbon-fixing bacteria Chao1 index were significantly positively correlated ($P < 0.05$). Slope, N-NO_3^- and the observed number were significantly positively correlated ($P < 0.05$). There was no significant correlation between slope, N level, soil physicochemical properties, microbial biomass, stoichiometric ratio, and Shannon diversity. This indicates that N addition at different slopes had a significant effect on the community structure and a non-significant effect on the diversity of carbon-fixing bacteria communities.

DISCUSSION

Effects of Nitrogen Addition on the Community of Carbon-Fixing Bacteria

Carbon sequestration by autotrophic microorganisms is the key to biogeochemical carbon cycling in soil ecosystems. In this paper, we compare the differences in the structure and diversity of soil carbon-fixing bacterial communities at different slopes after N addition in a degraded alpine meadow. We found that among the carbon-fixing bacterial communities, at the phylum level, Proteobacteria was the most abundant phylum belonging to the studied groups after N addition on both gentle and steep slopes and accounted for the largest proportion of the soil, which is consistent with the findings of previous studies (Gao et al., 2018). The strong environmental adaptability, rapid growth and reproduction, and the ability to take up substrate are the main reasons for the dominance of Proteobacteria in degraded alpine meadows (Salcher et al., 2010; Simek et al., 2011). The variation in abundance of each phylum of N-added carbon-fixing bacteria on the same slope was not significant. At the genus level, *Sulfurifustis* was the dominant genus. The dominant genus was not significantly different between the different N additions on the same slope, but N addition had a significant effect on some low abundance carbon-fixing genera, and the response of carbon-fixing microorganisms in soils varied between slopes after N application. This indicates that the degree of species specificity of carbon-fixing microorganisms in degraded alpine meadow soils is low under N addition. In terms of the effect of N addition on the abundance and diversity of carbon-fixing bacteria, middle N addition in gentle slope significantly reduced the Chao1 and observed species values, while any horizontal N addition on steep slope had no significant effect on Chao1 index, observed species

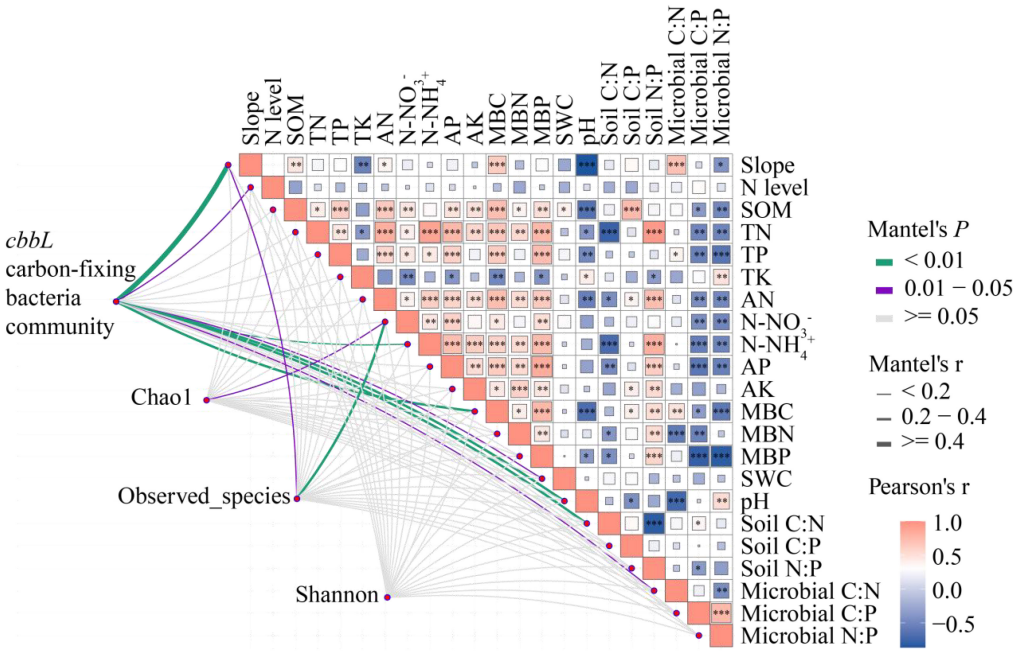

FIGURE 7 | Correlation of carbon-fixing microbial community and diversity with slope, nitrogen addition level, soil physicochemical properties, stoichiometric proportion, and microbial biomass. Mantel edge width corresponds to Mantel r value, and edge color indicates statistical significance. The color gradient of Pearson correlation coefficient r represents the paired correlation of variables. 27° for steep slope and 8° for gentle slope. N addition levels include CK: 0 g N·m^{-2}, LN: 2 g N·m^{-2}, MN: 5 g N·m^{-2}, and HN: 10 g N·m^{-2}. Carbon-fixing microbial community includes 28 bacteria genera with significant differences among different slopes. * indicates $0.01 < P < 0.05$, ** indicates $P < 0.01$, and *** indicates $P < 0.001$.

numbers, and Shannon diversity. Our previous results comparing the relationship between N addition and soil bacterial community diversity showed that soil bacterial richness and diversity tended to decrease and then increase with increasing N application, with moderate N addition significantly reducing soil bacterial richness and diversity, and high levels of N addition significantly inhibiting the decrease in soil bacterial richness and diversity (Li et al., 2020a). The results of the Mantel test showed a significant positive correlation between N level and carbon-fixing bacteria community abundance, and no effect of N level on Shannon diversity. The results of the Mantel test showed that N level and carbon-fixing bacteria community abundance were significantly and positively correlated. High levels of 10 g·m^{-2} N addition suppressed significant decreases in soil carbon-fixing bacteria abundance and observed species numbers, and could maintain soil ecosystem stability.

Effects of Slope on the Community of Carbon-Fixing Bacteria

There were no significant differences in soil microorganisms community structure and diversity in CK between gentle slope and steep slope, but there were significant differences in soil total potassium and microbiomass carbon. Compared to CK, the abundance of Cyanobacteria, organic matter, alkaline nitrogen, pH, soil C:N ratio, microbial C:N ratio, microbial N:P ratio, and the number of carbon-fixing bacteria species changed between the gentle slope and steep slope after N addition. The soil C:N ratio changed from significant to non-significant, and the abundance of Cyanobacteria, organic matter, alkaline nitrogen, microbial biomass C:N ratio, and number of species were significantly higher on steep slope than that on gentle slope, while pH and microbial biomass N:P ratio were significantly higher on gentle slope than that on steep slope. The variability between slopes after N application may be since slope is interfered with by microhabitat and microclimatic conditions such as light, which has an important influence on soil thickness, soil moisture content, and the degree of soil erosion in grassland (Zhang, 2019), resulting in soil total potassium and microbial carbon showing heterogeneity across slopes. The results of the Mantel test showed that slope and carbon-fixing bacteria community abundance and number of species observed were significantly positively correlated, with no significant effect on diversity. This indicates that the differences in carbon-fixing bacteria communities are related to slope, while Shannon diversity is not related to slope. In combination with the results of this study the three N addition levels on steep slopes had no significant effect on soil physicochemical properties, microbial biomass, stoichiometric ratios, or on the number of carbon-fixing bacteria Chao1, observed species, or diversity. The promoting effect of exogenous N addition was slope dependent and more appropriate for vegetation and soil restoration in degraded alpine grasslands on gentle slope, both for vegetation restoration (Li et al., 2020b) and for soil carbon-fixing bacteria richness and species number. There is an interdependent and competitive relationship between soil nutrients and plants. Nutrients play an

important role in the growth and development of plants and the succession of community structures and will directly affect plant nutrient uptake and plant growth, which may ultimately lead to differences in plant community productivity. Plant growth is accelerated when little N is applied, and most of the N applied to the soil may be taken up by plants, resulting in microorganisms not receiving enough N for their own reproduction, and therefore medium N leads to a decrease in microbial abundance and species numbers, thus affecting the stability of grassland systems. When N application is increased, the effective amount of soil nutrients is altered, the competition for nutrients between microbes and plants is weakened and carbon-fixing microbes may consume more of the fast-acting nutrients for their own reproduction. This leads to an increase in the abundance and number of species of carbon-fixing microorganisms under high N conditions. The need to mix other fertilizers with N application to maintain soil nutrient balance needs to be verified by further research.

Limiting Factors for Carbon-Fixing Microorganisms

There is a correlation between soil microbial community structure and soil physicochemical properties (Liu Q. et al., 2017). For example, it has been suggested that there is a correlation between soil carbon-fixing bacterial communities and all physicochemical properties of the soil, with soil pH and total N having the most significant effect on bacterial communities (Su et al., 2020). Li et al. (2020c) showed that soil pH, SOC, and bulk weight could help explain the changes in soil microbial composition observed in the study and that changes in soil fast-acting nutrients such as N-NO_3^-, AK, and AP had a significant effect on the abundance and diversity of carbon-fixing bacteria. The results of the Mantel test in this study showed a significant positive correlation between N-NH_4^+, MBC, SWC, pH, soil C:N, microbial C:N, and carbon-fixing bacterial community abundance, a significant positive correlation between N-NO_3^- and Chao1 index, and a significant positive correlation between N-NO_3^- and observed species number. Soil physicochemical properties, microbial biomass, and Shannon diversity of carbon-fixing bacteria were not significantly correlated, indicating that the contribution of the fast-acting nutrient N-NO_3^- to CO_2 fixing microorganisms was significant. In addition to the influence of soil physicochemical properties on microbial community structure and diversity, an increasing number of studies have shown that soil C, N, and P ratios are the main drivers of microbial diversity (Zhang et al., 2016; Yang et al., 2020, 2022).The present study also showed that carbon-fixing bacterial community abundance and soil and microbial C:N:P ratios were closely related. Specifically, soil and microbial C:N were strongly related to the carbon-fixing bacterial community, while soil and microbial C:P and N:P were weakly related to the carbon-fixing bacterial community. This suggests that soil and microbial C:N ratios have a greater effect on carbon-fixing bacteria. However, stoichiometric ratios had no significant effect on the Shannon diversity of carbon-fixing bacteria.

Our short-term results can reflect the transient kinetic response of alpine meadows to N addition. Whether these short-term results can be translated to longer time scales needs to be tested. In future studies, in addition to the Calvin cycle, some other CO_2 fixation pathways of autotrophic microorganisms should be considered, such as the reduced tricarboxylic acid cycle, the reductive acetyl CoA pathway, 3-hydroxypropionic acid cycle, 3-hydroxypropionic acid/4-hydroxybutyric acid cycle, and the 2-carboxylic acid/4-hydroxybutyric acid cycle (Thauer, 2007; Berg, 2011; Jeoung et al., 2014). Quantitative studies of key genes and their expression profiles in these biological carbon sequestration processes are needed to understand the specific contribution of these microorganisms to CO_2 fixation.

CONCLUSION

The dominant species composition of the soil carbon-fixing bacterial community in degraded alpine meadows on gentle/steep slopes after N addition was similar, with Amoeba being the most abundant phylum in the study and *Sulfurifustis* being the dominant genus. Exogenous N addition had a significant effect on soil nutrients (TK, AP), microbial biomass (P), stoichiometric ratios (soil C:N, microbial C:N, microbial C:P) and the abundance and number of species of carbon-fixing bacteria in degraded alpine meadows, and this effect was dependent on slope positions. Significant differences in abundance of Cyanobacteria and 28 genera of identified carbon-fixing bacteria, soil nutrients (SOM, TK, AN), microbial biomass (C), pH, and stoichiometric ratios (microbial C:N, microbial N:P) were found between gentle and steep slope after N addition. This result highlights the need for future research to integrate the effects of topographic factors on different degraded grasslands to better understand the mechanisms of degraded grassland restoration and management. For the management of degraded alpine meadows on steep slopes, N addition is not recommended for restoration, and the corresponding restoration and management options need to be studied in depth.

DATA AVAILABILITY STATEMENT

The raw sequence data from this study were deposited in the NCBI database with the study accession number: PRJNA843979 that are publicly accessible at http://www.ncbi.nlm.nih.gov/bioproject/843979.

AUTHOR CONTRIBUTIONS

CL carried out the field experiments and sampling and analyzed the data. CL wrote the original manuscript, with contributions from XL, YS, HL, and YY. All authors gave approval to the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

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Nitrogen-Induced Changes in Soil Environmental Factors Are More Important Than Nitrification and Denitrification Gene Abundance in Regulating N_2O Emissions in Subtropical Forest Soils

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Subtropical regions are currently experiencing a dramatic increase in nitrogen (N) deposition; however, the contributions of nitrification and denitrification processes to soil N_2O emissions and the underlying mechanisms under increasing N deposition remain unclear. Therefore, a ^{15}N-tracing laboratory experiment with four N application rates (0, 12.5, 25, and 50 μg ^{15}N g^{-1} soil) was conducted to investigate the response of nitrification- and denitrification-derived N_2O to N additions in an evergreen broad-leaved forest (BF) and a *Pinus* forest (PF) in the Wuyi Mountains in southeastern China. Moreover, the abundance of functional genes related to nitrification (*amoA*), denitrification (*nirK*, *nirS*, and *nosZ*), and soil properties were measured to clarify the underlying mechanisms. Results showed that nitrification-derived N_2O emissions were generally decreased with increasing N input. However, denitrification-derived N_2O emissions were a non-linear response to N additions, with maximum N_2O emissions at the middle N application rate. Denitrification-derived N_2O was the dominant pathway of N_2O production, accounting for 64 to 100% of the total N_2O fluxes. Soil NH_4^+-N content and pH were the predominant factors in regulating nitrification-derived N_2O emissions in BF and PF, respectively. Soil pH and the *nirS* abundance contributed the most to the variations of denitrification-derived N_2O emissions in BF and PF, respectively. Our results suggest that N application has the potential to increase the contribution of denitrification to N_2O production in subtropical forest soils. Changes in soil chemical properties induced by N addition are more important than the abundance of nitrification and denitrification functional genes in regulating soil nitrification- and denitrification-derived N_2O emissions.

Keywords: nitrification, denitrification, microbial functional genes, N_2O emissions, N application

INTRODUCTION

Nitrogen (N) deposition is continuously increasing primarily due to increased anthropogenic fossil fuel combustion, industrialization, and N fertilizer application. Elevated N deposition is expected to not only alter N cycling in forest ecosystems but also enhance N gas loss from the soils (Lu et al., 2011; Fang et al., 2015; Niu et al., 2016; Tang et al., 2018). As one of the primary forms of N gas loss from the soils, nitrous oxide (N_2O) is more than 298 times as effective at trapping atmospheric heat as CO_2 and is also one of the largest stratospheric ozone-depleting substances (IPCC, 2013). Terrestrial ecosystems contribute ∼60% of the global N_2O emissions (IPCC, 2013), of which about 15 to 55% are from tropical and subtropical forests that are believed to be the largest N_2O terrestrial source (Solomon, 2007; Zhang et al., 2019). Moreover, N deposition in tropical and subtropical areas has been steadily increasing in recent decades (ranging from 30 to 73 kg N ha^{-1} $year^{-1}$), and this trend is projected to continue over the coming decades (Zhang et al., 2008; Liu et al., 2013; Zhu et al., 2015).

N_2O is produced primarily during the soil microbial processes of nitrification and denitrification, the rates of which are influenced by the inputs of N. Recent studies in subtropical forests demonstrated that the contribution of nitrification-derived N_2O to the total N_2O emissions was $\leq$50% and that of denitrification were 53 to 100% under aerobic conditions (Zhang et al., 2011; Zhu et al., 2013a; Han et al., 2018; Tang et al., 2018). In addition, the rates of denitrification have generally increased following N application (Zhu et al., 2013b; Morse et al., 2015; Niu et al., 2016; Han et al., 2018). However, few studies have focused on the response of nitrification-derived N_2O emissions to N application, especially in subtropical forests (Han et al., 2018). Therefore, whether the response of nitrification-derived N_2O emissions to N application is more pronounced than that of denitrification-derived N_2O emissions or whether the contribution of denitrification to N_2O production under elevated N input is still dominant over that of nitrification remains unclear. Thus, quantifying the underlying nitrification and denitrification contributions to the N_2O production will help us understand the changes in the N transformation process with increasing N input in forest ecosystems.

Soil nitrification and denitrification can be influenced by different soil microbial groups, soil properties, and climate conditions (Levy-Booth et al., 2014; Cheng et al., 2019). Ammonia oxidation is not only the first but also the rate-limiting step of nitrification, which is performed by both ammonia-oxidizing archaea (AOA) and bacteria (AOB) (Chen and Peng, 2020). On the other hand, denitrification is a four-step microbial facilitated reduction process, whereby NO_3^- is reduced to NO_2^-, NO, N_2O, and eventually to N_2. The first step for the gas production during denitrification is the reduction of NO_2^- to NO, which is catalyzed by nitrite reductases encoded by *nirK* and *nirS* genes (Levy-Booth et al., 2014; Hu et al., 2015). N_2O can be further reduced to N_2 by nitrous oxide reductases, which are encoded by *nosZ* genes (Levy-Booth et al., 2014). While the importance of nitrification and denitrification microorganisms in regulating soil N_2O emission has been widely accepted (Chen and Peng, 2020), to what extent the abundance of nitrifier and denitrifier functional gene can be a good predictor of N_2O emissions under elevated N inputs remain unclear. Moreover, several recent studies have reported that soil environmental factors (e.g., NH_4^+, NO_3^-, pH, TN/TP, and TC/TN) can explain better the changes in soil nitrification and denitrification activities than soil nitrification and denitrification functional genes in fertilized forest soils (Pett-Ridge et al., 2013; Shcherbak et al., 2014; Tang et al., 2018, 2019). Although tropical and subtropical forest soils are the largest N_2O terrestrial sources (Solomon, 2007; Soper et al., 2018; Zhang et al., 2019), only a few studies so far in these regions have attempted to directly link the nitrification- and denitrification-derived N_2O emissions in fertilized soils to N functional genes (Tang et al., 2018, 2019), and more importantly, to investigate the relative contribution of soil biotic and abiotic factors on the nitrification- and denitrification-derived N_2O emissions. Greater insight into the regulation of these processes can help us understand the underlying mechanisms and adopt management practices to lower N_2O emissions.

To evaluate the changes in soil N_2O emissions resulting from nitrification and denitrification processes under N application in subtropical forests and clarify the underlying mechanisms, we carried out a ^{15}N-NO_3^- and ^{15}N-NH_4^+ labeling experiment under laboratory conditions. Mineral soils were collected from two subtropical forests, an evergreen broad-leaved forest (BF), and a *Pinus* forest (PF), which are located in the Wuyi Mountains in southeastern China. The evergreen BF was chosen because it is a product of the monsoon climate and it represents the climax vegetation type of subtropical regions (Zhou et al., 2013). *Pinus* forest was selected because of its strong contribution to afforestation purposes since the 1980s. It is worth highlighting that the selected pine species have a strong ability to adapt to poor soil conditions in the hilly red soil region of south China, which faced moderate to intense soil and water loss challenges in the 1980s. The forests have been widely planted since that time and account for about 30.5% of the subtropical forests in south China (Cao et al., 2015). Many previous studies have demonstrated that broadleaf/deciduous species produce more N_2O than conifers due to a large difference in the production and reduction rates of N_2O under the broadleaf/deciduous species (Zhang et al., 2008; Qin et al., 2019). However, some studies reported that coniferous stands emit more N_2O than the adjacent deciduous stands that were affected by N deposition. Therefore, the role of different forest types (or tree species) in regulating N_2O emissions remains unclear.

This study aimed to (1) investigate the responses of nitrification- and denitrification-derived N_2O emissions to elevated N inputs, (2) clarify the dominant factors that control nitrification- and denitrification-derived N_2O emissions, and (3) compare the differences in nitrification- and denitrification-derived N_2O emissions between the BF and PF. We hypothesized that (1) nitrification- and denitrification-derived N_2O emissions would be enhanced with the increased N application and that denitrification may contribute more to the increase in N_2O emissions than nitrification, (2) the abundances of functional genes could be more important than soil environmental factors in regulating nitrification- and denitrification-derived N_2O emissions, and (3) the nitrification- and denitrification-derived N_2O emissions would be greater in the BF than the PF.

MATERIALS AND METHODS

Study Site Descriptions and Soil Sampling Procedures

The experimental soil samples were collected at the Wuyishan National Nature Reserve (WNNR), located in Fujian Province (27°33′–27°54′N, 117°27′–117°51′E) in southern China. The climate of the WNNR (area: 56,527 ha) is humid monsoon, which is mainly characterized by the mean annual precipitation and temperature of 2,000 mm and 15°C, respectively, as well as 83.5% relative humidity and 100 fog days $year^{-1}$ (Xu et al., 2010; Wang et al., 2013). The WNNR has two typical vegetation types, that is, evergreen BF and pine forest (PF). The BF and PF are located at about 550 m a.s.l. and 1,100 m a.s.l., respectively. While *Castanopsis carlesii* Hay and *Castanopsis eyrie* Tutch are the dominant species in the BF, *Pinus taiwanensis* Hayata is the dominant species in the PF (Xu et al., 2010; Lyu et al., 2019). Background atmospheric N deposition in the WNNR is about 8 kg N ha^{-1} $year^{-1}$ (Xu et al., 2016; Gurmesa et al., 2022).

In April 2018, three 20 m × 20 m plots without N addition (plots were about 10 m away from each other) were randomly established in each vegetation type, and soil samples were collected with a soil core sampler (8 cm in diameter) from the surface soil layer (0–20 cm) after litter removal. Ten soil cores were randomly collected from each plot and there were a total of 30 soil cores collected in each forest stand. Soil samples from the same forest type were thoroughly mixed to form a composite sample. All soil samples were sieved through a 2-mm mesh sieve and were then divided into two sub-samples. The first sub-sample was maintained at 4°C for 1 week before being used for the incubation experiment. The second sub-sample was air-dried for the measurements of soil chemical properties. The soil at both sites is classified as yellow-red soil based on the Chinese Soil Classification System, equivalent to an Ultisol according to the United States Department of Agriculture Soil Taxonomy classification system (Lyu et al., 2019). Soil chemical properties were shown in **Table 1**. The concentrations of soil SOC, TN, NH_4^+-N, and NO_3^--N in the PF were significantly higher than those in the BF, whereas soil pH and $\delta^{15}N$ were lower in the PF and no significant difference was found in the soil C/N ratio in these two forest soils (**Table 1**).

^{15}N-Tracing Experiment and Gas Sampling Procedures

This study used a completely randomized factorial design experiment that consisted of four N application rate treatments (0, 12.5, 25, and 50 μg ^{15}N g^{-1} soil equivalent to 0, 16, 32, and 64 kg N ha^{-1} $year^{-1}$). We applied N as $^{15}NH_4NO_3$ (99.10 atom %) and $NH_4{}^{15}NO_3$ (99.21 atom %). The rates of N application were in the range of those of N deposition (10 to 73 kg N ha^{-1} $year^{-1}$) in subtropical forests (Zhang et al., 2011, 2019). For each forest soil type, 120 glass jars (500 mL) were prepared and divided into two groups: one group comprised of 60 glass jars containing $^{15}NH_4NO_3$ (4 N addition rates × 3 replications per N application rate × 5 destructive soil sampling) and the other group consisted of 60 glass jars containing $NH_4{}^{15}NO_3$. A total of 30 g (oven-dry basis) of fresh soil was weighed into each glass jar and pre-incubated for 7 days at 25°C to allow the recovery of soil microbial activities. Then, $^{15}NH_4NO_3$ or $NH_4{}^{15}NO_3$ was dissolved in 4 mL deionized water and added to the soil at a rate of 0, 12.5, 25, and 50 μg $^{15}NH_4^+$-N g^{-1} soil or $^{15}NO_3^-$-N g^{-1} soil. Soil without N addition was regarded as a control and was added with the same volume of deionized water to ensure its similarity in soil moisture content with the N-added soil. The soils were then incubated for 15 days at 25°C and their moisture content was maintained at 60% water-holding capacity throughout the entire experiment. Soils were corrected for water loss by weighing each glass jar plus soil every week and deionized water was added as required to maintain constant soil moisture. During gas sampling, three glass jars at each level of N treatment were randomly selected. The glass jars were closed with the gas-tight rubber lids, and gas sampling tubes were fixed into the middle of the rubber lids to enable the collection of the gas samples. Gas samples (20 mL) were collected from each glass jar with a 3-way stopcocks syringe on days 1, 2, 4, 9, and 15 after N application. Five millimeters were taken from each gas sample to determine the N_2O concentration (Shimadzu, GC-2014C, Japan) and the remaining 15 mL were used to determine the N_2O isotopic composition (IRMS, Isoprime 100, United Kingdom). Before each gas sampling, the glass jars were opened and flushed with ambient air for approximately 30 min and then resealed with stoppers for 30 min.

Quantification of the Contributions of Nitrification and Denitrification to N_2O Emissions

The fractions of N_2O derived from denitrification (*FD*) and nitrification (*FN*) processes were calculated using the following equations from Stevens et al. (1997):

$$FD = (a_m - a_n)/(a_d - a_n) \quad (1)$$

$$FN = 1 - FD \quad (2)$$

where a_m is the ^{15}N atom % of N_2O, a_n is the ^{15}N atom % of N_2O from the nitrification process (assumed to be equivalent to ^{15}N

TABLE 1 | Soil chemical properties in the BF and PF.

Forest type	SOC (%)	TN (%)	C/N	$\delta^{15}N$ (‰)	pH	NH_4^+-N (mg kg^{-1})	NO_3^--N (mg kg^{-1})
BF	4.10 ± 0.07b	0.21 ± 0.01b	19.80 ± 0.47a	10.84 ± 1.24a	4.80 ± 0.03a	91.89 ± 1.13b	9.40 ± 0.06b
PF	5.67 ± 0.20a	0.27 ± 0.01a	20.63 ± 0.12a	9.97 ± 1.56b	4.72 ± 0.01b	126.66 ± 3.07a	15.37 ± 0.56a

BF and PF indicate the broad-leaved forest and Pinus forest, respectively. Values are means ± standard error, n = 3. Different lowercase letters in the same column represent the difference was significant at P < 0.05.

atom % of the soil NH_4^+), and a_d is the ^{15}N atom % of N_2O from denitrification (assumed to be equivalent to ^{15}N atom % of the soil NO_3^-).

$$\text{The atom percent of}^{15}\text{N is defined as } {}^{15}\text{N atom\%} = {}^{15}\text{N}/({}^{15}\text{N} + {}^{14}\text{N}) \times 100\% \quad (3)$$

$$\text{Denitrification} - \text{derived } N_2O \text{ flux} = FD \times N_2O \text{ flux} \quad (4)$$

$$\text{Nitrification} - \text{derived } N_2O \text{ flux} = FN \times N_2O \text{ flux} \quad (5)$$

Soil Inorganic N, Net Nitrification Rate, Dissolved Organic Carbon, Dissolved Organic Nitrogen, and pH Measurements

Soil samples were also collected after gas sampling. Five times destructive soil sampling was used for the analysis of the soil NH_4^+ and NO_3^- contents, as well as the $\delta^{15}N$ values of soil NH_4^+ and NO_3^-. Dissolved organic carbon (DOC) and nitrogen (DON) contents and soil pH were also determined at the end of the experiment. Soil NH_4^+ and NO_3^- contents were extracted using 2 mol L^{-1} KCl for 1 h and then the extracted solutions were divided into two parts. One part was reacted with sodium salicylate (for NH_4^+ solution) and hydrazine sulfate solution (for NO_3^- solution) and measured, respectively, at 660 and 550 nm using a discrete wet chemistry analyzer (SmartChem 200, AMS Alliance, Italy) to determine the concentrations of soil NH_4^+-N and NO_3^--N. The other part was used for ^{15}N measurements by distillation using MgO and Devarda's alloy (Zhang et al., 2011). The DOC in the soil was extracted with 2 mol L^{-1} KCl at 1:5 w/v and measured using a TOC-TN analyzer (Shimadzu TOC, Japan). The soil DON concentration was determined by calculating the difference between soil total dissolved nitrogen (2 mol L^{-1} KCl extracted at 1:5 w/v) and soil mineral nitrogen (Jones and Willett, 2006). Soil pH was measured at a 1:2.5 soil to solution ratio using a pH detector (PHS-3C, Sheng Ci, Shanghai, China).

Quantification of the *amoA*, *nirK*, *nirS,* and *nosZ* Gene Abundances

At the end of the incubation experiment, soil samples for molecular analyses were immediately stored at −80°C before the aforementioned analysis. DNA was extracted from 0.3 g of frozen soils using a Mo Bio PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, United States) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were determined with a spectrophotometer at 260 nm (NanoDrop Technologies, United States). All extracted soil DNA samples were stored at −80°C before analysis. Quantitative polymerase chain reaction (qPCR) was used to estimate the abundance of nitrification and denitrification functional genes (*amo*A, *nirK*, *nirS*, and *nosZ*) using a real-time PCR detection system. The PCR primers used in this study are listed in **Supplementary Table 1**. The 10-fold serially diluted plasmids carrying each target gene were subjected to real-time PCR assays in triplicate to generate a standard curve. The qPCR efficiencies for AOA-*amoA*, AOB-*amoA*, *nirK*, *nirS*, and *nosZ* were 0.85, 0.94, 0.96, 0.91, and 0.87, respectively. The corresponding determination coefficients of the standard curve for AOA-*amoA*, AOB-*amoA*, *nirK*, *nirS,* and *nosZ* were 0.998, 0.993, 0.990, 0.999, and 0.996, respectively.

Statistical Analyses

The gene copy numbers were log-transformed to meet the homogeneity of variance requirements. Three-way ANOVA was used to examine the effects of forest type, N application rate, sampling date, and their interactions on soil N_2O flux, nitrification-, and denitrification-derived N_2O emissions, as well as on soil NH_4^+-N, NO_3^--N and their ratio (NH_4^+-N/NO_3^--N). Two-way ANOVA with Duncan's test was performed to determine the effects of forest type, N application rate, and their interactions on the soil properties (soil pH, DOC, and DON) and the abundances of soil nitrification and denitrification functional genes (*amoA*, *nirK*, *nirS*, and *nosZ*). All of the above statistical analyses were performed using SPSS 16.0 (SPSS Inc., Champaign, IL, United States).

To estimate the direct and indirect effects of soil biotic and abiotic factors on soil nitrification- and denitrification-derived N_2O emissions in the BF and MF, structural equation models (SEM) were performed based on our knowledge of soil biotic and abiotic properties in relation to soil nitrification- and denitrification-derived N_2O. Before the SEM analyses, the units of the predictor and dependent parameters were adjusted to obtain comparable parameter variances according to the standardized method. The quality of the SEM model was assessed by using a low χ^2 value and $P > 0.05$, the root-mean-square error of approximation (RMSEA, the RMSEA $\leq$0.08 indicates a model fit), and the model fit index (GFI > 0.90). All SEM analyses were using Amos 17.0 (IBM, SPSS, NY, United States).

RESULTS

Effects of N Addition on Total and Nitrification- and Denitrification-Derived N_2O Emissions

Soil N_2O fluxes decreased with increasing incubation period (**Figure 1**) and were significantly affected by forest type, N application rate, sampling date, and their interactions (**Table 2**). The N_2O flux values in the BF ranged from 0.05 to 3.85 μg N kg^{-1} day^{-1} throughout the entire incubation period (**Figure 1**), with the average fluxes across all the treatments being about 2.4 times greater than those in the PF (**Figure 1**). Non-linear responses of the soil N_2O fluxes to N addition were observed in both the BF and the PF. Specifically, the highest amount of soil N_2O emissions was noted at low and moderate N applications in the BF and PF, respectively (**Figure 2A**).

The application rates of N fertilizer altered not only the total amount of soil N_2O fluxes but also the nitrification- and denitrification-derived N_2O fluxes (**Figures 1**, **2**). The nitrification- and denitrification-derived N_2O emissions were significantly higher in the BF than in the PF (**Figure 2B**). The nitrification-derived N_2O fluxes were in the range of 0.03 to 2.66 μg N kg^{-1} day^{-1} and 0 to 0.45 μg N kg^{-1} day^{-1} in the BF

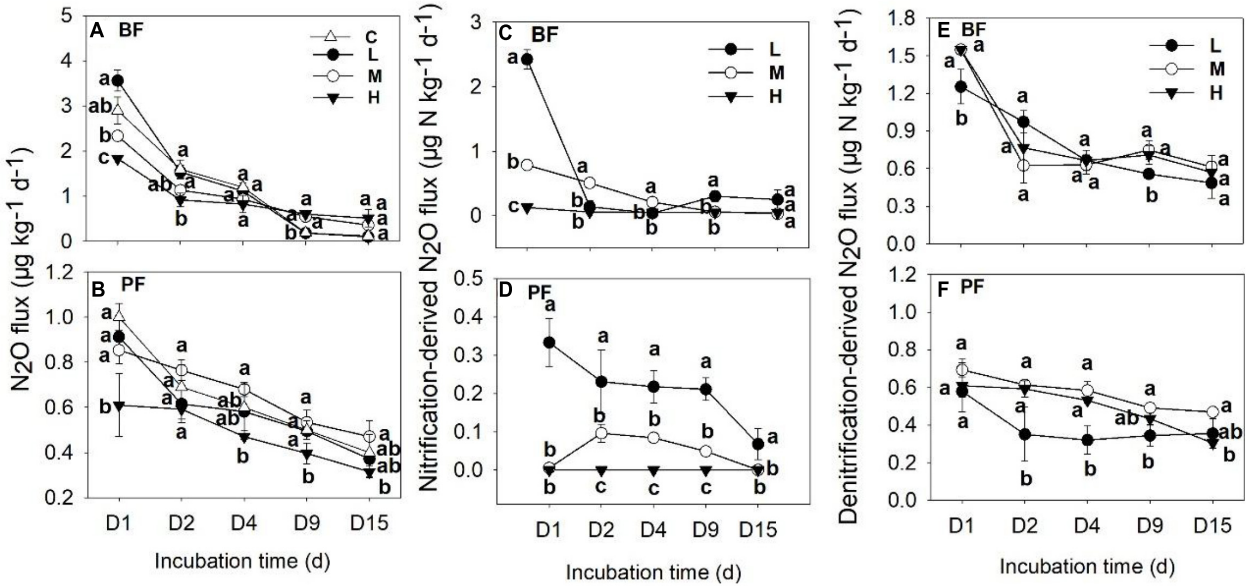

FIGURE 1 | Dynamics of soil N_2O fluxes **(A,B)**, nitrification- **(C,D)**, and denitrification-derived **(E,F)** soil N_2O fluxes under different treatments. Values are means ± standard error (n = 3). Different lowercase letters for the same incubation time represent significant differences between different treatments. C, L, M, and H represent N application rates of 0, 12.5, 25, and 50 $\mu g\ ^{15}N\ g^{-1}$ soil, respectively. BF and PF indicate broad-leaved forest and *Pinus* forest, respectively.

TABLE 2 | *P*-values from Repeated-measure ANOVA of the effects of forest type (*FT*), N application rates (*N*), sampling date (*D*), and their interaction on the N_2O fluxes, nitrification-, and denitrification-derived N_2O emissions, soil NO_3-N, and NH_4^+-N, n = 3.

	FT	*N*	*D*	*FT *N*	*FT *D*	*N *D*	*FT *N*D*
N_2O fluxes	<0.001	<0.01	<0.001	0.157	<0.001	<0.001	<0.001
Nitrification-derived N_2O	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Denitrification-derived N_2O	<0.001	<0.05	<0.001	0.123	<0.001	0.274	<0.05
NO_3-N	<0.001	<0.001	<0.001	0.605	<0.001	<0.001	<0.001
NH_4^+-N	<0.001	<0.001	<0.001	0.554	<0.001	<0.001	<0.01
NH_4^+-N/ NO_3^--N	0.222	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01

and PF, respectively (**Figures 1C,D**), and these fluxes decreased significantly as N application rates increased in both forests. At high rates of N addition, the decrease in nitrification-derived N_2O fluxes was approximately 84.6% in the BF and 100% in the PF relative to those observed at low N addition rates (**Figure 2B**). The denitrification-derived N_2O fluxes were in the range of 0.4 to 1.7 $\mu g\ N\ kg^{-1}\ day^{-1}$ and 0.2 to 0.8 $\mu g\ N\ kg^{-1}\ day^{-1}$ in the BF and PF, respectively (**Figures 1E,F**) and maximum average of denitrification-derived N_2O emissions was observed in middle N application rate in both BF and PF (**Figure 2B**). Moreover, denitrification was the dominant pathway of soil N_2O production in the present study, accounting for 64 to 90% and 81 to 100% of the total N_2O fluxes in the BF and PF, respectively (**Figure 2C**).

Effects of N Addition on Soil Inorganic N, pH, Dissolved Organic Carbon, and Dissolved Organic Nitrogen

Soil NO_3^--N and NH_4^+-N contents were significantly affected by forest type and N application rate (**Table 2**). The soil NO_3^--N and NH_4^+-N contents increased from day 1 to day 9 and then decreased significantly on the last day of the incubation experiment (**Figure 3**). These soil inorganic N contents increased with increasing N application rates, and they were significantly higher in the PF than in the BF (**Figures 3A–D**). Soil NH_4^+-N contents in the control treatments were, respectively, ∼9- and ∼7-fold greater in the BF and PF compared with soil NO_3^--N contents (**Figures 3E,F**). Moreover, the ratio of soil NH_4^+-N to soil NO_3^--N decreased with increasing N inputs. High N application significantly decreased soil pH in both forests. While high N addition significantly decreased soil DON content in PF, no significant effect of N addition on soil DOC was observed in this forest type (**Figure 4**).

Effects of N Addition on the Abundances of Nitrification and Denitrification Functional Genes

While the abundances of AOA- and AOB-*amoA* genes in the BF and PF were not significantly affected by N inputs (**Figures 5A–D**), those of *nirK* and *nirS* genes were increased substantially by N inputs (**Figures 5E,F**). The increase in abundance of *nirK* genes

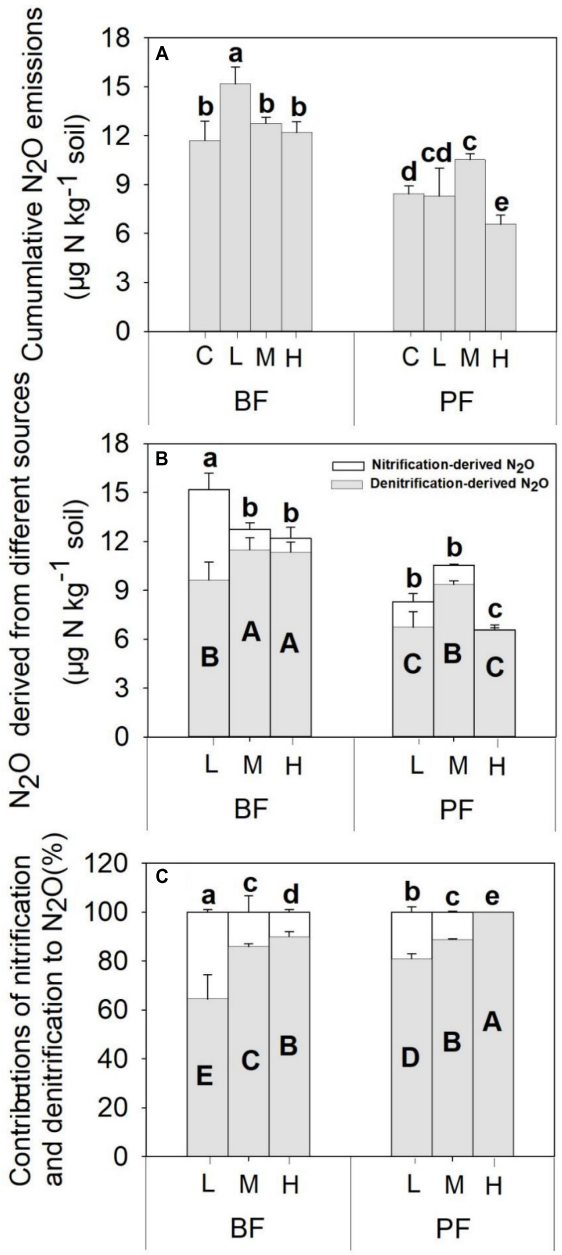

FIGURE 2 | Nitrification-**(A)** and denitrification-**(B)** derived soil N_2O emissions, as well as the contributions of nitrification and denitrification to total soil N_2O emissions **(C)**, under different treatments. Values are means ± standard error (n = 3). Different lowercase letters indicate that the nitrification-derived N_2O differed significantly between treatments ($P < 0.05$). Different uppercase letters indicate that the denitrification-derived N_2O differed significantly between treatments ($P < 0.05$). C, L, M, and H represent N application rates of 0, 12.5, 25, and 50 μg ^{15}N g^{-1} soil, respectively. BF and PF represent the broad-leaved forest and *Pinus* forest, respectively.

in the PF (12%) was greater than that in the BF (5%), whereas the increase in abundance of *nirS* genes in the BF (15%) was slightly higher compared with that in the PF (14%). Inputs of N had no significant effect on *nosZ* gene abundance (**Figure 5G**).

Controlling Factors of Nitrification- and Denitrification-Derived N_2O Emissions

According to the SEM, soil properties (soil NH_4^+-N, NO_3^--N, DON, and pH) and nitrification and denitrification functional genes (AOA-*amoA*, AOB-*amoA*, *nirK*, *nirS*, and *nosZ*) explained 99 and 95% of the variance observed in nitrification-derived N_2O emissions in the BF and PF, respectively (**Figures 6A,B**). These variables also could explain 55 and 88% of the variance of denitrification-derived N_2O emissions in the BF and PF, respectively (**Figures 6C,D**). The dominant factor in regulating soil nitrification-derived N_2O emission in the BF and PF was soil NH_4^+-N (standardized total coefficient = −0.93, $P < 0.001$) and soil pH (standardized total coefficient = −0.74, $P < 0.001$), respectively, (**Table 3**). The dominant factors in regulating soil denitrification-derived N_2O in BF and PF were significantly differently (**Figures 6C,D** and **Table 3**). In the BF, it was soil pH. However, in the PF, the dominant regulatory factor was *nirS* (**Table 3**).

DISCUSSION

Effects of N Addition on Nitrification- and Denitrification-Derived N_2O Emissions

In the present study, soil N_2O fluxes responded non-linearly to N addition. Low- and middle-level N application rates increased soil N_2O emissions which might be due to N application and increased N availability (**Figure 3**) for nitrifying and denitrifying microorganisms. However, high N input had no significant effect on soil N_2O emission from BF or even decreased soil N_2O emission from PF (**Figure 2B**). Similarly, previous studies carried out in a subtropical montane forest also reported that short-term low and medium N addition favored N_2O production but this effect became weaker or even decreased N_2O emissions at high N addition (Han et al., 2018). A possible reason may be that the positive effect of high N application on denitrification-derived N_2O in both forests was partially offset by its negative effect on nitrification-derived N_2O (**Figure 2B**). Another explanation may be that the consumption of N_2O may be greater than the production of N_2O at high N treatment (Senbayram et al., 2012).

The application of N not only affects total soil N_2O emissions but also the contribution of nitrification and denitrification processes to soil N_2O production. In the present study, the average nitrification-derived N_2O emissions under different N treatments ranged from 0 to 0.37 μg N kg^{-1} day^{-1} (**Figures 1C,D**), which is similar to a previous study in subtropical forests [0.06–0.40 μg N kg^{-1} day^{-1}, Zhang et al. (2011)]. Over 15 days of incubation, nitrification-derived N_2O contributed 0 to 36% of the total N_2O emissions (**Figure 2**). This range is within the range (<50%) that has been documented in previous studies carried out in the subtropical forest with similar N application rates (Zhang et al., 2011; Han et al., 2018). In contrast to our first hypothesis, our result showed a decrease in the contribution of the nitrification process to the total N_2O emissions following N application (**Figure 3**), and the magnitude of decrease was about 80 to 100% across the two forests. Deppe

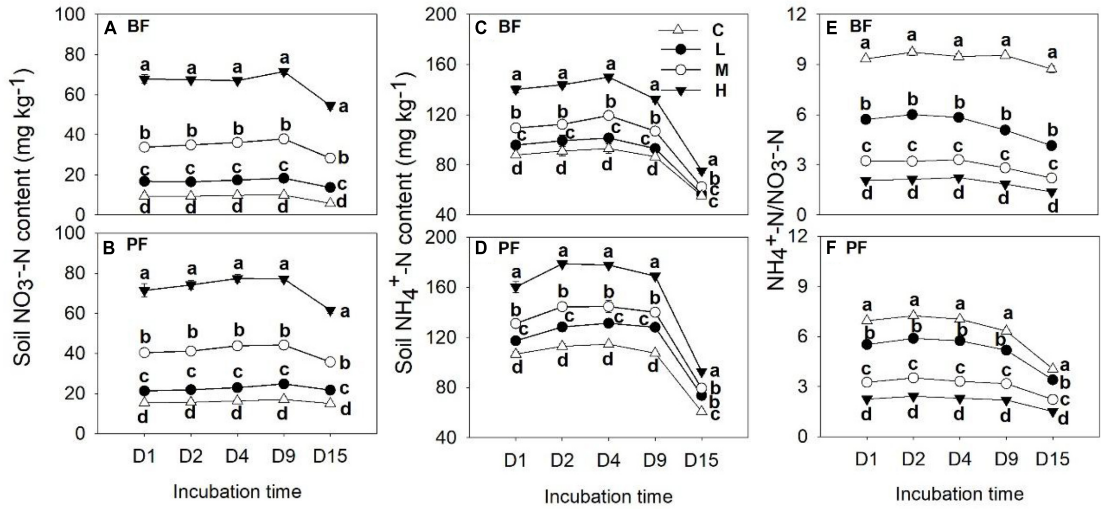

FIGURE 3 | Dynamics of soil NO_3^--N **(A,B)** and NH_4^+-N contents **(C,D)** and soil NH_4^+-N to NO_3^--N ratio **(E,F)** under different treatments. Values are means ± standard error (n = 3). Different lowercase letters indicate that the soil NO_3^--N and NH_4^+-N contents and their ratios differed significantly among treatments at each sampling time ($P < 0.05$). C, L, M, and H represent N application rates of 0, 12.5, 25, and 50 μg ^{15}N g^{-1} soil, respectively. BF and PF represent the broad-leaved forest and *Pinus* forest, respectively.

et al. (2017) also found that N addition can significantly decrease the contribution of nitrification to N_2O production in a Haplic Luvisol soil, but the magnitude of decrease (7 to 21%) was not as pronounced as the one observed in the present study. One possible explanation for this phenomenon may be that N application decreased soil pH (**Figure 4A**), and soil nitrification rates have been demonstrated to be relatively low in acidic soils and decrease with decreasing soil pH (Cheng et al., 2013; Wang et al., 2020).

In contrast to the response of nitrification-derived N_2O emissions to N input as explained above, the highest denitrification-derived N_2O emissions occurred at the middle N application rate in both BF and PF, and the denitrification process is the dominant pathway for the production of soil N_2O emission, accounting for 64 to 100% of the total N_2O fluxes (**Figure 3C**). Previous studies carried out across subtropical forests reported that 53 to 100% of the total N_2O emissions were derived from denitrification (Zhang et al., 2011; Zhu et al., 2013a; Han et al., 2018). Moreover, the relative contributions of denitrification to N_2O emissions in forest soils were found to increase significantly with increasing N application (Zhu et al., 2013a; Morse et al., 2015; Niu et al., 2016), with the magnitude of increase ranging from 51 to 130% in a subtropical forest (Zhu et al., 2013a). The significantly higher relative contribution of denitrification to total N_2O production in the PF compared with the BF in the present study can be partly attributed to the greater decrease in nitrification-derived N_2O emissions in the PF (100%) than in the BF (80%).

Moreover, both nitrification- and denitrification-derived N_2O emissions were significantly higher in the BF relative to the PF, which indicated that the BF was a more important N_2O emission source than the PF. This is consistent with our third hypothesis. A study by Zhang et al. (2008) in southern China also reported that the emissions of N_2O from a mature monsoon evergreen BF were significantly higher compared to those from a PF. The authors attributed their observation to the higher soil N availability in the monsoon evergreen BF. However, their explanation cannot be extended to our findings because the BF had significantly lower soil available N content than the PF (**Figure 3**). Considerable differences in the amount of N_2O emissions among the investigated forest soils might be attributed to the differences in tree species, or more specifically, their influences on the soil's abiotic and/or biotic properties (Butterbach-Bahl et al., 1997; Qin et al., 2019). It has been reported that trees in BF are associated with arbuscular mycorrhizal fungi, which can promote soil microbial communities with higher N cycling potential and activity relative to microbial communities in soils dominated by the trees associated with ectomycorrhizal fungi in the PF (Mushinski et al., 2021).

Overall, our results supported part of our first hypothesis as the denitrification process was the dominant pathway of soil N_2O production. However, the denitrification-derived N_2O emissions were observed as non-linear responses to N application in both forests. In addition, the nitrification-derived N_2O emissions decreased with increasing N application in both BF and PF, which is in contrast with our first hypothesis. An explanation for this phenomenon is presented in section "Controlling Factors Related to Nitrification-Derived N_2O Emissions in Forest Soils."

Controlling Factors Related to Nitrification-Derived N_2O Emissions in Forest Soils

Soil NH_4^+-N content and soil pH contributed the most to the variations of nitrification-derived N_2O emissions in the BF and

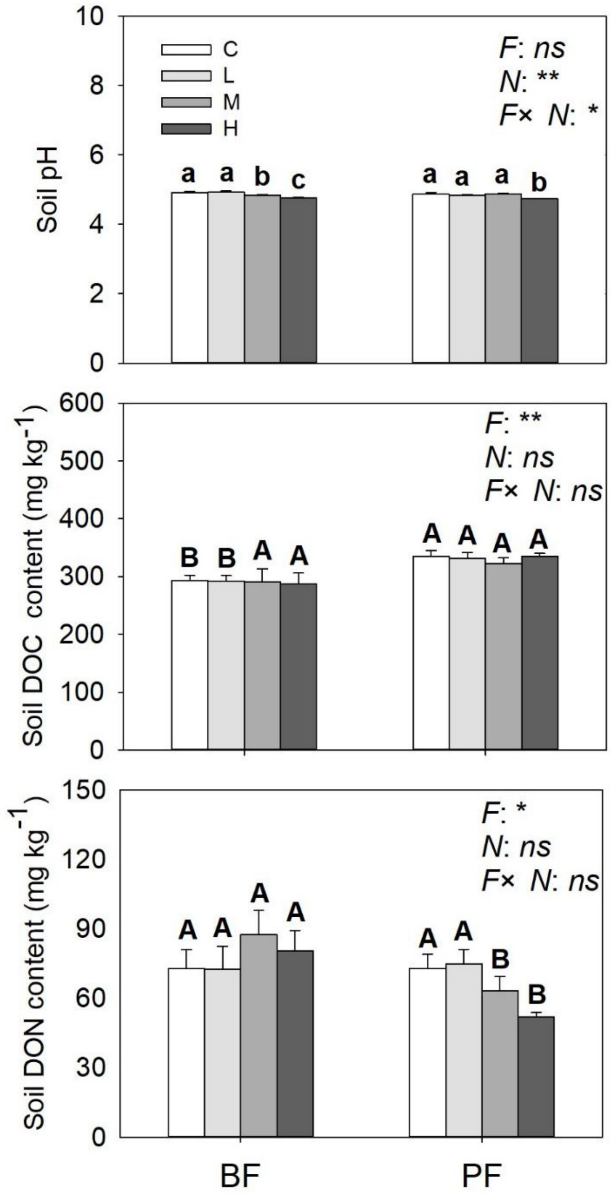

FIGURE 4 | Soil pH, soil DOC, and DON contents under different treatments. Values are means ± standard error ($n = 3$). Different lowercase letters indicate that soil pH, soil DOC, and DON contents differed significantly between treatments ($P < 0.05$). Different uppercase letters indicate that soil pH, soil DOC, and DON were significantly different between the two forest soils ($P < 0.05$). C, L, M, and H represent N application rates of 0, 12.5, 25, and 50 μg ^{15}N g^{-1} soil, respectively. BF and PF represent the broad-leaved forest and *Pinus* forest, respectively. *F*, *N*, and *F* × *N* represent the effects of forest type, N application, and their interactions on these indicators. * and ** represent the effects of forest type or N application or their interactions on these indicators were significant at $P < 0.05$ and $P < 0.01$, respectively. ns indicate their effects were not significant at $P < 0.05$.

PF, respectively (**Figure 6** and **Table 3**). Their effect was more pronounced than the effects of AOB-*amoA* on nitrification-derived N_2O emission. Therefore, in contrast to our second hypothesis, our results suggest that N-induced changes in soil environmental factors were more important than the abundance of *amo*A genes in regulating nitrification-derived N_2O emissions. Similarly, Tang et al. (2019) found that soil environmental factors (especially soil NH_4^+ content) accounted for 50 to 77% of the variation in potential nitrification activities, which was better explained than nitrification functional genes in fertilized soils. In the BF, high soil NH_4^+-N concentrations not only had a direct negative effect on nitrification-derived N_2O emission but also had an indirect negative effect *via* influencing soil pH and the abundance of AOB (**Figure 6A**). The reasons for high soil NH_4^+-N contents reducing nitrification-derived N_2O emission may be as follows: ammonia oxidizers are unable to tolerate high NH_4^+-N contents (>100 mg N kg^{-1} soil) in acidic soils (Gubry-Rangin et al., 2010; Carey et al., 2016; Farquharson, 2016; Breuillin-Sessoms et al., 2017; Li et al., 2018; Liu et al., 2018). In the current study, the average soil NH_4^+-N content in N-amended soils was much higher than not only the aforementioned 100 mg N kg^{-1} soil but also other soil NH_4^+-N content averages that have been reported in previous studies in various subtropical fertilized forest soils (ranging from 1 to 50 mg N kg^{-1} soil) (Han et al., 2018; Tang et al., 2018, 2019; Wang et al., 2018). This indicates the lack of efficient oxidation of NH_4^+ to NO_3^- during nitrification that has led to lower nitrification-derived N_2O emissions in N-amended soils (Farquharson, 2016; Wang et al., 2020).

Soil NH_4^+-N content also had a direct significant effect on nitrification-derived N_2O emission in the BF, but its total effect was offset by its indirect effect on soil pH (**Figure 6B**). This resulted in soil pH playing a more important role in regulating nitrification-derived N_2O emissions in the PF (**Table 3**). Previous studies have reported that soil with a lower pH support less microbial diversity and lower N-cycle potential (Cheng et al., 2019; Mushinski et al., 2021). A meta-analysis reports that N addition dramatically decreases the ratios of fungi to bacteria and microbial C to N on an average by 10 and 8%, respectively (Zhou et al., 2017). In addition, the nitrification rate is generally decreased with decreasing soil pH, and its rate can be inhibited in soil with a pH lower than 5 (Zhao et al., 2018; Cheng et al., 2019). This could explain why the nitrification-derived N_2O emission at high N addition in the PF is close to zero (**Figure 2B**). Overall, in terms of nitrification-derived N_2O emissions, changes in soil environmental factors (e.g., soil NH_4^+-N and pH) induced by N application play more important roles than changes in the abundance of AOA and AOB in controlling its emission.

Controlling Factors Related to Denitrification-Derived N_2O Emissions in Forest Soils

Soil pH is the most important controller of denitrification-derived N_2O emission in the BF (**Figure 6C** and **Table 3**), and high N application significantly decreased soil pH (**Figure 4**). The reasons for soil pH regulating denitrification-derived N_2O emissions in BF may be as follows. Soil pH can exert a direct and an indirect effect on biological properties (e.g., reductase activities, the abundance, and expression of functional genes,

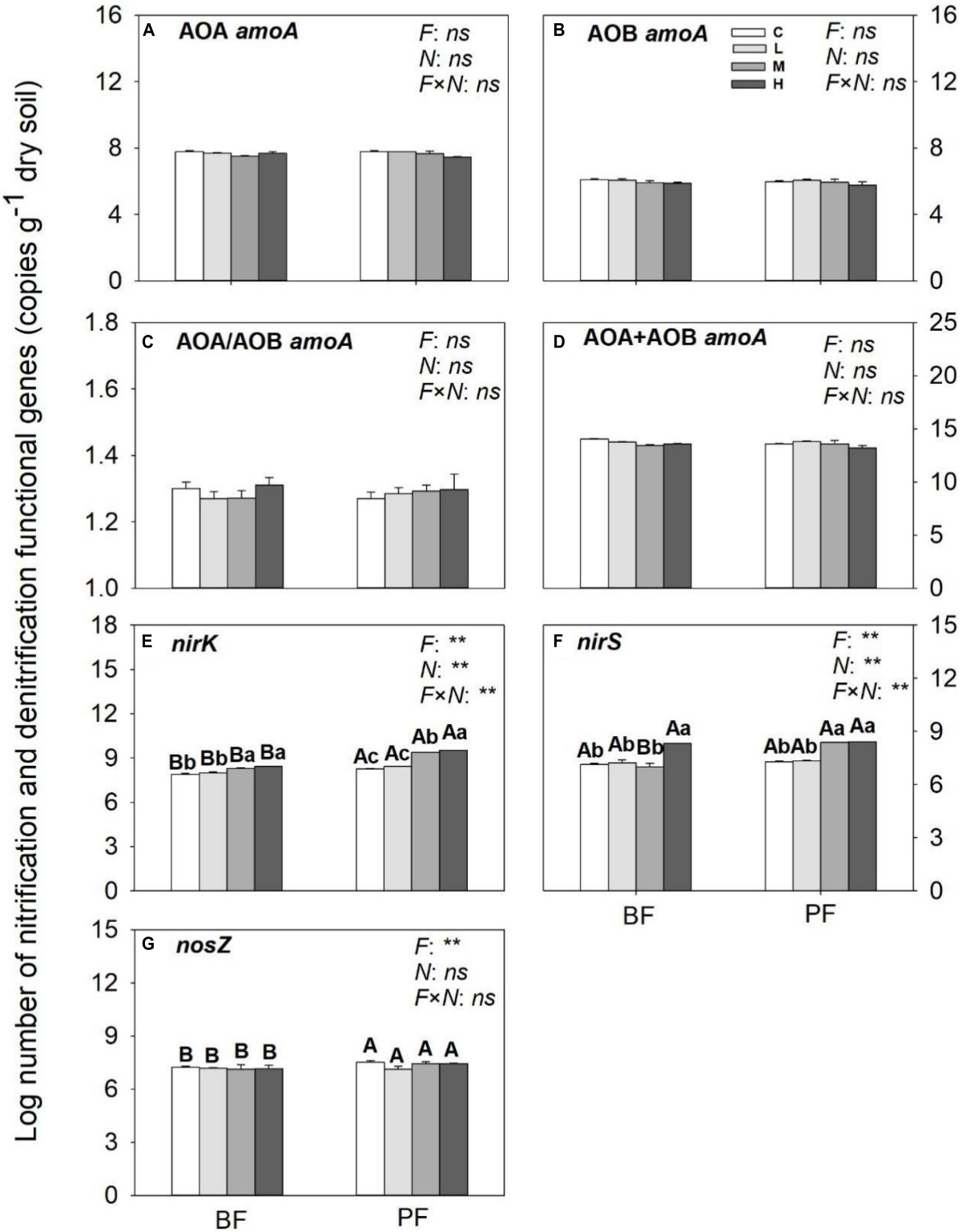

FIGURE 5 | Abundances of nitrification **(A–D)** and denitrification **(E,F)** functional genes under different treatments. Values are means ± standard error (n = 3). Different lowercase letters indicate that the functional gene abundance differed significantly among treatments ($P < 0.05$). C, L, M, and H represent N application rates of 0, 12.5, 25, and 50 μg ^{15}N g^{-1} soil, respectively. BF and PF represent the broad-leaved forest and *Pinus* forest, respectively. *F*, *N*, and *F* × *N* represent the effects of forest type, N application, and their interactions on these indicators. ** represent the effects of forest type or N application or their interactions on these indicators were significant at $P < 0.05$ and $P < 0.01$, respectively. ns indicate their effects were not significant at $P < 0.05$.

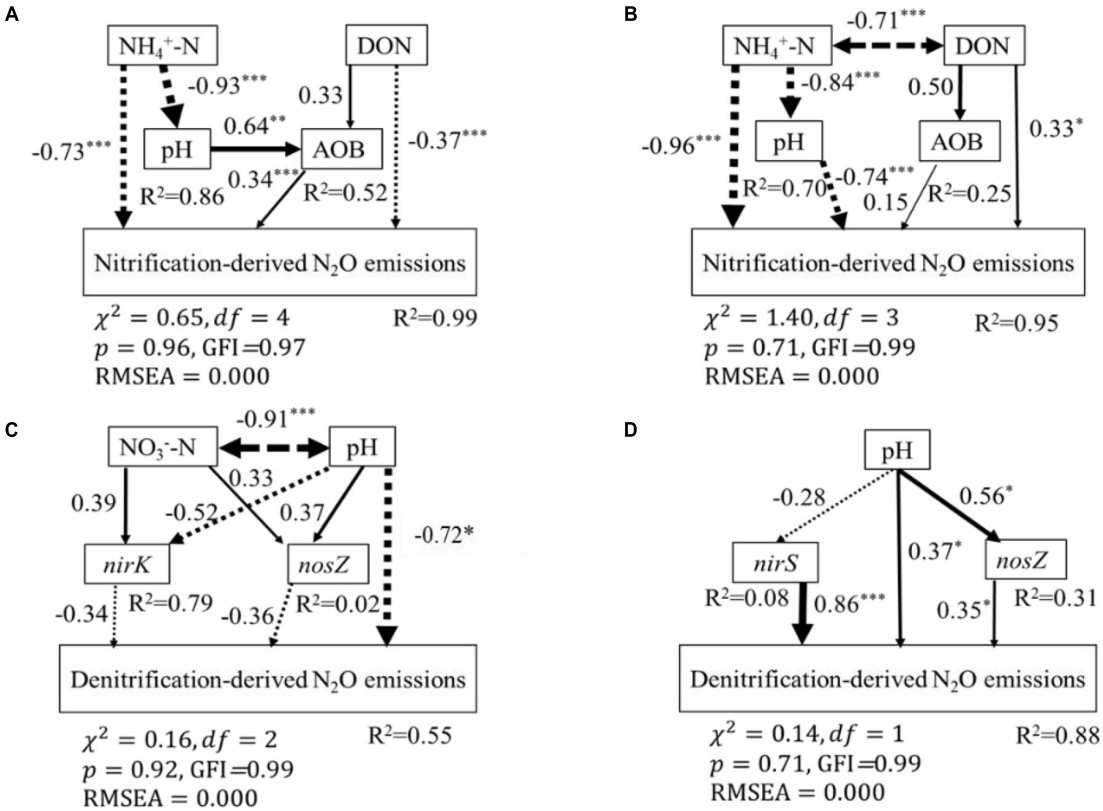

FIGURE 6 | Structural equation modeling (SEM) analysis of the effects of soil biotic and abiotic properties on soil nitrification-derived N_2O emissions in BF **(A)**, PF **(B)**, and on soil denitrification-derived N_2O emissions in BF **(C)** and PF **(D)**, respectively. Continued and dashed arrows indicated positive and negative relationships in a fitted SEM, respectively. Arrow line thickness indicates the strength of the causal relationship. Numbers adjacent to arrows represented standardized path coefficients of the relationships (*$P < 0.05$, **$P < 0.01$, ***$P < 0.01$). Double-headed arrows represented covariance between variables. The total variation explained by the model is indicated by R^2.

TABLE 3 | Standardized total effects of each explanatory variable on nitrification- and denitrification-derived N_2O emissions.

	Nitrification-derived N_2O emissions		Denitrification-derived N_2O emissions	
	Explanatory variable	**Standardized total effects**	**Explanatory variable**	**Standardized total effects**
BF	NH_4^+-N	−0.93	pH	−0.85
	AOB	0.34	*nosZ*	−0.36
	DON	−0.26	*nirK*	−0.34
	pH	0.22	NO_3^--N	−0.25
PF	pH	−0.74	*nirS*	0.86
	NH_4^+-N	−0.54	*nosZ*	0.35
	DON	0.41	pH	0.33
	AOB	0.15		

etc.) (Liu et al., 2010; Hu et al., 2015; Han et al., 2019). The reductases for nitrate, nitrite, and nitric oxide are more active at pH <7 (Gillam et al., 2008), while the N_2O reductase activities are inhibited in soil with low pH (Levy-Booth et al., 2014). A review covering 50 years of research on the effects of soil pH on denitrification also reports that there is a consistent negative relationship between denitrification-derived N_2O production and soil pH (ŠImek and Cooper, 2002). A close relationship between soil pH and denitrification-derived N_2O in the BF suggested that soil pH is a strong predictor of denitrifier activity in the BF. In the PF, the abundance of *nirS* is the dominant factor controlling denitrification-derived N_2O emissions. Previous studies have also reported that the abundance of denitrification genes correlated with *nirS* gene abundance (Morales et al., 2010).

The dominant factors in regulating soil denitrification-derived N_2O emission were different in these two forests. This may be because differences in the tree species lead to variations in soil environment and the community composition of functional microbes, and changes in these variations induced the differences

in the nitrification- and denitrification-derived N_2O emissions in these acidic forest soils (Barberán et al., 2015; Soper et al., 2018). Moreover, regardless of nitrification- or denitrification-derived N_2O emissions, soil environmental factors are more important than nitrifier and denitrifier abundance in regulating soil N_2O emissions in both forests (except denitrification-derived N_2O emission in PF). Previous studies also demonstrate that tree species-induced changes in community composition and environmental factors play more important roles in regulating soil N_2O emissions than the abundance of nitrifier/denitrifier functional genes (Qin et al., 2019). In the present study, we still do not know how the species diversity or the nature of individual species affect nitrification- and denitrification-derived N_2O production in the field, even though several studies have investigated N_2O emission from plantation forest soils (Arai et al., 2008; Wang et al., 2014; Kou-Giesbrecht and Menge, 2021). Further study needs to focus on the role of the identities of specific species in the production processes of N_2O under different N inputs.

CONCLUSION

Our results demonstrated clearly that nitrification-derived N_2O emissions decreased with N application rates, while denitrification-derived N_2O emissions were non-linear responses to N addition. The total soil N_2O emissions, nitrification-, and denitrification-derived N_2O emissions were significantly higher in the broadleaf forest than in the conifers forest. Changes in soil environmental factors (i.e., pH and soil NH_4^+-N content) induced by N addition are more important than the abundance of nitrification and denitrification functional genes in regulating the production process of soil N_2O. The incorporation of soil nitrogen substrates, soil pH, and forest type into the nitrogen cycling model will more precisely predict the global N_2O from soil under elevated N deposition.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

QQ: conceptualization, methodology, software, investigation, supervision, validation, formal analysis, writing–original draft, writing–reviewing and editing, visualization, data curation, and resources. AM: investigation and writing–reviewing and editing. SJ: writing–original draft, writing–reviewing and editing, visualization, and supervision. YH: conceptualization, methodology, software, validation, formal analysis, writing–original draft, writing–reviewing and editing, visualization, and supervision. All authors contributed to the article and approved the submitted version.

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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.2022.950367/full#supplementary-material

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Belowground Root Competition Alters the Grass Seedling Establishment Response to Light by a Nitrogen Addition and Mowing Experiment in a Temperate Steppe

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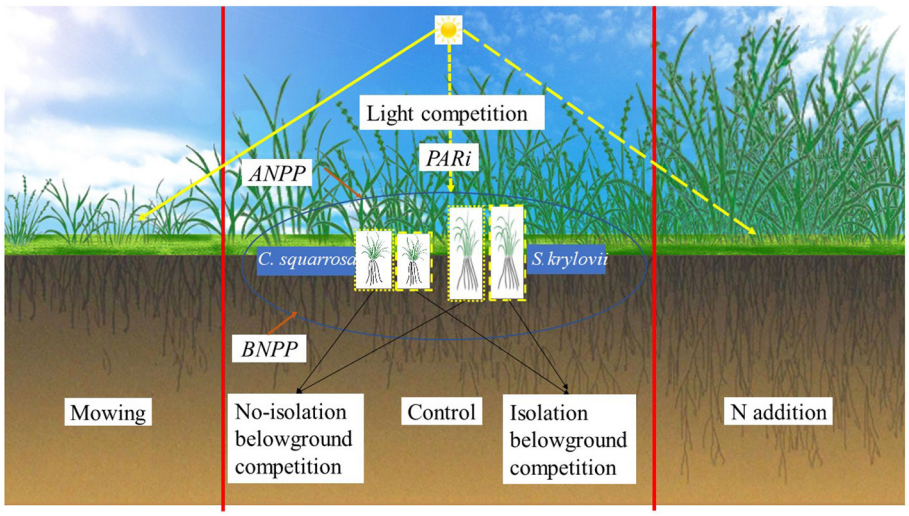
Predicting species responses to climate change and land use practices requires understanding both the direct effects of environmental factors as well as the indirect effects mediated by changes in belowground and aboveground competition. Belowground root competition from surrounding vegetation and aboveground light competition are two important factors affecting seedling establishment. However, few studies have jointly examined the effect of belowground root and light competition on seedling establishment, especially under long-term nitrogen addition and mowing. Here, we examined how belowground root competition from surrounding vegetation and aboveground light competition affect seedling establishment within a long-term nitrogen addition and mowing experiment. Seedlings of two grasses (*Stipa krylovii* and *Cleistogenes squarrosa*) were grown with and without belowground root competition under control, nitrogen addition, and mowing treatments, and their growth characteristics were monitored. The seedlings of the two grasses achieved higher total biomass, height, mean shoot and root mass, but a lower root/shoot ratio in the absence than in the presence of belowground root competition. Nitrogen addition significantly decreased shoot biomass, root biomass, and the survival of the two grasses. Regression analyses revealed that the biomass of the two grass was strongly negatively correlated with net primary productivity under belowground root competition, but with the intercept photosynthetic active radiation in the absence of belowground root competition. This experiment demonstrates that belowground root competition can alter the grass seedling establishment response to light in a long-term nitrogen addition and mowing experiment.

Keywords: belowground competition, land use change, light competition, nitrogen addition, seedling germination



GRAPHICAL ABSTRACT | Effects of light competition and belowground competition on the seedling of *S. krylovii* and *C. squarrosa* under the control, mowing, and N addition treatment.

INTRODUCTION

The seedling stage is a critical phase of plant growth that has a major effect on the structure and composition of natural communities (Ding et al., 2016; Peay and Clemmensen, 2018; Tomlinson et al., 2018; Zhou et al., 2021). Characterizing the responses of different plant seedlings to various factors affecting growth, survival, and biomass allocation can improve our understanding of community assembly and the mechanisms maintaining diversity in natural and disturbed ecosystems (Liu et al., 2012; Zhang et al., 2017a, 2020; Zhong et al., 2019). However, in recent years, relatively few experiments have been conducted to study the response of seedlings to global change, especially *in situ* experiments in the field.

Nitrogen (N) deposition is a major global driver of plant diversity loss that is predicted to increase in the future (Clark et al., 2007; Galloway et al., 2008; Bobbink et al., 2010; Liu et al., 2013b; Li et al., 2021). Most studies of nutrient-induced plant species loss have focused on competition-based mechanisms (Gilliam, 2006; DeMalach et al., 2017), including belowground competition and aboveground competition (light) (Ceulemans et al., 2017; Broadbent et al., 2018; Zheng et al., 2019). Among them, the effect of light competition on plant growth is a hot research topic in nutrient experiment in recent years (DeMalach et al., 2016, 2017; Xiao et al., 2021). Numerous studies have shown that light competition, with a lower light acquisition per unit biomass for small plants, has been proposed as a major mechanism of species loss after nutrient addition (DeMalach et al., 2016, 2017; Xiao et al., 2021). There are also studies showing that under nutrient addition, light is an important contributor affecting diversity replenishment, but not a decisive factor (Harpole et al., 2017). However, none of these studies discuss the role of belowground root competition. Belowground root competition is also an important factor affecting plant growth, especially in nutrient addition experiments (Träger et al., 2019; Wang et al., 2019). Differences in responses of large and small plants to belowground root competition may alter plant responses to light competition, affecting plant diversity. But how large and small plants respond to belowground root competition is unclear, especially in nutrient addition experiments.

Mowing for hay is a common land use type in grassland management that has a considerable effect on plant diversity and environment characteristic (Socher et al., 2012; Yang et al., 2012; Zhang et al., 2017b; DoleŽal et al., 2018; Huang et al., 2020). Mowing is often cited as an important mechanism for mitigating biodiversity loss from nutrient enrichment (Collins et al., 1998; Zhang et al., 2017b). Mowing can increase species richness by increasing light availability for small, subdominant plant species, thereby increasing germination rates and promoting seedling recruitment and plant growth (Collins et al., 1998; Yang et al., 2012; Stevens, 2016). Likewise, mowing alters belowground root competition, which in turn affects the establishment of seedlings of different plants. However, which competition is more important, we do not know.

Grasslands contain ∼37% of the vegetation in terrestrial ecosystems and are one of the most important ecosystems in terms of their contribution to global food production (O'Mara, 2012; Wang et al., 2021a). Nutrient enrichment and mowing are two common management practices for increasing the use of grassland ecosystems (Humbert et al., 2016; Zhang et al., 2017b; DoleŽal et al., 2018), but their effect on seedling establishment remains unclear, especially in the nutrition experiment. Although most grassland species are perennials, seedling establishment is still an important factor affecting the structure of grassland plant communities. Here, we conducted a seedling transplant experiment within a multi-year N addition and mowing experiment, simulating seedling builds respond to aboveground light and belowground root competition. Our study species were

Stipa krylovii (large plant) and *Cleistogenes squarrosa* (small plant), which are the most common grasses at the grassland study site. By studying the responses of plants of different sizes to aboveground and belowground competition in nutrient addition and mowing experiments, the mechanism of plant diversity loss under nutrient addition was explored.

MATERIALS AND METHODS

Site Description and Species Selection

This experiment was conducted at Duolun Restoration Ecology Station, which is located in southern Inner Mongolia Autonomous Region (42°02'N, 116°17'E, 1,324 m a.s.l). The long-term (1954–2013) mean annual precipitation is 385 mm, and the mean annual temperature is 2.1°C. Ninety percent of the precipitation occurs between May and October. Monthly mean temperature ranges from −17.6°C in January to 19.2°C in July. The soil is classified as chestnut according to the Chinese classification. Dominant plant species in the temperature steppe include the perennial herbs *Stipa krylovii*, *Cleistogenes squarrosa,* and *Agropyron cristatum* (Yang et al., 2012).

In this study, the two most common grasses, *S. krylovii* and *C. squarrosa*, were selected as the research objects in the grasslands of Inner Mongolia. *Stipa krylovii* is a grass that tends to grow in clusters with large individual (high: 30–80 cm), which is advantageous under nutrient enrichment (Zhao et al., 2016). By contrast, *C. squarrosa* is a lower cluster grass with small individual (high: 10–30 cm), which makes it more prone to loss under nutrient enrichment. Moreover, with a fibrous root system, *C. squarrosa* is considered as a key species for sustainable grassland development (Liang et al., 2002).

Experimental Design

Our experiment was nested within an existing long-term mowing and N addition experiment that began in 2012 (Wang et al., 2020, 2021b). Five 24 × 4 m blocks were arranged into one row and five columns. Each block was randomly assigned to four plots, each 4 × 4 m, with four treatments: (1) control (C), (2) mowing (M), (3) N addition (N, ambient plus 10 g N m^{-2} $year^{-1}$, NH_4NO_3), and (4) combined M with N addition (MN, Wang et al., 2020). The subset of 15 plots of control, mowing, and N addition treatments were used in this experiment.

We collected the seeds of our two study species from a natural community in 2016. These seeds were sown in a seedbed in the field on May 10, 2017, and were carefully nursed for 20 d. Previous observations indicated that the roots of these two species were distributed in the top 15 cm of soil (Yang et al., 2011). Cylindrical root ingrowth containers were made from rigid plastic mesh (diameter = 7 cm, length = 15 cm, mesh = 4 × 4 mm square) (Chen et al., 2017). These root ingrowth containers contain two specifications, one is made of dense mesh (15 cm long, 5 cm width, 50 μm mesh) and the other is made of sparse mesh (15 cm long, 5 cm width, 2 mm mesh). Dense mesh can isolation root competition of surrounding plants for target plants, but sparse mesh cannot. On June 1, 2017, 20 root ingrowth containers were installed in each subset plot, including 10 dense mesh and 10 sparse mesh, and filled with *in situ* soil. Ten *S. krylovii* seedlings were placed in five root ingrowth containers with dense mesh and five root ingrowth containers with sparse mesh in each subset plot. Ten *C. squarrosa* seedlings were placed in the other half of root ingrowth containers in each subset plot. The in each subset plot were watered with 200 ml every day and dead seedlings were replaced for the first week. The seedlings were left to grow naturally until the end of September when they were sampled. The results under the C, M and N treatment were analyzed in this experiment.

Measurements of the Microenvironment

Photosynthetic active radiation (PAR) on the ground was measured three times per month near the seedling within each plot using a Li-Cor Quantum Sensor (Li-Cor, Lincoln, NE, USA) on clear days. Two PAR values of the upper part of the canopy (PAR*u*) and the surface (PAR*s*) were measured at each site. Intercept photosynthetic active radiation (PAR*i*) was calculated using the following formula: PAR*i* = (PAR*u* – PAR*s*)/PAR*u*.

Plant Sampling

On September 30, 2017, we recorded the number of surviving individuals and measured the maximum height of each plant. All seedling in each plot were then taken out from the ingrowth containers. Because of the short time of the experiment, all the roots were located inside the ingrowth containers. Each seedling was separated into shoots and roots. Roots were gently washed from the soil. All samples were oven-dried at 65°C for 48 h and weighed.

In the middle of August 2017, we harvested the biomass of surrounding vegetation at the peak aboveground plant biomass according to species in a 1 × 1 m square in each plot. Aboveground net primary productivity was estimated using standing biomass. Two 50-cm-deep holes were excavated with a soil auger (5-cm internal diameter) in each plot. Soil was sieved through a 2-mm screen, and roots were washed to measure the belowground net primary productivity. All samples were oven-dried at 65°C for 48 h and weighed. Net primary productivity (NPP) is equal to aboveground net primary productivity plus belowground net primary productivity.

Statistical Analyses

We used three-way ANOVAs to assess the effects of species, root isolation, and management strategy on biomass production, height, survival, shoot, root, and shoot/root ratio. Duncan's multiple range test was used to compare differences between treatments. Regression analyses were used to assess the contributions of NPP and PAR*i* to seedling characteristics of the two species. All statistical analyses were performed in R 3.5.0 (Team, 2018).

RESULTS

Individual Biomass, Height, and Survival

The belowground root isolation (RI) treatment significantly increased mean individual biomass and height by 207 and 46% (**Table 1**; **Figure 1**) across both *S. krylovii* and *C. squarrosa*, respectively. N addition significantly decreased

TABLE 1 | Results (*F*-values) of three-way ANOVA on the effects of species (SP), root isolation (RI), management strategy (MS: control, N addition, mowing), and their interactions on total biomass, height, survival, shoot and root biomass, and root/shoot.

Source of variation	Total biomass	Height	Survival	Shoot	Root	Root/shoot
SP	0.00	103.354***	0.21	0.92	4.089*	1.06
RI	58.761***	36.372***	1.28	59.886***	44.29***	13.18**
MS	14.315***	2.76	6.169*	13.27***	13.154***	0.31
SP*RI	0.01	1.79	1.99	1.01	5.454*	17.116***
SP*MS	0.17	3.616*	0.65	0.43	1.39	3.311*
RI*MS	7.606**	1.66	0.33	7.496**	6.221**	0.90
SP*RI*MS	0.08	0.35	1.27	0.40	1.19	1.61

*Significant level of F-value: *p < 0.05, **p < 0.01, ***p < 0.001.*

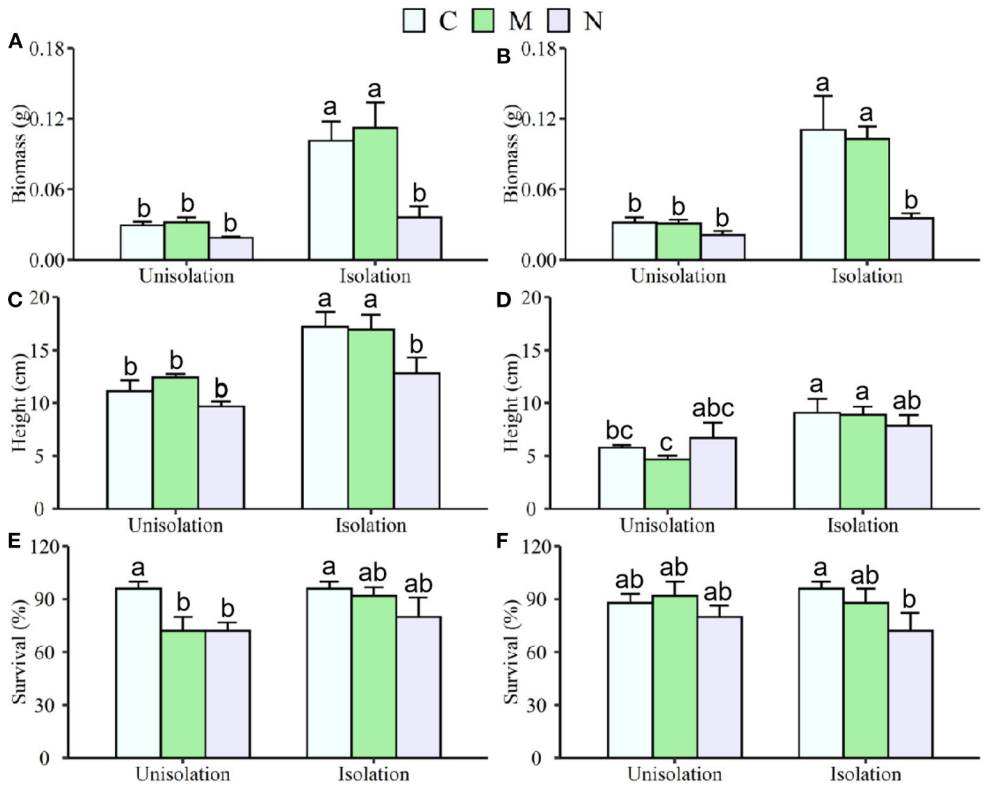


FIGURE 1 | Effects of root isolation7 on biomass, height, and survival of *S. krylovii* **(A,C,E)** and *C. squarrosa* **(B,D,F)** in control (C), mowing (M), and N addition (N) plots. Different lowercases indicate significant differences among the three treatments at $p < 0.05$.

mean individual biomass and survival by 60 and 18%, respectively (**Supplementary Table S1**; **Figure 1**). There was a significant interaction effect between RI and N on individual biomass (**Supplementary Table S1**). RI significantly increased the individual biomass of *S. krylovii* and *C. squarrosa* by 226 and 189% and their height by 47 and 50%, respectively (**Table 2**; **Figure 1**). N addition significantly decreased the individual biomass of *S. krylovii* and *C. squarrosa* by 57 and 63% and their survival by 20 and 15%, respectively (**Supplementary Table S2**; **Figure 1**). Mowing did not affect the individual biomass of *S. krylovii* and *C. squarrosa*. However, the survival of *S. krylovii* was reduced by 14% under the mowing treatment (**Supplementary Table S2**; **Figure 1**). There was a significant interaction between RI and N addition on the biomass of *S. krylovii* (**Supplementary Table S2**).

Shoot and Root Mass and Root/Shoot Ratio

Across the two species, RI treatment significantly increased mean shoot and root mass by 231 and 168%, respectively, and reduced the root/shoot ratio by 0.12 (**Table 1**; **Figures 2**, **3**). N addition significantly decreased mean shoot and root mass by 61 and 58%, respectively (**Supplementary Table S1**; **Figure 2**). There was a significant interaction effect between RI and N on

TABLE 2 | Results (*F*-values) of two-way ANOVA on the effects of root isolation (RI), management strategy (MS: control, N addition, mowing), and their interactions on total biomass, height, survival, shoot and root biomass, and root/shoot of *S. krylovii* and *C. squarrosa*, respectively.

Source of variation		Total biomass	Height	Survival	Shoot	Root	Root/shoot
S. krylovii	RI	31.515***	21.266***	3.333	36.926***	24.738***	0.002
	MS	0.385	0.707	6.533*	0.387	0.372	2.107
	RI*MS	0.034	1.14	3.333	0.052	0.017	1.134
C. squarrosa	RI	24.944***	12**	0.004	25.399***	20.612***	21.153***
	MS	6.926**	0.282	2.641	6.594**	7.685**	2.152
	RI*MS	3.342	1.22	0.882	3.615*	2.239	1.152

*Significant level of F-value: *p < 0.05, **p < 0.01, ***p < 0.001.*

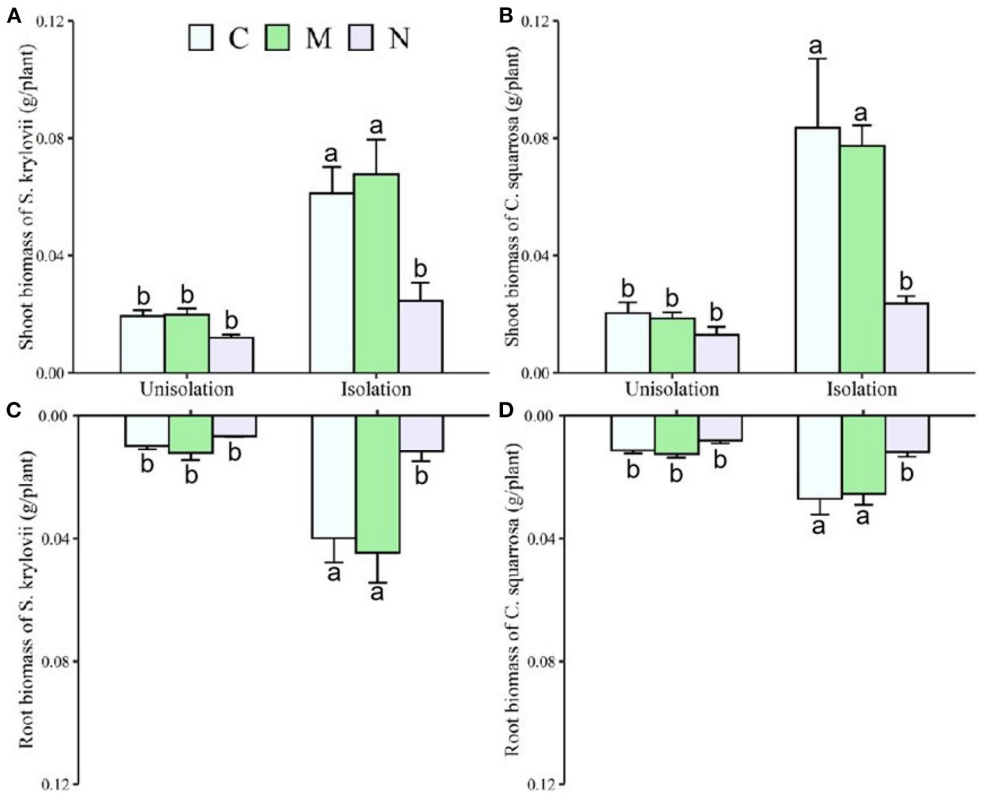


FIGURE 2 | Effects of root isolation on shoot and root biomass of *S. krylovii* **(A,C)** and *C. squarrosa* **(B,D)** in control (C), mowing (M), and N addition (N) plots. Different lowercases indicate significant differences among the three treatments at $p < 0.05$.

shoot and root mass (**Supplementary Table S1**). RI significantly enhanced the shoot mass of *S. krylovii* and *C. squarrosa* by 216 and 224% and root mass by 249 and 98%, respectively (**Supplementary Table S2**; **Figure 2**). N addition significantly decreased the shoot mass of *S. krylovii* and *C. squarrosa* by 54 and 68% and root mass by 63 and 52%, respectively (**Supplementary Table S2**; **Figure 2**). RI significantly decreased the root/shoot ratio of *C. squarrosa* by 0.25 (**Table 2**; **Figure 3**). N addition significantly increased the root/shoot ratio of *C. squarrosa* by 0.15 (**Supplementary Table S2**; **Figure 3**). There were significant interaction effects between RI and N on the shoot and root mass of *S. krylovii* and between RI and M on the shoot/root ratio of *C. squarrosa* (**Supplementary Table S2**).

Relationships of Plant Performance With NPP and PARi

Simple linear regression analyses showed that the biomass of *S. krylovii* was negatively correlated with NPP under the no-isolation treatments ($R^2 = 0.29$, $P = 0.040$, **Figure 4A**). The biomass of *S. krylovii* was negatively correlated with PARi under the isolation treatments ($R^2 = 0.35$, $P = 0.021$, **Figure 4B**). The biomass of *C. squarrosa* was negatively correlated with NPP ($R^2 = 0.29$, $P = 0.039$, **Figure 4C**) and PARi ($R^2 = 0.27$, $P = 0.045$, **Figure 4D**) under the no-isolation treatments but was only negatively correlated with PARi under the isolation treatments ($R^2 = 0.31$, $P = 0.029$, **Figure 4D**).

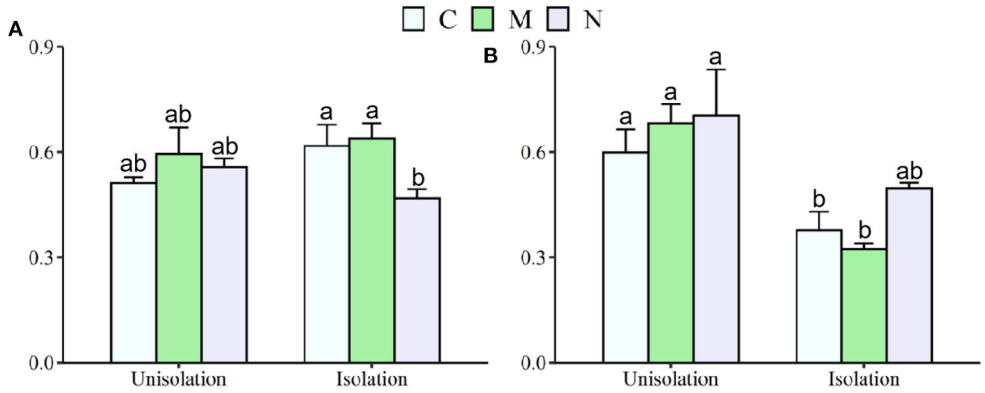


FIGURE 3 | Effects of root isolation on root/shoot rate of *S. krylovii* **(A)** and *C. squarrosa* **(B)** in Control (C), mowing (M), and N addition (N) plots. Different lowercases indicate significant differences among the three treatments at $p < 0.05$.

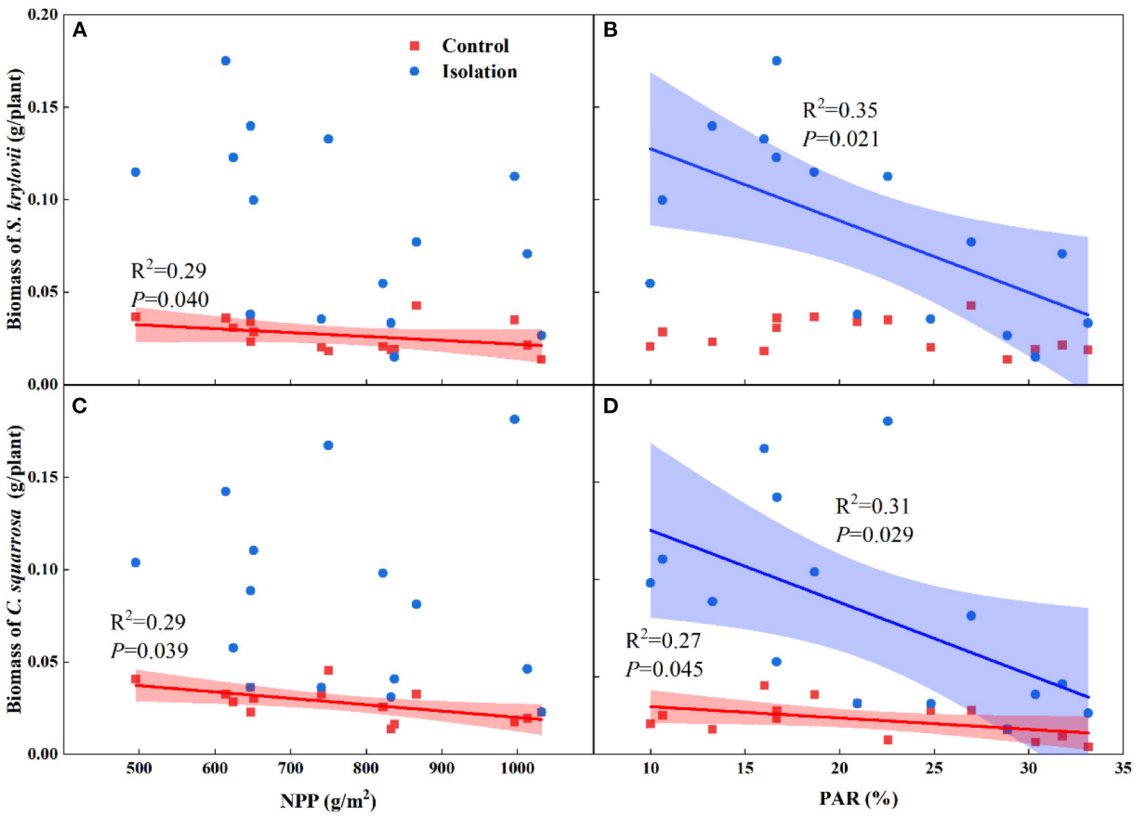


FIGURE 4 | Relationships of total biomass of *S. krylovii* **(A,B)** and *C. squarrosa* **(C,D)** with net primary productivity (NPP) and lower canopy intercept photosynthetic active radiation (PARi). Each data point represents mean biomass of each specie in each plot.

DISCUSSION

Effects of N Addition and Mowing on Seedling Biomass

Nitrogen addition significantly decreased the individual seedling biomass of the two species, whereas mowing did not affect the biomass of these species. The negative responses of seedling biomass of the two species can be explained by indirect factors. Planting experiments showed that N addition increased biomass of seedling because of higher soil N availability (Ceulemans et al., 2017; Luo et al., 2020). But, these studies did not competition from surrounding plants. We found that N addition increased

NPP, which was consistent with other studies conducted *in situ* ecosystems (DeMalach et al., 2017; Wang et al., 2017; Zhao et al., 2018). Therefore, N addition inhibits the growth of seedlings by increasing competition from surrounding vegetation (Jensen and Löf, 2017). On the one hand, N addition increase the height of the surrounding vegetation, increasing light competition (DeMalach et al., 2017). On the other hand, N addition can also increase the belowground root competition (Wang et al., 2019). Therefore, both aboveground light and belowground root competition combines to reduce biomass of seedling. Mowing can have a positive effect on seedling establishment (Bissels et al., 2006; Gibson et al., 2011). In some cases, mowing can decrease vegetation cover or NPP and increase ground light intensity (Collins et al., 1998; Gibson et al., 2011). However, we did not find a significant effect of mowing on the seedling biomass of the two species. This may stem from that the frequency of mowing was only once a year, which thus did not affect NPP and PAR*i*.

Effects of N Addition and Mowing on Seedling Survival

Nitrogen addition and mowing decreased seedling survival in this experiment. Our results were inconsistent with previous studies on herbs (Jutila and Grace, 2002; Bissels et al., 2006; Zhang et al., 2018) or woody plants (Walters and Reich, 2000). This inconsistency might be explained by the different approaches used. Many previous studies have conducted planting experiments in greenhouses or fields in which the surrounding vegetation was absent (Walters and Reich, 2000; Zhang et al., 2018). However, the plots in our study were nested within a long-term N addition and mowing experiment. Both root and light competition are important factors that affect seedling survival (Gunaratne et al., 2011; Tomlinson et al., 2018; Hu and Wan, 2019). For example, nutrient enrichment can decrease seedling establishment in grassland by enhancing light asymmetry and interspecific competition (Xia and Wan, 2013; DeMalach et al., 2017). N enrichment can also increase the availability of toxic metals, which decreases seedling survival (Bobbink et al., 2010). Mowing can increase seedling establishment by removing the most productive plants and decreasing light competition (Collins et al., 1998; Gibson et al., 2011). But the decrease in seedling establishment due to mowing observed in this study may be caused by the lower soil nutrient content and soil quality after long-term clipping (Wang et al., 2020).

Belowground Root Competition on the Seedling Characteristic

In our study, the performance of seedlings significantly increased in the RI treatment. These findings are consistent with previous studies showing that a low level of belowground root competition can maximize the success of seedling recruitment (Haugland and Tawfiq, 2001; Liu et al., 2013a). McConnaughay and Bazzaz (1991) suggested that root competition in the soil not only depletes water and nutrient but also creates physical barriers to root growth. The isolation of neighboring roots may, therefore, increase the physical space available for the growth of target seedling roots as well as reduce competition for other resources (Liu et al., 2013a). However, the effects of neighboring interactions on community structure differ at different phases of population growth. For example, competition associated with neighbors can accelerate seedling emergence (Dyer et al., 2008) but decrease seedling survival and biomass (Fayolle et al., 2009).

Belowground Root Competition Alters the Relationship Between Light and Seedling Establishment

We used a simple correlation analysis to assess the relationship between seedling biomass and environmental factors. The negative relationships between seedling biomass and NPP are consistent with the results of many theoretical and empirical studies under the no-isolation treatments (Liu et al., 2007, 2013a). However, interspecific interactions can be complex (Martorell et al., 2014). Negative interspecific competition can occur when one species occupies the space required for another species to establish (e.g., mats of vegetation), and positive interactions can occur when, for example, adult plants create an optimal microclimate that facilitates the recruitment of small seeds and seedlings (Martorell et al., 2014). In our study, most species share similar ecological niches, so the relationship between species is more competitive than mutually reinforcing. Root and light competition are considered two important aspects of interspecific competition. Previous studies show that light competition is one of the main factors affecting seedling growth (Liu and Han, 2007; Fayolle et al., 2009; Liu et al., 2013a). However, other studies show that belowground root competition has been found to be more important than light competition in grasslands (Cook and Ratcliff, 1984; Haugland and Tawfiq, 2001). In our study, light competition becomes an important factor affecting seedling biomass when belowground root competition is isolation (**Figure 4**). Further analysis of previous studies found that the experiments that considered light competition as the main factor were mostly greenhouse experiments or planting experiments (Hautier et al., 2009), while the experiments that considered belowground root competition to be the main factor were mostly *in situ* experiments (Cook and Ratcliff, 1984; Haugland and Tawfiq, 2001; Wang et al., 2021c). Therefore, our study suggests that belowground root competition alters the relationship between light and seedling establishment (**Graphical Abstract**).

CONCLUSIONS

Nitrogen addition significantly decreased the biomass and survival of seedlings. Grass seedlings achieved higher biomass and height under belowground root competition. NPP was negatively related to biomass under belowground root competition. However, the intercept PAR significantly affected the biomass of the two grass species in the absence of belowground root competition. The differential effects of belowground root competition and management strategy on seedling characteristics were largely attributed to the indirect effects of changes in NPP and light. Our findings provide insight

into the mechanisms underlying the response of seedlings to aboveground and belowground root competition, information that is crucial for predicting the responses of species to global change.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

AUTHOR CONTRIBUTIONS

ZY designed the research. MZ, DW, and ZY collected data and performed the analysis. All authors wrote the article, 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.2022.801343/full#supplementary-material

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Effect of Nitrogen Application on the Sensitivity of Desert Shrub Community Productivity to Precipitation in Central Asia

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Precipitation variability and nitrogen (N) deposition caused by anthropogenic activities could profoundly impact ecosystem productivity and carbon cycling. In desert ecosystems, vegetation is sensitive to changes in precipitation and N deposition. However, the impacts of large changes in precipitation, especially with a concurrent increase in N content, on plant community remain unclear. In this study, we carried out experiments to monitor the impacts of five precipitation levels and two N levels on the plant community function and composition from the Junggar desert in Central Asia during the period 2018–2019. Our results showed that: (1) Aboveground net primary production (ANPP) significantly increased with increasing precipitation, it followed a positive linear model under normal precipitation range, and nonlinear mode under extreme precipitation events; (2) N application led to an increase in ANPP, but did not significantly improve the sensitivity of ANPP to precipitation change; (3) Changes in N content and precipitation, and their impacts on ANPP were mainly driven by plant density. These results provide a theoretical basis for predict the future dynamics of terrestrial vegetation more accurately under climate change and increasing nitrogen deposition.

Keywords: aboveground net primary productivity, nitrogen application, precipitation variation, desert ecosystem, sensitivity

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INTRODUCTION

In arid and semi-arid areas, water shortage and nitrogen (N) deficit are often considered to be the two key factors limiting plant growth and community formation (Noy-Meir, 1973; Avolio et al., 2014). Human activities have intensified the terrestrial hydrological cycle (IPCC, 2018), leading to an increase in the frequency of extreme drought and extreme precipitation events (Swain et al., 2018). Moreover, they have also contributed to a 3–5 fold increase in active N over the past century (Gruber and Galloway, 2008; Boutin et al., 2017). Thus, such changes are bound to have profound effects on productivity and other ecosystem processes.

Aboveground net primary productivity (ANPP) is a functional indicator closely related to energy flux and carbon cycle (Melillo et al., 1993; Haberl et al., 2014). Therefore, the relationship between ANPP and precipitation has always been a long-term concern (Sala et al., 2012; Felton et al., 2019). Most research studies have shown that there is a positive asymmetry between ANPP and

precipitation at the same location, indicating that the positive impacts of wet years on ANPP tend to be much greater than the negative impacts of dry years (Knapp and Smith, 2001; AhlstrÖm et al., 2015; Zhang et al., 2020). However, some studies also showed that there is a negative asymmetry (Luo et al., 2008; Wu et al., 2018) or a positive linear relationship (Wilcox et al., 2016; Felton et al., 2019) between ANPP and precipitation. In addition, these models do not fully consider extreme drought and extreme precipitation events; therefore, Knapp et al. (2017b) proposed a "non-linear double asymmetric" model, which has wider applications and considers both positive asymmetry under nominal precipitation conditions and negative asymmetry under extreme precipitation events.

As noted above, the relationship between ANPP and precipitation is inconsistent, and the differences in vegetation community composition, the sensitivity of species to precipitation change, and biogeochemical factors in different areas under study are the major reasons for the inconsistent relationship between ANPP and precipitation (Knapp et al., 2017b; Deng et al., 2021). Previous studies on the relationship between ANPP and precipitation focus primarily on temperate steppe ecosystems (Knapp et al., 2017a). As an important part of ecosystem system, desert ecosystems are areas that need urgent attention. However, the changing patterns of ANPP in desert ecosystems having a broad range of precipitation are still unclear (Knapp et al., 2017a; Song et al., 2019; de Boeck et al., 2020).

Besides precipitation, N has long been considered one of the major factors affecting plant growth, according to Liebig's law of the minimum (Thomas, 1929). Several studies have shown that N deposition could increase ANPP (Xu et al., 2016b; Liu et al., 2019), and the response of ANPP to N application is regulated by environmental factors, especially precipitation (Harpole et al., 2011). Precipitation could affect the mineralization rate and uptake of N; thus, regulating N turnover and its effect on plant community structure and function (Gebauer and Ehleringer, 2000). In turn, N application could also affect the sensitivity of plant community structure and function to precipitation change (Meng et al., 2021).

The synergistic interactions between multiple limiting resources have been widely studied (Harpole et al., 2011; Marleau et al., 2015). Those studies indicated that soil moisture and N content are the major factors limiting vegetation growth in most ecosystems. Therefore, N application generally increased the sensitivity of ANPP to increased precipitation (de klein et al., 2015; Ma et al., 2020). When compared to the vegetation community sensitivity to increased precipitation after N application, the sensitivity to reduced precipitation is still uncertain. N application could reduce the drought tolerance of plants by decreasing root shoot ratio and increasing stomatal conductance (Gessler et al., 2017), and improve the sensitivity of vegetation community to drought (Xu et al., 2014). However, certain mechanisms suggest a reduction in the sensitivity of ANPP to drought conditions after N application. For example, higher soil available N could increase the consumption of N-based compounds by plants for osmotic regulation, thus alleviating the nutrient deficiency caused by drought (He and Dijkstra, 2014). In recent years, nitrogen deposition has increased from 1.3 to 2.1 g N m^{-2} y^{-1} in China (Liu et al., 2013). Therefore, elucidating whether nitrogen application increases the sensitivity of ANPP to precipitation change will predict the future dynamics of terrestrial vegetation more accurately.

The mechanism governing the impact of precipitation and N content on ANPP is not yet well-understood (Weltzin et al., 2003; Ogle and Reynolds, 2004; Luo et al., 2021; Guo et al., 2022). Generally, the changes in precipitation determine the changes in ANPP by triggering physiological responses of individual plants and based on community attributes (plant density, height, species richness, etc.) (Smith et al., 2009). When there are changes in the precipitation pattern, the plant features initially affected are the leaf water potential and degree of stomatal opening and closing in plant leaves, thus affecting the photosynthetic capacity of plant leaves. Further, they could lead to a change in the biomass of individual plants by altering the plant height and tillering number (Weltzin et al., 2003; Yahdjian and Sala, 2006). However, this change is often limited. The demographic mechanisms suggest that the plant density increases to overcome meristem constraints and increase ANPP with the increase in precipitation, especially in arid and semi-arid regions (Hu et al., 2018; Felton et al., 2020). The diversity hypothesis suggests that greater species diversity leads to more functional strategies and complementary resource utilization to increase ANPP (Diaz and Cabido, 2001). However, most studies have shown that N deposition has increased ANPP but decreased species diversity (Siddique et al., 2010; Isbell et al., 2013b; Soons et al., 2017). Further studies are necessary to understand the effect of species diversity on productivity in resource-limited arid and semi-arid areas.

The Junggar Desert in Northwest China forms an important part of the temperate desert biome in Central Asia and has not been studied as much as deserts in other regions (Song et al., 2019). Similar to many drylands, the Junggar Desert is expected to experience an increased frequency of extreme drought and extreme precipitation events (Ma et al., 2015; Huang et al., 2017). Several studies have shown that water and N are the two major factors affecting vegetation productivity in the drylands (Yahdjian et al., 2011; Dijkstra et al., 2018). However, the effects of precipitation and N deposition and their impacts on vegetation productivity in arid ecosystems are not well-understood. In this context, the present study aims to analyze the sensitivity of plant community structure and functions to large changes in precipitation and to assess the influence of N application on ecosystem sensitivity. While conducting the study, we hypothesized that: (1) the relationship between ANPP of shrub communities in the Southeastern Junggar Desert and precipitation in the growing season conforms to the non-linear double asymmetric model, (2) N application increased the sensitivity of ANPP to precipitation change, and (3) ANPP was primarily driven by the plant density.

MATERIALS AND METHODS

Study Site

The study was conducted in the Mori Wildlife Ecological Monitoring and Experimentation Station, Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences, located

on the southeastern edge of the Junggar Basin, Northwest China (43°59′ N, 90°48′ E, 1,000 m AMSL). The climate of the study area is arid and cold with the monthly average temperature varying from-15°C in January to 20°C in July and annual precipitation of 150 mm. Winds are strong in spring and summer, and gales above force 7 are frequent and occur for over 80 days annually. The soil in the region is mainly clayey and sandy (Xu et al., 2016a). The vegetation is co-dominated by shrub species such as *Anabasis salsa* and *Seriphidium borotalensis* with the plant height ranging from 10 to 15 cm. The most common herbaceous plants found in the region are *Ceratocarpus arenarius, Salsola paulsenii, Halogeton glomeratus, and strigosella (Malcolmia) Africana* (Yang et al., 2008).

Experimental Design

The experiments were conducted according to the split-plot design in randomized complete blocks. From early May 2016, the main plots were subjected to the precipitation treatment, while the subplots were treated with N. The precipitation treatment had five levels: one level as the precipitation control, i.e., treatment with the ambient amount of precipitation while the precipitation was reduced and increased by 60 and 30% relative to the ambient precipitation in the remaining four levels. The five levels are marked here as Control, −60, −30, +30, and +60%. The N treatment had two levels: without N application as the N control (N0) and with N application (N10) at the rate of 10 g N m^{-2} yr^{-1}. This value is the critical load point to influence plant community structure and function (Bai et al., 2010). The main plots (8 × 3 m) subjected to the five-level precipitation treatment were randomly allocated to each block with five replicates, and the subplots (half of the main plot) subjected to the two-level N treatment were randomly placed in each main plot with a 3 m buffer zone separating contiguous main plots. During each early growing season (1–15 April), N was applied using ammonium nitrate (NH_4NO_3) dissolved in 33.75 L water (equal to 0.15 mm rainfall), and applied to each plot using a sprayer to distribute the fertilizer evenly. An equivalent amount of water was applied to each N control plot.

For drought plots, passive rainfall shelters were constructed to create experimental drought conditions based on the design given by Yahdjian and Sala (2002). We chose the same design as used in that study due to its effectiveness and because it exerted only minimal impacts on the energy balance and microclimatic conditions (Hoover et al., 2015). The shelters constructed were 0.6 m high at the short end and 1.6 m high at the long end. These rainout shelters were composed of 3 × 3 m steel and transparent polycarbonate plastic strips. An adequate buffer of 50 cm was set at the shelter edges to prevent the edge effects. The collected rainwater after each rainfall event was added manually to subplots which are to be treated with increased precipitation. The steel sheets were installed at the edges of each plot at a depth of 30 cm to prevent the horizontal exchange of soil water and nutrients between plots and isolated areas. The transparent polycarbonate plastic strips for the shelters will be removed in October and reinstalled in March of the following year.

Precipitation Data

Long-term annual precipitation data were obtained from Drought-Net (http://www.drought-net.org/). During the growing season, the precipitation at the experimental site was obtained from an automatic meteorological observation station located about 50 m away from the experimental site. A lognormal function was used to calculate the estimated probability density function of precipitation.

Vegetation Survey and Plant Aboveground Biomass

From April to September in the years 2018 and 2019, we recorded the number and height of plants were recorded twice a month, and calculated plant density and height using data from early August. The density of each plant species was calculated by dividing the number of plants by the plot area. The average plant height of the three densest species was considered the height of the community. Species richness was annually recorded as the total number of constituent plant species within the 1 m^2 quadrat. Community cover was conducted using the method developed by Yang et al. (2011).

Aboveground biomass was measured during the peak biomass period in 2018 and 2019 (i.e., the first 2 weeks of August) by harvesting all the aboveground plant material in a 1 × 1 m quadrat in each plot. After harvesting, the shrubs and herbs were separated. All the plant samples were oven-dried at 75°C until the weights remained constant. We measured sensitivity as the change in ANPP per unit change in rainfall (Smith et al., 2017) and the sensitivity of shrubs and herbs were calculated as the mean of sensitivity from different precipitation treatments.

Statistical Analyses

A generalized linear mixed model (GLMM) was used to evaluate the individual and interactive effects of precipitation change, N application, and year on community attributes (plant density, height, community cover, and species richness), ANPP and the sensitivity of ANPP. During the analyses, the precipitation change, N application, year, and their interactive effects were considered fixed factors, while plots were considered random effects. The model fitting was carried out by selecting the standard link function corresponding to the variables. Plant cover, density, height, ANPP and the sensitivity of ANPP data were found to be normally distributed. The species richness data showed binomial distribution and Poisson distribution. The standard link functions for these distributions are identity function, logit function, and logarithmic function, respectively (Zou et al., 2013). One-way ANOVA was used to determine the effect of different plant life forms on ANPP sensitivity.

The least significant difference (LSD; $P < 0.05$) test was used to compare the differences in the mean values when the GLMM and ANOVA results were significant. Regression analysis was used to evaluate the response of ANPP with precipitation change for N0 and N10. The final regression models were selected according to Akaike information criterion (AIC) and coefficient of determination (R^2). All the data analyses were performed using SPSS 25.0 (SPSS Inc., Chicago, IL, USA), and the graphs

were prepared using the software Origin 9.0 (OriginLab Corp., Northampton, MA, USA).

Finally, the structural equation modeling (SEM) technique was adopted to estimate the direct and indirect effects of precipitation change and N application and their integrated effects on ANPP using the Amos 21.0 software (Smallwaters Corporation, Chicago, IL, USA) based on the hypothesized causal relationships and the results from the previous studies (**Supplementary Figure 1**). The fitness of the SEM model was evaluated by the non-significant χ^2 test ($P > 0.05$), low Akaike value (AIC), high goodness of fit index (GFI) (>0.90), and low root mean square error of approximation (RMSEA) (<0.05) (Hooper et al., 2008).

RESULTS

Changes in Precipitation

Based on the meteorological data from the study area, the mean growing-season (end of March to September) precipitation (GSP) was 50.1 mm, whereas the GSP in 2018 and 2019 was 62.5 and 47.7 mm, respectively (**Figure 1**). The mean GSP values for −60 and +60% precipitation treatments in our study were 25.0 and 100.0 mm, respectively, in 2018. The former GSP value was marginally near the 10th percentile, while the latter exceeded the 95th percentile of the historical GSP distribution. In 2019, the GSP of −60% precipitation treatment was reduced to 19.1 mm, below the 5th percentile, whereas the GSP of +60% precipitation treatment increased to 76.*3 mm, exceeding the 90th percentile (**Figure 1**).

Changes in Community Attributes

According to the GLMM, precipitation, N addition, and year led to significant changes in the community cover, plant density, height, and species richness (**Table 1**). Community cover, plant density, height, and species richness increased gradually with the increasing precipitation (**Figure 2**). Precisely, −60, −30, +30, and +60% precipitation treatments led to −38.7, −29.4, +4.7, and +20.3% change in community cover; −23.9, −11.6, +24.3, and +23.3% change in plant density; −14.5, −5.8, −3.7, and +10.7% change in height and −8.9, −34.3, +69.8, and +56.7.0% change in species richness relative to the control, respectively (**Figure 2**). N addition also led to a notable ($P < 0.05$) increase in the community cover (+15.7 %), plant density (+15.7 %), and height (+22.4 %); however, it caused a significant ($P < 0.05$) decline in the species richness (−4.4%) (**Figure 2**). In addition, the interactive effect of precipitation and N application led to significant changes in community plant height, density and species richness (**Table 1**). N addition promoted the increase of plant density and height induced by precipitation, but inhibited the increase of species richness (**Figure 2**).

Relationship Between Precipitation and ANPP After N Application

Precipitation and N application resulted in significant changes in ANPP (**Table 1**, **Figure 3**, $P < 0.01$). Precipitation treatments led to a significant change in ANPP (**Figure 3**). More specifically, −60, −30, +30, and +60% precipitation treatments led to −20.9, −7.4, +11.7, and +3.8% change in ANPP relative to the control, respectively (**Figure 3**). N addition also led to a notable increase (+25.0%) in the ANPP (**Figure 3**).

In the normal precipitation range, ANPP increased gradually with increasing precipitation, and a linear model was fitted better than nonlinear model (**Figure 3** and **Supplementary Table 1**). However, a nonlinear model was fitted better than linear model for the ANPP and precipitation relationship under extreme precipitation events and all precipitation gradients (**Figure 3** and

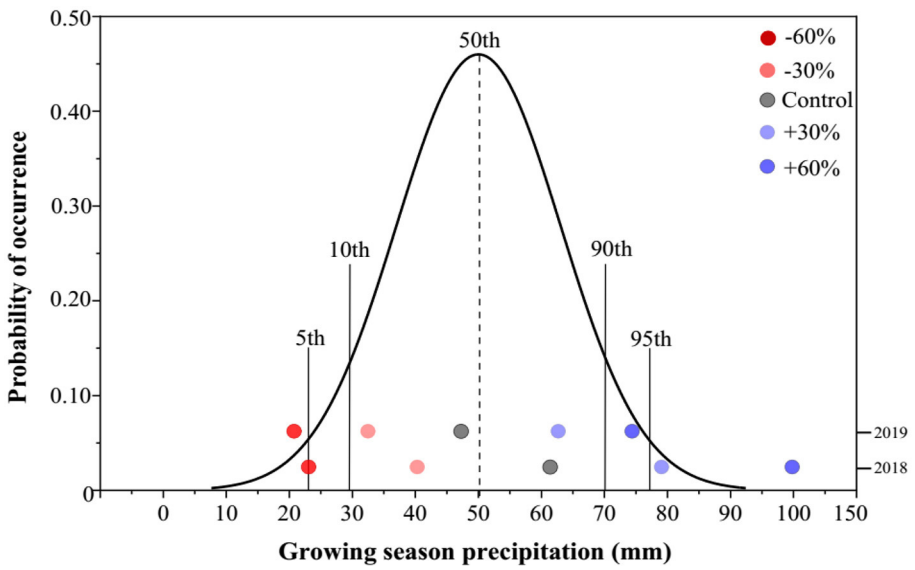

FIGURE 1 | Probability distribution of annual precipitation in the study area during the plant growing season over the past 100 years. The precipitation treatments include five levels, namely, −60%, −30%, Control, +30%, and +60% precipitation relative to the ambient level.

TABLE 1 | GLMM results for the effects of precipitation change (P), nitrogen application (N), year (Y), and their interactive effects on ANPP, the sensitivity of ANPP (Sensitivity) and community attributes.

Effect	Cover		Density	Height	Richness	ANPP	Sensitivity	
	Df	*F*	*F*	*F*	*F*	*F*	df	*F*
P	4	11.14**	36.89**	40.52**	47.74**	19.37**	3	1.8
N	1	4.49*	185.22**	173.07**	173.26**	78.56**	1	1.31
Y	1	10.94**	18.92**	24.99**	70.66**	0.16	1	0.05
P × N	4	0.56	2.76*	6.85**	3.01*	0.17	3	1.03
P × Y	4	3.43*	4.35**	1.94	7.16**	0.08	3	0.06
N × Y	1	7.86**	0.14	9.30**	6.05*	0.07	1	0.02
P × N × Y	4	2.76*	7.33**	3.13*	5.61**	0.12	3	0.09

*Indicates $P < 0.05$ and **indicates $P < 0.01$.

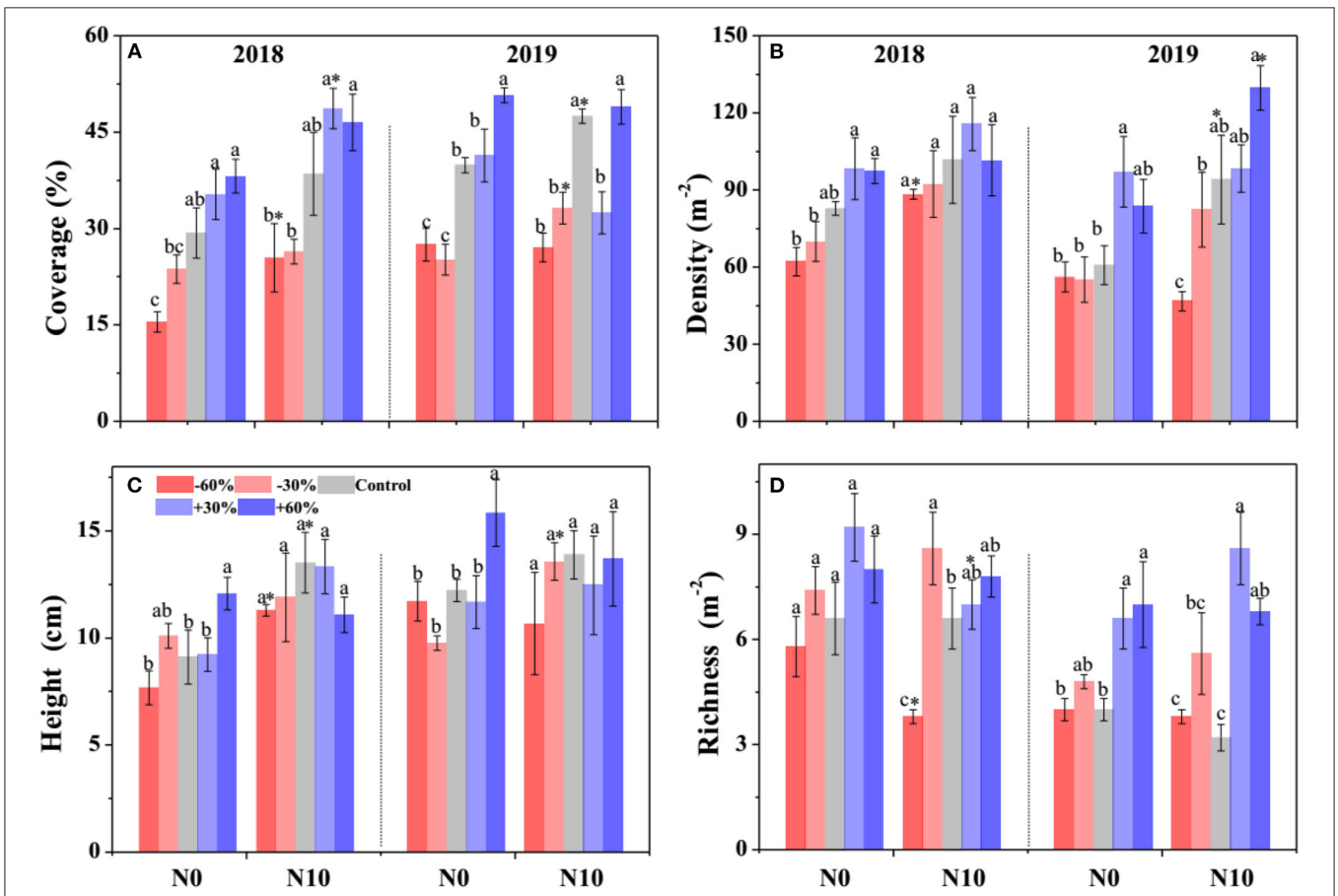

FIGURE 2 | Effects of precipitation change and nitrogen application on community attributes **(A–D)** in 2018 and 2019. Bars are represented as mean ±1 SE. Different lowercase letters indicate significant differences ($P < 0.05$) between precipitation treatment groups. *Indicates significant differences ($P < 0.05$) between the N0 and N10 treatments. The precipitation treatments include five levels, namely, −60%, −30%, Control, +30%, and +60% precipitation relative to the ambient level. N0 and N10 represent the treatments without N addition (Control) and with 10 g N m^{-2} yr^{-1} application of nitrogen, respectively.

Supplementary Table 1). Although N application increased the sensitivity of ANPP to precipitation change, it was not significant (**Figure 4** and **Table 1**). N application did not change the fitting relationship between ANPP and precipitation, that is, the fitting relationship between ANPP and precipitation is a linear model in the normal precipitation range, and nonlinear model in the extreme precipitation events and all precipitation gradients (**Figure 3**).

With precipitation application from 19.1 to 100.0 mm, the relative ANPP of shrubs declined from 77.0 to 51.1%, while the

relative ANPP of herbaceous plants increased from 22.9 to 40.8% (**Supplementary Figure 2**). N application further increased the proportion of herbaceous plants (19.1 mm: from 22.9 to 31.7%; 100.0 mm: from 40.5 to 48.8%, **Supplementary Figure 2**). In addition, the sensitivity of ANPP of herbaceous plants to precipitation change was significantly ($F = 96.2$, $P < 0.01$) greater than that of shrubs (**Figure 4**). N addition increased the sensitivity of herbaceous and shrub ANPP to precipitation change, but neither of them was significant (**Figure 4**).

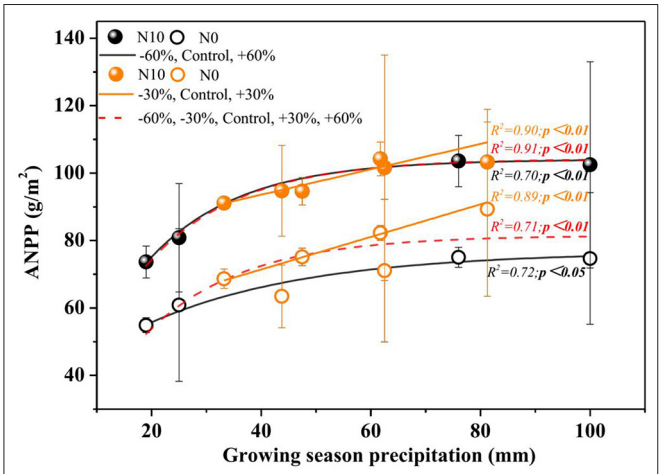

FIGURE 3 | Relationship between ANPP and amount of precipitation under two nitrogen levels. *P-values* smaller than 0.05 are presented in bold. Points are represented as mean ±1 SE. The precipitation treatments include five levels, namely, −60%, −30%, Control, +30%, and +60% precipitation relative to the ambient level. N0 and N10 represent the treatments without N addition (Control) and with 10 g N m^{-2} yr^{-1} application of nitrogen, respectively. The fitting function is shown in **Supplementary Table 1**.

Effects of Precipitation and N Application on ANPP

The SEM was used to assess the integrated effects of precipitation, N application, and community attributes (plant density, height, and species richness) on ANPP. Overall, the precipitation and N application increased the ANPP mainly by increasing the plant density (**Figure 5**). Further, quantification of the indirect effects (product of path coefficients) implied that the major factor affecting ANPP was plant density (0.112), followed by species richness (0.072), and height (0.065), as shown in **Figure 5**. N application increased the ANPP also mainly by increasing the plant density (0.097) followed by height (0.080, **Figure 5**). In addition, N application had a negative effect on species richness (**Figure 5**).

DISCUSSION

The Relationship Between ANPP of Shrub Communities and Precipitation in the Growing Season

In this study, the relationship between ANPP and precipitation is not completely consistent with our hypothesis. In the normal

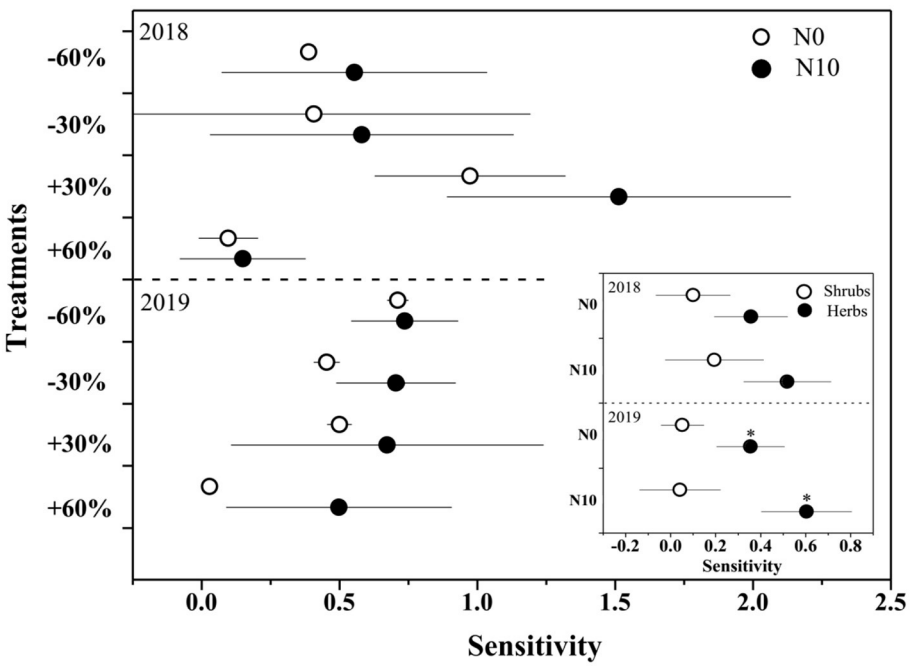

FIGURE 4 | The sensitivity of ANPP and shrubs and herbs (inserted panels) to precipitation change and nitrogen application in 2018 and 2019. Points are represented as mean ±1 SE. The precipitation treatments include five levels, namely, −60%, −30%, Control, +30%, and +60% precipitation relative to the ambient level. N0 and N10 represent the treatments without N addition (Control) and with 10 g N m^{-2} yr^{-1} application of nitrogen.

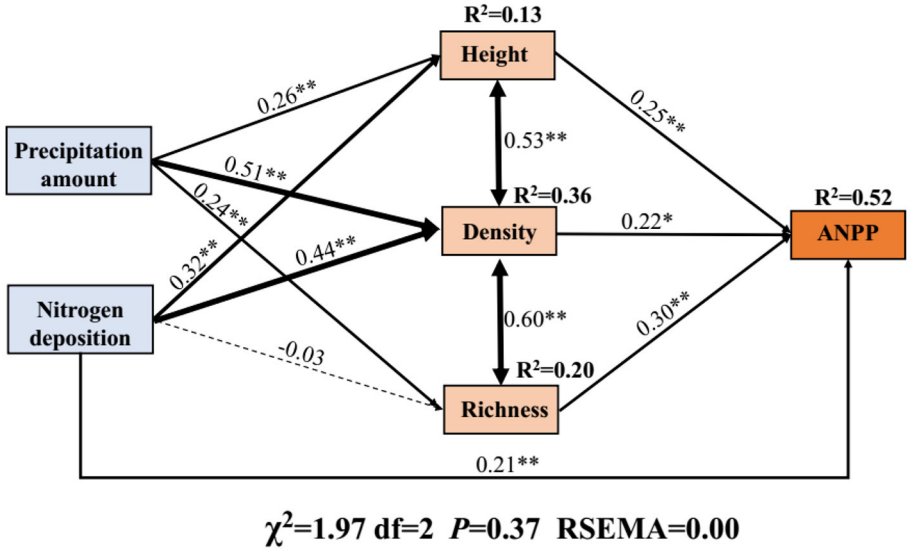

FIGURE 5 | Structural equation model (SEM) representing the effects of precipitation change, nitrogen application, and their integrated impacts on ANPP. The SEM considered all plausible pathways through which plant traits influence ANPP. Solid lines represent the positive paths, and dashed lines indicate negative paths. Arrow width is proportional to the strength of the relationship. *Indicates $P < 0.05$ and **indicates $P < 0.01$. R^2 represents the proportion of the variance for each dependent variable in the model.

precipitation range, a linear model was fitted better than nonlinear models for the ANPP and precipitation relationship (**Figure 3**). Both recent studies conducted in the Central Great Plains of Colorado, USA and Damao County, Inner Mongolia, China, showed that linear relationship often best explains the ANPP responses to changes in precipitation (Felton et al., 2019; Ma et al., 2020), and they suggested that the rapid plant compositional shifts may be the reason for the linear relationship between ANPP and precipitation.

Our results are consistent with non-linear double asymmetric model proposed by Knapp et al. (2017b) only under extreme precipitation events, that is, the increase of ANPP of extreme precipitation is less than the decrease of extreme drought (**Figure 3**). In general, plant physiological processes, meristem tillering, density constraints and soil nutrients could well account for the non-linear double asymmetric model in both extreme drought and extreme precipitation (Yahdjian and Sala, 2006; Smith et al., 2009; Xu and Zhou, 2011; Felton et al., 2019).

The "hierarchical response framework" suggested by Smith et al. (2009) postulates that general drought conditions could trigger the physiological response of plants. However, drought conditions for a long time or extreme drought conditions might lead to plant physiological responses exceeding the threshold limit. Such extreme conditions result in a significant increase in community mortality, a decrease in the plant density (Niu et al., 2014; Felton and Smith, 2017). The results of our study showed that plant density was the most important driving factor of ANPP (**Figure 5**). Therefore, the decrease of plant density caused by extreme drought would inevitably increase the rate of ANPP decline (**Figures 2B**, **3**).

In addition, according to Liebig's law of the minimum (Thomas, 1929), the factors limiting plant growth are often the ones present in small quantities relative to the plant demand. Therefore, when there are extreme precipitation events, soil nutrient distribution may become the primary factor limiting plant growth in general, especially in N-deficient desert ecosystems (Huxman et al., 2004; Eskelinen and Harrison, 2015). As a result, the rate of ANPP rise declines, and the relationship between ANPP and precipitation shows a negative asymmetry during extreme precipitation.

The experimental area under study is characterized by poor soil nutrient content (Wu et al., 2020). However, interestingly, N application did not change the relationship between ANPP and precipitation in the growing season, which still made a nonlinear model was fitted better than linear model for the ANPP and precipitation under N10 treatments (**Figure 3**).

In addition to soil nutrients, vegetation constraints may also play an important role in mediating ANPP-precipitation relationship in the drylands (Lauenroth and Sala, 1992; Sala et al., 2012; Felton et al., 2019). Vegetation constraints, such as leaf area, meristem, tiller density, and overall growth limitations responses to water availability is a key mechanism limiting ANPP, particularly with respect to responses to increases in precipitation (Yahdjian and Sala, 2006; Estiarte et al., 2016; Knapp et al., 2017b). Unfortunately, our analyses did not include these indicators (e.g., leaf area, meristem). Moreover, the community composition discussed in detail below may also be one of the important factors regulating the relationship between ANPP and precipitation.

Interactive Effects of Precipitation Change and N Application on ANPP

In contrast to our hypothesis, although N application increased ANPP (**Figure 3** and **Table 1**), it did not significantly improve the sensitivity of ANPP to precipitation change (**Figure 4** and **Table 1**). Terrestrial ecosystems are generally limited by nitrogen, so increased nitrogen could improve community productivity (Hooper and Johnson, 1999; Vitousek et al., 2002; Xu et al., 2016b; Liu et al., 2019). Furthermore, several recent studies have demonstrated that N deposition could also improve the sensitivity of ANPP to precipitation reduction or increase (Bharath et al., 2020; Ma et al., 2020; Meng et al., 2021).

N application not only increases the nutrient availability in the soil but also decreases the drought tolerance of plants by reducing the root to shoot ratio, increasing the stomatal conductance and plant height, and impairing the symbiotic relationship between plants and mycorrhiza (Gessler et al., 2017). Xu et al. (2014) found that long-term N application resulted in a 33% increase in the grassland productivity response to drought conditions in Duolun Grassland, Inner Mongolia, China. The multiple resource co-limitation theory proposed by Harpole et al. (2011) states that soil N dynamics and the effect of N on plant communities largely depend on water availability (Delgado-Baquerizo et al., 2013; Xu et al., 2016b). Therefore, N application generally increased the sensitivity of ANPP to increased precipitation (de klein et al., 2015). While, in the present study, N application did not significantly improve the sensitivity of ANPP to precipitation change (**Figure 4**), which may be related to community composition.

The dominant species in our study area are shrubs *A. salsa* and *S. borotalensis*. In general, desert shrubs, as perennial woody plants, grow at a slow rate and have stronger resistance to stress and poor environment than herbaceous plants, therefore, they are relatively insensitive to environmental changes (Peters et al., 2012; Baze et al., 2013). Gonzalez-Paleo and Ravetta (2018) found that shrubs are less sensitive to high levels of nitrogen availability than annual herbs with acquisitive resource use strategies. Therefore, nitrogen-induced changes in herbs productivity tend to account for most of the total changes in ANPP in arid shrub communities (Hall et al., 2011; Peters et al., 2012). A recent meta-analysis (Xu, 2021) also found that the response of ANPP of woody plants (36.15%) to nitrogen addition was significantly lower than that of herbaceous plants (56.11%). In this study, our results also showed that herbaceous plants were significantly more sensitive to precipitation change and N application than shrubs (**Figure 4**). However, according to the mass ratio hypothesis (Grime, 1998), dominated species occupy the majority of the niche space and have disproportionate effects on ecological processes, so the change of community productivity mainly depends on the dominant species (Smith et al., 2020). Therefore, due to the large proportion of shrubs biomass (**Supplementary Figure 2**), N application did not significantly improve the sensitivity of community ANPP to precipitation change.

The evidence above suggests that shrub-herb composition, rather than soil nutrients, may be the main factors regulating the sensitivity of community productivity to precipitation change and ANPP-precipitation relationship in the arid shrub communities.

Precipitation Change and N Application Drive ANPP Mainly Through Plant Density

Our results showed that plant density had the greatest effect on ANPP under precipitation change and N application (**Figure 5**). The results confirmed our hypothesis. It is well-known that ANPP is closely related to the plant density, height (Smith et al., 2009) and species richness (Hooper et al., 2005; Isbell et al., 2013a), and precipitation and N application change the values of these parameters, further driving changes in ANPP (Zang et al., 2021).

Plant height is a trait associated with plant growth ability, so community productivity usually increases with plant height (Moles et al., 2009; Luo et al., 2021). However, in our study area, winds are strong in the growing season, and gales above force 7 are frequent and occur for over 80 days annually, which leads to plants widespread dwarfing (Zhang et al., 2013; Xu et al., 2017). Therefore, the precipitation and nitrogen could only increase plant height limited. The seeds in deserts have flexible germination strategies due to high precipitation variability and unpredictability (Noy-Meir, 1973). They can quickly acclimatize to the external environment and make adjustments with respect to the germination time (Koller, 1972; Chen et al., 2020). With increased precipitation, higher germination of seeds was observed, led to an increase in species richness and plant density (**Figures 2B,D** and **Table 1**). Many biodiversity ecosystem functioning experiments have shown that community productivity increases with the species richness, and the species added are generally rare species (Duncan and Young, 2000; Tilman et al., 2001; Hooper et al., 2012). Although rare species can contribute significantly to total community diversity, their contribution to total primary production is relatively low (Smith et al., 2020).

Two major factors may be responsible for plant density had the greatest effect on ANPP under precipitation change and N application. On the one hand, as mentioned above, when a long time drought conditions or extreme drought occurs, the threshold of plant physiological response is broken, and the mortality rate of community increases and the plant density decreases, leading to the decline of ANPP (Smith et al., 2009; Niu et al., 2014; Felton and Smith, 2017). On the other hand, when the availability of water and nitrogen resources increases significantly, the demographic mechanisms suggest that communities increase ANPP mainly by increasing plant density to overcome meristem limitations, especially in desert ecosystems with limited meristem and tillering potential (Hu et al., 2018; Felton et al., 2020).

CONCLUSIONS

In the present study, field experiments were conducted considering water and N as treatment factors. The study attempts to assess the potential mechanisms by which large precipitation change and N application elicit a notable ecological response. In addition, the interactive effects of

precipitation and N application were also evaluated. The study revealed that the relationships between ANPP and precipitation conform to a linear model under normal precipitation range, and nonlinear mode under extreme precipitation events. N application increased ANPP, but it did not significantly improve the sensitivity of ANPP to precipitation change. Moreover, the individual effects of N application and precipitation and their integrated effects on ANPP were mainly driven by plant density, rather than by height or species richness. The ecosystem sensitivity to future changes in precipitation may be regulated by N application. Thus, it can be concluded that multiple constraints should be considered while assessing the sensitivity of terrestrial ecosystems to climate change.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

W-KY and W-XX designed the experiment. KW and Y-XZ performed the field and laboratory work. Y-XZ analyzed the data. Y-XZ and W-XX wrote the manuscript. W-KY provided valuable comments and suggestions on draft. All authors discussed the results and commented on 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.2022.916706/full#supplementary-material

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The effects of changes in flowering plant composition caused by nitrogen and phosphorus enrichment on plant–pollinator interactions in a Tibetan alpine grassland

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Soil eutrophication from atmospheric deposition and fertilization threatens biodiversity and the functioning of terrestrial ecosystems worldwide. Increases in soil nitrogen (N) and phosphorus (P) content can alter the biomass and structure of plant communities in grassland ecosystems; however, the impact of these changes on plant–pollinator interactions is not yet clear. In this study, we tested how changes in flowering plant diversity and composition due to N and P enrichment affected pollinator communities and pollination interactions. Our experiments, conducted in a Tibetan alpine grassland, included four fertilization treatments: N (10 g N m^{-2} $year^{-1}$), P (5 g P m^{-2} $year^{-1}$), a combination of N and P (N + P), and control. We found that changes in flowering plant composition and diversity under the N and P treatments did not alter the pollinator richness or abundance. The N and P treatments also had limited effects on the plant–pollinator interactions, including the interaction numbers, visit numbers, plant and pollinator species dissimilarity, plant–pollinator interaction dissimilarity, average number of pollinator species attracted by each plant species (vulnerability), and average number of plant species visited by each pollinator species (generality). However, the N + P treatment increased the species and interaction dissimilarity in flowering plant and pollinator communities and decreased the generality in plant–pollinator interactions. These data highlight that changes in flowering plants caused by

N + P enrichment alter pollination interactions between flowering plants and pollinators. Owing to changes in flowering plant communities, the plant–pollinator interactions could be sensitive to the changing environment in alpine regions.

KEYWORDS

climate change, grassland ecosystems, biodiversity loss, nutrition addition, pollination network, Qinghai-Tibet Plateau

Introduction

The structure and function of biodiversity, such as the biomass and diversity of plants and the animals that depend on them for survival, are vital for the sustainability of ecosystems (McCann, 2007). However, biodiversity loss caused by anthropogenic nutrient enrichment and climate change threatens the functions and services of terrestrial ecosystems, particularly grasslands (Hautier et al., 2009; Hooper et al., 2012; Isbell et al., 2013). For example, land-use change and environmental pollution have contributed to a decline in biodiversity worldwide (IPBES, 2018). To restore grassland services and functions, specific agricultural management strategies, such as livestock exclusion and chemical fertilizer application, are employed to improve grassland productivity and other ecological services, such as pollination (IPBES, 2016). Plant–pollinator interactions can change through an increase in dominance and a decrease in the diversity of plants in grasslands under nutrient enrichment through agricultural fertilization and atmospheric deposition (Zarzycki and Kopec, 2020; Villa-Galaviz et al., 2021). In addition, the number and diversity of wild pollinators in natural and agricultural ecosystems have declined because of climate change, habitat loss, pathogen transport, commercially managed pollinators, agrochemicals, and nutrient enrichment (Potts et al., 2010; Ghazoul, 2015; Baude et al., 2016; Ollerton, 2017), which alter the stability of pollination ecosystems. However, since recent reviews predict that N enrichment might disrupt or enhance individual plant–pollinator interactions in grassland communities (Stevens et al., 2018; David et al., 2019), it is not yet clear how the changes in plants due to multiple nutrient enrichment in soil alter plant–pollinator interaction networks.

The availability of nitrogen (N) is considered to be the most important nutritionally limiting factor for primary productivity in grasslands (Elser et al., 2007; Harpole et al., 2011; Xiao et al., 2020). Although the N supply is increasing worldwide (Galloway et al., 2008; Liu et al., 2013; Wang et al., 2017), N availability has decreased in many regions of the world due to increased temperatures and CO_2 (Mason et al., 2022; Olff et al., 2022), dramatically reducing the biodiversity of terrestrial ecosystems (Hooper et al., 2012; Storkey et al., 2015). Nutrient co-limitation is common in the biomass of plant communities (Elser et al., 2007; Harpole et al., 2011). Phosphorus (P) enrichment can increase soil P bioavailability and accelerate N uptake by plants, which can increase plant biomass and change the structure of plant communities (Ren et al., 2017; Xiao et al., 2020). This reshaping of the composition of plant communities through nutrient enrichment can change the animal and microbial diversity and abundance above and below ground (Liu et al., 2021; Villa-Galaviz et al., 2021; Zi et al., 2022), in addition to potentially altering biodiversity at multiple trophic levels within the community's food webs (Tylianakis et al., 2008; Burkle and Irwin, 2009; Xiao et al., 2020; Villa-Galaviz et al., 2021). Some studies on the responses of pollinator communities and plant–pollinator interaction networks to nutrient supply in grassland ecosystems have been conducted, but their results have been inconsistent (Burkle and Irwin, 2009; Carvalheiro et al., 2019; Villa-Galaviz et al., 2021). For example, Burkle and Irwin (2009) found that N enrichment exerted no effect on pollinator assemblages and the community network structure between plants and pollinators, but Villa-Galaviz et al. (2021) showed that fertilizer addition decreased plant species richness, floral abundance and bumblebee richness. Therefore, there is an urgent need to focus on the consequences of soil nutrient intake on pollinator assemblages and plant–pollinator interaction networks in other grassland ecosystems from agricultural activities and environmental changes.

The Tibetan grassland ecosystem covers over 60% of the Qinghai-Tibet Plateau; however, this region is experiencing increased atmospheric N deposition and local warming (Chen et al., 2013; Liu et al., 2013). These grasslands are fragile ecosystems owing to their high altitude and low temperature and are sensitive to human activities and global changes (He et al., 2016; Liu et al., 2021; Dong et al., 2022). Previous studies have shown that adding N and P can increase the net primary productivity of grasses, while predominantly reducing the biomass and abundance of legumes on the alpine grasslands (Ren et al., 2016; Ren et al., 2017; Luo et al., 2019). Many recent studies have focused on the effects of N and P addition on plants and soil microorganisms in the alpine grasslands (Zong and Shi, 2019; Liu et al., 2021; Dong et al., 2022; Zi et al., 2022), showing

that combined N and P enrichment decreased the diversity of plants but increased net primary productivity (Ceulemans et al., 2013; Xiao et al., 2020). However, because bottom-up effects driven by changes in flowering plant diversity and flower abundance could have a major impact on the plant–pollinator interactions, few studies have focused on how changes in plant community structure due to N and P enrichment affect the richness and abundance of pollinators in this alpine grassland ecosystem.

Here, we experimentally manipulated the addition of nutrients to investigate whether and how the addition of P and N affected flowering plant communities, pollinator assemblages, and plant–pollinator interactions (**Figure 1**). In addition, we used a network approach to describe the interaction networks between plants and pollinators, which is commonly used to study changes in plant–pollinator interactions in communities (Kaiser-Bunbury et al., 2017; Gao et al., 2021; Villa-Galaviz et al., 2021). Previous research showed that nutrient addition reduced the diversity of plants in grasslands (Stevens et al., 2018; Xiao et al., 2020; Villa-Galaviz et al., 2021). Therefore, we hypothesized (**Figure 1D**) that the co-addition of N and P (1) would decrease the diversity of flowering plants and alter the composition of flowering plant communities (Hypothesis 1); (2) decrease the diversity of pollinators and change the structure of pollinator assemblages due to reduced legume biomass and relative abundance (Hypothesis 2); and (3) change plant–pollinator interactions across trophic levels due to changes in the relative abundances of flowering plants and pollinators (Hypothesis 3). The insights gained from this research will improve our understanding of how global changes, such as atmospheric nitrogen deposition and fertilization, can maintain and improve ecosystem biodiversity and the structure of interaction networks between plants and pollinators and make grassland ecosystems more sustainable.

Materials and methods

Study area

This study was conducted at the Haibei Alpine Grassland Ecosystem Research Station (37° 36′ N, 101° 12′ E, 3,250 m above sea level), Qinghai, China (**Figure 1A**). The research station has a typical plateau continental climate (short and cool summers, and long and cold winters), with a mean annual temperature and precipitation of −1.1°C and 480 mm, respectively. The precipitation (about 80%) is mainly concentrated in the growing season (May–September). The soil in this area is classified as Mat-Gryic Cambisols (WRB, 1998). In 2011, the soil content 10 cm underground in the station sampling site contained total carbon, 78.2 g kg^{-1}; organic carbon, 63.1 g kg^{-1}; nitrogen, 5.75 g kg^{-1}; and phosphorus, 0.79 g kg^{-1} (Zhang et al., 2021). The plant community mainly consists of grasses and forbs (**Figure 1B**). The flowering plant species are mainly *Gentiana straminea*, *Gentiana aristata*, *Morina kokonorica*, *Angelica nitida,* and *Dasiphora fruticosa* (Liu et al., 2019), which are primarily pollinated by insects such as honey bees, bumble bees, butterflies and flies (Zhang et al., 2015; Wang et al., 2021).

Nitrogen and phosphorus manipulations

From May 2011, four nutrient-addition experiments were conducted in an alpine grassland: N addition, P addition, a combination of N and P addition, and no nutrient addition (**Figure 1C**). The fertilizers were 100 kg of urea (CH_4N_2O) and 50 kg of triple superphosphate [$Ca(H_2PO_4)_2{\cdot}CaHPO_4$] per hectare per year. Our experiment included 24 plots (4 treatments × 6 blocks) at 6 m × 6 m each (**Figure 1C**), which is comparable to other studies (Burkle and Irwin, 2009; Villa-Galaviz et al., 2021). In the experimental plots, the legumes and forbs mainly include *Oxytropis kansuensis*, *Gentiana straminea, A. nitida, Aster farreri*, *Tibetia himalaica*, and *Thermopsis lanceolata*. To maintain the plant species' similarity and reduce the impairment caused by different fertilization treatments on adjacent plots, the blocks were spaced 2 m apart, and the plots within each block were spaced 1 m apart. We divided the fertilizer into three portions and spread it evenly from June to August each year (Ren et al., 2017).

Flower and pollinator survey

To examine the change in the diversity and flower abundance of flowering plants after the N and P addition treatments for eight consecutive years (2011–2018), we created four transects (1 m × 5 m) within each plot in 2019. From early July to late August 2019, we counted the number of flowering plant species and flowers for each plant four times per half month along the four transects of each plot.

To examine the diversity and number of pollinating insects, from early July to late August 2019, we monitored flowers for 30 min in each transect (1 m × 5 m) four times on clear days in the presence of strong wind (09: 00–19: 00). Using this observation method, each plant species was observed in each transect for 120 min. If an insect encountered the sexual components of the flowers (anthers or stigmas), we recorded a pollination visit without considering the effectiveness of the pollination (Memmott, 1999). As few pollinators visited the flowers in alpine meadows at low temperatures, we did not observe pollinator visits at night (Fang and Huang, 2016). The pollinator abundance depended on the number of visits to flowering plants per plot for each pollinator species. With the

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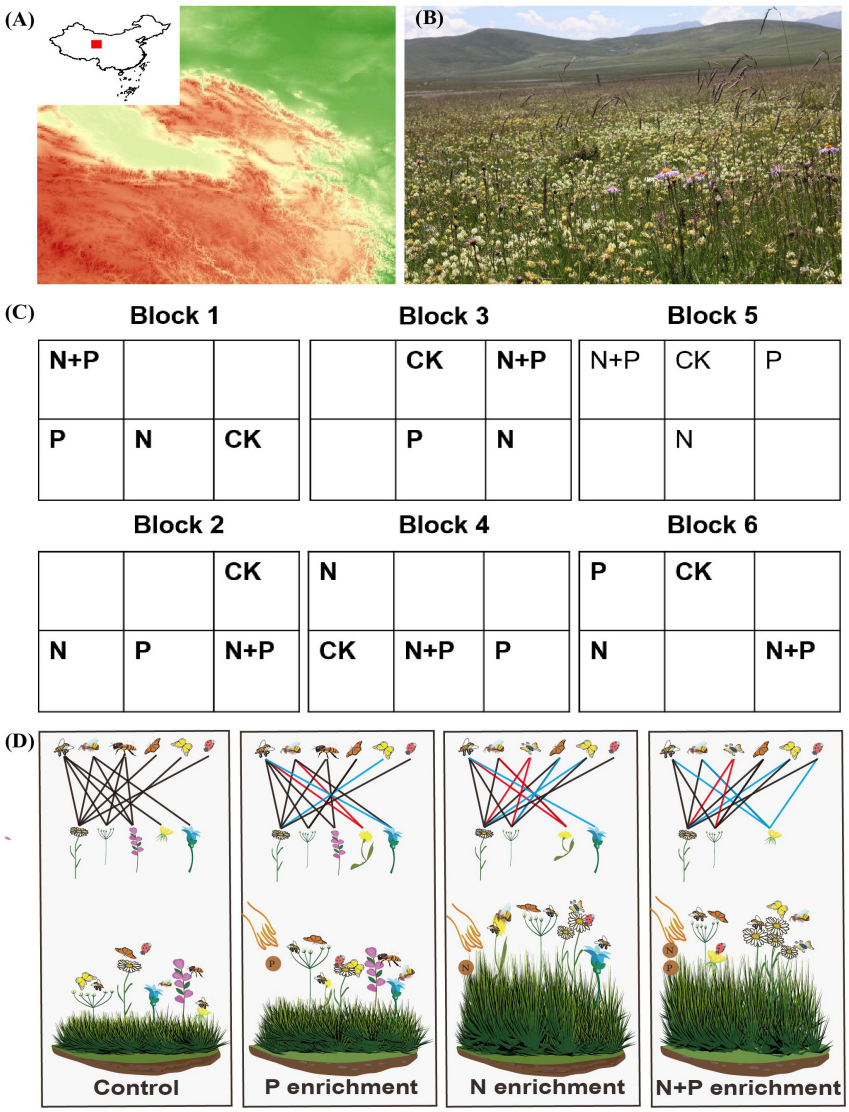

FIGURE 1

Description of study area and experiment design. **(A)** Location of the study area in the northeastern Qinghai-Tibet Plateau, Qinghai, China. **(B)** Alpine grassland landscape of the study site and legumes, which are in full bloom (taken by Lin-Lin Wang, July 30, 2017). **(C)** Study design shows 24 plots in the N and P enrichment plots. CK represents the control plots. Two empty plots in each block indicate not included in this experiment. **(D)** Schematic framework of how N and P enrichment affects the diversity of flowering plants, pollinator communities, and plant–pollinator interactions. The colored links identify the changes in plant and pollinator interactions due to nutrient enrichment. Pictures of **(D)** are available at https://pixabay.com.

help of taxonomic experts, we identified the species or higher-level groups of unknown visitors.

Pollination network construction and parameter calculation

To estimate the completeness of the sample, we divided the observed richness by the estimated abundance-based richness estimator, Chao1, using the vegan package (v2.5-7) with the R statistical software (v4.0.3, R Core Team, 2020).

To determine the effects of nutrient addition on the diversity and abundance of flowering plants and pollinators, we counted the flowering plant species, pollinator species, flowers, pollinator individuals, plant–pollinator unique interactions, and visits per plot.

To determine whether the core generalist flowering plant and pollinator species changed with different nutrient treatments, we used UCINET (v6.0) to calculate the eigenvector centrality score (network > centrality > eigenvector in UCINET) of each plant and pollinator species of different nutrient treatments (Alarcón et al., 2008). In addition, when

one plant or pollinator species with a high eigenvector centrality score participated in more than 5% of the visits and interacted with more than 25% of the taxa in each nutrient treatment, we delimited them as core generalists (Alarcón et al., 2008). We then analyzed the core generalized plant and pollinator species to determine whether they had changed due to the nutrient addition treatments.

To determine the effects of nutrient addition on the dissimilarity between flowering plants and pollinators, we quantified the species and interaction dissimilarity (species or link differences in plant–pollinator interaction networks) based on the methods of Poisot et al. (2012). We ran all combinations of networks using the "betalinkr" function in the bipartite package (Dormann et al., 2021). According to Poisot et al. (2012), the dissimilarity of a pair of networks was divided into the dissimilarity due to differences in species composition (species dissimilarity) and dissimilarity due to interaction rewiring (interaction dissimilarity). We focused on the change in species and interaction composition involving species of plants and pollinators that were present for the control and nutrient-addition treatments. These two metrics quantify species and link changes in species interaction networks under different nutritional treatments (Poisot et al., 2012). A value of 0 indicates that the species/interaction compositions are identical, and a value of 1 indicates that the species/interaction compositions are completely different.

To determine the impact of nutrient addition on the changes in links between plants and pollinators, we calculated two quantitative network metrics, vulnerability and generality, using the methods of Villa-Galaviz et al. (2021). We used the "networklevel" function in the bipartite package (Dormann et al., 2021) to calculate the values of vulnerability and generality. The vulnerability and generality were weighted according to their marginal sums (Bersier et al., 2002). The vulnerability corresponds to the average number of pollinator species attracted by each plant species, and the generality corresponds to the average number of plant species visited by each pollinator species (Villa-Galaviz et al., 2021). The changes in vulnerability and generality reflect the effects of nutrient addition on the distribution of links between plants and pollinators (Villa-Galaviz et al., 2021). An increase in vulnerability indicates that pollinator communities are increasingly dependent on fewer plant species. Conversely, an increase in generality demonstrates that the range of plant species visited by pollinators has increased, or that generalized pollinator species have increased their abundance.

Data analysis

The data were checked for normality and homogeneity of variance before performing the ANOVA. If the assumptions of normality and variance were not met, the data were log-transformed. We then used two-way ANOVAs in the R basic package to determine the effects of nutrient enrichment on plants, pollinators, and their interactions. The N and P treatments were fixed factors, and the block was an error term. The variables included five flowering plant and pollinator variables (the flowering plant diversity, flower abundance, pollinator diversity, pollinator abundance, and species dissimilarity of plants and pollinators) and five pollination interaction variables (the interaction numbers, number of visits, interaction dissimilarity, vulnerability, and generality). In addition, three other statistical analyses (one-way ANOVA, the pairwise permutational multivariate analysis of variance, and piecewise structural equation modeling) were performed.

First, we used a one-way ANOVA to examine the effects of the nutrient-addition treatments (control, N, P, and N + P treatments) on each response variable (the flowering plant diversity, pollinator diversity, flower abundance, pollinator abundance, interaction numbers, number of visits, species dissimilarity, interaction dissimilarity, vulnerability, and generality).

Second, we applied the pairwise permutational multivariate analysis of variance (PERMANOVA) using the vegan package (v2.5-7) (Oksanen et al., 2020) to evaluate the effects of the addition of N and P on the plant and pollinator diversity. To illustrate the impacts of the N and P treatments on changes in the structure of plant and pollinator communities, we performed paired Bray–Curtis distance principal coordinate analysis (PCoA).

Finally, we applied piecewise structural equation modeling in the piecewise SEM package (v2.1.2) (Lefcheck, 2016) to clarify the direct and indirect effects of the addition of N and P on changes in plant–pollinator interactions (**Supplementary Figure 1**). The hypothesized direct effects of N and P enrichment, and the indirect effects mediated by plant and pollinator community (i.e., plant diversity, flower abundance, pollinator diversity, and pollinator abundance) on vulnerability and generality are included in **Supplementary Figure 1**. The piecewise SEM included several linear mixed-effects models, and the block was a random effect. The full piecewise SEM comprised the effects of N and P's effects on four mediators of plant and pollinator communities (plant diversity, flower abundance, pollinator diversity, and pollinator abundance), as well as the direct and indirect effects on the vulnerability and generality of plants and pollinators. To simplify the model, we did not consider the interactions between the N and P treatments.

Results

In total, we found that 20 flowering plant species (**Supplementary Table 1**) were visited by 54 pollinator species

(Supplementary Table 2) that participated in 4954 individual interactions (Figure 2). Based on the sample completeness analysis, these data represented about 64% of the pollinator species for each treatment.

Structural changes from N and P addition to flowering plant communities

The N treatment decreased the flowering plant diversity, and the N + P treatment increased the negative effect on the flowering plant diversity (Figure 2 and Supplementary Figure 2A). In particular, the N treatment reduced the legume diversity (Figure 2, e.g., *O. kansuensis*, *Oxytropis ochrocephala*, and *Oxytropis qinghaiensis*). By contrast, the N + P treatment further reduced the diversity of rare species (Figure 2, e.g., *Ranunculus membranaceus*, *Euphrasia regelii*, and *Galium verum*). The abundance of flowers was similar to that for the control and N or P treatments (Supplementary Figure 2B). However, the N + P treatment increased the abundance of flowers (Supplementary Figure 2B), primarily by increasing the number of flowers of *Descurainia sophia*.

The N + P treatment altered the numbers and identities of the core generalist plants (Supplementary Figure 3A). For example, four plant species (*Morina kokonorica*, *A. nitida*, *Anaphalis lacteal*, and *Aster farreri*) were core generalists with the control treatment. By contrast, only two species (*A. nitida* and *A. lacteal*) were core generalists with the P treatment (Supplementary Figure 3A). Additionally, *Potentilla saundersiana* dominated the N treatment group as the core plant species at the expense of *A. nitida*. To the detriment of *M. kokonorica* and *A. lacteal*, *D. sophia* became a core plant species in the N + P treatment (Supplementary Figure 3A). PERMANOVA analyses revealed that the N + P treatment changed the structure of the flowering plant communities (Figure 3A).

Effects of structural changes in flowering plants on pollinator communities

Flies were the most abundant pollinators (Figure 2). The N and P treatments did not affect the diversity or abundance of pollinators (Supplementary Figures 2C,D). The N, P, and

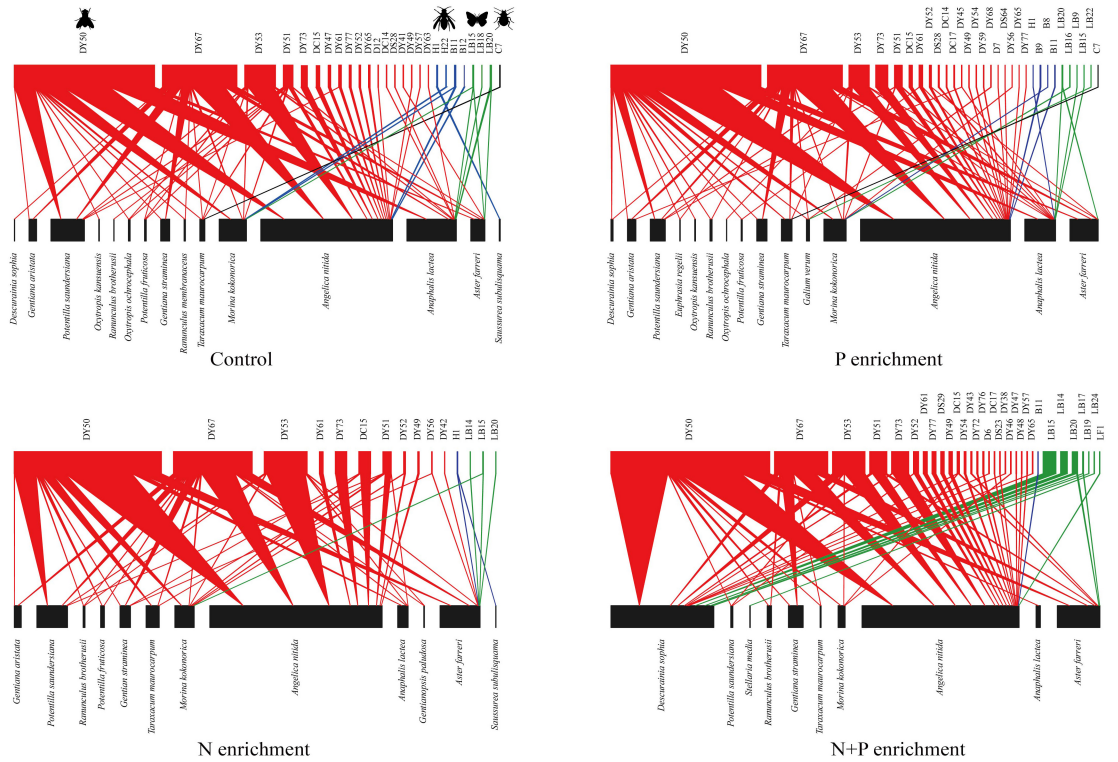

FIGURE 2
Plant–pollinator interaction networks for N and P enrichment on an alpine grassland. These pollination networks include flowering plant species (bottom blocks), pollinator species (top blocks), and their pollination interactions (color triangles). The widths of the blocks and triangles reflect the numbers of plants and pollinators or the numbers of pollination interactions, respectively. Colors represent the pollinator groups: green, Hymenoptera; red, Diptera; blue, Lepidoptera; black, Coleoptera.

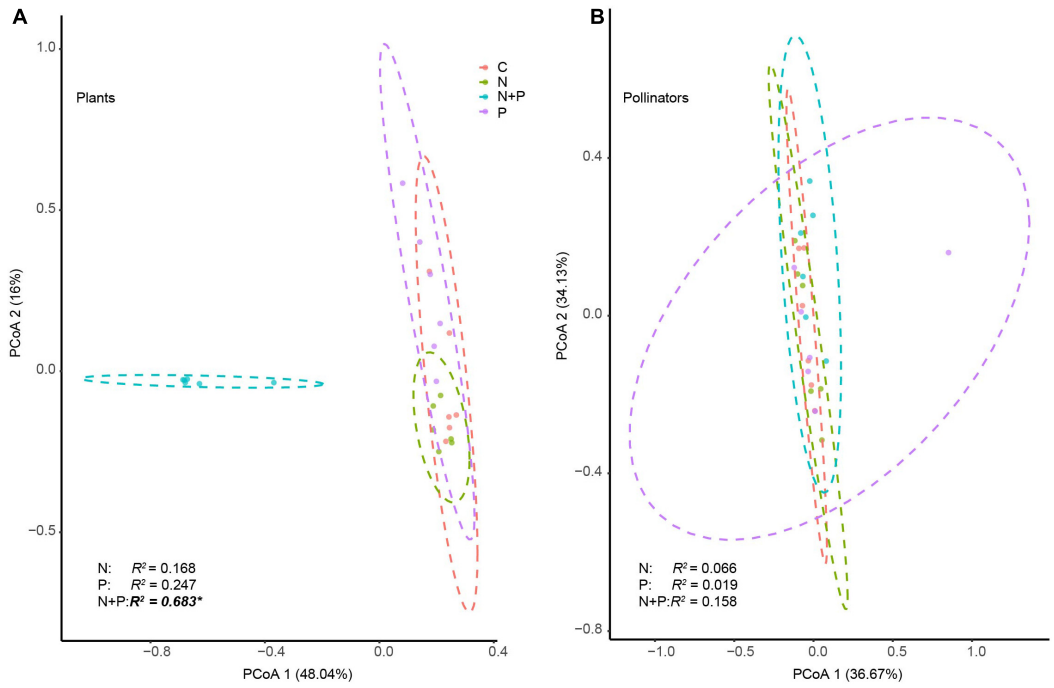

FIGURE 3
The effects of nutrient enrichment on the community structure of plant **(A)** and pollinator species **(B)**. The structure is based on a principal coordinate analysis. Permutation analysis of variance was used to evaluate the influence of N addition, P addition, and N and P co-addition on the community structure of plants and pollinators. The asterisk (*) indicates a significant difference at the 0.05 level.

N + P treatments did not change the numbers and identities of the core pollinators (Supplementary Figure 3B), except that the N treatment decreased the diversity of the bees and the N + P treatment increased the diversity of the butterflies (Figure 2). None of the nutrient additions changed the structure of the pollinator communities (Figure 3B).

The impacts of nutrient additions on plant–pollinator interactions

The N, P, and N + P treatments did not change the number of unique interactions between plants and pollinators (Figure 4A) or the total number of visits to the pollination networks (Figure 4B). The N or P treatment alone did not change the species and interaction dissimilarity. However, compared to the control treatment, the N + P treatment increased the species and interaction dissimilarity in the pollination networks (Figures 4C,D). None of the treatments altered the vulnerability (Figure 4E). However, the N + P treatment decreased the generality (Figure 4F).

The full piecewise SEM (Supplementary Figure 4 and Supplementary Table 3; χ^2 = 1.629, df = 6, P = 0.95, and AICc = −108.163) and the final model are qualitatively similar (Figure 5 and Supplementary Table 3; $\chi 2$ = 7.819, df = 14, P = 0.899, AICc = −123.88, and ΔAICc = 15.72), which explained about 75% of the variance of the generality (marginal R^2 = 0.75). The final model revealed that the change in flowering plant species caused by the N and P treatments indirectly affected the generality through changing the flower abundance and vulnerability (Figure 5 and Supplementary Figure 4). The N or P treatment alone did not affect the richness and abundance of the pollinators and vulnerability (all $P > 0.05$, Figure 5). However, the N treatment directly decreased the flowering plant diversity (standardized path coefficient β = −0.809, $P < 0.0001$) and increased the generality (β = 0.323, P = 0.03). In addition, the P treatment directly increased the abundance of flowers (β = 0.481, P = 0.004) and deceased the generality (β = −0.654, P = 0.001).

Discussion

Examining how eutrophication due to climate change and anthropogenic activities affects communities of plants, animals, and species interactions is vital for understanding how biological communities respond to global changes (David et al., 2019). In the current study, we investigated the effects of N and P addition on plant–pollinator interactions in an alpine grassland. Our findings revealed that N and P addition changed the structure of flowering plant communities. Although pollinator communities were resilient to nutrient

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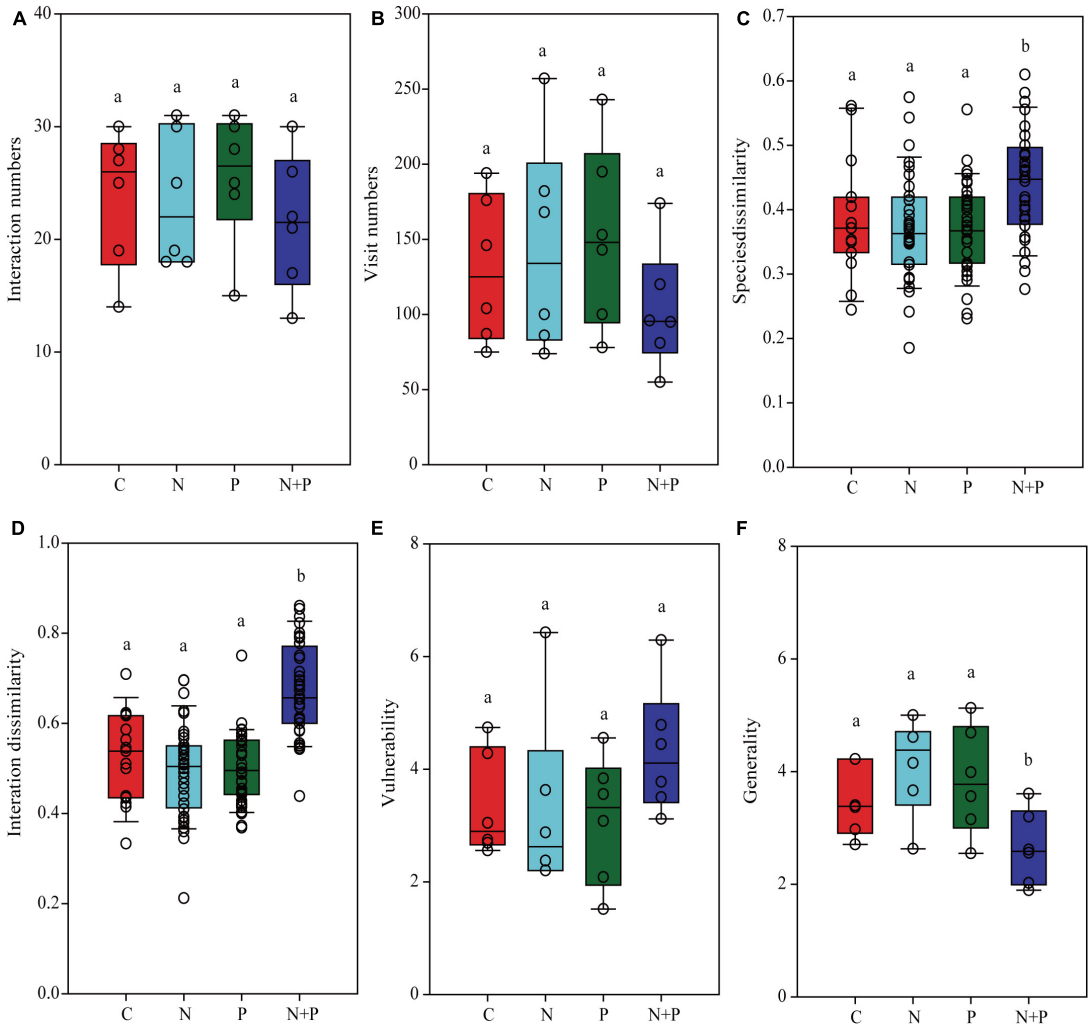

FIGURE 4
The effects of nutrient enrichment on the links between plants and pollinators. **(A)** plant–pollinator interaction numbers, **(B)** pollinator visit numbers, **(C)** plant–pollinator species dissimilarity, **(D)** plant–pollinator interaction dissimilarity, **(E)** vulnerability, and **(F)** generality. Each panel shows a one-way analysis of variance used to examine the differences between different nutrient treatments. Different lowercase letters on the bars indicate significant differences in nutrient supply at the 0.05 level.

addition, the plant–pollinator interactions were unstable under the N + P treatment in alpine grassland ecosystems. These results suggest that N- and P-fertilization-induced changes in flowering plant composition have a minor effect on pollinator communities. However, the plant–pollinator interactions can be sensitive to nutrient addition through agricultural activities and environmental changes.

Structural changes in flowering plant communities under N and P addition

Soil eutrophication due to N and P addition can increase the biomass of grasses and decrease the biomass and diversity of flowering plant species, such as forb and legumes, in nutrient-limited grasslands (Harpole et al., 2016; David et al., 2019). For example, fast-growing grasses can become taller, denser, and more competitive for sunlight under N enrichment. As a result, the biomass and diversity of nitrophobous forb species are often reduced and they can become locally extinct because of their small sizes and slow growth rates (Crawley et al., 2005; Storkey et al., 2015). The results of the present study support our first hypothesis that N enrichment reduces the diversity of flowering plants, primarily that of legumes (**Figure 2**). However, our findings did not agree with those of Ren et al. (2017), who found that the richness of legumes did not change under N enrichment in alpine grassland (Ren et al., 2017). A possible explanation is that the diversity of legumes deceased and disappeared over time, suggesting that the time and intensity of N addition played a vital role in their

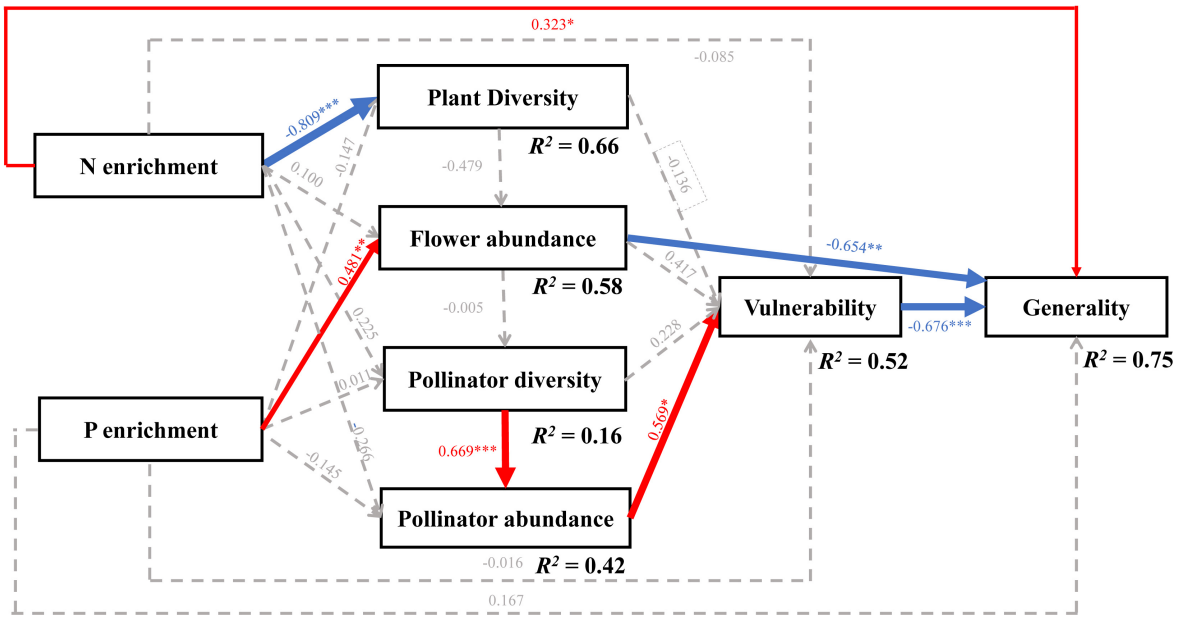

FIGURE 5
The results of the final piecewise structural equation model (SEM). The final SEM fitted the data: χ^2 = 7.819, df = 14, P = 0.899, and AICc = −123.88. The numbers on the arrows indicate standardized path coefficients, and the asterisks show statistical significance: ***P < 0.001; **P < 0.01; *P < 0.05. Red, blue, and gray arrows indicate positive, negative, and insignificant relationships. The arrow width shows the strength of the relationship, and the marginal R^2 shows the variance, which is explained by fixed effects in the model.

local extinction. For example, the effect of fertilization was not fully visible within only three years (Ren et al., 2017) because the perennial legumes (e.g., *O. kansuensis*, *O. ochrocephala*, and *O. qinghaiensis*) can only change their distribution over generations, so they can become locally extinct under long-term fertilization.

Additionally, this research revealed that P addition further enhanced the negative effect of N enrichment on flowering plant species in alpine grasslands (**Figure 2**). For example, *R. membranaceus*, *E. regelii*, and *G. verum* disappeared after eight years of N + P enrichment. N enrichment affects the N and P balance in the soil, changing the situation from N limitation to P limitation (Hooper et al., 2012; Harpole et al., 2016; Zhan et al., 2017). As P addition can also reduce the limitations for crucial nutrient resources (David et al., 2019), this could consequently alter the competitive dynamics of plants, typically leading to a reduction in legumes and endangered forbs (Suding et al., 2005; Clark et al., 2007). For example, N and P co-addition increased flower production because of the increased number of *D. sophia* plants (**Supplementary Figure 2B**), as N enrichment can significantly increase the height, biomass, and seed yield of *D. sophia* (Mokhtassi-Bidgoli et al., 2013). Furthermore, the changes in flowering plant diversity and flower abundance led to a further reorganization of the flowering plant community structure (**Figure 3A**), indicating that flowering plant communities are unstable under long-term P and N enrichment in alpine grassland ecosystems. However, future experiments with more time scales and fertilization intensities are still needed to examine the effects of nutrient enrichment on the structure of plant communities.

Weak effects of structural changes in flowering plants on pollinator communities

Contrary to our second hypothesis, our findings revealed that N and P addition did not alter the pollinator diversity and abundance and the core pollinator species (**Supplementary Figures 2, 3**). Burkle and Irwin (2009) also found that N addition did not affect the richness of pollinator communities. A possible explanation might be that most of the remaining flowering plant species, such as Asteraceae, Ranunculaceae, and Rosaceae, had an open morphology with radially symmetrical flowers in the nutrient-addition plots (**Figure 2** and **Supplementary Table 1**), which could attract many generalized pollinator species to visit these flowering plants (Duan et al., 2007; Zhang et al., 2015; Wang et al., 2021). For example, we found that the flowers of *D. sophia* and *Angelica nitida* could attract more than ten pollinator species. Additionally, our results showed that the main pollinator species were flies, bees, and butterflies (**Figure 2**), which are generalized pollinators that can visit many flowering plants in alpine grasslands (Wang et al., 2021). Therefore, we could not determine any changes

in pollinator diversity and abundance due to P and N addition (Burkle and Irwin, 2009; Villa-Galaviz et al., 2021).

It is important to note that, similarly to in other studies (**Supplementary Table 5**), the scale of the experimental treatments for the plants was relatively small, which would probably have not affected the populations of pollinators but, rather, simply their behavior in terms of which plots they decided to forage in. However, our results showed that N and P co-addition decreased the richness of bumble bees but increased the diversity of butterflies (**Figure 2**). Changes in the flowering plant species composition can negate the ability of pollinators to choose different resource supplies (Burkle and Irwin, 2009; Roger et al., 2017; Carvalheiro et al., 2019). Our results showed that bumble bees mainly visited the legumes, suggesting that they have specialized diets, which may explain why they are more susceptible to declines than flies and butterflies. For example, researchers have suggested that significant spatial changes in flowering plant species due to N enrichment would decrease the diversity of pollinators, such as bees and butterflies (Carvalheiro et al., 2019). Due to the mismatch in the scales at which pollinators and plants respond to the nutrient treatments, large-spatial-scale studies on the effects of different nutrient enrichments on the populations of pollinators will be investigated in the future.

The impacts of nutrient additions on plant–pollinator interactions

Contrary to the results of previous studies, Burkle and Irwin (2009) found no effects of N addition on the identity and frequency of plant–pollinator interactions. Our results demonstrate that pollinators increasingly rely on fewer flowering plant species and that pollinators visit a reduced range of plant species. Thus, plant–pollinator interactions are sensitive to changes in flowering plant composition and flower abundance, and the variation in pollinator behavior among different plants that were affected by the N, P, and N + P enrichment treatments. These results support our third hypothesis that nutrient enrichment can change plant–pollinator interactions.

Plant–pollinator interaction networks always exhibit nested properties and are centered around a core of generalized plant and pollinator species (Bascompte et al., 2003; Jordano et al., 2006). Thus, changes in the composition of the core generalized plant species and floral abundance can directly affect the structure of the pollination network, such as nestedness (i.e., interactions between the most generalist plants and animals create a dense core that includes other specialized plants and animals in its community). The nested structure of the network can reduce the extent to which species and interactions are affected by perturbation (Bascompte et al., 2003; Pawar, 2014), indicating that pollination networks are highly tolerant of plant extinctions due to their nestedness structure.

Furthermore, the addition of N or P alone did not change the core generalized flowering plants, which may not change the nestedness of plant–pollinator interaction networks, because of the generalized characteristics of pollination networks (Waser et al., 1996; Johnson and Steiner, 2000) and the rapid turnover of interactions due to the loss and gain of non-core plant and pollinator species (Burkle and Irwin, 2009; CaraDonna et al., 2017). We also cannot rule out the possibility that the experimental plots were too small to attract different pollinators or that the pollinators are not specific for a given flowering plant species, as mentioned above. Although previous studies revealed that N and P enrichment reduces the biomass and abundance of legumes and the overall diversity of plant species in alpine grasslands (Ren et al., 2017; Luo et al., 2019), the loss of non-core species did not change the structure of the pollination networks (Weiner et al., 2011). Thus, changes in the composition and flower abundance of the core generalized flowering plant species due to the addition of N and P can influence the link distribution of the pollination networks.

Conclusion

We tested the importance of nutrient enrichment for changes in pollination interactions by combining network theory and pollination ecology. Our study revealed that changes in flowering plant diversity and composition, both of which are reduced by nitrogen and phosphorus enrichment, alter plant–pollinator interactions in a Tibetan alpine grassland. Future experiments in manipulating nutritional resources, and the composition and abundance of plant and pollinator species on larger temporal and spatial scales will provide important insights into how nutrient enrichment affects the response of pollinator assemblages.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

Author contributions

L-LW, Y-PY, Y-WD, and J-SH conceived the ideas and designed methodology. L-LW, FR, CZ, Z-HZ, and X-JH collected the data. L-LW analyzed the data. L-LW, Y-PY, and

Y-WD led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.964109/full#supplementary-material

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Characteristics of nitrogen deposition research within grassland ecosystems globally and its insight from grassland microbial community changes in China

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As global change continues to intensify, the mode and rate of nitrogen input from the atmosphere to grassland ecosystems had changed dramatically. Firstly, we conducted a systematic analysis of the literature on the topic of nitrogen deposition impacts over the past 30 years using a bibliometric analysis. A systematic review of the global research status, publication patterns, research hotspots and important literature. We found a large number of publications in the Chinese region, and mainly focuses on the field of microorganisms. Secondly, we used a meta-analysis to focus on microbial changes using the Chinese grassland ecosystem as an example. The results show that the research on nitrogen deposition in grassland ecosystems shows an exponential development trend, and the authors and research institutions of the publications are mainly concentrated in China, North America, and Western Europe. The keyword clustering results showed 11 important themes labeled climate change, elevated CO_2, species richness and diversity, etc. in these studies. The burst keyword analysis indicated that temperature sensitivity, microbial communities, etc. are the key research directions. The results of the meta-analysis found that nitrogen addition decreased soil microbial diversity, and different ecosystems may respond differently. Treatment time, nitrogen addition rate, external environmental conditions, and pH had major effects on microbial alpha diversity and biomass.

The loss of microbial diversity and the reduction of biomass with nitrogen fertilizer addition will alter ecosystem functioning, with dramatic impacts on global climate change. The results of the study will help researchers to further understand the subject and have a deep understanding of research hotspots, which are of great value to future scientific research.

KEYWORDS

nitrogen deposition, grassland, keyword co-occurrence, meta-analysis, microbial community

Introduction

Nitrogen (N) is an essential element for the growth of organisms. Haber-Bosch's nitrogen conversion project (Erisman et al., 2008) has effectively addresses the growing food demand of billions of humans. However, the unreasonable utilization of N has led to a series of ecological and environmental problems, the most important of which is the rapid increase of atmospheric N deposition. Atmospheric nitrogen deposition is mainly caused by the emission of excess reactive nitrogen compounds from human activities and atmospheric transport processes (Fowler et al., 2013). Grasslands, as the largest terrestrial ecosystem in the world, account for about 30–40% of the total land area (O'Mara, 2012). Grasslands are limited by nutrient availability, due to the relatively poor nutrient background of soils, the relatively low historical N deposition, and nutrient loss induced by grazing and mowing (Chen et al., 2016a). Thus, even chronic N deposition can cause soil eutrophication and acidification (Tian and Niu, 2015). Excess N levels directly affect plant diversity (Payne et al., 2017) and net primary productivity (NPP) (LeBauer and Treseder, 2008), which in turn affect the biogeochemical cycles of C, N, and P (Quinn Thomas et al., 2010; Peñuelas et al., 2013). The profound impact of atmospheric N deposition on grassland ecosystems has generated extensive research interest. Researchers have conducted different types of long-term or short-term experimental simulation and computer model simulations in global grassland ecosystems (Luo et al., 2016), and these studies have become a research hotspot in global change ecology.

Throughout the history of research on the ecological effects of N deposition (including simulated N deposition, hereafter the same) in grassland ecosystems, relevant research work has focused on the effects of N deposition on the composition, structure, function, and processes of grassland ecosystems. It can be summarized in the following three aspects, as shown in the **Figure 1**.

(1) Effects of N deposition on the composition, structure and function of vegetation.

Nitrogen deposition significantly alters the species composition of native grasslands because increased the availability of nitrogen in the soil. Enriched nitrogen may be interacting with mycorrhizal feedbacks, allowing a few nitrophilic plant species become dominant while other species to disappear (Johnson et al., 2008). The plants species composition is significantly species-specific in response to nitrogen deposition and is influenced by species properties (Cleland et al., 2006; Shen et al., 2022), environmental factors (e.g., light, temperature, and soil acidity) (Tian and Niu, 2015), and mycorrhizal symbiotic status (Quinn Thomas et al., 2010). Nitrogen deposition can alter ecosystem function and stability by enhancing nitrogen competition between dominant and dependent species, especially in highly dominant grasslands (Liu et al., 2019). Studies have found that changes in species composition led to a decrease in the proportion of forb at high nitrogen addition rates (Bai et al., 2010).

Nitrogen deposition could reduce the plant diversity in grassland ecosystems (Duprè et al., 2010; Payne et al., 2017; Li et al., 2020). Some mechanisms may explain changes in plant diversity, including accumulation pressures with large amounts of N (Payne et al., 2017), mild competition (Ma et al., 2020), soil acidification and its ionic toxicity (Tian et al., 2016), nutrient imbalance (Payne et al., 2017).

Nitrogen deposition significantly reduced the height and coverage of plant (Payne et al., 2013; Wu et al., 2020), richness (Stevens et al., 2004; Bai et al., 2010; Roth et al., 2013), and the Pielou's index (Tang et al., 2017). Nitrogen deposition could affects ecosystem productivity through its effects on community structure. Nitrogen enrichment could increase plant productivity. When nitrogen deposition lifts the nitrogen restriction of plant growth, plants invest less carbon in the underground part and more carbon in the above-ground part to obtain other restricted resources (e.g., light). Root growth and above-ground net primary productivity (ANPP) accumulation tend to exhibit different response characteristics to nitrogen deposition. For example, Wang et al. (2019) found that in temperate grasslands, at lower N addition levels, ANPP increase with N additions, while below-ground net primary productivity (BNPP) decreases. Nitrogen enrichment could reduce the stability of ANPP on local and larger spatial scales but

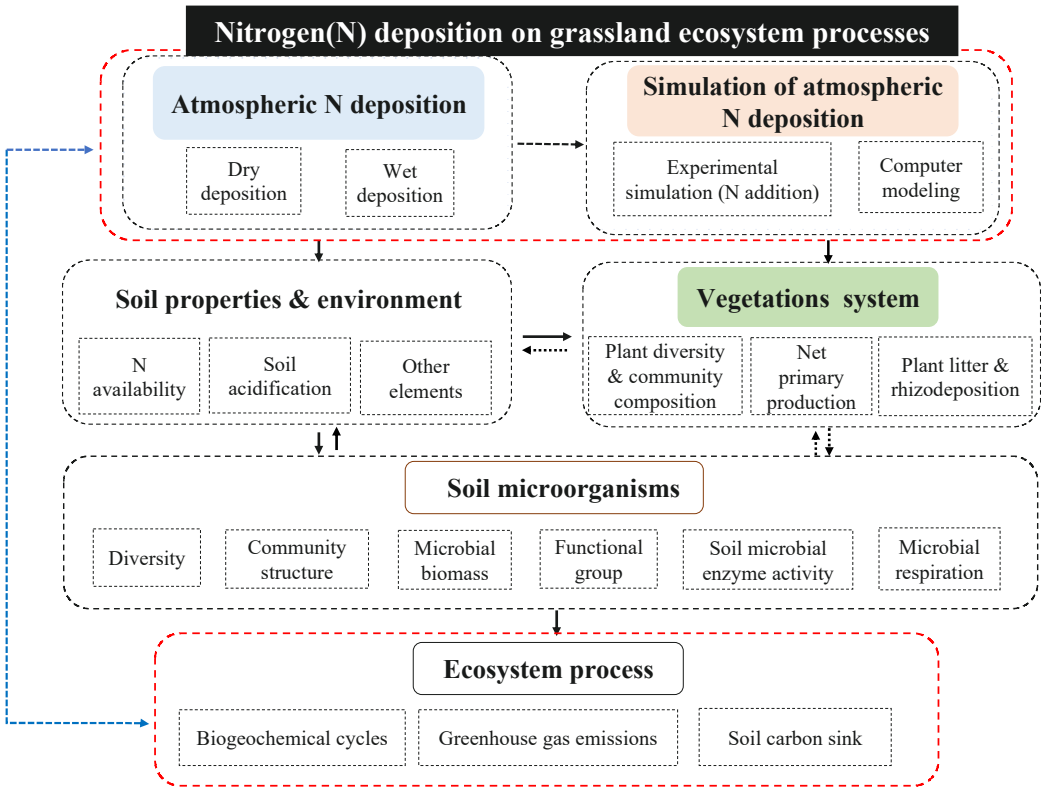

FIGURE 1
Conceptual diagram of the ecological effects of N deposition in grassland ecosystems. Nitrogen deposition can be divided into atmospheric nitrogen deposition (dry deposition and wet deposition) and artificially simulated nitrogen deposition (artificial addition experiments and computer simulations). The main impacts on grassland ecosystems include soil systems, plant systems, and ecosystem processes, with soil microorganisms in turn playing an important role as drivers affecting the soil-plant-ecological process continuum.

does not affect the stability of BNPP or NPP at the scale studied (Yang et al., 2022).

(2) Effects of nitrogen deposition on the composition, structure and function of soil systems.

In nitrogen limited ecosystems (e.g., grassland ecosystems in northern China), nitrogen deposition generally relieves the nitrogen limitation of plants and microorganisms (Han et al., 2012). It has been shown that N addition significantly increased the biomass and respiration of plant roots in the nitrogen limited grasslands of Inner Mongolia (Chen et al., 2014, 2016b; Zhang et al., 2022). Nitrogen deposition could directly and indirectly cause a series of changes in soil physicochemical properties (e.g., N effectiveness, soil acidification, base cation composition, etc.) (Zhou et al., 2017) and soil enzyme activity (Henry et al., 2005). Simulated increased nitrogen deposition promoted the activity of soil hydrolytic enzymes (sucrase, cellulase, acid phosphatase, and urease) (Marklein and Houlton, 2012; Jian et al., 2016), inhibited oxidase activity (polyphenol oxidase and peroxidase) (Treseder, 2008; Jian et al., 2016). Nitrogen deposition could directly affect soil microbial biomass, diversity and community structure by changing soil environmental conditions, resulting in a significant decrease in soil microbial respiration (Zhou et al., 2014). It could also indirectly affect the structure of the soil microbial community through the physiological and ecological responses of above-ground vegetation (Chen et al., 2017). In contrast to the consistent response of overall microbial diversity to nitrogen deposition, there are significant differences in the response of different functional groups of microorganisms involved in the N cycle to nitrogen deposition. Key enzyme-related genes encoding N transformation processes, such as the *nifH* gene for N fixation, the *chiA* gene for mineralization, the *amoA* gene for ammonia-oxidizing archaea (AOA) and ammonia-oxidizing bacterial (AOB), and the abundance of *narG*, *nirS*, *nirK* and *nosZ* genes for nitrification is bound to change with increasing N deposition (Chon et al., 2011; Zhang et al., 2013; Zhou et al., 2021).

(3) The influence of N deposition on biogeochemical cycles.

Numerous studies have shown that the accumulation of reactive nitrogen could change the stoichiometric balance of C:N:P in ecosystems. Greenhouse gas (CO_2, N_2O, and

CH_4) emissions are ultimately controlled by altering the biogeochemical cycles of carbon, nitrogen and phosphorus (LeBauer and Treseder, 2008; Quinn Thomas et al., 2010). Nitrogen deposition could alleviate nitrogen limitation of soil microorganisms and increase microbial activity and decomposition rate of soil organic matter, which may lead to loss of soil carbon. A global meta-analysis showed that CH_4 was increased and N_2O was released due to nitrogen deposition (Liu and Greaver, 2009; Gomez-Casanovas et al., 2016).

In summary, nitrogen deposition not only has a profound effect on soil systems, plant systems, and ecosystem functions and processes, but also has a significant impact on soil microbial communities and presents diverse mechanisms of influence. Some studies have shown that nitrogen deposition has an inhibitory effect on microbial growth and that nitrogen deposition may reduce the activity of plant inter-rooted microorganisms. Different nitrogen deposition classes have different effects on soil microbial load. Microbial biomass carbon (MBC) can reflect subtle changes in the soil environment due to its high sensitivity (Eisenhofer et al., 2019). Therefore, an in-depth understanding of the response of soil microbial biomass to nitrogen deposition in grasslands and its mechanisms is extremely important to maintain the ecological function of grassland ecosystems. Therefore, in addition to a holistic understanding of the response of grassland ecosystems to nitrogen deposition, the importance of microbial biomass response to N deposition and its mechanisms in maintaining the ecological functions of grassland ecosystems should be better understood.

Although a lot of research has been done in the field of grassland nitrogen deposition, there is still a lack of research on the publication situation and quality of in-depth literature in this field. Bibliometric scientific mapping is a quantitative method that analyzes the full range of terms present in scientific publications in their titles, abstracts, and keywords. Meta-analysis is a statistical method used to compare and synthesize the results of studies on the same scientific question. Whether the conclusions are meaningful or not depends on the quality of the included studies. It is often used for quantitative pooled analysis in systematic reviews. The combination of these two analyses allows us to analyze the evolution of grassland nitrogen deposition research and to predict emerging themes within the discipline. Despite the comprehensive and objective nature of the analysis, bibliometric analysis and meta-analysis has been used in many studies within grassland ecosystem, such as remote sensing (Li et al., 2021), soil metagenomics (Vieira et al., 2021), pasture modeling (Nduku et al., 2021). We used bibliometrics to analyze the responses of global nitrogen deposition to grassland ecosystems, and used a meta-analysis method to analyze the effects of nitrogen deposition on soil microbial biomass, taking China's grassland ecosystems as an example. We focused on the following four scientific questions: (1) What are the publication patterns and status of N deposition in global grassland ecosystems? (2) What are the hot topics of current research? (3) What are the future research trends? (4) Taking grassland ecosystems in China as an example to explore the effects of nitrogen additions on microbial communities? This research could provide a global perspective on the international dynamics of global grassland nitrogen deposition research, and to provide a reference for understanding the interaction and feedback mechanisms between microbial processes and nitrogen deposition in grassland ecosystems from a local perspective.

Materials and methods

Data sources and retrieval strategies

The Web of Science database Science Citation Index Expanded (SCI-EXPAND) was used as a data source, with the keywords "grassland" and "N deposition" as search terms. The search time was 1990–2021 by advanced search, and a total of 2021 was retrieved, the total number of publications is 2,786. We performed a bibliometrics analysis based on these literatures. When we further take China's grassland ecosystem as an example to explore the meta-analysis of the effect of nitrogen addition on soil microorganisms. Therefore, our data is mainly obtained by searching for literature from ISI Web Science, Science Direct, Google, Google Scholar, and CNKI for peer-reviewed journal articles since December 31, 2021. The keywords searched are (nitrogen deposition OR nitrogen addition OR nitrogen enrichment OR nitrogen fertilizer OR nitrogen amendment OR nitrogen elevated) AND (microbial biomass OR microbial communities OR fungi OR bacteria) AND (soil) AND (China), mainly to observe the changes of microbial diversity and biomass related indicators in five major grassland ecosystems in China under nitrogen addition. We have strict standards for the selection of articles, please refer to the **Supplementary material** for specific standard information.

Research methodology

Citespace and VOSviewer are commonly used scientific knowledge mapping tools that are important for a systematic and in-depth understanding of a research field. In this paper, we use VOSviewer to explore the basic knowledge, research hotspots, and frontier knowledge in this field, and CiteSpace to analyze keywords burst of research. Keywords can accurately reflect the hot spots of research in the field. In bibliometric analysis, the frequency of keywords is usually used to determine the focus and development trend of a research field, where the size of the circle represents the frequency of keywords; the higher the frequency is, the larger the circle will be (Van Eck and Waltman, 2010). The keywords in this study were

cleaned by thesaurus_terms.txt, which comes with VOSviewer software, and visualized using VOSviewer and Paulek64 5.14 software for further analysis and visual debugging. Keyword burst statistics can reflect research hotspots in the field (Chen, 2017). This study used CiteSpace software to map the keyword burst for N deposition.

To further explore the dynamic evolution of N deposition research over time, we introduced a local citation score (LCS) and a global citation score (GCS) to identify the most-cited references at each stage, with higher LCS values implying that the literature is more important to the field of study. In contrast to a literature with a high GCS, this indicates that the literature has attracted the attention of scientists worldwide, including citations from scholars in other fields of study not related to the discipline (Garfield, 2004). Therefore, the LCS is more appropriate for selecting core literature in the field than the GCS, but the GCS value should also be considered, as it reflects the impact of the work beyond the field. In this study, we used HistCite Pro 2.1 software to calculate the LCS and GCS values.

Meta-analysis requires a large amount of data to calculate. Finally, our dataset includes 251 pairs of observations from 95 published literatures. Among them, microbial carbon has 142 observations, bacterial PLFA has 83 observations, fungal PLFA has 87 observations, Shannon index has 99 observations, and Chao1 has 126 observations. Our metadata covers major grassland ecosystems in China, including alpine grasslands, alpine meadows, meadow grasslands, typical grasslands, and desert grasslands. These data cover a wide range of climatic conditions and soil properties for grasslands in northern China. For example, altitude, annual mean temperature, and precipitation are 475–4,745 m, -5.3 to 8.9°C, and160–900 mm, respectively. Soil properties such as pH (4.0–10.0) also showed a wide range. The average nitrogen application rate was 116 kg ha^{-1} yr^{-1} (ranging from 7.5-640 kg ha^{-1} yr^{-1}), the average experiment duration was 5.5 years (1–15 years), and the main type of fertilization was the commonly used fertilizer ammonium nitrate (NH_4NO_3) and urea.

We chose Hedges'd to calculate effect sizes rather than the natural log response ratio (ln*RR*) because the frequency distribution of bacterial diversity is more unimodal when Hedges'd is used. The Hedges'd is a unitless metric ranging from $-\infty$ to $+\infty$. The corresponding magnitude and direction of change can be estimated, and this parameter is not affected by small sample sizes. As suggested by Hedges et al. (1999) for meta-analysis, we used a weighted random-effects model because, when dealing with ecological data, when combining data from individual studies, using a random-effects model can reduce the variability of different studies, and when the weights of studies. The larger the value, the higher the weight of these studies, the higher the replication and the lower the variance.

Overall effect sizes were calculated by weighted resampling method from random effects models using MetaWin version 2.0 (Sinauer Associates, Inc., Sunderland, MA, United States). Computational biases were corrected using a bootloader (Dixon, 2020). Missing standard deviations were calculated using the mean coefficient of variation of data sets with known standard deviations according to the method of Geisseler and Scow (2014). If the 95% confidence interval does not overlap with zero at the $\alpha = 0.05$ level, we consider the effect to be significant at $P < 0.05$ (Hedges and Olkin, 2014). Mineral fertilization type, experimental time, fertilization amount, and ecosystem effects on microbial biomass and diversity were calculated using the procedure described above.

When calculating a random value associated with the between-group ($Q_{between}$) statistic, this value describes the heterogeneity of the effects of different groups. The magnitude of the effect is related to the difference in the eigenvalue categories of the research method, based on the chi-square test. In this analysis, grassland ecosystem types are divided into alpine meadow, alpine grassland, meadow grassland, typical grassland, desert grassland; fertilizer types are divided into NH_4NO_3 and urea; fertilization time is divided into $\leq$5, 5–10, >10; application rates $\leq$50, 50–100, 100–150, 150–200 and >200 kg ha^{-1} yr^{-1}. We performed Kendall's tau rank correlation test (Sokal and Rohlf, 1995) on the data to examine the relationship between the number of replicates per study and standardized effect size (Rosenberg et al., 2000). This relationship suggests publication bias, where larger N effects are more likely to be published than smaller N effects, but we found no associated bias. Analyses were performed using the "metafor" package in R version 4.1.0 (Viechtbauer, 2010). Publication bias for each response variable across the database was assessed using funnel plots. We also detected funnel plot asymmetry by performing Egger's regression test (Dieleman and Janssens, 2011). All meta-analysis and statistical comparisons were performed using software R 4.1.0.

Results and discussion

Analysis of global literature publication characteristics and bibliometric analysis

Annual publication trend

A total of 2,815 papers were obtained after cleaning the subject dataset and eliminating irrelevant papers. The distribution of publications on N deposition in global grassland ecosystems from 1990 to 2021 (**Figure 2A**). The results show that research on N deposition in grassland ecosystems shows an "exponential growth" model, indicating that N deposition is developing rapidly in grassland ecosystem research. Based on the changes in the annual literature volume, the research on N deposition in grassland ecosystems can be divided into three stages: (1) the nascent period (1990–1997), where the

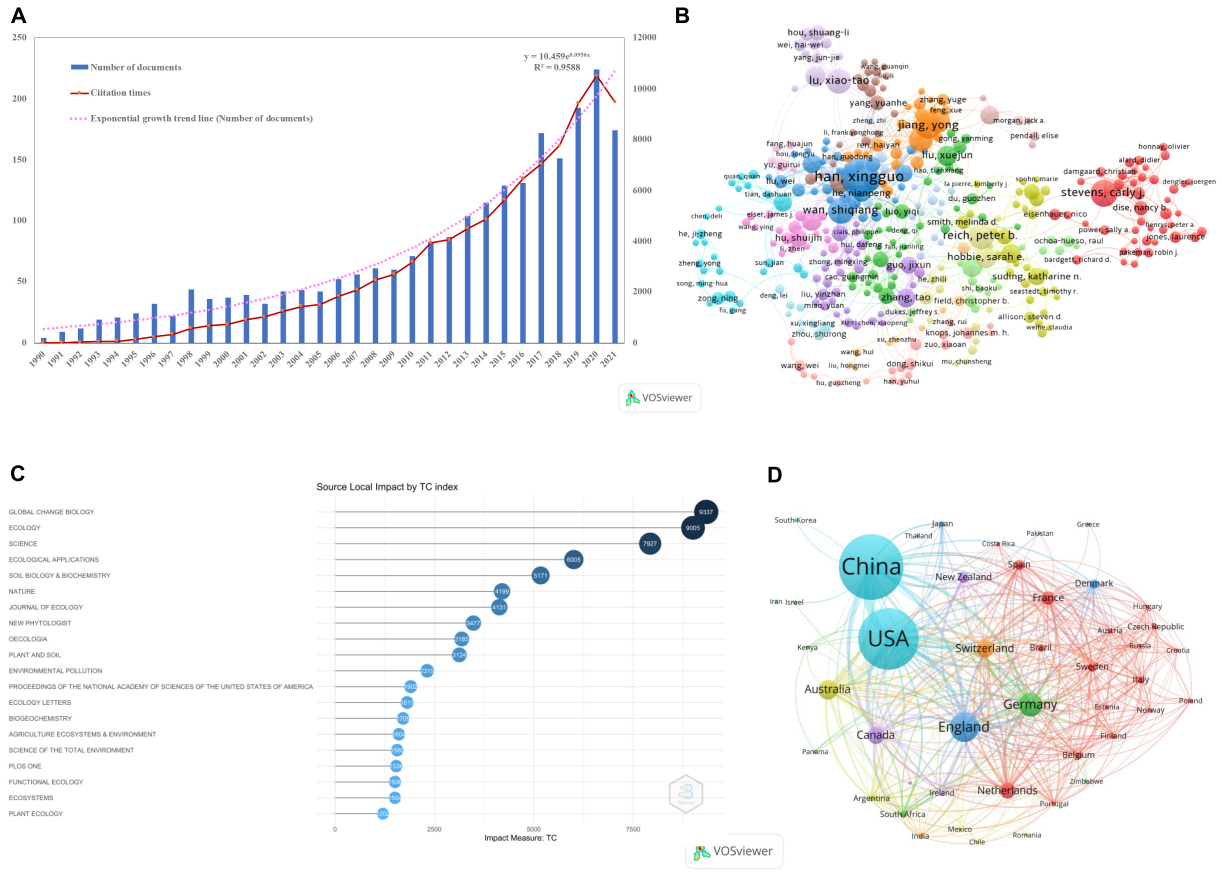

FIGURE 2
Temporal trend and change point of all papers and cumulative papers in terms of annual publications **(A)**; the network visualization map of 598 authors out of 8,173 authors with more than five publications as produced by VOSviewer **(B)**; top 20 journals based on total local citations as produced by R **(C)**; the network visualization map of 43 countries out of 89 countries with more than five publications as produced by VOSviewer **(D)**.

number of publications increased steadily with time, and the average annual number of publications was less than 20, indicating that the topic of N deposition in grassland ecosystems did not attract extensive attention from scholars in the early stage; (2) the basic exploration period (1998–2011), the number of articles published increased rapidly, but the average annual number of articles published was less than 60; and (3) The number of articles published in the period of rapid development (2012–2021) exceeded 100 and showed a significant upward trend overall, indicating that this research area has stimulated the interest of scientists in recent years and that N deposition in grassland ecosystems has become a hot research area in academia.

Supplementary Table 1 lists the five most cited references that have constructed the knowledge base for N deposition research over three consecutive periods beginning in 1990. These core publications will be identified and listed according to their LCS values. During the nascent period (1990–1996), we identified the five core references with the highest LCS values, which included Tilman (1993), Morecroft et al. (1994), Wilson et al. (1995), Vitousek et al. (1997) reviewed the negative impacts of accelerated input rates and alterations in the terrestrial ecological N cycle from human activities leading to increased N_2O, a potent greenhouse gas, loss of soil nutrients, potassium and calcium, soil acidification and loss of biodiversity, with the highest LCS values at this stage (268), and Mountford et al. (1993), Tilman (1993), Morecroft et al. (1994) and others found a decrease in species diversity through the application of N treatments by modeling N deposition, and these studies suggest that early on they attracted the attention of scholars in the field of N deposition research. During the fermentation phase (1998–2011), Stevens et al. (2004) had the highest LCS values (380), followed by Clark and Tilman (2008), LeBauer and Treseder (2008), and Bai et al. (2010), and these studies inspired the field of this study during this phase, which focused on N deposition studies on species richness (Stevens et al., 2004; Clark and Tilman, 2008; LeBauer and Treseder, 2008; Bai et al., 2010). In the developmental "leapfrog" phase (2012–2021),

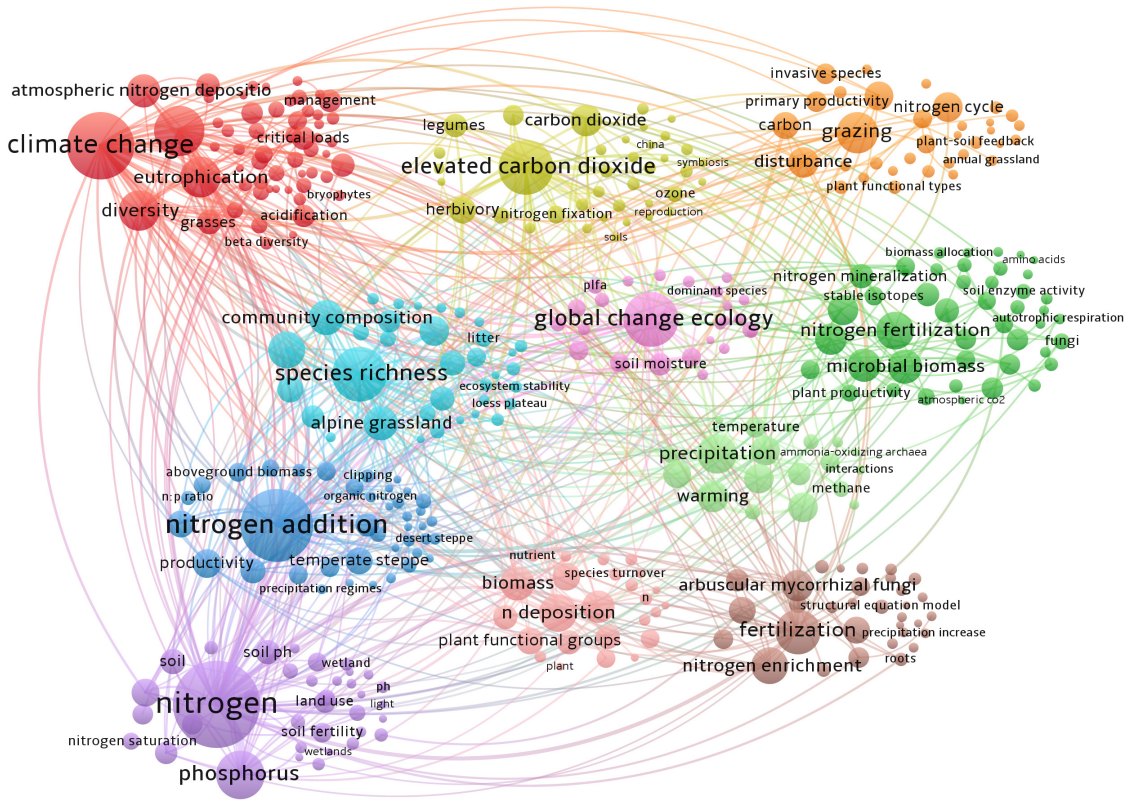

FIGURE 3
Network of author keywords based on the co-occurrence method on N deposition research from 1990 to 2021.

Phoenix et al. (2012), Isbell et al. (2013), and Borer et al. (2014) were the three highest LCSs in this phase, followed by Wei et al. (2013) and Yang et al. (2012). The N deposition studies at this stage did not only focus on the impact of N deposition on biodiversity (Phoenix et al., 2012; Isbell et al., 2013; Borer et al., 2014), and there are many studies concerned with the effects of N deposition on plant–soil–microbial systems (Wei et al., 2013). Notably, Stevens CJ's work is the most cited of the N deposition studies for the entire period (1990-2021), and his findings show that long-term N deposition significantly reduces plant species richness and that species richness decreases linearly with the rate of inorganic N deposition (Stevens et al., 2004).

Author analysis

A total of 9,384 authors were involved in the study of N deposition in grassland ecosystems (**Figure 2B**). A total of 434 authors from 18 clusters published at least five articles on N deposition in grassland ecosystems (**Figure 2B** and **Supplementary Table 2**), and the top ten most published authors were Han XG (95), Lu XT (61), Reich PB (53), Stevens CJ (50), Wan SQ (47), Jiang Y (38), Hobbie SE (36), Wang RZ (36), Xu ZW (34), and Tilman D (31). The TLS (total link strength) calculated by VOSviewer reflects the extent of close collaboration between authors. Han XG's TLS value was 86, which was twice that of Jiang Y (37), indicating that Han XG had more cooperative work. His most representative work was trade-offs and thresholds for modeling the effects of N deposition on grassland biodiversity and ecosystem function in Inner Mongolia (Bai et al., 2010), which has been cited >500 times.

Countries and institutes' analysis

There were 43 out of 89 countries that published a minimum of five publications on N deposition in grassland ecosystems (**Figure 2D**). China was the most productive country, with 1,053 articles, 5,025 citations, and 4.8 citations per article. The second was the United States, with 968 articles, 7,764 citations, and 8.0 citations per article. The United Kingdom ranked third, with 338 articles, 3,307 citations, and 9.8 citations per article, while Germany and Australia ranked fourth and fifth, respectively (**Supplementary Table 2**). The TLS (total link strength) calculated by VOSviewer reflects the degree of close cooperation between countries. The United States ranks first with a TLS value of 809, followed by China with 669, indicating that although China has the largest number of publications, it is lower than the United States in terms of international cooperation. China is working more closely with the United States, Canada, the United Kingdom, Germany, and France (**Figure 2D**). A total

of 2,173 institutions worldwide were found to contribute to N deposition in grassland ecosystems (**Supplementary Table 2**). The top 10 research institutions ranked according to a total national publication, a volume published a total of 1,548 articles, with Chinese research institutions, accounting for 70%, with the Chinese Academy of Sciences (CAS) (660) and the University of Chinese Academy of Sciences (UCAS) (275) leading the way, followed by the University of Minnesota System (134). The CAS is ranked first in terms of TLCS at 4,140, followed by the University of Minnesota System with a TLCS value of 2,027, while Germany and Australia, the top five countries in terms of total publications, do not have institutions in the top 10 list.

Influential journals

The 440 journals included in SCIE, the top 20 (4.54%) journals in terms of productivity published a total of 1,211 papers, representing 43.81% of the total literature, with *Plant and Soil* (127, 4.59%), *Global Change Biology* (124, 4.49%), and *Soil Biology & Biochemistry* (112, 4.05%) being the three most published journals; however, 201 journals (45.68%) published only one paper, and 382 journals (86.81%) published fewer than 10 papers (**Figure 2C** and **Supplementary Table 3**). *Global Change Biology* ranked first with 9,337 total citations, followed by *Ecology* with a total of 9,005 citations, but the *Ecology* journal ranked first in terms of average citations per paper (120.07), confirming that research on N deposition in grassland ecosystems is focused on global change and ecological issues (Sala et al., 2000; Yang et al., 2019). The impact factors of the journals *Global Change Biology* (10.86) and *New Phytologist* (10.15) are greater than 10, which means that the papers published in these two journals have the highest academic value and are the most referred to by the academic community. Therefore, the three journals *Global Change Biology*, *Ecology*, and *New Phytologist* should be focused on and tracked in the study of N deposition in grassland ecosystems.

Co-occurrence keyword and burst keyword analysis of research hotspots and trend analysis

Co-occurrence keyword analysis

A total of 389 keywords co-occurrence map (**Figure 3**) was obtained from 6,201 keywords, with each color cluster on the map reflecting a different research theme in the field of N

TABLE 1 Identified clusters of author keywords on N deposition research from 1990 to 2021.

ID	color	Cluster name	L	TLS	W	Top 10 author keywords
#1	Red	Climate change	198	438	157	Biodiversity, eutrophication, diversity, atmospheric, N, deposition, grasses, critical loads, species composition, land-use change, acidification, management
#2	Yellow	Elevated carbon dioxide	131	292	111	Carbon dioxide, herbivory, legumes, photosynthesis, N fixation, soil N, ozone, CO_2 enrichment, respiration
#3	Orange	Grazing	125	181	68	Disturbance, N cycle, nutrient limitation, carbon, invasive species, primary productivity, restoration, invasion, plant functional types, water
#4	Green	N fertilization	101	155	60	Microbial biomass, soil respiration, carbon sequestration, carbon cycle, nitrate, N mineralization, soil organic carbon, ^{15}N tracer, soil organic matter, ammonium
#5	Red Purple	Global change ecology	166	328	109	Soil moisture, plant community, fire, microbial community, soil properties, ecosystem functioning, PLFA, precipitation change, coastal sage scrub, lignin
#6	Light blue	Species richness	135	340	110	Alpine meadows, community composition, alpine grassland, Tibetan plateau, species diversity, Qinghai-Tibetan plateau, functional groups, community structure, functional traits, nutrient addition
#7	Blue	N addition	179	420	176	Productivity, ecological stoichiometry, temperate steppe, nutrient enrichment, semiarid grassland, above-ground biomass, soil microbial biomass, steppe, temperate grassland, stoichiometry
#8	Purple	Nitrogen	225	709	239	Phosphorus, nutrients soil, soil pH, decomposition, land use, pasture, soil carbon, soil fertility, biogeochemistry
#9	Pink	N deposition	92	148	58	Biomass, competition, drought, plant functional groups, litter decomposition, nutrient cycling, soil microbial community, litter quality, plant-soil (below-ground) interactions, N
#10	Brown	Fertilization	133	256	80	Nitrogen enrichment, arbuscular mycorrhizal fungi, plant diversity, soil acidification, tallgrass prairie, mowing, global warming, extracellular enzymes, nutrient availability, structural equation model
#11	Light green	Precipitation	115	221	64	Warming, greenhouse gas emissions, meta-analysis, nitrification, nitrous oxide, denitrification, temperature, climate warming, N_2O emission, methane

deposition. The results show that N deposition research could be divided into 11 clusters within different colors, with the red section focusing on climate change and biodiversity. The yellow section represents studies of increasing CO_2 concentrations. The orange section represents studies of grazing disturbances in the N cycle. The green section focuses on the effects of N fertilizer application on soil microbial biomass, C and N nutrient cycling, and soil physicochemical properties. The fuchsia section is mainly related to global ecosystem change. The light blue section mainly represents studies of species richness and diversity in places such as alpine grasslands and the Tibetan Plateau. The blue section is mainly related to studies of N application on productivity and econmetrics in temperate grasslands, semiarid grasslands, and Inner Mongolia. The purple section focuses on the study of N and phosphorus nutrients. The pink section mainly represents studies of N deposition on biomass, plant functional groups, and species regeneration. The coffee color mainly represents the effect of fertilizer application on N enrichment and the clumping of mycorrhizal fungi. The light green color is mainly related to precipitation, N_2O, and methane, the main greenhouse gas emissions. Details of the top 10 keywords for each cluster are shown in **Table 1**.

Burst keyword analysis

The analysis of the development path and publication time of the keywords indicates a gradual evolution of N deposition research (**Figure 4**). From 1990 to 2000, the grasslands that were the focus of N deposition research were calcareous grassland and Heathland (Johnson et al., 1998; Lee and Caporn, 1998), and the types of plants of interest was *Lolium Perenne* (Bardgett et al., 1999). The indicators of interest were plant growth, photosynthesis and gas exchange (Morecroft et al., 1994; Van Der Heijden et al., 2000), as well as soil C and N cycle dynamics and root system dynamics (Thornley et al., 1995; Norby and Jackson, 2000), were in response to N deposition, with a particular focus on grassland ecosystems in the context of atmospheric CO_2 (burst value of 26.21) and N deposition (Lloyd, 1999).

From 2000 to 2010, there was a continued focus on the impact of N deposition on CO_2, which increases net soil CO_2 emissions and offsets carbon sequestration by vegetation and soil fractions (Xu et al., 2004). Jiang et al. (2010) showed that N deposition tended to reduce CO_2 emissions and CH_4 uptake and increase N_2O emissions. Attention was also paid to the effects of N deposition on the biodiversity and richness of grassland ecosystems. Stevens et al. (2004) found that long-term N deposition significantly reduced plant species richness through a study of 68 acid grasslands in the United Kingdom and that the rate of this decline was linearly related to the rate of inorganic N deposition. The main cause is the accumulation of N, which increases the competitiveness between species and creates unfavorable soil conditions unfavorable for species growth

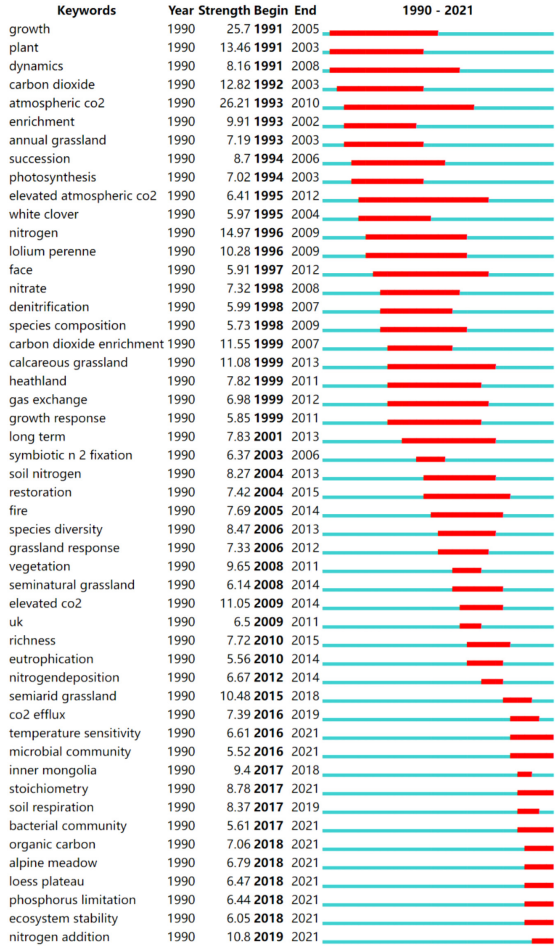

FIGURE 4
Top 50 keywords with the strongest citation bursts (the red bars indicate some keywords cited frequently; the green bars indicate keywords cited infrequently).

(Bobbink et al., 2010), such as consumption of soil cations leads to soil acidification, metal migration, and eutrophication (Van Landuyt et al., 2008), which results in changes in flora, and in addition, increases in soil N levels caused by atmospheric N deposition or other means may increase the dominance of annuals and may promote the invasion of new species, which may reduce the diversity of native annuals, while increases in the biomass of exotic annual grasses may also increase the frequency of fire (Brooks, 2003).

From 2010 to 2021, scientists continued to focus on the study of N deposition on CO_2 fluxes in grassland ecosystems. Long-term atmospheric N deposition promotes soil CO_2 emissions from alpine meadows on the Qinghai-Tibet Plateau by increasing the soil fast-acting N content and promoting plant growth (Fang et al., 2012). By meta-analysis, Deng et al. (2020) showed that soil organic carbon content and CO_2 emission fluxes increased by 3.7 and

0.3%, respectively, under N deposition at the global scale and that grassland ecosystems are important greenhouse gas sinks. The results indicate that researchers in this period were more concerned with the study of the effects of N deposition on the physicochemical properties (soil stoichiometric characteristics, soil respiration, soil organic carbon, P limitation) of grassland soils.

Since 2017, the keywords Inner Mongolia, alpine meadow, Loess Plateau, and other locations have become the dominant bursts with burst values of 9.4, 7.06, and 6.79. The research areas of N deposition in grassland ecosystems at this stage are mainly concentrated in Inner Mongolia temperate grassland (Tian et al., 2016), semiarid grasslands (Wang et al., 2019), alpine grassland (Jiang et al., 2018), and the Loess Plateau (Chen et al., 2018) in China. The effect of nitrogen deposition on microbial is an important hotspot in current academic research (Zhang et al., 2013; Zhao et al., 2021; Wang et al., 2022).

Effects of N addition on microbial biomass and alpha diversity in grassland ecosystems—A case study of Chinese grassland ecosystems

Effect of nitrogen addition on soil microbial growth

The forest plot showed (**Figure 5**) that in the grassland ecosystem, the biomass of bacteria increased significantly under nitrogen addition, while the biomass of fungi did not change

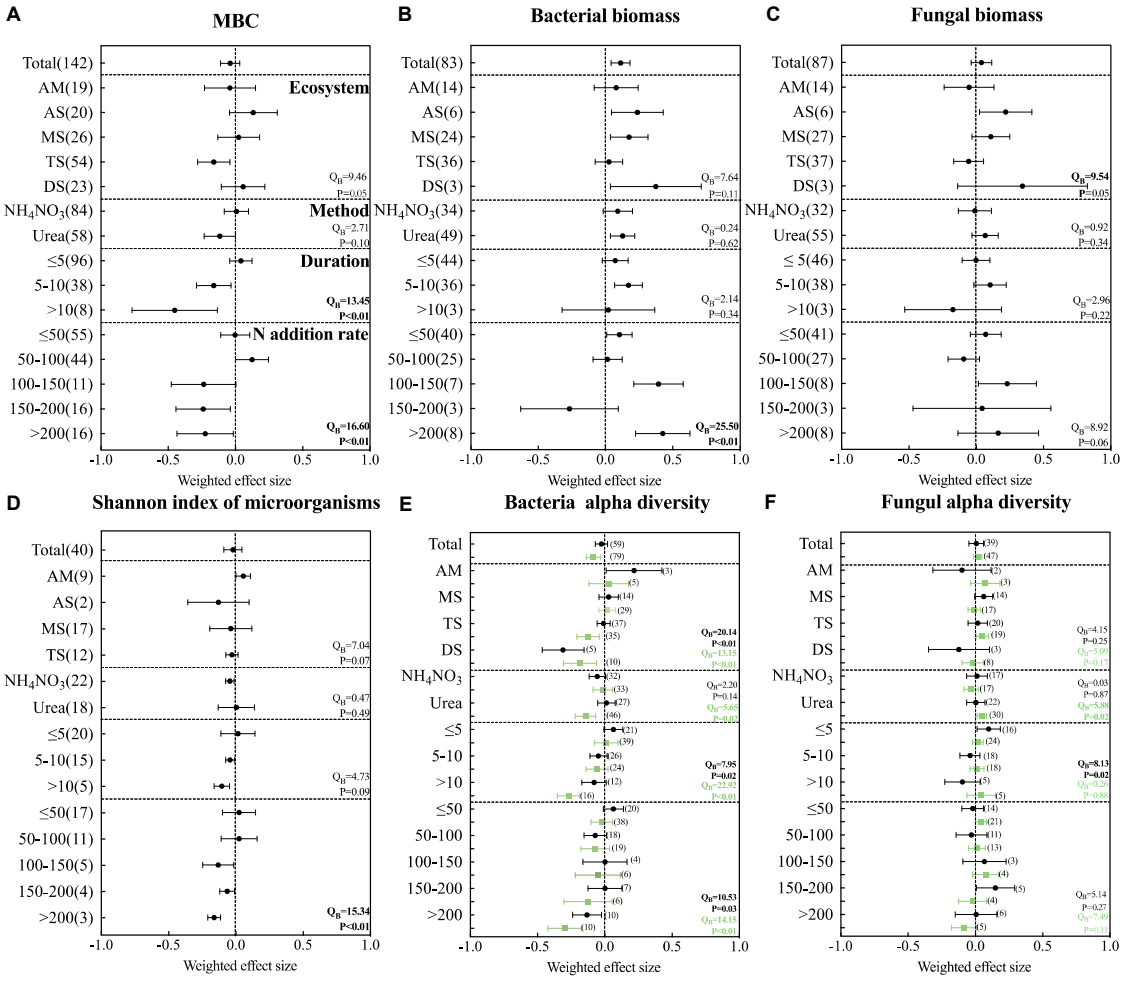

FIGURE 5
Effects of N addition on **(A)** microbial carbon, **(B)** bacterial biomass, **(C)** fungal biomass, **(D)** shannon index of microorganisms, **(E)** bacterial alpha diversity and **(F)** fungal alpha diversity. The variables are categorized into different ecosystems (AM, alpine meadow; AS, alpine steppe; MS, meadow steppe; TS, typical steppe; DS, desert steppe); fertilizer types (NH_4NO_3 and urea), experiment durations, and N application rates. Error bars present 95% confidence intervals. The dashed line was drawn at a mean response ratio = 0. If 95% CI does not overlap zero, then microbial diversity in fertilized soils differed significantly from those in unfertilized soils. The numbers in parentheses next to the point represent the number of studies. In Panels **(E,F)**, black represents Shannon index and green represents chao1 index.

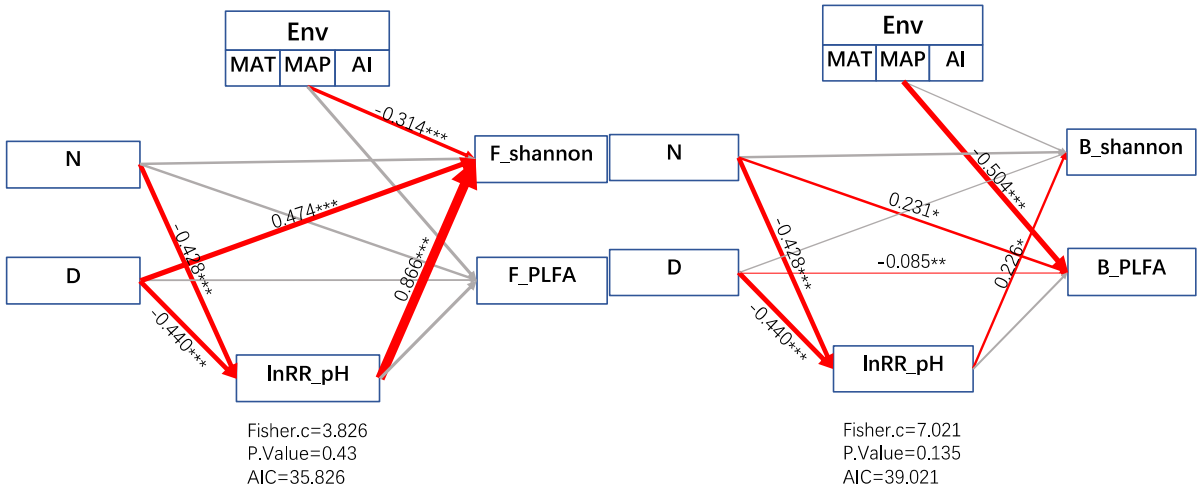

FIGURE 6
Structural equation modeling (SEM) depicting the effects of multiple drivers on bacterial (B) and fungal (F) Shannon index response ratios and PLFA. D: duration of N fertilizer addition experimental time (years); N: fertilizer addition amount (kg ha^{-1} yr^{-1}); ln*RR*_pH is the response ratio of soil pH to microbial PLFA and Shannon diversity under N addition. Environmental factors (ENV) include annual mean temperature (MAT), annual rainfall (MAP), and aridity index (AI). Red arrows and gray arrows indicate significant or insignificant correlations, respectively. The numbers adjacent to the arrows are the normalized path coefficients, which are like relative regression weights and represent the magnitude of the effect of the relationship. The thickness of the arrows is proportional to the magnitude of the normalized path coefficients or covariance coefficients. Fisher.C: Fisher's C statistic; *P* Value: probability level; AIC: Akaike's information criterion.

significantly. Simultaneous nitrogen addition did not alter soil microbial carbon (MBC).

Among them, we found that different grassland ecosystem types responded differently to nitrogen addition. Nitrogen addition significantly increased bacterial biomass in alpine steppe, meadow steppe, and desert steppe ecosystems, while nitrogen addition had no significant effect on bacterial biomass in alpine meadow and typical steppe. Fungal biomass increased only with nitrogen fertilizer addition in alpine steppe ecosystems. Meanwhile, microbial carbon only decreased with nitrogen addition in typical grassland ecosystems and was not significant in other ecosystems. The bacterial biomass is more sensitive to urea, and the addition of urea could significantly increase the bacterial biomass. Fungi are not affected by the type of fertilization. With the increase of fertilization years, the microbial carbon decreased significantly. Bacterial biomass increased significantly at fertilization time 5–10 years. When the fertilization time is more than 10 years, the results here are not significant, probably because of less research. Overall, with the increase of fertilization years, the bacterial biomass increased significantly. However, the biomass of fungi did not change much with the increase of fertilization time. The biomass of bacteria increased significantly with the increase of fertilization rate, while the microbial carbon decreased significantly with the increase of nitrogen application rate.

These results represent that in nitrogen-limited grassland ecosystems, nitrogen addition relieves microbial nitrogen limitation, providing evidence that nitrogen addition can increase bacterial biomass in grassland ecosystems. It was also found that bacteria were more sensitive to nitrogen addition than fungi.

Effect of nitrogen addition on soil microbial alpha diversity

In all studies, the effect of nitrogen addition on the Shannon index of soil microorganisms was not significant. Nitrogen addition only significantly decreased the Chao 1 index of bacteria. Different grassland ecosystem types had no significant effect on the total microbial diversity and fungal diversity. Changes in bacterial diversity respond inconsistently across ecosystem types. In a typical ecosystem, the Chao1 index of bacteria decreased significantly with nitrogen addition, but the Shannon index did not change significantly. In the desert-grassland ecosystem, both the Shannon index and Chao 1 index of bacteria decreased significantly. The addition of urea significantly decreased the Chao 1 index of bacteria and significantly increased the Chao 1 index of fungi. The microbial diversity and bacterial diversity decreased gradually with the increase of fertilization time. The diversity of microorganisms and bacteria also decreased significantly with the increase of fertilization rate. Fungal diversity was not significantly affected by the time and nitrogen fertilizer addition rate.

Therefore, our results show that the addition of nitrogen fertilizer could significantly reduce the microbial diversity and bacterial diversity to a certain extent. Fungal diversity was not significant for nitrogen addition.

Multivariate relationships between the responses of soil microbes and nitrogen addition

According to the obtained structural equation model (**Figure 6**), it can be obtained that changes in the environment will reduce the alpha diversity of fungi ($r = -0.314$) and will reduce the biomass of bacteria ($r = -0.504$). And the duration of nitrogen addition and the amount of fertilizer addition will change the alpha diversity of bacteria and fungi by reducing the pH of the soil environment. The Shannon index of fungi will be increasesd by an increase in fertilization time ($r = -0.474$). The time of fertilization directly reduces the biomass of bacteria ($r = -0.085$), and the amount of fertilization positively affects the biomass of bacteria ($r = 0.231$). The Shannon index of bacteria was positively influenced by the ln*RR* of pH indirectly through the regulation of fertilization time and fertilization amount ($r = 0.226$).

Our structural equation model showed that for nitrogen-limited grassland ecosystems, the addition of nitrogen fertilizers could alleviate the nitrogen limitation of microorganisms to a certain extent and increase the diversity and biomass of microorganisms. At the same time, it was also proved that the reduction of soil pH with nitrogen fertilizer addition can positively affect the alpha diversity of microorganisms.

Conclusion

Publications on N deposition in grassland ecosystems were analyzed by bibliometric analysis from 1990 to 2021 based on data from SCI-EXPAND. The results show that (1) The topic of "grassland" and "N deposition" studies have grown exponentially ($R^2 = 0.9588$), and the publication trend can be divided into three periods. Before 1997, there was slow, but steady progress (<20 publications per year) in the field. But thereafter, with over 100 publications, and the average annual number increased by more than 5-fold from 2012 to 2021. (2) Research teams in China and United States make the greatest contributions and have a relatively high influence on this research field. Among them, the CAS, the UCAS, and the University of Minnesota System have performed prominently in the number of literature and citations in recent years. (3) The journals with the highest academic value articles by researchers of "N deposition in grassland ecosystems" are global change biology, ecology, and new phytologist. (4) The co-occurrence keyword analysis shows that "N deposition in grassland ecosystems" studies can be categorized into 11 main research themes, including climate change, elevated CO_2, grazing, species richness, and diversity, etc. (5) Moreover, based on burst keyword analysis, we identified mainly three potential or not fully explored topics: CO_2 efflux, fragile grassland ecosystems (Inner Mongolia temperate grassland, semiarid grasslands, alpine grassland, and the Loess Plateau), and microbial community. Our further meta-analysis results showed that the addition of N changed the living environment of microorganisms by changing the pH value of the soil, resulting in the rapid growth of acidophilic microorganisms, and the rapid reduction of some microorganisms that were not suitable for the acidic environment, resulting in a decrease in biomass. The diversity of microorganisms will change. Due to the action of hyphae and mycorrhizal fungi, they can perform good self-regulation, so the biomass and diversity of fungi are not as obvious as those of bacteria. This comprehensive study can provide a preliminary analysis of the progress and development trend of N deposition research on global grassland ecosystems. Meanwhile, meta-analysis as a specific case study can further understand the process and mechanism of N deposition impact on grassland microorganisms, which provides an important and important reference for the overall improvement of basic research and ecological risk management of N deposition ecological effects, as well as the grasp of future hotspot areas and key microbial processes. In summary, this research could provide a global perspective on the international dynamics of global grassland N deposition research, and to provide a reference for understanding the interaction and feedback mechanisms between microbial processes and N deposition in grassland ecosystems from a local perspective.

Data availability statement

The original contributions presented in this study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

This study was reviewed and approved by the University of Chines Academy of Sciences of Ethics Committee.

Author contributions

TL, ZX, YW, and XC: conceptualization. TL, LC, and LL: data curation, investigation, and visualization. XC and YW: funding acquisition. TL, ZX, XS, and RC: methodology. HW and YW: resources. CL: software. ZX, YW, and XC: supervision. TL, YW, and XC: validation. TL: writing—original draft. TL, LC, LL, JQD, HW, YH, XC, LT, CL, FW, ZX, and XC: writing—review and editing. HW, JFD, and XC: academic editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.947279/full#supplementary-material

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Tipping point of plant functional traits of *Leymus chinensis* to nitrogen addition in a temperate grassland

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It has widely been documented that nitrogen (N) enrichment stimulates plant growth and modifies plant functional traits in the terrestrial ecosystem. However, it remains unclear whether there are critical transitions or tipping points for the response of plant growth or traits to N enrichment, and how these responses differ to different N forms. We chose the native, perennial clonal grass, *Leymus chinensis* in Inner Mongolia steppe, and conducted a field experiment, in which six N addition rates (0, 2, 5, 10, 20, and 50 g N m^{-2} $year^{-1}$) and five N compound types [NH_4NO_3, $(NH_4)_2SO_4$, NH_4HCO_3, $CO(NH_2)_2$, slow-release $CO(NH_2)_2$] are considered. Here, we found that the different N compound types had no significant effect on the growth of *L. chinensis*. N addition rate significantly increased plant aboveground biomass and leaf nitrogen concentration, whereas decreased leaf dry matter content. The tipping point for N-induced aboveground biomass increase was at 10 g N m^{-2} $year^{-1}$, and the changes in functional traits were at N addition rates of 20 g N m^{-2} $year^{-1}$. Our findings suggested that the responses of aboveground biomass and functional traits to N addition were asymmetric, in which responses in aboveground biomass were more sensitive than that in functional traits. The differential sensitivity of aboveground biomass and functional traits of *L. chinensis* occurred to N deposition highlights the importance of functional traits in mediating ecosystem functioning in the face of N deposition, regardless of which chemical forms dominate in the deposited N.

KEYWORDS

nitrogen deposition, threshold, functional traits, productivity, steppe

Introduction

Nitrogen (N) is a major limiting resource for plant growth in diverse natural ecosystems (Vitousek and Howarth, 1991; Elser et al., 2007) and plays a crucial role in photosynthesis and all enzymatic activities (Reich et al., 2004). Human activities such as fossil fuel combustion and fertilizer application have dramatically increased reactive N being deposited into many terrestrial ecosystems (Galloway et al., 2008). There likely exist ecosystem-specific thresholds for N enrichment, beyond which ecosystem start to transit substantially, such as the critical threshold for N-induced species loss to mature Eurasian grasslands is 1.75 g N m^{-2} $year^{-1}$ (Bai et al., 2010). An ecological threshold or tipping point is the point at which there is an abrupt change in an ecosystem quality, property, or phenomenon (Groffman et al., 2006). Identifying ecological thresholds in response to climate change such as N deposition is important to guide ecosystem management and maintain ecological services (Rockström et al., 2009).

Plant functional traits reflect plants' capacities for resource capture and adaptations to environmental changes (Wright et al., 2004). As plant economic spectrum predicts, species with the traits capable of acquiring resource rapidly (e.g., high N concentration, specific leaf area, and photosynthetic rate) would compete for resource more strongly under N enrichment (Wright et al., 2004; La Pierre and Smith, 2015). Recent studies have revealed inconsistent responses of plant functional traits to nutrient addition, while some studies showed that N addition enhanced leaf N concentration, chlorophyll concentration and specific leaf area, thus leading to a marked increase in photosynthetic rates (Tatarko and Knops, 2018; Zheng et al., 2018; Sun et al., 2022). Others found no effect of N addition on leaf mass area, area-based concentrations of foliar N and chlorophyll concentrations (Hu and Wan, 2019). One possible explanation for the inconsistency among these studies is that the response of plant functional traits to N enrichment may be non-linear (Zhang et al., 2019; Song et al., 2022), such that plant functional traits may not necessarily show a significant change until a threshold N level is reached. Nevertheless, it remains unclear whether there is tipping point for the effect of N inputs on plant traits. Moreover, plasticity in plant traits is predicted to play a key role in affecting plant production. For example, the study conducted in the central French alpine grassland found that the productivity was positively associated with fertilization-induced increases in plant height and leaf area (Lavorel and Grigulis, 2012). Another study conducted in an Chinese alpine steppe found that increasing leaf N content and leaf area under N addition significantly promoted plant community productivity (Peng et al., 2017; Zhang et al., 2021). However, to date, very few studies have considered whether plant traits can be associated with the response of plant production to increased N inputs.

In addition to plant functional traits, N enrichment could influence plant growth and productivity by changing soil environmental conditions such as soil N availability and acidification (Bobbink et al., 1998). Increasing soil N availability was the key direct factor driving the plant growth under N addition (Tao et al., 2022). In addition, N deposition can result in soil acidification both directly as a result of acid deposition (nitric acid) and indirectly through processes and reactions in soil and water (Stevens et al., 2010), which results in toxicity through mobilization of metals, leaching of base cations and changing the balance between nitrogenous compounds, and hence causes cascading effects on plant growth (Roem et al., 2002; Stevens et al., 2010).

In this study, we investigated the plant functional traits in a multi-level (0, 2, 5, 10, 20, and 50 g N m^{-2} $year^{-1}$) N addition experiment in a temperate grassland in Inner Mongolia, China, to test whether there will be a N threshold for plant functional traits in response to N deposition. The temperate grassland in our study site is dominated by a native perennial rhizomatous grass *Leymus chinensis*, which is palatable for grazing animals and has high forage value and drought tolerance (Bai et al., 2004; Huang et al., 2015). Here, *L. chinensis* was chosen as a model plant, we hypothesized that (a) when the N addition level reaches a certain level, aboveground biomass and plant functional traits will cross the critical threshold and show dramatic response, and the threshold will be at similar N addition rate for aboveground biomass and traits, (b) changes in traits and soil conditions will together drive the response of plant production to increasing N addition.

Materials and methods

Study site

The experiment was conducted at the Erguna Forest-Steppe Ecotone Research Station (N50°10'46.1″, E119°22'56.4″), Institute of Applied Ecology, the Chinese Academy of Sciences. The grassland had been used for forage harvest before 2013. The long-term mean annual precipitation of the site is 363 mm and the mean annual temperature is -2.45°C (1957–2016). The soil is classified as chernozem according to the Food and Agricultural Organization of the United Nations classification. The dominant species in this ecosystem is *Leymus chinensis*, which makes up > 45.6% of the total aboveground biomass.

Experimental design

The N addition experiment was started in 2014, with a randomized complete block design to study the effects of rates and types [NH_4NO_3, $(NH_4)_2SO_4$, NH_4HCO_3, $CO(NH_2)_2$, slow-release $CO(NH_2)_2$] of N addition on plant biomass and leaf

functional traits. There were six N addition rates (i.e., 0, 2, 5, 10, 20, and 50 g N m^{-2} $year^{-1}$), and five types of N compounds. Each of the 30 treatments had eight replications, leading to 240 treatment plots in total. The area of each plot was 10 m × 10 m. Nitrogen fertilizers were added annually since 2014, in late May. Fertilizers were mixed with sand (because of the low amount of added fertilizer at low addition rates) and added uniformly by hand. Sand was sieved through less than 2 mm in size, washed in water, and then heated at nearly 250°C for 60 min in an iron pan. To avoid potentially confounding effects, all plots received the same amount of sand (0.5 kg per plot).

Field sampling and measurement

At each plot, 15 healthy and mature plant individuals were randomly selected from different locations between 1st and 5th August, 2015. These plant individuals were clipped at the ground level and immediately placed in a portable cooler. In the laboratory, all samples were soaked in water for 6 h to ensure full rehydration. Each leaf was then cut from the stem and gently dried with tissue paper before measurement. Water-saturated leaf mass was weighed, scanned the leaf with a scanner (Canon LiDE120), and determined the leaf area by the ImageJ software. All leaves were then dried for 48 h at 65°C and then weighed. The leaf dry matter content (LDMC), specific leaf area (SLA), and stem: leaf ratio were calculated. After being ground in a ball mill (Retsch MM 400; Retsch, Haan, Germany), the leaf total N concentration was analyzed on an elemental analyzer (Vario MACRO cube, Elementar Analysensysteme, Germany). The grinded leaf samples (50 mg) were digested with H_2SO_4–H_2O_2 (Bennett et al., 2002), and measured colorimetrically for leaf P concentration at 880 nm after reaction with molybdenum blue. N: P ratio was reported as mass ratios.

Aboveground biomass of *L. chinensis* was sampled between August 10th and 20th by clipping plants at the soil surface in a 1 m × 1 m quadrat, which was randomly placed in each plot without a spatial overlap of quadrats among different years and at least 50 cm inside the border of each plot to avoid edge effects. All plant materials were oven-dried at 65°C for 48 h and weighed. After clipping above-ground biomass, three soil cores (0–5 cm depth and 50 cm apart) were collected using a 7 cm diameter soil auger adjacent to each aboveground plant sample plot and mixed *in situ* into one composite sample. Fresh soil samples were sieved through a 2-mm sieve to remove visible roots, plant residues, and stones, and taken to the laboratory for analysis of soil water content, soil inorganic N and pH. Fresh soil was extracted by 2 M KCl solution, 10 g of soil was extracted in 50 mL of 2 M KCl solution, and then inorganic N concentrations were analyzed with a FLAstar 5000 Analyzer (Foss Tecator, Hillerød, Denmark). Subsamples were air−dried and analyzed soil pH using a pH meter (Thermo Fisher Scientific, America).

Statistical analysis

Linear mixed effect model analysis of variance was performed using the lme function from the nlme package with N addition rate and N compounds type as fixed factors and block as a random factor. Given N compounds showed no significant effect on plant biomass and functional traits (Table 1), we pooled data from different N compounds under a certain N addition rate to simplify our analysis and focus on the effect of N addition rate. We used Duncan's test to evaluate aboveground biomass, SLA, LDMC, stem: leaf ratio, leaf N concentration, P concentration, leaf N: P ratio of *Leymus chinensis*, and SIN, soil pH and SWC under varying N addition rates. To examine how aboveground biomass of *Leymus chinensis* was correlated with SIN or soil pH across different N addition rate, a linear regression model was performed. All analyses were conducted using R version 4.0.2 (R Development Core Team, 2020).

Results

Effects of N addition on plant functional traits of *Leymus chinensis*

The compound types of added N had no significant effects on aboveground biomass, SLA, LDMC, stem: leaf ratio, leaf N content, leaf P content, leaf N: P ratio of *L. chinensis* (Table 1, all $P > 0.05$). By contrast, N addition rates showed dramatic effects

TABLE 1 Results of mixed model analysis of variance for aboveground biomass, SLA (specific leaf area), LDMC (leaf dry matter content), stem: leaf ratio, leaf N concentration, leaf P concentration, leaf N: P ratio of *Leymus chinensis*, and soil inorganic nitrogen content (SIN), soil pH, soil water content (SWC).

	N addition rate	N compounds type	N addition rate × N compounds type
Aboveground biomass	**55.64*****	0.39^{ns}	1.74^{ns}
SPAD	**14.22****	1.06^{ns}	0.45^{ns}
SLA	**16.38****	0.79^{ns}	0.64^{ns}
LDMC	**5.75***	0.43^{ns}	2.27^{ns}
Stem: leaf ratio	0.19^{ns}	1.56^{ns}	1.17^{ns}
Leaf N content	**30.84*****	2.11^{ns}	0.49^{ns}
Leaf P content	0.03^{ns}	0.53^{ns}	0.28^{ns}
Leaf N: P ratio	**13.83*****	2.25^{ns}	2.53^{ns}
SIN	**136.19*****	**11.32*****	2.35^{ns}
Soil pH	**54.32*****	**6.4*****	0.405^{ns}
SWC	**6.62***	0.52^{ns}	**3.19***

N addition rate and N compounds type were used as fixed factors and block as a random factor. The *F*-values were shown. Asterisks denote significant levels: ns, $P > 0.05$; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; and $^{***}P \leq 0.001$, respectively.

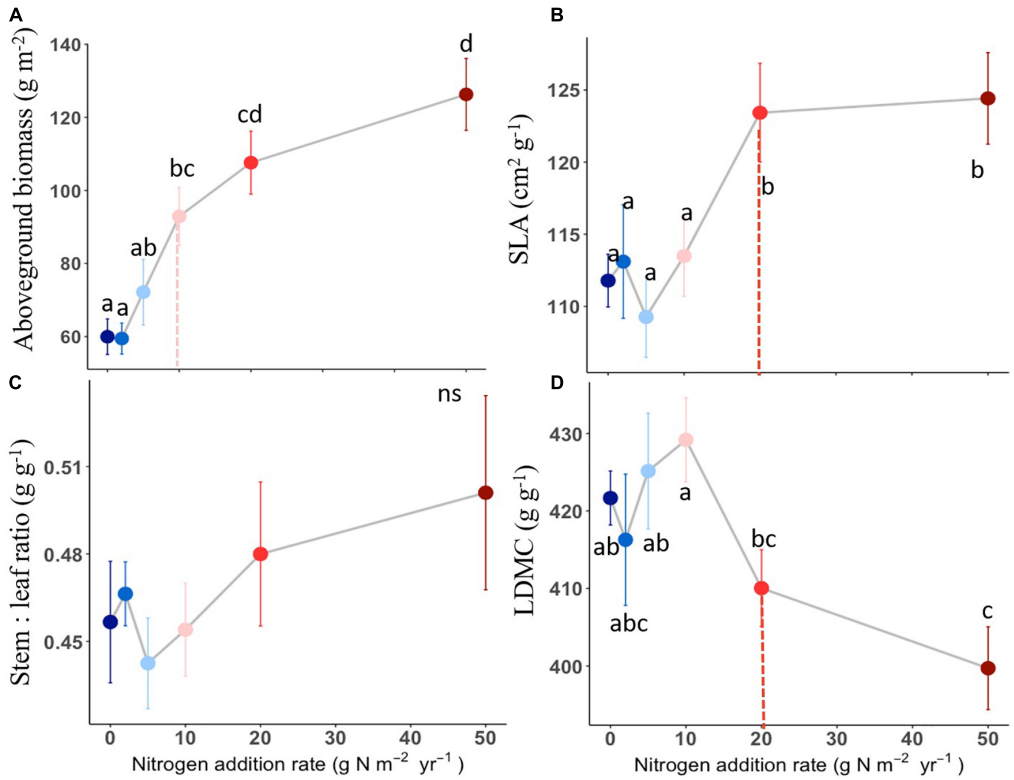

FIGURE 1
Effects of N addition on plant biomass and leaf traits of *L. chinensis*. Trait abbreviations are aboveground biomass **(A)**, SLA (specific leaf area) **(B)**, LDMC (leaf dry matter content) **(C)** and stem: leaf ratio **(D)**. Different lower-case letters indicate significant differences ($P < 0.05$) among treatments, and ns indicates non-significant ($P > 0.05$). The dashed line in each panel indicate for tipping points. The data shown are the means with 40 replications ± standard error.

on aboveground biomass, leaf morphology and stoichiometry. Aboveground biomass was not different among the control and N addition treatments up to 5 g N m^{-2} $year^{-1}$ but significantly increased at the three highest N addition levels (10, 20, and 50 g N m^{-2} $year^{-1}$; Figure 1A). N addition rate increased SLA and leaf N concentration whereas decreased LDMC (Figures 1B,D, 2A), with the tipping point of N-induced changes being at the rate of 20 g N m^{-2} $year^{-1}$. The stem: leaf ratio and leaf P concentration were not affected by N addition (Figures 1C, 2B).

Effects of N addition on soil characteristics

Soil inorganic nitrogen increased with N addition once the N addition rate passed the 2 g N m^{-2} $year^{-1}$ (Figure 3A). Soil pH in the plots receiving 10, 20, and 50 g N m^{-2} $year^{-1}$ was lower than the one receiving N below 10 g N m^{-2} $year^{-1}$ level (Figure 3B). However, there was no detectable difference in soil water content among N addition rates (Figure 3C). The effects of N addition on soil inorganic N and soil pH significantly different among N compounds ($P < 0.05$, Table 1 and Supplementary Figure 1), with the $(NH_4)_2SO_4$ treatment showing stronger effect than other N compounds.

Correlations between aboveground biomass and soil conditions

The aboveground biomass was positively correlated with soil inorganic N (Figure 4A, $R^2 = 0.29$, $P < 0.001$), but negatively correlated with soil pH (Figure 4B, $R^2 = 0.23$, $P < 0.001$). No significant relationship, however, was found between aboveground biomass and functional traits response to N addition (all $P > 0.05$).

Discussion

Non-linear response of aboveground biomass to N addition

Consistent with our hypothesis, we found that aboveground biomass of *L. chinensis* was not affected by low levels of N

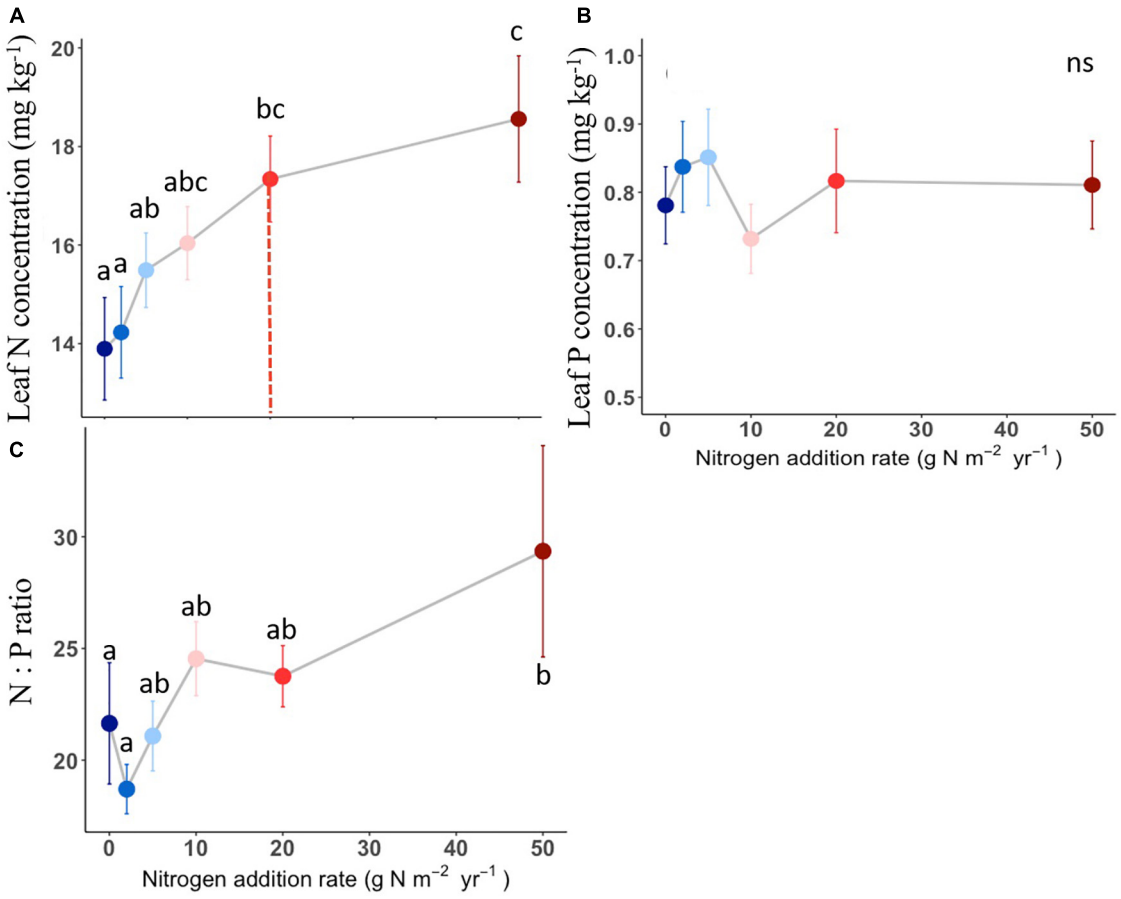

FIGURE 2
Effects of N addition on leaf stoichiometry of *L. chinensis*. Trait abbreviations are leaf N concentration **(A)**, leaf P concentration **(B)**, leaf N: P mass ratio **(C)**. Different lower-case letters indicate significant differences ($P < 0.05$) between treatments, and ns indicates non-significant ($P > 0.05$). The dashed line in each panel indicate for tipping points. The data shown are the means with 40 replications ± standard error.

addition (i.e., < 5 g N m^{-2} year^{-1}), but sharply increased between 10 and 50 g N m^{-2} year^{-1}. These results suggested that plant growth in Inner Mongolia grassland was strongly limited by N and N addition could stimulate plant growth by relieving N limitation, which agreed with observations in Hautier et al. (2009). Moreover, another field experiment in Inner Mongolia at the typical steppe found that increase in N availability also stimulated community aboveground biomass, with the significant positive response occurred at the N addition rate of 1.75 g N m^{-2} year^{-1} by the fourth year (Bai et al., 2010). It is lower than that in our study where N threshold was ≥ 10 g N m^{-2} year^{-1}. This may be mainly due to that our study have been performed just for 2 years, which has lower soil N availability. So the change of *L. chinensis* under long-term N addition should be investigated in future studies.

Nitrogen-induced changes in soil conditions could mediate aboveground biomass responses to the increasing N addition rate. Correlations between aboveground biomass and soil N availability vs. soil pH indicate that aboveground production is sensitive to changes in soil properties (**Figure 4**). First, the increase in soil N availability could contribute to plant growth (Gough et al., 2000), especially in our experiment. This because the model species *L. chinensis* is typical clonal plant. It has been reported that clonal plants have competitive advantages in acquiring soil nutrients and light, which cause their response to N addition to be more positive than non-clonal plants (Gough et al., 2012; Dickson et al., 2014). Second, the soil acidification under N addition could be another reason for the loss of aboveground production. For example, previous study found that decreased soil pH may lead to an increase in mortality of acid−sensitive plants (van den Berg et al., 2005), and induce decreases in plant productivity (Chen et al., 2013). Many studies have documented that soil acidification increases Al^{3+}, which can be directly toxic to plants (van den Berg et al., 2005). However, a previous study showed that *L. chinensis* was positively correlated with the degree of acidification in a temperate steppe (Lan, 2014), which was in line with our results here. Both results suggested *L. chinensis* could be tolerant to acid

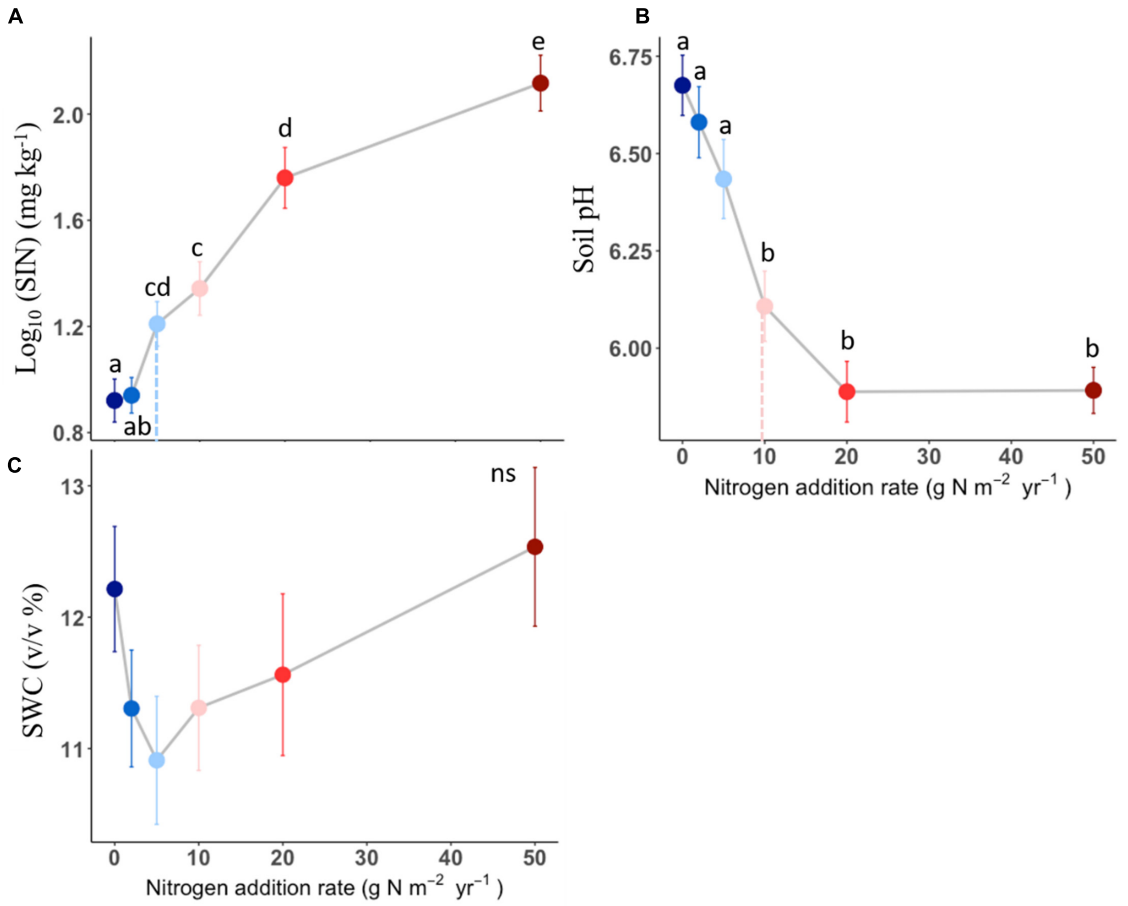

FIGURE 3
Effects of different N addition on soil inorganic nitrogen (SIN, log_{10}-transformed, **A**), soil pH **(B)**, soil water content (SWC, **C**). Different lower-case letters indicate significant differences ($P < 0.05$) between treatments, and ns indicates non-significant ($P > 0.05$). The dashed line in each panel indicate for tipping points. The data shown are the means with 40 replications ± standard error.

conditions and its relative biomass could continue to increase with increasing N addition even though such a scenario soil acidification became severe (Yang et al., 2019).

Non-linear response of plant functional traits to N addition

Contrary to our hypothesis, we did not find a similar threshold in response to N addition between plant biomass and functional traits. For example, the threshold for N-induced plant traits changes was at N addition rate of 20 g N m^{-2} $year^{-1}$, higher than that of aboveground biomass (**Figures 1**, **2**). It reflected that plant traits was relatively less sensitive than the aboveground biomass to N addition rate from the short-term N deposition. The distinct N threshold between the aboveground biomass and plant traits could be explained by the following two aspects. First, N is the limiting nutrient for productivity in the temperate steppe, and N enrichment could increase aboveground production (Bai et al., 2010). The enhanced plant growth induced by N addition could increase the intensity of light competition among plant species (DeMalach et al., 2017). Plants usually develop leaves with high SLA to enhance light capture (Freschet et al., 2015). Therefore, changes of SLA could reflect their adaptive capacities to low light conditions after the enhanced plant growth induced by N addition. Second, the response of traits to one global change driver may also be associated with other drivers. Given that water and N availability can co-limit plant growth in semiarid regions (Hooper and Johnson, 1999; Niu et al., 2010). It is expected that drought would restrain plant N uptake, thus the effects of changing precipitation regimes and global N enrichment will be interdependent (Shen et al., 2020). The long-term mean annual precipitation of the study site is 363 mm, and our study was conducted in a drought year (the mean annual precipitation is 148.2 mm). Previous study found that drought had a stronger effect on leaf traits than short-term N deposition, and the effect of drought on traits is opposite to

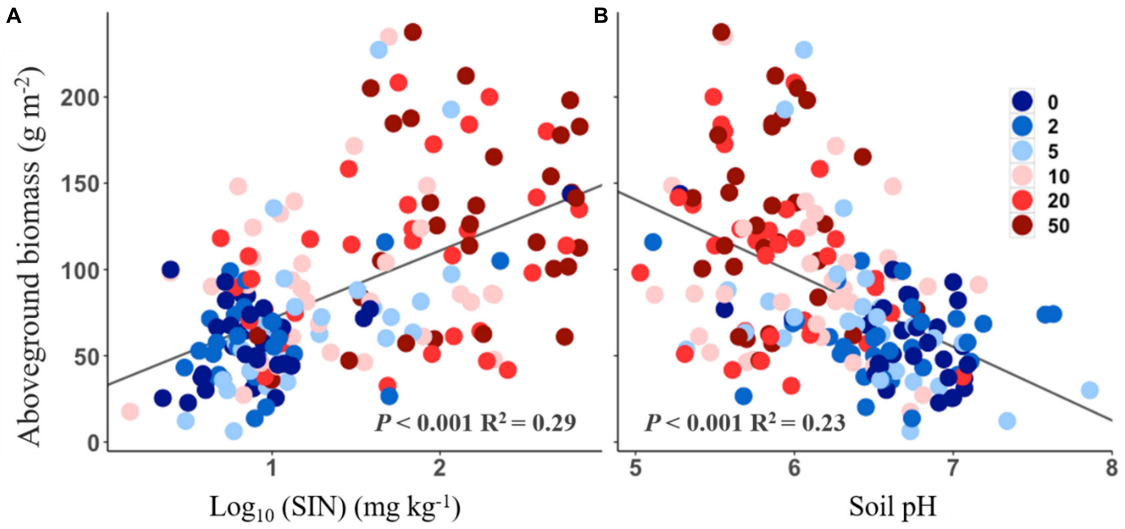

FIGURE 4
Relationships between aboveground biomass and soil inorganic nitrogen (SIN, log_{10}-transformed, **A**) and soil pH **(B)**.

that of N addition, for example drought had significant negative effects on the leaf N concentrations and net photosynthetic rate (Yu et al., 2019). Therefore, the threshold for N-induced plant traits changes was higher in this study.

High N addition rates increased SLA and leaf N content, decreases LDMC, which is consistent with many previous findings (La Pierre and Smith, 2015; Wang et al., 2016; Valliere et al., 2017; Zheng and Ma, 2018). For those traits, first, SLA indicates captured light resources on LA per unit leaf dry matter investment, which is closely related to plants' light interception efficiency (Reich, 2014). Previous studies have shown that N addition increased SLA, possibly because it promoted growth and thus increased leaf photosynthesis, or reduced the availability of canopy light to increase the SLA (Zeng et al., 2009; Palmroth et al., 2014). Second, Leaf N is an integral component of the photosynthetic machinery, and changes in leaf N concentration may underpin the greater competitive capability of *L. chinensis* in the community under enhanced N input (Zheng et al., 2018). In line with our results, a recent work using 2683 observations showed that N addition enhanced leaf N concentration both on mass basis and area basis (Liang et al., 2020). LDMC is an index of conservatism in life history. We found that LDMC decreased under N addition, and low LDMC represent rapid nutrient acquisition, which is conducive to the growth of plants in a nutrient-rich environment (Wilson et al., 1999). Overall, elevated SLA and leaf N content, declined LDMC caused by N enrichment suggests that *L. chinensis* have a resource acquisitive strategy and such strategy become stronger under exogenous N input.

Here we targeted one single species under short-term N addition by focusing on traits of aboveground tissue, which raised few limitations that need to be addressed in future studies. First, it has been reported that clonal plants response more positively than non-clonal plants to N addition (Dickson et al., 2014). Therefore, further research is needed to explore how other species respond to N addition, whether the responses of aboveground biomass and functional traits to N addition were asymmetric for other species. Second, this study is based on a relatively short-term N-enrichment experiment. The response of functional traits and productivity may differ between short-term and long-term experiments. Therefore, long- term N addition experiments are needed. Thrid, root traits also play pivotal roles in resource acquisition, as plant economic spectrum predicts, species with the traits of high specific root length and low tissue density would more strongly compete for belowground resource (Weemstra et al., 2016; Erktan et al., 2018). Recognition of the different response between above- and below-ground traits could improve our understanding of plant growth to N addition. Thus we need explore further how the root traits would affect plant growth under N deposition scenarios.

Conclusion

By conducting a multi-level of rates and chemical forms of N addition at the field condition in Chinese temperate grassland, here we show evidence to support that there indeed exist tipping points for plant biomass and functional traits in response to N addition. In particular, the tipping point for N-induced increase in *L. chinensis* aboveground biomass is at N addition rates of 10 g N m^{-2} $year^{-1}$, while that critical points in functional traits

are at 20 g N m^{-2} $year^{-1}$, suggesting that the responses to N addition were more sensitive in aboveground biomass than that in plant functional traits. Our findings can help to understand to what extent and in which rate of N deposition will start to cause the transition of plant functional traits and biomass production. These insights certainly can be useful in forecasting changes in ecosystem service and maintaining ecosystem management under future N deposition scenarios.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding authors.

Author contributions

GY, ZZ, and GZ collected the data. GY, ZZ, PZ, and RW developed the research questions. GY and QL analyzed the data. GY wrote the draft with contributions and input from all authors.

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Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.982478/full#supplementary-material

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Nitrogen addition alters plant growth in China's Yellow River Delta coastal wetland through direct and indirect effects

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In the coastal wetland, nitrogen is a limiting element for plant growth and reproduction. However, nitrogen inputs increase annually due to the rise in nitrogen emissions from human activity in coastal wetlands. Nitrogen additions may alter the coastal wetlands' soil properties, bacterial compositions, and plant growth. The majority of nitrogen addition studies, however, are conducted in grasslands and forests, and the relationship between soil properties, bacterial compositions, and plant growth driven by nitrogen addition is poorly understood in coastal marshes. We conducted an experiment involving nitrogen addition in the *Phragmites australis* population of the tidal marsh of the Yellow River Delta. Since 2017, four nitrogen addition levels (N0:0 g • m^{-2} • $year^{-1}$, N1:5 g • m^{-2} • $year^{-1}$, N2:20 g • m^{-2} • $year^{-1}$, N3:50 g • m^{-2} • $year^{-1}$) have been established in the experiment. From 2017 to 2020, we examined soil properties and plant traits. In 2018, we also measured soil bacterial composition. We analyzed the effect of nitrogen addition on soil properties, plant growth, reproduction, and plant nutrients using linear mixed-effect models. Moreover, structural equation modeling (SEM) was utilized to determine the direct and indirect effects of nitrogen addition, soil properties, and bacterial diversity on plant growth. The results demonstrated that nitrogen addition significantly affected plant traits of *P. australis*. N1 and N2 levels generally resulted in higher plant height, diameter, leaf length, leaf breadth, and leaf TC than N0 and N3 levels. Nitrogen addition had significantly impacted soil properties, including pH, salinity, soil TC, and soil TS. The SEM revealed that nitrogen addition had a direct and positive influence on plant height. By modifying soil bacterial diversity, nitrogen addition also had an small indirect and positive impact on plant height. However, nitrogen addition had a great negative indirect impact on plant height through altering soil properties. Thus, nitrogen inputs may directly enhance the growth of *P. australis* at N1 and N2

levels. Nonetheless, the maximum nitrogen addition (N3) may impede *P. australis* growth by reducing soil pH. Therefore, to conserve the coastal tidal marsh, it is recommended that an excess of nitrogen input be regulated.

KEYWORDS

nitrogen addition, coastal wetland, soil properties, bacterial diversity, plant traits, SEM

Introduction

Agriculture and industrialization are major contributors to the global release of reactive nitrogen into the environment. For example, the utilization of nitrogen fertilizer in agriculture increased from 12 Mt in 1961 to 110 Mt in 2014, and a considerable proportion of nitrogen inputs ended up in terrestrial and aquatic ecosystems, resulting nitrogen enrichment and contamination (Bodirsky et al., 2014; Yu et al., 2019). Then, nitrogen overloading may alter soil properties, soil microorganisms, and plant compositions, including species richness, and abundance, leading to a shift in ecosystem functions (He et al., 2021a; Zhong et al., 2022). These consequences will challenge ecosystem conservation and management. Therefore, simulative nitrogen addition studies have been done in numerous ecosystems, and outcomes from these experiments can shed light on the consequences and mechanisms of nitrogen enrichment on ecosystems.

Nitrogen enrichment can directly and substantially affect soil physical and chemical properties. The experiments focus primarily on measuring soil pH, which is a crucial and comprehensive component. The soil pH responds sensitively to nitrogen enrichment, particularly in acidic soils. Nitrogen addition can reduce soil pH, because NH_4^+ is coupled with OH^- in the soil and then released into the air as NH_3; as a result of the depletion of OH^- in the ground, the pH value decreases, and the earth becomes more acid (Zhou et al., 2017). The drop of soil pH following nitrogen addition caused variations in soil enzyme activity (Liu and Zhang, 2019), microbial communities (Zhou et al., 2017), and plant community composition (Liu et al., 2020). In addition, an increase in nitrogen input resulted in stoichiometric imbalances (Li et al., 2016). For instance, nitrogen addition increased the amount of available N (AN) but lowered the amount of soil available P (AP) in the soil (Touhami et al., 2022), which might increase the N:P ratio in the soil and hinder the growth of microorganisms or plants. Yuan et al. (2019) reported that in the non-linear relationship between nitrogen addition and C:N ratio, C:P ratio, and N:P ratio, the greatest stochiometric imbalance occurred at the intermediate nitrogen addition level, which promoted the microbial C limitation but not P limitation.

Nitrogen addition has detrimental effects on the microbial community. For example, nitrogen addition decreased soil fungal richness (Leff et al., 2015; Yuan et al., 2020) and soil bacterial diversity (Lu et al., 2021). Furthermore, soil acidification and the modification of resources by nitrogen addition are recognized as the primary mechanisms underlying the diversity in microbial communities. Chen et al. (2015) demonstrated that nitrogen-induced soil acidification was the primary factor inhibiting bacterial, fungal and actinobacteria biomass. In a long-term nitrogen addition experiment, Liu et al. (2020) also revealed that the decrease in soil pH was attributable to the loss of soil bacterial diversity. However, Zhou et al. (2017) demonstrated through a meta-analysis that nitrogen addition promoted the increase of resources, whereas soil acidification had no effect on microbial communities.

Depending on the amount of nitrogen added, nitrogen addition has both beneficial and negative impacts on plant growth and community productivity. High and abrupt nitrogen addition was toxic for plant growth (Gao et al., 2014) whereas, intermediate nitrogen addition increased leaf nutrients, total leaf biomass (Liang et al., 2020), and carbon in plant shoots (Sun et al., 2020; Wang et al., 2020), seedling performance (Shi et al., 2020), and the stability of aboveground net primary productivity plant community (Chen et al., 2016b; Yang et al., 2022).

Few studies have investigated the effects of nitrogen enrichment on coastal wetland ecosystems, whereas numerous studies have examined the effects of nitrogen enrichment on grasslands and forests (Zhou et al., 2017). Coastal regions account for only 4% of the worldwide geographical area, although they are home to one-third of the global population. Diversity of fish, birds, and benthic animals inhabit coastal wetlands. Coastal wetlands also provide human with numerous ecosystem services, such as carbon sequestration, storm mitigation, and coastline protection. Similar to other ecosystems, nitrogen is a limiting nutrient for plant growth in coastal wetlands, despite the considerable nitrogen storage capacity of coastal wetland. Thus, the coastal wetland often was frequently utilized as a buffer zone or reservoir for the removal excess nitrogen from agricultural lands prior to its

discharge into the ocean. Empirical research indicating that the excessive nitrogen inputs may severely impair the coastal wetland vegetation (Deegan et al., 2012), suggesting that the vegetation of the coastal wetland is susceptible to nitrogen fluctuations. Contradictory evident indicated that 13-year nutrient enrichment did not affect the ecosystem stability (e.g., soil strength and structural integrity of the soil matrix) in the coastal marsh, whereas the belowground root biomass was reduced in the coastal marsh (Graham and Mendelssohn, 2014). Therefore, further research is required to determine the impact of nitrogen enrichment on soil properties and vegetation growth in the coastal wetlands. Furthermore, the linkage among nitrogen addition, soil properties, bacterial composition, and plant growth in the coastal wetland could be established. In this study, we addressed the following scientific question: how do soil properties, bacterial composition, and plant growth respond to nitrogen addition in the coastal wetland? This study will deepen our understanding of the effect and the underlying mechanism of nitrogen addition on plant growth in the coastal wetland. It also will provide suggestions on the conservation of coastal wetlands in the context of environmental change.

Methods

Study site

The study site was located in the salt marsh of the Yellow River Delta (YRD) in Shandong Province (37°44'5" N, 119° 12'56" E). The climate of this region is warm temperate, and the annual average temperature is 11.7-12.6°C. The average yearly precipitation is 530-630 mm, and most of the precipitation occurs in July and August. The average yearly evaporation is 1750-2430 mm, and tidal flooding occurs through irregular semilunar and semidiurnal tides. In the YRD, the dominant native species are *P. australis* and *Suaeda salsa*; and *P. australis* distribute in the high marsh while *S. salsa* dominates the low marsh. During the growth season (May to November), the total N deposition rate in the YRD was estimated to be around 22.64 kg/hm^2 (Guan et al., 2019).

Field experiments

In 2017, the experiment plots were randomly established in the *P. australis* vegetation of the high marsh; the nitrogen addition levels were 0 $g \bullet m^{-2} \bullet year^{-1}$ (N0), 5 $g \bullet m^{-2} \bullet year^{-1}$ (N1), 20 $g \bullet m^{-2} \bullet year^{-1}$ (N2), and 50 $g \bullet m^{-2} \bullet year^{-1}$ (N3) (Zhao et al., 2021). Urea [$CO(NH_2)_2$ containing 46% nitrogen] was added in May and July each year and it was scattered evenly in the plot. Each level had six replicates. The plot size was 2×2 m^2. The distance between the two plots was greater than 10 m. The height of *P. australis* individuals in the initial plots was similar in May 2017. During 2017 and 2020, the diameter, height, leaf numbers, leaf length, and leaf breadth of each individual, and the total individuals in the plot were measured in the middle of May, Jul., and Sep. In Sep. 2017~2020, the soil at a depth of 0~10 cm and mature leaves from each plot were sampled. One portion of the fresh soil was refrigerated at -20°C and then used to determine the NO_3-N, and NH_4-N contents, the other portion of the soil was air-dried to measure soil pH, electrical conductivity (EC), total C (TC), total N (TN), total P (TP), AP, and total S (TS). In Sep. 2018, one portion of the fresh soil was frozen at -80°C, and utilized to determine the bacterial composition. In addition, the leaves were collected to determine leaf nutrients, including TC, TN, TP, and TS.

Soil and plant analysis

The elemental analyzer (Vario Micro cube, Elementar Co., Germany) was employed to measure TC and TN in soil and leaves. The pH meter (QT-PH220S, Beijing Channel Scientific Instrument Co., Ltd, China) was used to determine soil pH (soil: water = 1: 5). The continuous flow analyzer (AutoAnalyzer III, Seal Co., Germany) was used to measure NO_3-N and NH_4-N contents [extraction: 3 g of fresh soil using a 15 ml KCl solution (2 mol/L)]. The total amount of AN was the sum of NO_3-N and NH_4-N contents. The ultraviolet spectrophotometer (T6NewCentury, Beijing Persee General Instrument Co., Ltd, China) was employed to determine TP and AP content by the Oslen method.

Soil bacterial composition

The soil bacterial diversity was determined by 16S rRNA. 0.5 g of soil was used to extract DNA. The genomic DNA of the sample was extracted by the CTAB method. Then, the purity and concentration of DNA were detected by agarose gel electrophoresis. A suitable sample of DNA was used in a centrifuge tube and diluted with sterile water to 1 ng/μl. Using the diluted genomic DNA as the template and according to the selection of sequencing region, the specific primers with barcodes were used. Phusion® High-Fidelity PCR Master Mix with GC Buffer and high efficiency and high-fidelity enzyme were used for PCR to ensure amplification efficiency and accuracy. PCR products were detected by electrophoresis with agarose gel of 2% concentration. According to the concentration of PCR products, the samples were mixed equally, and then the PCR products were detected by 2% agarose gel electrophoresis. The target bands were recovered from the gel recovery kit provided by the Qiagen company. Truseq ® DNA PCR Free Sample Preparation Kit was used for library construction. The constructed library was quantified by Qubit and Q-PCR. After the library was qualified, NovaSeq6000 was used for computer sequencing. According to the Barcode sequence and PCR amplified primer sequence, each sample data was separated from the off-line

data. After the Barcode and primer sequence were intercepted, FLASH (v1.2.7, http://ccb.jhu.edu/software/FLASH/) spliced the reads of each sample (Magoc and Salzberg, 2011), and the splicing sequence obtained was raw tags data (raw tags). Raw tags obtained by splicing need to be strictly filtered to obtain high-quality tags data (clean tags) (Bokulich et al., 2013). Referring to Qiime (v1.9.1, http://qiime.org/scripts/split_libraries_fastq.html), the tag quality control process was as follows (Caporaso et al., 2010). a) tags interception: cut raw tags from the first low-quality base site with a continuous low-quality value (the default quality threshold is < = 19) and a set length (the default length value is 3); b) Tags length filtering: the tags data set obtained by intercepting tags further filters out tags in which the continuous high-quality base length is less than 75% of the tag's length. The tags obtained after the above processing need to be processed to remove the chimera sequence, and the tags sequence passed Vsearch (https://github.com/torognes/vsearch/) (Rognes et al., 2016) were compared with the species annotation database to detect chimeric sequences, and finally the chimeric sequences were removed to obtain the final effective tags.

The Uparse software (Uparse v7.0.1001, http://www.drive5.com/uparse/) was used to cluster all effective tags of all samples (Haas et al., 2011). By default, the sequence was clustered into OTUs (operational taxonomic units) with 97% identity. At the same time, the representative sequence of OTUs will be selected according to its algorithm principles. The sequence with the highest frequency in OTUs was selected as the representative sequence of OTUs. The bacterial OTUs was detected in Novogene Co.,Ltd. The bacterial diversity index, including OTUs and beta diversity. The beta diversity was represented by the loadings of the first two principal components in the result of PCA (principal components analysis).

Data analysis

Because the mixed effect models can handle the repeated measurement data, linear mixed-effect models (lmer () function) were utilized to analyzed the effects of nitrogen, month, and year on the soil properties and plant traits. The correlations between repeated measurements within each plot were explicitly accounted for through mixed models. Nitrogen addition, month, and year were set as the fixed factors, and the year was appointed as the random factor. The ls. Means () function was used to compare the means of different treatments. The distance-based redundancy analysis (db-RDA) was employed to test the relationship between soil properties and bacterial diversity.

Using the sem () in package "lavaan", the SEM model was developed to figure out the direct and indirect factors affecting plant height growth. "soil", "bacterial", and "plant" were set as latent variables. The measured variables of "soil" were soil pH, and soil TC, while the measured variables of "bacteria" included bacterial OTUs and beta diversity. The Chi-Square test statistic, CFI (the comparative fit index), SRMR (the standardized root mean square residual), and RMSEA (the root mean square error of approximation) were used to evaluate the goodness of structural equation model fit. The model fits well if it meets the following criteria: (*P*-value of Chi-Square test statistic > 0.05, CFI > 0.95, SRMR < 0.08, and RMSEA < 0.05).

All data were presented as mean ± SE in the text. We deposited the bacterial data in National Library of Medicine (the BioProject ID is PRJNA871067), We performed statistical analyses and drew the figures using in R i386 4.1.1 (R Core Team, 2021) packages "lmerTest", "lavaan", "vegan", and "semPlot", respectively.

Results

The effect of nitrogen addition on soil properties and bacterial compositions

The result showed that nitrogen additions significantly affected soil properties, including pH, salinity, soil TN, soil TS, and soil AP. However, nitrogen additions had little effect on soil total N, soil AN, and soil TP (Figure 1, Supporting information Table S1). N2 (8.46 ± 0.03) and N3 (8.48 ± 0.04) treatments had lower pH than N0 (8.71 ± 0.09) and N1 (8.72 ± 0.09) (Figure 1A), a high level of nitrogen addition increased the soil EC which indicated the salinity level (Figure 1B), N2 level quadrats (22.30 ± 0.31 g/kg) had a higher soil TC than N0 (20.65 ± 0.24 g/kg), N1 (21.37 ± 0.20 g/kg), and N3 (21.55 ± 0.27 g/kg) (Figure 1E). The nitrogen additions (N1, N2, and N3) increased the soil TS (N0: 0.39 ± 0.08 g/kg, N1: 0.55 ± 0.07 g/kg, N2: 0.48 ± 0.07 g/kg, and N3: 0.48 ± 0.10 g/kg, Figure 1F). The level of nitrogen addition N2 resulted in the greatest soil AP (Figure 1H).

The first two axes of the dbRDA plot accounted for 52.62 percent of the variance in bacterial compositions (OTUs), and the dbRDA plot showed that the most important factors influencing the bacterial compositions were pH (R^2 = 0.322, *P*-value=0.014), soil TC (R^2 = 0.415, *P*-value=0.004), soil TN (R^2 = 0.361, *P*-value=0.006; Figure 2; Table S2). Soil TC and TN determined the variation in bacterial compositions quadrats of N2 and N3 nitrogen addition levels, whereas pH related to the variation in bacterial compositions of N0 and N1 treatments (Figure 2).

The effect of nitrogen addition on plant traits

For the plant nutrient traits, nitrogen addition did not affect the leaf TN and leaf C: N ratio, but it altered the leaf TC and TS (Figure 3; Table S3). Individuals with N1 (446.08 ± 3.56 g/kg) and N2 (447.18 ± 3.28 g/kg) nitrogen addition levels exhibited higher leaf TC than N0 (439.93 ± 2.52 g/kg) and N3 (442.66 ± 2.71 g/kg), but nitrogen additions decreased the leaf TS

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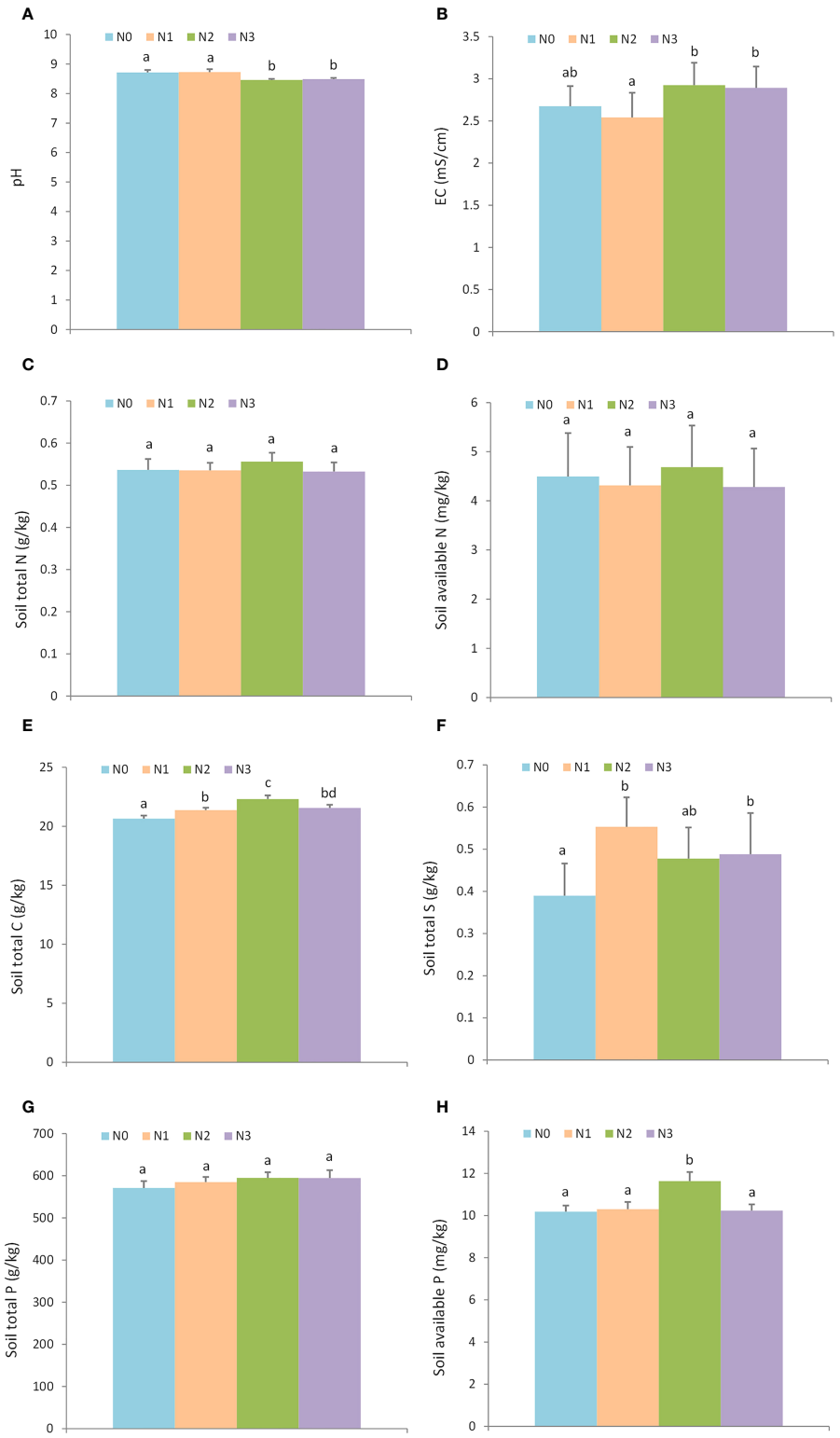

FIGURE 1
The effect of nitrogen addition on soil physical and chemical properties. N0: 0 g • m^{-2} • $year^{-1}$; N1: 5 g • m^{-2} • $year^{-1}$; N2: 20 g • m^{-2} • $year^{-1}$; N3: 50 g • m^{-2} • $year^{-1}$. Different letters denote statistically significant differences. **A**: pH; **B**: EC (mS/cm); **C**: Soil total N (g/kg); **D**: Soil available N (mg/kg); **E**: Soil total C (g/kg); **F**: Soil total S (g/kg); **G**: Soil total P (g/kg); **H**: Soil available P (mg/kg).

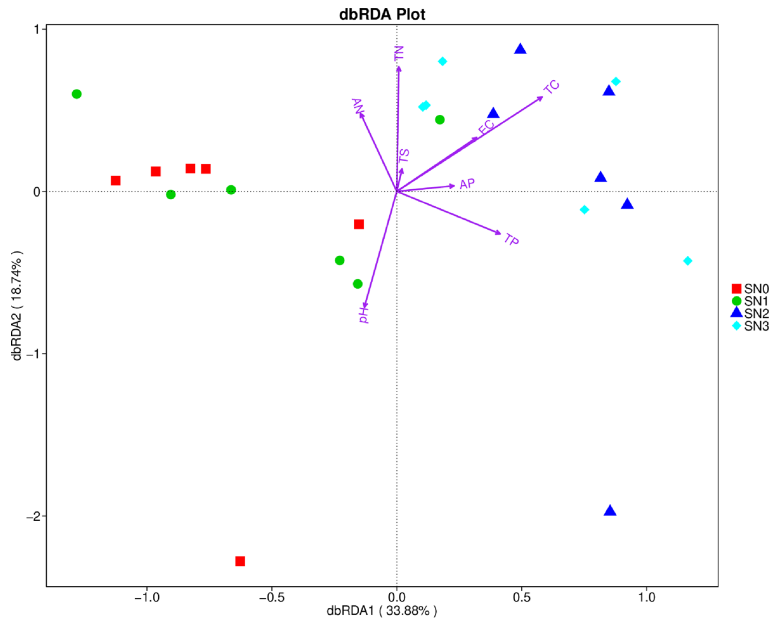

FIGURE 2
The distance-based redundancy analysis (dbRDA) plot. The relationship of soil physical and chemical properties and bacterial OTUs composition. SN0 is N0 in September 2018, SN1 denotes N1 in September 2018, SN2 means N2 in September 2018 and SN3 indicates N3 in September 2018. AN, soil available N; TN, soil total N; EC, electrical conductivity; TC, soil total N; AP, Soil available P; TP, soil total P; TS, soil total S.

compared to the control treatment (N0: 2.26 ± 0.36 g/kg, N1: 1.86 ± 0.20 g/kg, N2: 1.65 ± 0.15 g/kg, and N3: 1.52 ± 0.09 g/kg).

At the end of the growing season, plants in the N1 and N2 nitrogen addition quadrats (N1: 81.62 ± 0.98 cm; N2: 82.41 ± 0.96 cm) grew taller than those in N0 and N3 quadrats (N0: 75.45 ± 0.76 cm; N3: 75.36 ± 0.89 cm) (Figure 4A; Table 1). N1, N2, and N3 addition levels promoted diameter growth, but N1 had the greatest diameter excess in May, Jul., and Sep. (Figure 4B; Table S4A). The effect of nitrogen addition on leaf numbers varied by month (Figure 4C; Table S4B). In May, N3 reduced the leaf numbers, but N1 and N2 did not significantly differ from N0. In Jul., the leaf number in nitrogen addition treatments (N1, N2, and N3) were significantly greater than N0, and N2 had the greatest leaf numbers. However, in Sep., higher level of nitrogen additions reduced the leaf numbers. The N2 had the greatest leaf length and breadth (Figures 4D, E; Tables S4C, D). Individuals per plot and spike length decreased as nitrogen addition levels increased (Figures 4F, G; Tables S4E, F).

The direct and indirect impacts of nitrogen addition on plant growth

The goodness of model fit satisfied the SEM model criteria (Chi.sq=4.546, df=5, *P*-value=0.474, CFI=1.000, SRMR=0.063, and RMSEA=0.000). From the SEM model (Figure 5), we found that nitrogen addition had a positive direct effect on the plant height (path coefficient=0.36), and soil properties also had a positive direct effect on the plant height (path coefficient=0.64. However, the bacterial diversity had a negatively direct effect on the plant height (path coefficient=-0.25). The soil properties and bacterial diversity were negatively affected by nitrogen enrichment (path coefficient=-0.61; path coefficient=-0.17). By altering bacterial diversity, nitrogen addition had a marginally positive and indirect effect on plant height (path coefficient=0.04). Additionally, nitrogen enrichment also had a small positive impact on the plant height *via* altering both soil properties and bacterial diversity (path coefficient=0.03). However, nitrogen addition decreased plant height through modifying soil properties (path coefficient=-0.39).

Discussion

The effect of nitrogen addition on the soil properties in the tidal marsh

Our study was conducted in the tidal marsh, which was flooded frequently by seawater (Zhang et al., 2021). Seawater may reduce the amount of added nitrogen. However, nitrogen

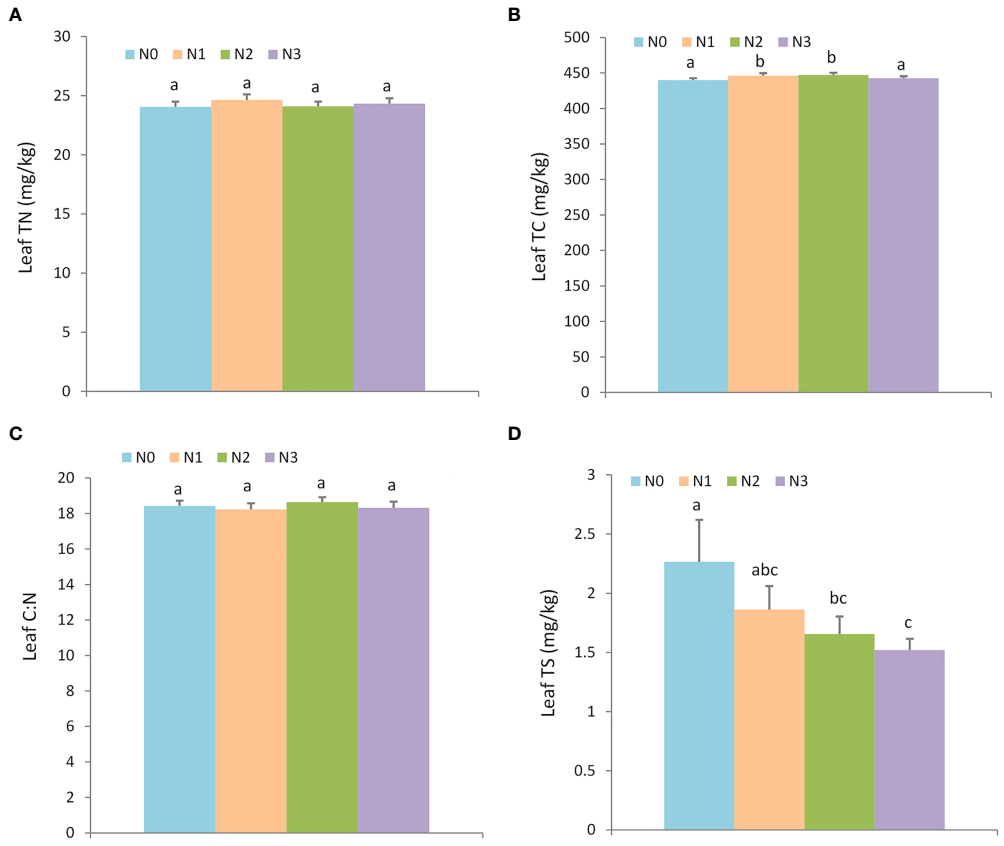

FIGURE 3
The effect of nitrogen addition on plant nutrients. Different letters denote statistically significant differences. N0: 0 g • m^{-2} • $year^{-1}$; N1: 5 g • m^{-2} • $year^{-1}$; N2: 20 g • m^{-2} • $year^{-1}$; N3: 50 g • m^{-2} • $year^{-1}$. **A**: Leaf TN (mg/kg); **B**: Leaf TC (mg/kg); **C**: Leaf C:N; **D**: Leaf TS (mg/kg).

addition had a significant impact on soil properties, soil bacterial compositions, and plant growth. Similar to acid soils, nitrogen addition also decreased pH in alkaline soils. This result is inconsistent with study by He et al. (2021b), which revealed that soil pH responded to nitrogen addition modestly in alkaline soil, and nitrogen addition buffered soil acidification by calcium carbonate (Qin et al., 2018). In our study, the pH significantly fell in N3 and N4 compared to N0 and N1, and high nitrogen addition may neutralize the calcium carbonate buffer effect.

The soil salinity did not consistently decrease with increasing of nitrogen addition constantly, and it exhibited a non-linear relationship with nitrogen addition levels. N1 and N0 had lower salinity than N2 and N3. This result is partly consistent with findings of Guan et al. (2019), and they conducted the nitrogen addition experiment in the non-tidal coastal wetland of YRD and found that salinity had a negative relationship with nitrogen addition. This experiment had a lower nitrogen addition rate than our work, and the nitrogen substance used in the experiment was NH_4NO_3 (we used urea) which may immediately increase soil NH_4^+ and NO_3^- after nitrogen addition. Because the association of soil exchangeable base cations and soil NH_4^+ and NO_3^- was negative, salinity decreased with nitrogen addition (Qin et al., 2018).

According to our study, neither soil TN nor AN changed after nitrogen addition. This result may have been caused by three factors. Initially, plants and microorganisms absorbed and consumed nitrogen, thereby reducing the amount of added nitrogen. Secondly, the seawater may have washed away some fertilizer, but the amount of fertilizer washed away has not yet been quantified. Thirdly, a portion of the urea had not been converted into inorganic nitrogen but had instead remained in organic form.

In accordance with findings from other studies, nitrogen addition increased soil TC (Guan et al., 2019; Sun et al., 2020). The primary reason may be that nitrogen addition increased the productivity of plants and microbes, resulting in a greater return of litter or microbial carbon to the soil. The highest soil AP was found in N2, which may be associated with the decomposition

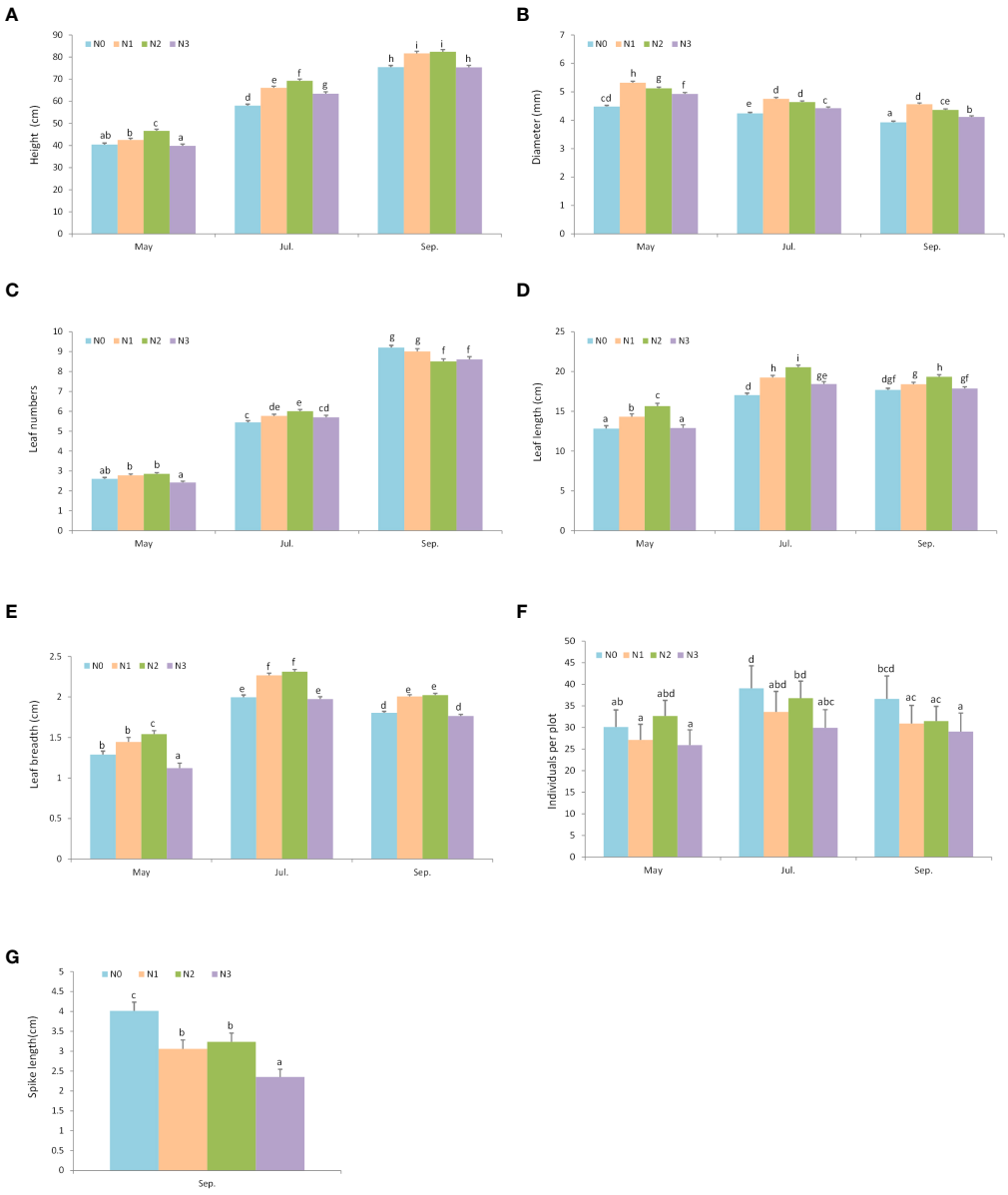

FIGURE 4
The effect of nitrogen addition on plant growth and reproductive traits in different months. Different letters denote statistically significant differences. N0: 0 g • m^{-2} • $year^{-1}$; N1: 5 g • m^{-2} • $year^{-1}$; N2: 20 g • m^{-2} • $year^{-1}$; N3: 50 g • m^{-2} • $year^{-1}$. **A**: Height; **B**: Diameter (mm); **C**:Leaf numbers; **D**: Leaf length (cm); **E**: Leaf breadth (cm); **F** Individuals per plot; **G**: Spike length (cm).

rate of plants and microorganisms. However, the highest nitrogen addition (N3) rate inhibited phosphatase and consequently decreased soil organic P mineralization (Olander and Vitousek, 2000). Few studies have examined the effect of nitrogen addition on soil S. Sulfur is an essential element in the coastal wetland, and its influence on plant growth depends on the chemical species present (sulfate or sulfide). Intriguingly, nitrogen addition increased soil TS. A previous study demonstrated that nitrogen addition can inhibit the activity of soil arylsulfatase due to a decrease in pH, which could mineralize the organic S into inorganic form (Chen et al., 2016a). Wang et al. (2016) found that nitrogen addition to the grassland had no effect on soil TS but increased soil available S. The outcome of the grassland differs from that of the coastal wetland. The mechanism underlying the response of coastal wetland soil TS to nitrogen addition requires further investigation.

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TABLE 1 Linear mixed-effects model predicting influences of nitrogen, month (including May, July, and September) on the plant height of the common reed.

Effects	Sum square	Mean square	Num Df	Den Df	F-value	*P*-value
Fixed effects						
Nitrogen	9.61×10^{4}	3.20×10^{4}	3	9.19×10^{3}	64.6	<0.001***
Month	1.93×10^{6}	9.65×10^{5}	2	9.19×10^{3}	1.94×10^{3}	<0.001***
Nitrogen: Month	1.42×10^{4}	2.37×10^{3}	6	9.19×10^{3}	4.77	<0.001***
Random effect						
	Npar	**LogLik**	**AIC**	**LRT**	**Df**	***P*-value**
<none>	14	-4.16×10^{4}	8.32×10^{4}			
(1 |Year)	13	-4.16×10^{4}	8.33×10^{4}	27.06	1	<0.001***

Nitrogen and Month were considered fixed factors; Year was treated as random factors. Npar: number of model parameters; LogLik: the log-likelihood for the model; AIC: the AIC for the model evaluated as -2×(logLik - Npar), and smaller is better; LRT: the likelihood ratio test statistic. '***' denotes P-value < 0.001.

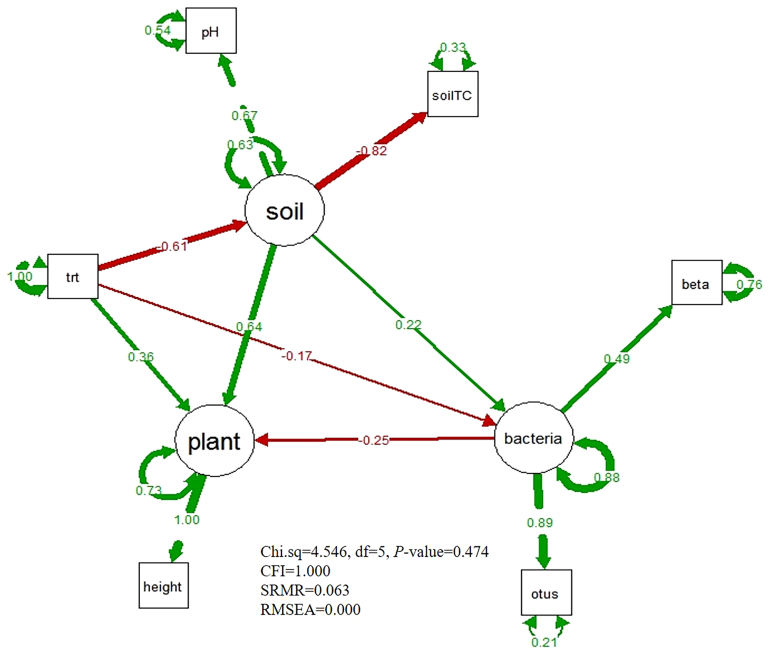

FIGURE 5
The Structural equation model of nitrogen addition treatment, soil physical and chemical property, bacterial diversity and plant height. The red arrows mean negative effect, the green arrows mean positive effect. Thicker lines denote higher effects. "trt" is the nitrogen addition treatment, "SoilTC" is soil total carbon, "beta" means bacterial beta diversity, "otus" indicates bacterial OTUs.

The effect of nitrogen addition on bacterial compositions and plant traits in the tidal marsh

Nitrogen addition altered soil physical and chemical factors, and bacterial compositions was also affected by environmental variables. Significantly influenced by nitrogen addition, soil pH and TC played critical roles in the variation of soil bacterial compositions. This result is consistent with previous studies. Soil acidification caused by nitrogen addition can reduce bacterial, fungal, and actinobacteria biomass (Chen et al., 2015) as well as bacterial diversity (Zhou et al., 2022). In a non-tidal coastal wetland nitrogen addition experiment, Lu et al. (2021) also found that soil TC was one of the most crucial factors influencing soil bacterial diversity. Thus, soil acidification and resource availability contributed to the altered bacterial composition following nitrogen addition.

The leaf TC and TS also changed as a result of nitrogen addition. The moderate nitrogen addition level promoted carbon assimilation in plant leaves, but the positive effect disappeared at higher levels. It suggests that excessive nitrogen addition can inhibit the carbon

dioxide adsorption or organic carbon synthesis, thereby suppressing plant growth. Sun et al. (2020) and Jing et al. (2017) revealed that nitrogen addition increased the carbon content of plant leaves. While the leaf TS decreased with nitrogen addition, this suggests that plants absorb less S as nitrogen addition levels increase. There are two possible causes for this outcome. Firstly, the decrease of inorganic S due to a decrease in soil arylsulfatase activity in higher nitrogen addition levels. Secondly, the plant allocated fewer resources for S acquisition because it must allocate more resources for P acquisition, which is the first limited resource (Chen et al., 2016a). It contradicts the findings of Li et al. (2019), and they discovered that plant S uptake increased with nitrogen addition in a S-deficient temperate steppe. This discrepancy may be caused by varying S supply levels.

Furthermore, our study revealed the linkages between nitrogen addition, soil environment, bacterial diversity, and plant growth in the coastal wetland. Few studies have investigated these connections in the coastal wetland ecosystems. Our study may partially fill this knowledge gap. In a coastal wetland, nitrogen addition had a direct and positive effect on plant growth, and a positive or negative indirect effect on aboveground plant growth, primarily by altering soil properties (i.e., soil pH and TC), and bacterial composition. Totally, the indirect effect contributed similarly but contrarily to the direct effect. The direct effect is the result of the added nitrogen being utilized by plants. Liu et al. (2020) demonstrated that nitrogen addition increased plant aboveground biomass directly in the grassland, but they did not investigate the effect of soil property and bacterial richness on plant growth. Nevertheless, numerous studies have documented the substantial effect of plant growth or diversity resulting from nitrogen addition on soil properties and the bacterial community (Li et al., 2013; Zeng et al., 2016; Liu et al., 2020). Consequently, the bi-directional interactions between plant community, soil properties and bacterial community is crucial for the response of ecosystem structure to nitrogen deposition.

Finally, in the tidal marsh, even at the lowest input level (5 g • m^{-2} • $year^{-1}$), the nitrogen addition had a significant effect on soil physical and chemical properties, soil bacterial compositions, and plant growth despite the flush of the sea tide. At the maximum level of nitrogen input, plant growth was inhibited. Consider the tidal flush, when nitrogen supply greater than 20 g • m^{-2} • $year^{-1}$ and less than 50 g • m^{-2} • $year^{-1}$ inhibited plant growth. Therefore, under the scenario of future high nitrogen inputs, the growth of coastal wetland vegetation, including tidal marshes, will be hampered. It is suggested that the regulation of agricultural nitrogen inputs is still necessary to protect vegetation.

Conclusion

The addition of nitrogen significantly altered pH, salinity, soil TC and soil TS in the coastal tidal marsh. Soil pH and TC were the most influential environmental influences on the soil bacterial compositions. Intermediate nitrogen addition enhanced plant growth and reproductive traits significantly, with N1 and N2 having greater values for these traits than N0 and N3. Nitrogen addition influenced plant height in the coastal wetland both directly and indirectly, and the indirect but negative effect was mainly caused by soil properties change induced by nitrogen addition. Additional research is required to investigate different ecological functions of the coastal wetland under future nitrogen input scenarios.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: NCBI, PRJNA871067, available at https://www.ncbi.nlm.nih.gov/bioproject/PRJNA871067.

Author contributions

LWZ: Conceptualization, Methodology, Investigation, Data curation, Visualization, Writing Original draft preparation, and Funding acquisition. LJZ, HPY, SQL & LC: Methodology and Investigation. GXH: Conceptualization and Supervision. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1016949/full#supplementary-material

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Long-term water use efficiency and non-structural carbohydrates of dominant tree species in response to nitrogen and water additions in a warm temperate forest

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Nitrogen (N) deposition tends to accompany precipitation in temperate forests, and vegetation productivity is mostly controlled by water and N availability. Many studies showed that tree species response to precipitation or N deposition alone influences, while the N deposition and precipitation interactive effects on the traits of tree physiology, especially in non-structural carbohydrates (NSCs) and long-term water use efficiency (WUE), are still unclear. In this study, we measured carbon stable isotope ($\delta^{13}C$), total soluble sugar and starch content, total phenols, and other physiological traits (*e.g.*, leaf C:N:P stoichiometry, lignin, and cellulose content) of two dominant tree species (*Quercus variabilis* Blume and *Liquidambar formosana* Hance) under canopy-simulated N deposition and precipitation addition to analyze the changes of long-term WUE and NSC contents and to explain the response strategies of dominant trees to abiotic environmental changes. This study showed that N deposition decreased the root NSC concentrations of *L. formosana* and the leaf lignin content of *Q. variabilis*. The increased precipitation showed a negative effect on specific leaf area (SLA) and a positive effect on leaf WUE of *Q. variabilis*, while it increased the leaf C and N content and decreased the leaf cellulose content of *L. formosana*. The nitrogen–water interaction reduced the leaf lignin and total phenol content of *Q. variabilis* and decreased the leaf total phenol content of *L. formosana*, but it increased the leaf C and N content of *L. formosana*. Moreover, the response of *L. formosana* to the nitrogen–water interaction was greater than that of *Q. variabilis*, highlighting the differences between the two dominant tree species. The results showed that N deposition and precipitation obviously affected the tree growth strategies by affecting the NSC contents and long-term WUE.

Canopy-simulated N deposition and precipitation provide a new insight into the effect of the nitrogen–water interaction on tree growth traits in a temperate forest ecosystem, enabling a better prediction of the response of dominant tree species to global change.

KEYWORDS

nitrogen and water additions, water use efficiency, non-structural carbohydrates, nutrients stoichiometry, $\delta^{13}C$ stable isotope

Introduction

Atmospheric nitrogen (N) deposition, an important threat to plant biodiversity, is mainly derived from natural processes and anthropogenic activities (Sala et al., 2000; Payne et al., 2017). Galloway (2004) predicted that, by 2050, global N deposition would double that of the early 1990s (Galloway et al., 2004). A modest rate of N deposition boosts forest abundance and foliar growth (Mao et al., 2018). However, excessive N deposition can lessen the diversity of plant species (Stevens, 2004; Schrijver et al., 2011; Payne et al., 2017), threaten plant growth (Galloway et al., 2008; Mao et al., 2018), weaken plant resistance (Bobbink et al., 2010), and even change the community structure of forests (Song et al., 2017). Additionally, temperate forests are regarded as N-limited, and N deposition affects the carbon (C): N: phosphorus (P) balance of forest vegetation and the C cycle process (Thomas et al., 2010). Generally, N deposition and precipitation occur simultaneously under natural conditions (Jia et al., 2014). Thus, the research of N-precipitation interaction is more accord with natural status. Precipitation affects the changes in belowground and aboveground communities, and its intensity is a critical factor in habitat alteration—for example, increasing water can increase vegetation production (Fu et al., 2018) and decrease the conductance of leaves (Sellin et al., 2019).

The long-term water use efficiency (WUE), which can be regarded as a comprehensive index of plant growth suitability under water stress, is generally expressed by the ratio of photosynthesis and stomatal conductance (Eamus, 1991; Jennings et al., 2016). Previous studies have shown that WUE is related to average precipitation, the length of plant growing seasons, and the specific leaf area (SLA) (Wright et al., 1994; Xiao et al., 2013; Easlon et al., 2014). N application can promote plant WUE (Martin et al., 2010; Zhang et al., 2018; Hu et al., 2019). The impact of rainfall on WUE is complex, and there are differences in WUE among various plant species (Xiao et al., 2013). Some studies have shown that WUE increases with water deficit (Ying et al., 2015) and decreases with increasing precipitation (Niu et al., 2011; Huang et al., 2017). Moreover, the WUE has been used to describe the coupling relationship between vegetation productivity and carbon supply and water consumption, which is an important part of the coupling cycles of carbon–nitrogen–water (Felzer et al., 2011; Yu et al., 2014).

Non-structural carbohydrates (NSCs) are crucial substrates for plants' growth and metabolism, which consist of total soluble sugar and starch, and both are from photosynthesis (Hartmann and Trumbore, 2016; Hao et al., 2021). They participate in many important metabolic processes (*e.g.*, photosynthesis and respiration), while non-metabolic processes include osmotic regulation, vascular transport, and cold tolerance (Sala et al., 2012; Dietze et al., 2014). Plant carbon dynamics can be inferred *via* quantifying the changes in NSC concentrations (Mitchell et al., 2013). When plant carbon demand exceeds supply, the starch in plant organs decomposes into soluble sugars, providing energy for and maintaining the growth of plants (Dietze et al., 2014; Hartmann and Trumbore, 2016). When carbon supply is over demand, more NSCs are stored for later use (Hoch et al., 2003; Würth et al., 2005; Smith and Stitt, 2007). Soluble sugars are indispensable for the metabolism, osmotic regulation, and energy conversion of plants. The soluble sugar content would increase for osmotic regulation to relieve stress under external stress (Dietze et al., 2014; Hao et al., 2021). Starch is an effective and relatively stable storage molecule that not only provides energy and sugar but also promotes plant growth (Dietze et al., 2014; Schiestl-Aalto et al., 2019). The NSC concentration reflects the adaptability of plants to a changeable environment (Hoch et al., 2003; Mo et al., 2020). When the NSC content is depleted, plants would face death (Hartmann and Trumbore, 2016; Santos et al., 2021). When the external environment changes, such as N deposition and increasing precipitation, the content of NSCs would change (Li et al., 2018; Du et al., 2020). N application reduces the soluble sugar concentration in leaves and roots (Wang et al., 2019) and decreases the starch and NSC concentrations in roots under high N deposition (Li et al., 2018).

WUE reflects the carbon–water relationship (*i.e.*, photosynthesis and respiration), and NSCs reflect the balance between carbon demand and carbon supply (Smith and Stitt, 2007). The carbon stable isotope ($\delta^{13}C$) is the most common method for determining the long-term WUE (Farquhar and Richards, 1984; Farquhar et al., 1989; Livingston et al., 1999;

Saurer et al., 2004; Easlon et al., 2014; Wang et al., 2018). The amount of C, N, and P in leaves represents the amount of nutrients that were accessible to plants and had a significant effect on plant growth (Li et al., 2008). The stoichiometric ratios of C, N, and P reflect the interaction between plants and the environment (Gruber and Galloway, 2008), which are important ways to know the reason of change of NSCs and WUE. In addition, N and P contents were the main limiting factors for plant growth, which not only affected species richness but also influenced biochemical cycles. Moreover, leaf NSC variables and C:N:P stoichiometric variables exhibit a substantial association (Xie et al., 2018). NSCs are the product of photosynthesis, and WUE is the ratio of photosynthetic rate to stomatal conductance, both of which reflect plant photosynthetic capacity. Although there have been many studies on the response of NSCs and WUE to external environmental disturbances, relatively few studies have been conducted on how NSCs and WUE respond to canopy N–water interaction.

The traditional method is mainly to add N directly to the understory, but this ignores the N interception effect of the forest canopy (Tian et al., 2019; Tang et al., 2020). In this experiment, we selected two dominant tree species in a temperate forest to conduct multi-year canopy N addition and precipitation enhancement, and we determined the effect of N and water addition on NSCs and leaf long-term WUE in the dominant tree species in the growing season. We hypothesized that (1) increasing the precipitation and N addition alone would decrease the leaf WUE (1-year period) of *Q. variabilis* and *L. formosana*, and the N–water interaction would have no significant effect on leaf WUE; (2) N addition has different effects on leaf NSCs and root NSCs of the same tree species; and (3) increasing the precipitation could alleviate the effects of N deposition on leaf WUE and NSCs.

Materials and methods

Study site

This study was conducted at the Henan Dabieshan National Field Observation and Research Station of Forest Ecosystem (31° 51′ N, 114°05′ E), which is a natural, deciduous, and broad-leaved mixed forest in Henan Province, China. The field station was located in the transitional zone between the subtropical and warm–temperate climates. The mean annual temperature is 15.2°C, and the mean annual precipitation is 1,119 mm; the background rate of N deposition in atmospheric precipitation is approximately 19.6 kg N ha^{-1} $year^{-1}$ in this region (monitoring data from August 2010 to July 2011). *Quercus variabilis* Blume and *Liquidambar formosana* Hance are the dominant tree species of the forest ecosystem. The forest is about 45 years old and has yellow–brown loam soil (Zhang et al., 2015).

Experimental design

The setting of the experimental platform comprehensively considered all factors (*e.g.*, vegetation and slope). We chose two dominant tree species in this forest: *Q. variabilis* and *L. formosana*. Both of them are dominant tree species in the temperate forest ecosystem and grow up to 30 m tall. *Q. variabilis* is shade tolerant with well-developed root systems, and *L. formosana* is modestly shade tolerant and drought resistant. This experiment was designed using random distribution within four blocks, and each block was set randomly with four different treatments, namely: (1) control treatment (CK, ambient environment and without N addition), (2) canopy N addition with 50 kg N ha^{-1} $year^{-1}$ (CN), (3) canopy water addition with increasing 30% greater than local mean annual precipitation (CW), and (4) interaction of canopy N application (50 kg N ha^{-1} $year^{-1}$) and water addition (30% of local annual precipitation) (CWN). The control (CK) plots were squares with a side length of 30 m, and the total area of each plot was about 900 m^2. The canopy treatments (CN, CW, and CWN) were circular plots with a diameter of 34 m, and the total area of each plot was about 907 m^2. A triangular iron tower with a height of 35 m was built in the center of each circular quadrat, about 5 m above the forest canopy, and a set of rocker nozzles was installed on top of the tower to drive the rocker nozzles to rotate 360° to ensure the uniformity and accuracy of spraying and accessibility (Zhang et al., 2015; Zhao et al., 2020; Supplementary Figure S1). The experiment began in April 2013, and the CN treatment was administered monthly from May to mid-October and repeated annually. The CW treatment was performed weekly at the same time. In August 2018, we randomly selected six healthy and sunny leaves of each dominant tree species from all treatments. Fine root samples (diameter <2 mm) of the two dominant tree species (*Q. variabilis* and *L. formosana*) in all plots were collected from branch roots and washed with purified water. The leaf and root samples were placed in labeled paper bags, dried in the oven at 70°C for 48 h, and analyzed in the laboratory. The basic soil properties of these four locations are shown in Supplementary Table S1.

Long-term water use efficiency analysis

$\delta^{13}C$ was determined in the sample leaves collected from each treatment, and the samples were placed in an oven (70°C for 48 h) and ground into powder with a ball mill. The $\delta^{13}C$ analysis was performed using 5 mg of sample leaves, which were burned in an elemental analyzer (Vario EL, Elementar, Hanau, Germany) and analyzed using an isotope ratio mass spectrometer (Finnigan Mat, typr Deltas). The relationship between carbon isotopic composition and WUE was determined using the equations in the following discussion.

Following Farquhar et al. (1989), the discrimination of isotopic composition was defined as:

$$\Delta = \left(\delta^{13}C_a - \delta^{13}C_p\right)/(1 + \delta^{13}C_p/1,000) \quad (1)$$

where $\delta^{13}C_a$ represents the isotopic composition of atmospheric CO_2, $\delta^{13}C_p$ is the CO_2 isotope value of plant leaves, and discrimination expresses the discrepancy between the $\delta^{13}C$ values of atmospheric CO_2 and plants to some extent. Farquhar et al. (1989) illustrated the relationship between $\triangle$ and C_p/C_a as follows:

$$\Delta = a + (b - a)\left(C_p/C_a\right) \quad (2)$$

where C_p/C_a is the ratio of intercellular to environment CO_2 concentrations, a (=4.4‰) represents fractionation due to air diffusion, and b (=27‰) is net fractionation due to carboxylation. Equation (2) can be converted into equation (3) as follows:

$$C_p = C_a(\Delta - a)/(b - a) \quad (3)$$

W: WUE, the ratio of net photosynthesis A to stomatal conductance for water vapor g_s, was used following Saurer et al. (2004):

$$W = A/g_s = \left(C_a - C_p\right)/1.6 \quad (4)$$

where 1.6 is the ratio of the CO_2 and water vapor diffusivity in the air. Equations (2) and (4) were combined to obtain the relationship between WUE, W, and isotopic discrimination, $\triangle$:

$$W = C_a(b - \Delta)/1.6\,(b - a) \quad (5)$$

The relevant items were determined according to this formula, and WUE was calculated using the carbon isotopic composition.

Determination of non-structural carbohydrate content in leaves and roots

NSC content was determined using the colorimetrical phenol–sulphuric acid method (Yemm and Willis, 1954; Dubois et al., 1956). In the study, the sum of total soluble sugar and starch was regarded as the total amount of NSCs. Briefly, the total soluble sugar content was determined by weighing 50 mg of dry fine power into centrifuge tubes, which was added with 5 ml of 80% ethanol, incubated in a water bath at 80°C for 30 min, and then centrifuged at 7,000 *g* for 5 min. The supernatants were transferred to centrifuge tubes. This procedure was repeated twice, and the supernatants were pooled together; then, colorimetric analysis was performed for measuring the soluble sugar. The residue was used for measuring the starch content. The solid residues left in tubes after total soluble sugar extraction were oven-dried at 80°C (24 h). Then, 2 ml water was added, and the samples were boiled in boiling water for 15 min. Subsequently, 2 ml of 9.2 M perchloric acid was added, and supernatants were collected after centrifuging at 7,000 *g* for 10 min. Then, the solution was used to measure the starch content.

Determination of C, N, and P content in leaves

A mesh with a pore size of 250 μm was used to crush the dried biomass of samples into fine powder. The dry powdered sample (0.5 g) was used to determine the C, N, and P content using fast dichromate oxidation, an elemental analyzer (Vario EL, Elementar, Hanau, Germany), and induced plasma emission spectroscopy (Hötscher and Hay, 1997), respectively. To calculate the C:N:P stoichiometry relationship, the elemental leaf C/N ratio and N/P ratio were calculated.

Determination of total phenols, lignin, and cellulose content in leaves

The total phenol content in the leaves was determined using the Folin–Ciocalteu reagent method as described by Khokhar and Magnusdottir, 2002 and Gundale et al. (2010), as the Folin-Ciocalteu reagent reacts with total phenol to produce a blue color that represents the total phenol content and can be determined spectrophotometrically. The leaf lignin and cellulose contents were measured according to the National Renewable Energy Experimental Procedures using two-step acid hydrolysis methods (Sluiter et al., 2008; Hou et al., 2020). A Muffle furnace (Neytech3-550; Lab-Pro Inc., Sunnyvale, CA, USA) and UV–vis spectroscopy TU-1901 (Purkinje General Instruments Ltd., Beijing, China) were used after the separation of leaf lignin into soluble and insoluble acids. Leaf cellulose was quantified using high-performance liquid chromatography Agilent-1260 (Agilent Technologies, Santa Clara, CA, USA).

Determination of specific leaf area

The SLA was determined by dividing the single-sided area of a single leaf by its dry weight using the leaf area analysis system to read the single-sided area of each leaf. The dry weight of a single leaf was measured after being placed in a drying box at 70°C for 48 h (Ramalho et al., 2013).

Determination of chlorophyll a and b content

The chlorophyll content was measured using spectrophotometry (Wang et al., 2015), following the protocol of Lichtenthaler (1987). Each sample (0.5 g) was extracted and ground into slurry, and 80% (v/v) acetone solution was used to extract the chlorophyll. Absorbance was measured using a spectrometer (Unicam UV-330, Unicam, Cambridge, UK) at a certain wavelength (Chl a at 663 nm and Chl b at 646 nm).

Data analyses

We used control variables and comparison methods to conduct experimental processing and conducted the model analysis to verify the hypotheses. The three-way analyses of variance (ANOVA) were used to evaluate the effects of species, N addition, and water addition on physiological properties. One-way ANOVA with the least significant difference ($P< 0.05$) was used to analyze the differences among CK, N addition, water addition, and N–water interaction treatments on the concentration of NSCs in leaves and roots, C:N:P stoichiometry, $\delta^{13}C$, and other indices. A correlation analysis was used to analyze the relationship between WUE and NSCs of *Q. variabilis* and *L. formosana*. Principal component analysis (PCA) of the two dominant trees' physiological traits was used to show the most discriminatory changes under canopy N and water addition. Data analysis was performed in SPSS 26.0 (SPSS Inc., Chicago, IL, USA) and Canoco 5.0 (Microcomputer Power, Ithaca, NY, USA).

Results

Changes in leaf C:N:P stoichiometry under N and water additions

C:N:P stoichiometry in leaves was affected by the treatments and species (Table 1). Leaf C, N, and P content and their stoichiometry showed no significant change across the treatments in *Q. variabilis*. For *L. formosana*, canopy N addition (CN) increased the leaf C/N ratio. The leaf C and N content of *L. formosana* was significantly increased, but the leaf C/N ratio was decreased under canopy water addition (CW) and water–nitrogen interaction (CWN) treatments. There was no difference in leaf P content and C/P ratio between the two species under CK treatment however, which showed an opposite trend between *Q. variabilis* and *L. formosana* under the treatments. The responses of leaf C, N, and P to N addition and rainfall enhancement were different, and the differences in leaf C:N:P stoichiometry of the two tree species showed different strategies of resource utilization.

TABLE 1 Changes in leaf C, N, and P content and C:N:P stoichiometry of *Q. variabilis* and *L. formosana* under canopy N application and water addition conditions.

Species	Treatments	LTC (mg g^{-1})	LTN (mg g^{-1})	LTP (mg g^{-1})	Leaf C/N ratio	Leaf C/P ratio	Leaf N/P ratio
Q. variabilis	CN	508.18 ± 9.70 abc	18.03 ± 0.92 a	1.44 ± 0.24 ab	28.25 ± 1.10 c	361.91 ± 58.94 ab	12.87 ± 2.34 bc
	CW	501.86 ± 13.52 abc	17.75 ± 1.01 a	1.17 ± 0.08 b	28.33 ± 0.88 c	431.98 ± 32.92 ab	15.27 ± 1.35 ab
	CNW	512.30 ± 5.35 ab	18.6 ± 1.15 a	1.11 ± 0.09 b	27.65 ± 1.56 c	466.00 ± 40.35 a	16.92 ± 1.79 a
	CK	497.39 ± 8.77 abc	17.65 ± 2.08 a	1.22 ± 0.12 ab	28.51 ± 2.75 c	411.88 ± 44.04 ab	14.56 ± 1.79 ab
L. formosana	CN	489.60 ± 10.76 c	13.66 ± 0.51 b	1.08 ± 0.06 b	35.92 ± 1.92 a	455.35 ± 25.56 a	12.72 ± 1.11 bc
	CW	515.72 ± 13.64 a	18.01 ± 0.92 a	1.44 ± 0.15 ab	28.68 ± 0.73 c	360.95 ± 37.64 ab	12.58 ± 1.20 bc
	CNW	513.87 ± 8.32 a	17.70 ± 0.58 a	1.65 ± 0.17 a	29.05 ± 0.71 c	314.38 ± 29.22 b	10.83 ± 1.03 c
	CK	493.56 ± 11.40 bc	15.27 ± 1.31 b	1.36 ± 0.20 ab	32.45 ± 1.96 b	371.05 ± 56.30 ab	11.46 ± 1.79 c
	F_S	ns	***	*	***	*	***
	F_N	ns	ns	ns	ns	ns	ns
	F_W	**	***	ns	***	ns	ns
	$F_{S \times N}$	ns	ns	ns	*	ns	ns
	$F_{S \times W}$	*	**	***	***	**	*
	$F_{N \times W}$	ns	ns	ns	ns	ns	ns
	$F_{S \times N \times W}$	ns	ns	**	ns	**	*

Values followed by the same letter in the same column are not significantly different at P< 0.05 according to least significant difference test. The values are expressed as means ± SE (n = 4). The significance values of the factorial analysis (ANOVA) are shown as follows: ns, not significant; *0.01< P ≤ 0.05; **0.001< P ≤ 0.01; ***P ≤ 0.001.
CK, control group; CN, canopy N addition; CW, canopy water addition; CNW, canopy N and water addition; LTC, leaf total carbon; LTN, leaf total nitrogen, LTP, leaf total phosphorus; F_S, species effect; F_N, nitrogen effect; F_W, water effect; $F_{S \times N}$, species × nitrogen effect; $F_{S \times W}$, species × water effect; $F_{N \times W}$, nitrogen × water effect; $F_{S \times N \times W}$, species × nitrogen × water effect.

Changes in long-term WUE under N and water additions

The response trends of the WUE of the tree species to N and water addition were consistent (Figure 1). CN significantly decreased the WUE and $\delta^{13}C$ of *L. formosana* and reduced the WUE of *Q. variabilis*. The WUE and $\delta^{13}C$ of *Q. variabilis* significantly increased in CW (Figures 1A, B). Compared with *L. formosana*, the WUE of *Q. variabilis* was more impressionable under CW condition. The WUE of the two trees showed no obvious differences under CWN (Figure 1A).

Changes in non-structural carbohydrates under N and water additions

The leaf NSCs of tree species had no change among the four treatments but showed differences between leaf and root responses to all treatments. CN had a significantly negative effect on the root NSCs, starch, and total sugar of *L. formosana* (Figures 2B, D, F). The leaf NSCs, starch, total sugar, and root NSCs of the two tree species were not significantly affected by CW and CWN (Figures 2 A, C, E, F). Compared with CK, the leaf and root starch content decreased in CW for *Q. variabilis*. The root starch

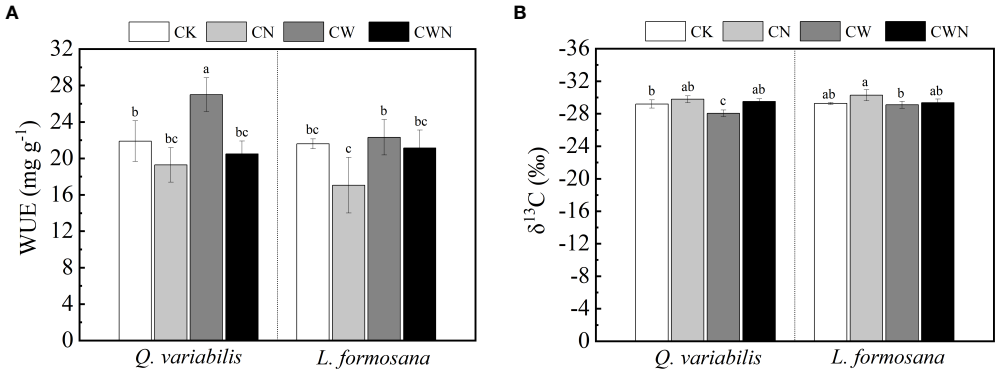

FIGURE 1
Response of long-term water use efficiency **(A)** and stable isotope carbon **(B)** of two tree species under canopy N and water additions. Values are means ± SE (n=4). Different letters indicate that the differences in means among the treatments are significant ($P < 0.05$).

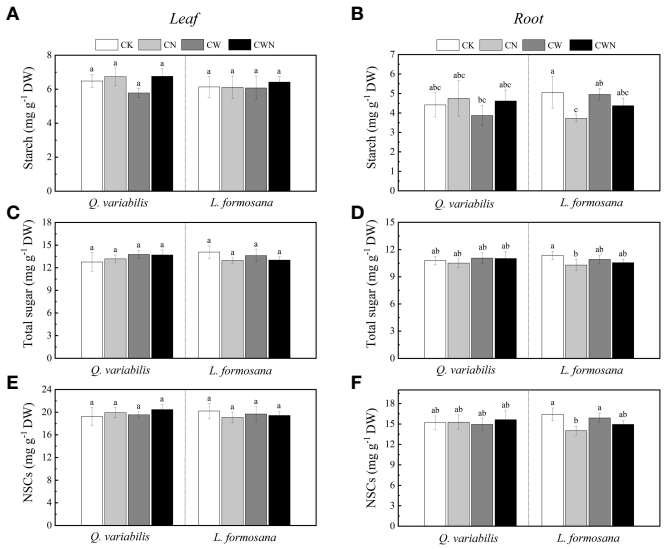

FIGURE 2
Changes in leaf and root starch **(A, B)**, leaf and root total soluble sugar **(C, D)**, leaf and root NSCs **(E, F)** concentration of two tree species under canopy N and water additions. Values are means ± SE (n=4). Different letters indicate that the differences in means among the treatments are significant ($P < 0.05$).

and total sugar contents of *L. formosana* were lower in CN, CW, and CWN treatments than in CK (Figure 2). N application and increased rainfall affected the total sugar and starch in the leaves and roots, but the total NSCs remained relatively stable.

Changes in leaf chlorophyll a and b under N and water additions

The N and water interaction obviously affected the leaf chlorophyll a and b of *Q. variabilis* and *L. formosana* (Figures 3A–D). CN did not affect the leaf total chlorophyll (Chl a + b) content of the two trees species, but CW decreased the leaf total chlorophyll content of *Q. variabilis* (Figure 3D). In contrast to CK, the leaf total chlorophyll content of the two tree species was higher under CWN treatment. CWN had the greatest leaf total chlorophyll content than that in CK, CN, and CW treatments, and the total chlorophyll content of *Q. variabilis* was higher than that of *L. formosana* (Figure 3D). The leaf chlorophyll b content of the trees was lower than that of chlorophyll a in all treatments (Figures 3A, B).

Changes in specific leaf area and total phenol, lignin, and cellulose content

The SLA of the two tree species was lower under CW than that of CK treatments, and CW significantly decreased the SLA of *Q. variabilis* (Figure 4A). CN and CWN significantly reduced the leaf lignin content of *Q. variabilis*. The leaf lignin content of *L. formosana* differed between CW and CWN (Figure 4B). CW obviously decreased the leaf cellulose of *L. formosana*, and the leaf cellulose content of the two trees was lower in CW treatment than in CWN (Figure 4C). The leaf total phenol content was significantly decreased under CWN compared with CK treatment (Figure 4D). The leaf lignin and cellulose contents of *L. formosana* were higher than those of *Q. variabilis*, while the total phenol content of leaves was lower than that of *Q. variabilis*. The interactive effects of nitrogen and water additions significantly affected specific leaf area, total phenols, lignin, and cellulose contents (Table 2).

Principal component analysis

The PCA showed the relationship between leaf physiological traits and the adaptability of trees to changes in N and water addition in *Q. variabilis* and *L. formosana*. Each treatment of both *Q. variabilis* and *L. formosana* was scattered (Figure 5), and the PCA for the two tree species alone is shown in the appendix (Supplementary Figures S2, S3). The PCA model with two components explained 45.31% of the observed total variance. PC1 was strongly influenced by leaf total soluble sugar, starch, NSCs, leaf C and N content, chlorophyll a, C/N ratio, root NSCs, starch, and total soluble sugar. PC2 was strongly influenced by leaf P content, C/P and N/P ratios, $\delta^{13}C$, lignin, total phenol, SLA, cellulose, and chlorophyll b (Figure 5). In addition, leaf N content showed a positive correlation with NSCs in leaves and roots and leaf WUE, whereas negative correlations were observed with the C/N ratio, SLA, lignin, and cellulose content.

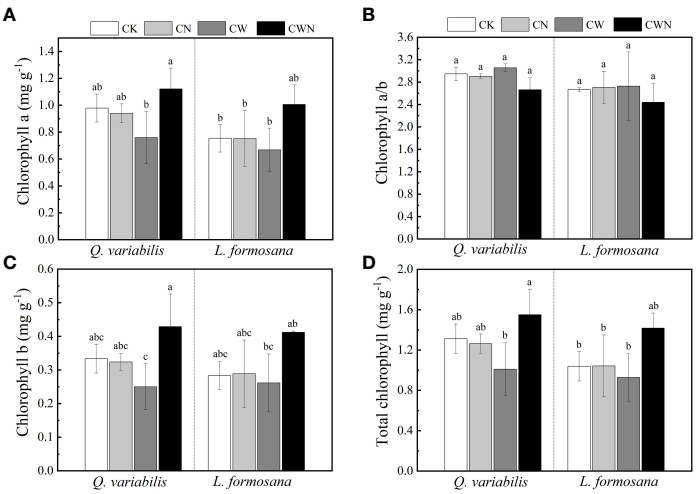

FIGURE 3
Effects of leaf chlorophyll a **(A)**, chlorophyll a/b **(B)**, chlorophyll b **(C)**, and total chlorophyll **(D)** of two tree species under canopy N and water additions. Values are means ± SE (n=4). Different letters indicate that the differences in means among the treatments are significant ($P < 0.05$).

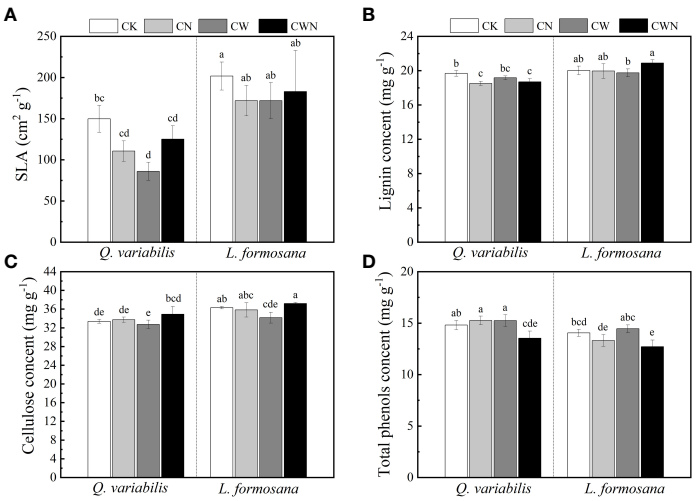

FIGURE 4
Effects of specific leaf area **(A)**, lignin **(B)**, cellulose **(C)**, and total phenol content **(D)** of two tree species under canopy N and water additions. Values are means ± SE (n = 4). Different letters indicate that the differences in means among the treatments are significant (P< 0.05).

Discussion

Long-term water use efficiency response to N and water additions

WUE is an indicator closely related to plant growth under water deficit conditions (Monclus et al., 2006), and water stress increases the WUE of plants (Rahimi et al., 2013; Liu et al., 2015; Yang et al., 2016; Wang et al., 2018). In general, enhanced precipitation increases ambient humidity (Fu et al., 2018) and reduces the WUE (Niu et al., 2011). In this study, increasing precipitation elevated the WUE of *Q. variabilis*, while it did not have a significant effect on *L. formosana*, indicating that different tree species have different water use strategies under

TABLE 2 Effects of species, nitrogen, water, and their interactions on non-structural carbohydrates, water use efficiency, and other physiological properties using three-way ANOVA.

Variable	F_S	F_N	F_W	$F_{S \times N}$	$F_{S \times W}$	$F_{N \times W}$	$F_{S \times N \times W}$
$\delta^{13}C$	**0.010**	**<0.001**	**0.004**	0.123	0.767	0.790	**0.023**
WUE	**0.010**	**<0.001**	**0.004**	0.124	0.770	0.793	**0.023**
Chl a	**0.014**	**<0.001**	0.936	0.984	0.456	**<0.001**	0.146
Chl b	0.603	**<0.001**	0.167	0.591	0.591	**0.003**	0.073
TChl	0.053	**<0.001**	0.682	0.830	0.460	**0.001**	0.103
Chl a/b	**0.003**	0.685	**0.027**	0.188	0.578	0.831	0.155
Leaf NSCs	0.674	0.914	0.707	0.105	0.568	0.537	0.719
LTS	0.829	0.259	0.358	0.096	0.113	0.990	0.405
Leaf starch	0.239	0.074	0.605	0.281	0.280	0.205	0.671
Root NSCs	0.893	0.091	0.743	**0.01**	0.844	0.167	0.583
RTS	0.774	0.061	0.501	0.224	0.300	0.300	0.590
Root starch	0.637	0.391	0.901	**0.005**	0.210	0.241	0.732
Lignin	**<0.001**	0.293	0.776	**0.001**	0.186	**0.026**	0.319
Cellulose	**<0.001**	**0.003**	0.754	0.931	0.541	**0.003**	0.275
Total phenol	**<0.001**	**<0.001**	0.178	0.139	0.382	**0.001**	0.225
SLA	**<0.001**	0.629	0.089	0.628	0.437	**0.005**	0.341

Significant (P < 0.05) effects are presented in bold.
$\delta^{13}C$, carbon stable isotope; WUE, water use efficiency; Chl a, chlorophyll a; Chl b, chlorophyll b; TChl, chlorophyll a + b; Chl a/b, chlorophyll a/b; NSCs, non-structural carbohydrates; LTS, leaf total soluble sugar content; RTS, root total soluble sugar content; SLA, specific leaf area.

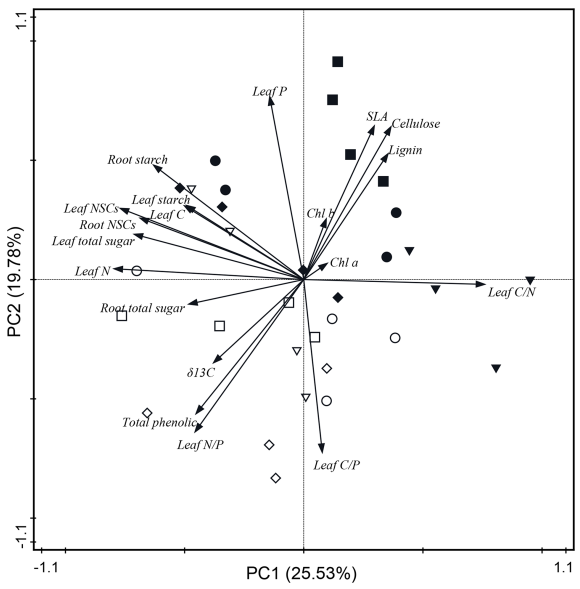

FIGURE 5
Principal component analysis based on the physiological traits of both *Q. variabilis* and *L. formosana* under canopy N and water additions. CK, control group; CN, canopy N addition; CW, canopy water addition; CWN, canopy N and water interaction; white circle, CK with *Q. variabilis*; white inverse triangle, CN with *Q. variabilis*; white diamond, CW with *Q. variabilis*; white square, CWN with *Q. variabilis*; black circle, CK with *L. formosana*; black inverse triangle, CN with *L. formosana*; black diamond, CW with *L. formosana*; black square, CWN with *L. formosana*; Chl a, chlorophyll a; Chl b, chlorophyll b; SLA, specific leaf area; NSCs, non-structural carbohydrates.

precipitation change. An increase in WUE may be related to a drop in SLA or total chlorophyll under increased precipitation. The SLA reflects the ability of the leaf to intercept light, and WUE is negatively correlated with SLA (Wright et al., 1994). In addition, the response of WUE to precipitation is primarily controlled by carbon rather than hydraulic processes (Niu et al., 2011). N is an important component involved in photosynthesis and chlorophyll synthase (Savitch et al., 2002), and the increased content of leaf N implies the enhancement of photosynthesis (Bauer et al., 2004). In this study, canopy N deposition decreased the WUE of the two dominant tree species, and N deposition and increased precipitation had no significant effect on WUE, which was partly consistent with the first and third hypotheses. This suggested that increasing precipitation and N deposition can affect the response of WUE. In the results, N deposition or increasing precipitation had no obvious effect on leaf N content, and there was also no direct association between leaf N and WUE, which suggested that WUE was driven more by abiotic environmental changes than by plant nutrients (Wang et al., 2016).

In addition, the interception of N deposition by the canopy leaves and stems can cause more canopy N accumulation and reduce the N input in soil. N trapped by the canopy is retained or assimilated through the leaf and bark surface (Gaige et al., 2007). Studies have shown that the retention N rate of the plant canopy is 44% at the same N concentration (Liu et al., 2020), and an excessively high N supply reduces a plant's photosynthetic capacity, which may reduce the plant WUE (Bauer et al., 2004; Rahimi et al., 2013). Previous research has verified that canopy N addition does not affect the carbon assimilation rate of *L. formosana* and *Q. variabilis* (Hu et al., 2021). Moreover, different N forms are absorbed differently by tree leaves with various N uptake strategies—for example, deciduous trees absorbed more ammonium N content than nitrate N in the winter (Ma et al., 2021). In this study, we found that N deposition significantly reduced the soil pH, which affected the soil chemical properties and soil microbes, and reduced the leaf WUE. However, soil pH value had no obvious change under N and water interaction (Supplementary Table S1). Shi et al. (2018) indicated that increased precipitation exacerbated the effect of N deposition. The inconsistency with previous studies could be due to differences in latitude region, studied species, and local climate feature.

Non-structural carbohydrate response to N and water additions

In this study, leaf NSCs, starch, and total soluble sugar concentrations showed less variation with treatments (Figure 2), which was consistent with the findings of other studies (Li et al., 2018; Mo et al., 2020; Tang et al., 2020). Previous studies have implied that the N and P levels in leaves can influence the production of NSCs (Xie et al., 2018). Interestingly, only the N addition treatment had an obviously negative effect on the starch, total soluble sugar, and NSC content of the roots of *L. formosana*, which suggested that the response of tree root NSCs to N application was related to tree species (Li et al., 2018). Moreover, the leaf starch of *Q. variabilis* had a negative relationship with the leaf WUE. On the contrary, the leaf total sugar of *L. formosana* was positively related with leaf WUE (Table 3). Previous research has shown that fine-root non-structural carbohydrates decrease with increasing N application (Zhu et al., 2021). When plants were under stress, the starch content decreased and total soluble sugar content increased to maintain osmotic changes (Dietze et al., 2014; Hartmann and Trumbore, 2016). In the present study, both root starch and total soluble sugar content decreased. This indicates that plants consume NSC content to promote plant growth based on increased leaf C/N and root respiration, as NSCs have a connection to the respiration of fine roots and can supply energy for the emergence of new roots and nutrient uptake (Zhu et al., 2021). Huttunen et al. (2013) showed that N fertilization reduced the leaf and root NSC concentrations. A meta-analysis showed that N fertilization significantly reduced

TABLE 3 Correlation coefficients of long-term WUE and NSC values for *Q. variabilis* and *L. formosana*.

Species		Leaf NSCs	Leaf starch	Leaf TS	Root NSCs	Root starch	Root TS	WUE
Q. variabilis	Leaf NSCs	1.000						
	Leaf starch	0.559*	1.000					
	Leaf TS	0.885**	0.206	1.000				
	Root NSCs	0.581*	0.516*	0.502*	1.000			
	Root starch	0.703**	0.767**	0.458	0.788**	1.000		
	Root TS	0.212	0.068	0.343	0.732**	0.210	1.000	
	WUE	-0.324	-0.603*	-0.027	0.072	-0.282	0.341	1.000
L. formosana	Leaf NSCs	1.000						
	Leaf starch	0.794**	1.000					
	Leaf TS	0.812**	0.384	1.000				
	Root NSCs	-0.047	-0.428	0.255	1.000			
	Root starch	0.424	0.097	0.508*	-0.038	1.000		
	Root TS	0.182	0.012	0.485	-0.165	0.430	1.000	
	WUE	0.450	0.199	0.638**	0.120	0.410	0.257	1.000

The single asterisk indicates a significant correlation at P< 0.05 level (bilateral), while the double asterisks indicate an extremely significant correlation at P< 0.01 level (bilateral).
Q. variabilis, Quercus variabilis Blume; L. formosana, Liquidambar formosana Hance; TS, total soluble sugar; NSCs, non-structural carbohydrates; WUE, water use efficiency.

the total root NSCs but did not affect the leaf NSC content (Mo et al., 2020), revealing that plants distribute large amounts of carbon to organs with the most limited resources (Poorter et al., 2012). Plants allocate more carbon to grow aboveground than belowground, which is driven by carbon allocation patterns when the nutrients are sufficient (Liu and Greaver, 2010; Li et al., 2018). In addition, the decrease in root NSCs may be due to the competition for nutrients between the roots and the microorganisms, as nitrogen fertilization increases soil microbial carbon and soil organic matter (Shi et al., 2018). CWN did not obviously affect the leaf and root NSCs in our study. This suggests that, by altering the retention and absorption of N and water, canopy processes have an impact on the effects of simultaneous N deposition and enhanced precipitation on NSCs.

Furthermore, the effect of N addition on tree species NSCs in trees might be related to changes in the content of defense structures (Koricheva et al., 1998; De Long et al., 2015). In this study, N deposition reduced the leaf lignin content of *Q. variabilis*, which would be linked to the higher allocation of C to plant growth rather than structural carbohydrates. N addition reduces the total phenol content in plants, thus affecting plant growth (Lu et al., 2008). CWN did not affect the leaf and root NSCs in our study (Figure 4). This suggests that, by altering the retention and absorption of N and water, canopy processes have an impact on the effects of simultaneous N deposition and enhanced precipitation on NSCs. In this study, CWN decreased the total phenol content of the two tree species (Figure 4D). The leaf N/P ratio of *Q. variabilis* was above 16 under N application and increased precipitation, which indicates P-limited biomass. Then, N and water interaction affected tree defense by changing the leaf nutrient limit, as reduced plant nutrient limitation leads to a lower C allocation to secondary metabolite production (Bryant et al., 1983). Factors such as tree taxonomic type, leaf habit, and tree ages can affect plant responses to the driver of external factors (Ramírez-Briones et al., 2017; Mo et al., 2020; Tixier et al., 2020). Moreover, the root NSCs of the two dominant tree species had no significant change under CWN treatment, indicating that increasing precipitation can alleviate the effect of N addition on root NSCs. This finding supported our hypotheses, which stated that N addition decreases the root NSC concentration of tree species, while increased precipitation mitigates the effect of N deposition. The physiological characteristics of the two dominant tree species had different responses to the nitrogen–water interaction, which could be due to their growth strategies to external disturbances.

Conclusion

The leaf WUE and NSCs of two dominant tree species showed different responses to canopy N deposition and water addition in a warm temperate forest. Elevated N deposition reduced the root NSC (total soluble sugar and starch) concentration of *L. formosana*, while it did not obviously change the leaf NSCs of the two dominant tree species. N deposition decreased the leaf WUE of *Q. variabilis* and *L. formosana*. Increased precipitation showed a positive effect on the leaf WUE of *Q. variabilis*. However, the N–water interaction did not obviously affect the leaf WUE and NSC contents of the two dominant tree species. This presented the dominant tree species regulated growth traits when faced with changes in external factors, such as enhanced tree adaptability by altering the tree NSCs and long-term WUE. Additionally, the leaf WUE was affected by changes of the abiotic environment rather

than leaf nutrients. When examining the impacts of N deposition on the physiological responses of the dominant tree species, it is crucial to consider the canopy nitrogen and water interactive effects, as the two natural processes frequently occur at the same time. The simulated canopy N deposition and precipitation addition provided a new method for researching the physiological responses of dominant tree species to global change.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

XJ and MS had the main responsibility for data collection, analysis, and writing. YQ, ML, and LM contributed to data and manuscript preparation. MS and SF (the corresponding authors) had the overall responsibility for experimental design and project management. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.1025162/full#supplementary-material

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Effect of different planting pattern arrangements on soil organic matter and soil nitrogen content under a maize/soybean strip relay intercropping system

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Assessing the spatial distribution of organic matter and total nitrogen in soil is essential for management and optimum utilization of fertilizers. Therefore, the present field experiment was conducted to evaluate the impact of different planting pattern arrangements on the spatial distribution of soil total nitrogen and organic matter content under a maize/soybean strip relay intercropping system. The planting was arranged in a manner such that soil sampling could be done from continuous maize/soybean relay strip intercropping (MS1), maize/soybean relay strip intercropping in rotation (MS2), traditional maize/soybean intercropping (MS3), sole maize (M), sole soybean (S), and fallow land (FL) from 2018 to 2020. The results showed significant variations for soil organic matter and total nitrogen content under different planting pattern arrangements of maize and soybean in the strip relay intercropping system. Across all systems, the highest soil organic matter (29.19 g/kg) and total nitrogen (10.19 g/kg) were recorded in MS2. In contrast, the lowest soil organic matter (1.69 g/kg) and total nitrogen (0.64 g/kg) were observed in FL. Soil organic matter and total nitrogen in MS2 increased by 186.45% and 164.06%, respectively, when compared with FL. Soil organic matter and total nitrogen in MS2 increased by 186.45% and 164.06%, respectively, when compared with FL. Furthermore, under MS2, the spatial distribution of soil organic matter was higher in both maize and soybean crop rows as compared with other cropping patterns, whereas the soil total nitrogen was higher under soybean rows as compared with maize in all other treatment. However, correlation analysis of the treatments showed variations in organic matter content. It can be concluded that different planting patterns can have varying effects on soil organic matter and total nitrogen distribution under the strip relay intercropping system. Moreover, it is recommended from this

study that MS2 is a better planting pattern for the strip relay intercropping system, which can increase the spatial distribution of soil organic matter and total nitrogen, thereby improving soil fertility, C:N ratio, and crop production. This study will serve as a foundation towards the scientific usage of chemical fertilizers in agricultural sector.

KEYWORDS

intercropping, soil organic matter and total nitrogen, spatial distribution, maize (Zea mays L.), soybean

1 Introduction

Soil organic matter and total nitrogen content are important indexes to evaluate soil fertility and soil quality. These indexes are essential sources and sinks of the global carbon cycle and have become one of the research hotspots in soil and environmental sciences (Johannes et al., 2020; Roberta et al., 2020; Wu et al., 2021). Although soil organic matter and total nitrogen content only account for a small part of the total soil volume, they play a vital role in balancing soil fertility, environmental protection, and sustainable agricultural production (Struijk et al., 2020). China feeds approximately 20% of the world's population, with less than 9% of the world's arable land area (Guo et al., 2010). The world's population is increasing rapidly; therefore, it must produce more from the limited arable land to meet the needs of the growing population (Altieri, 1999; Fan et al., 2012). Strengthening the utilization of chemical fertilizers is an essential step in obtaining bumper crop yields (Hassanein et al., 2019; Mohamed et al., 2020); nevertheless, the indiscriminate use of fertilizers has led to increased soil nutrient imbalance in various regions of the world, notably many Asian countries (Zhou et al., 2017; Wang et al., 2020). In economically developed areas of China, a markedly disproportionate dose of fertilizers is being administered, i.e., averaging 339 kg/hm^2, which is 1.29 times higher than the national average of 262 kg/hm^2; however, in underdeveloped areas, the fertilization rate is only 178 kg/hm^2 (Cho, 2007; Zhang and Zhang, 2008; Zhang et al., 2013).

Regarding the limited utilization of fertilizer resources, China adopted the concept of ecological agriculture in the last century (Shao et al., 2019; Tourn et al., 2019). In this regard, a lot of research on soil nutrient status has been carried out, and intercropping of different crops was one of the priorities. Intercropping is a cropping pattern in which two or more crops are cultivated simultaneously on the same piece of farmland (Godfray et al., 2010), which proves to be economically, ecologically, and socially profitable (Du et al., 2018; Gitari et al., 2020). According to statistics, the universal intercropping area is more than 1,109 hm^2, which is about 3% of the total cultivated area (Li et al., 2007; Gautam et al., 2014). Common intercropping patterns principally comprise intercropping or relay strip intercropping, and strip intercropping refers to growing two or more crops in strips within a specific width, allowing alternate planting of different crops. The main difference between intercropping and strip intercropping lies in the fact that, in strip intercropping, different kinds of crops are not grown in a single row, but two or more rows of the same crop are grown together; one crop is formed in a "strip" and the interval is cultivated with another "strip" of crop, having a relatively fixed line number, line spacing, and strip width. Strip intercropping can make full use of limited land resources, improve the nutrient absorption and utilization efficiency of crops, and enhance soil fertility as well as soil quality (Zhang et al., 2019; Ahmed et al., 2020).

Under intercropping, soil organic matter and total nitrogen content, like other soil characteristics, have high spatial variabilities in various regions. Similarly, in China, there are significant differences in soil organic matter and nitrogen contents at different spatial locations at the same time (Liu et al., 2011; Yao et al., 2019). The changes in soil organic matter and total nitrogen content are dependent on farming practices such as fertilization, incorporation of crop residues, crop rotations, soil utilization, and tillage method (Huang et al., 2007). The tillage method has a great influence on soil organic matter and total nitrogen content. It was reported that intercropping could enhance the distribution and content of soil organic matter and total nitrogen (Leonard et al., 2012). Cereal/legume intercropping is widely recognized as a sustainable agricultural production system, as it can improve the symbiotic nitrogen fixation of legumes and reduce the input of chemical fertilizers (Yao et al., 2019). Intercropping of maize and soybean represents a new cereal/legume pattern, which farmers are rapidly adopting in Southwest China (Yao et al., 2019). However, there is no available literature on soil organic matter and total nitrogen spatial distribution in the maize/soybean strip relay intercropping system so far. Therefore, the objectives of the present study were to identify better

management practices that could optimize land use efficiency; to understand the mechanism underlying the increased soil fertility under maize/soybean relay strip intercropping systems, especially Southwest China and similar areas; and, to quantify the relationship between soil organic matter, total nitrogen content, and planting patterns in a maize/soybean strip relay intercropping system. The results will serve as a foundation for the scientific usage of chemical fertilizers in the maize/soybean relay strip intercropping system.

2 Materials and methods

2.1 Experimental location

Field experiments were conducted from 2018 to 2020 at the research farm of Sichuan Agricultural University in Ya'an city located in Southwest Sichuan Province of China (101°56′26″E, 28°51′10″N). This region comprised of a hilly and mountainous topography (Figure 1). The climate was humid subtropical monsoon, with an average annual temperature of 16.2°C, an average annual rainfall of 1,250 to 1,750 mm, an average annual sunshine duration of 1,005 hours, and an average annual frost-free period of 300 days. The soil type was gley soils according to FAO-UNESCO 1988 (Rolf and Eddy de, 2011) with pH 6.6, organic matter 29.8 $g{\cdot}kg^{-1}$, total nitrogen 1.6 $g{\cdot}kg^{-1}$, total phosphorus 1.28 $g{\cdot}kg^{-1}$, and total potassium 14.28 $g{\cdot}kg^{-1}$.

2.2 Experimental design and treatments

The experimental design was a randomized block design with three replications (Figure 2). In this experiment, maize variety Denghai-605 and soybean variety Nandou-12 were used. Treatments were arranged MS1 = Continuous maize/soybean relay strip intercropping, MS2 = maize/soybean relay strip intercropping in rotations, MS3 = Traditional maize/soybean intercropping (MS3: A conventional planting method in Southwest of China), M = Sole maize, S = Sole soybean, or FL = Fallow land. The size of the experimental plots under MS1, MS2, MS3, M, and S was 6 × 6 (36 m^2), whereas the plot size for FL was 2 × 6 (12 m^2). The total width of MS1 and MS2 was "160 cm + 40 cm", i.e., the relay intercropping combination of two crop strips with a total width of 200 cm, consisting of two rows of maize and two rows of soybean with a 40-cm row width for maize and soybean, and 60-cm spacing between the adjacent rows of maize and soybean. MS3 had a total width of 100 cm with a 1:1 row ratio, and distance between maize/soybean rows was 50 cm. In sole planting of maize and soybean, the distance between two rows was 100 cm for maize and 50 cm for soybean. (Note: The difference between MS2 treatment and MS1 treatment was that the maize belt and soybean belt were rotated in MS2, i.e., maize belts turned into soybean and soybean belts turned into maize each year.)

Different basal fertilizers, including urea (CH_4N_2O, including 46% N), calcium superphosphate [$Ca(H_2PO_4)_2H_2O$, including 14% P_2O_5], and potassium chloride (KCl, including 52% K_2O)

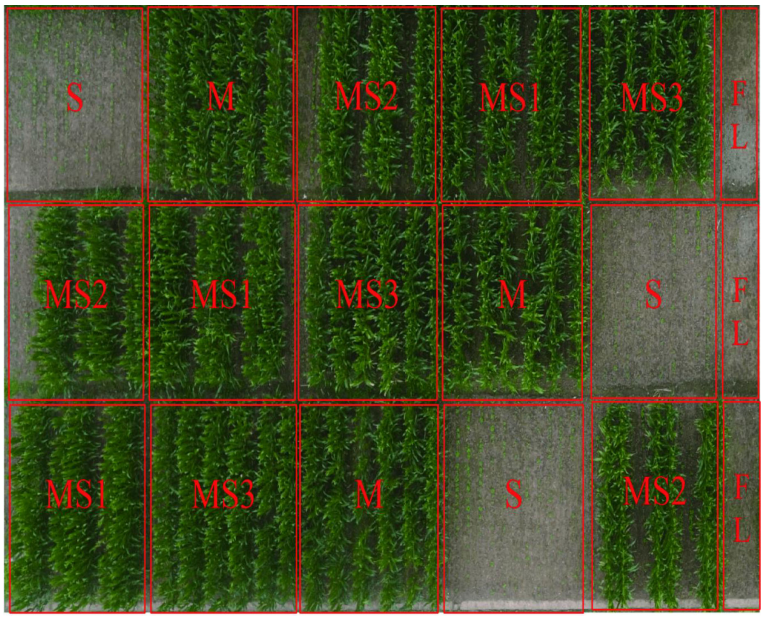

FIGURE 1
Location of the research site aerial photo. Note: MS1, MS2, MS3, M, S, FL represent the Continuous planting of maize/soybean relay strip intercropping, Planting of maize/soybean relay strip inter-cropping in rotation, Traditional maize/soybean inter-cropping, Sole maize planting, Sole soybean planting, Fallow land, respectively.

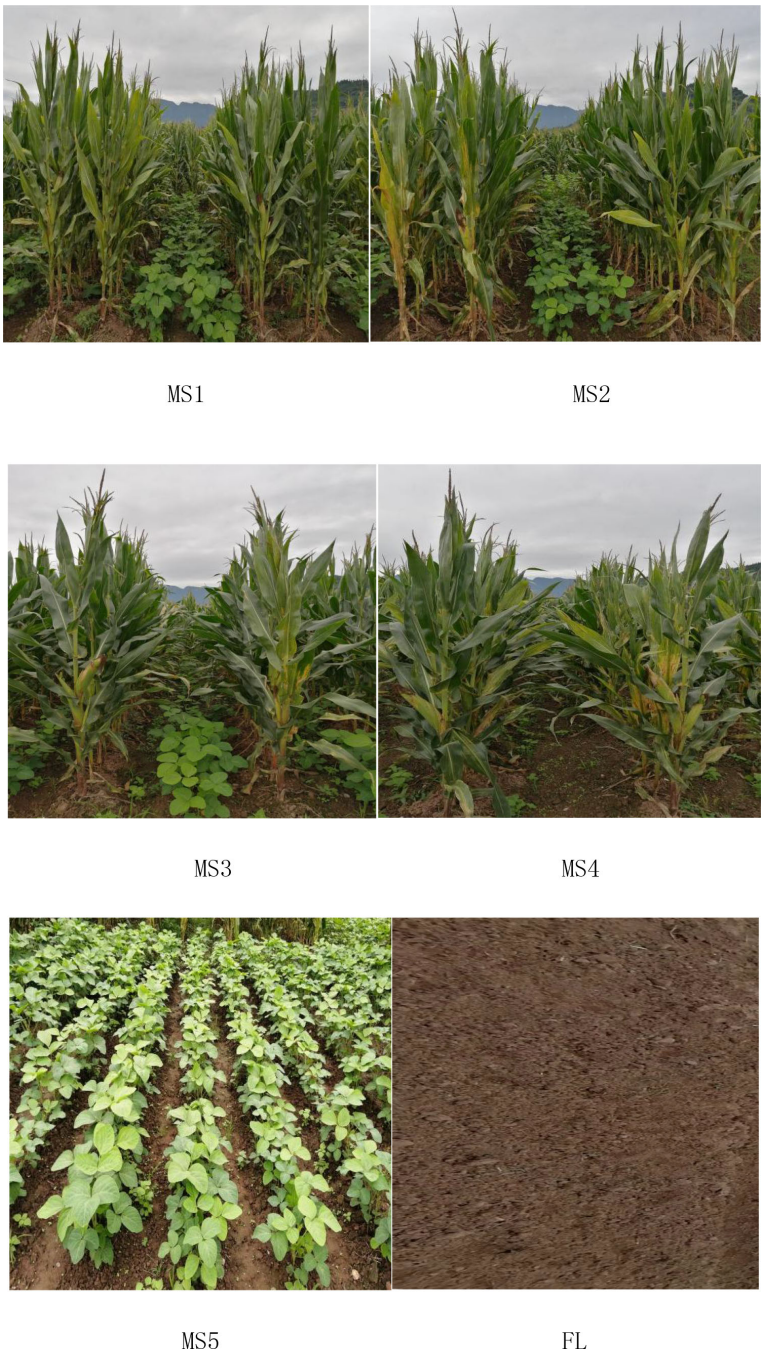

FIGURE 2
Spatial layout of different planting patterns.

were used for maize and soybean. Maize was fertilized with pure nitrogen at 120 kg/ha, P_2O_5 at 105 kg/ha, and K_2O at 135 kg/ha, and soybean was fertilized with pure nitrogen at 60 kg/ha, P_2O_5 at 63 kg/ha, and K_2O at 52.5 kg/ha in 2018, 2019, and 2020. Maize crop was sown on 24 March, 23 March, and 29 March, in 2018, 2019, and 2020, respectively, and harvested on 25 July, 6 August, and 8 August in 2018, 2019, and 2020, respectively. Soybean was sown on 7 June, 8 June, and 13 June, in 2018, 2019, and 2020, respectively, and harvested on 30 October, 23 October, and 22 October 22 in 2018, 2019, and 2020, respectively.

2.3 Sample collection and measurement

In this experiment, soil samples from all the cropping patterns were collected after soybean harvesting. For soil sampling, the fixed-point sampling procedure was adopted to collect the soil sample from 0 to 20 cm soil layer (Figure 3). To collect the sample, a soil core was inserted vertically into the ground. All the collected soil samples were mixed, and approximately 1 kg of soil was taken for further analysis. Undisturbed soil samples were placed in a tray and stored in a clean indoor ventilation area for natural air drying. After drying, samples were put into a sample bag for the determination of soil organic matter and total nitrogen. All sample bags were labeled with a number, sampling place, soil type, sampling depth, sampling date, and time.

2.3.1 Soil organic matter and total nitrogen content determination

The soil organic matter (SOM) was determined by the potassium dichromate volumetric method – external heating method (Geng and Wei, 2020).

$$\mathrm{SOM} = c\frac{(v_0\text{-}v) \times 0.003 \times 10724 \times 1.1}{m \times k} \times 100\,\% \quad (1),$$

where c (mol/L) is the molar concentration of consuming ferrous sulfate, V_0 is the volume (ml) of consuming ferrous

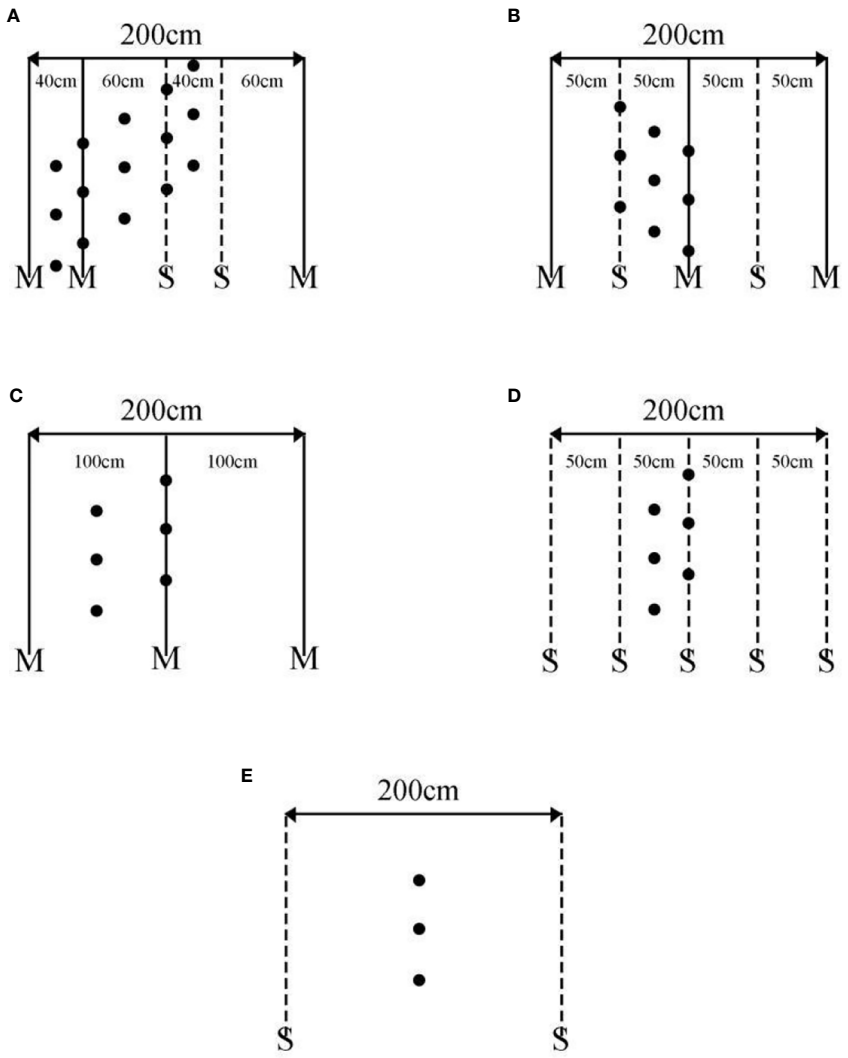

FIGURE 3
Spatial distribution of soil sample sites in different cropping patterns. **(A)** Maize/soybean relay strip intercropping (continuous and rotation cropping), **(B)** traditional maize/soybean intercropping, **(C)** maize monoculture, **(D)** soybean monoculture, and **(E)** fallow land. M, sole maize planting; S, sole soybean planting; ●, for the soil sample collection point.

sulfate in a blank test, V is the volume (ml) of consuming ferrous sulfate in the titrating soil sample, 0.003 is ¼ mmol/g of carbon, 10,724 is the conversion coefficient from soil organic carbon to organic matter, 1.1 is a correction factor (the oxidation rate in this method is 90%), m (g) is the air dry soil quality, and k is the coefficient of drying soil to drying soil.

The total nitrogen content (TN) was determined by the Kjeldahl method (Wu, 2004).

$$TN = \frac{(v_0\text{-}v) \times c \times 14 \times 10^{-3}}{w} \times 10^3 \quad (2),$$

Where V_0 is the volume (ml) of standard acid used for titrating the sample, V is the volume (ml) of normal acid used for titrating the blank, C is the normal acid concentration (mol/L), 14 is the molar mass of N (g/mol), and W is the sample weight (g).

2.3.2 Soil organic matter and total nitrogen reference standards

At present, there are many soil nutrient grading standards in China (Geng and Wei, 2020). The results of this experiment mainly refer to the national soil nutrient classification standard (Table 1). The Chinese soil nutrient classification standard divides soil organic matter and soil nutrient into six grades from 1 to 6. Soil organic matter and soil nutrient are the highest in grade 1 and the lowest in grade 6 (Wu, 2004). Furthermore, the spatial variation of soil organic matter and nutrient availability in China is relatively high. For example, soil organic matter in China can be as high as 200 g/kg or more, and as low as 5 g/kg or less, and the total nitrogen content can be as high as 35 g/kg and as low as 5 g/kg (Zhang et al., 2008). Therefore, further refinement of the soil organic matter and soil nutrient grade is needed to compare differences in soil nutrient grading in China (Yan et al., 2017).

The classification of coefficient of variation: coefficient of variation is considered weak under <10%, moderate between 10% and 100%, and strong when it is >100% (Duan, 2000; Zhang et al., 2003).

TABLE 1 National soil nutrient standard grade.

Standard grade[a]	Nutrient elements	
	Organic matter (g/kg)	Total nitrogen (g/kg)
1	>40.0	>2.00
2	30.1–40.0	1.51–2.00
3	20.1–30.0	1.01–1.50
4	10.1–20.0	0.76–1.00
5	6.0–10.0	0.50–0.75
6	<6.0	<0.50

[a]1, 2, 3, 4, 5, and 6 represent the first standard, second standard, third standard, fourth standard, fifth standard, sixth standard, respectively.

2.4 Data statistics and analysis

All the experimental data were managed in Microsoft Excel 2016, and the figures were constructed with Origin Pro 2018. Differences between intercropping systems and soil organic matter and total nitrogen content were identified by analyzing variance (ANOVA) using SPSS 22.0 software (SPSS Inc., Chicago, IL, USA). The mean values were compared with a least significant difference (LSD) test at the $p < 0.01$ significance level. Linear regression techniques were used to describe the relationships between soil organic matter and total nitrogen content. The effectiveness of cropping patterns was determined by regression analysis with p-values (Tukey's test) and the coefficient of determination (R^2).

3 Results

3.1 Soil organic matter content and spatial distribution

The different planting patterns showed significant ($p < 0.01$) variations for soil organic matter content in both maize and soybean at all sampling times across the 3 years of this experiment (Table 2 and Figure 4). During the 3 years, the average SOM of each treatment order was MS2 > MS1 > MS3 > S > M > FL (Table 2 and Figure 4). Furthermore, it was observed that the maximum soil organic matter (39.72 g kg^{-1}) was recorded in MS2, whereas the minimum soil organic matter (8.71 g kg^{-1}) was recorded in FL per system. The soil organic matter content in MS2 increased by 186.45% when compared with FL (Table 2 and Figure 4). At the same time, the spatial distribution of organic matter in maize and soybean rows was most dense in MS2. The obtained results were also graded by the coefficient of variation, where we found that MS1, MS2, MS3, M, and S demonstrated moderate variations when compared, while FL showed weak variation, and overall MS2 showed the most significant variations (Table 2).

3.2 Total soil nitrogen content and spatial distribution

Across all treatments, MS2 exhibited the most significant variation in total soil nitrogen content (Table 3 and Figure 5). On average, the order of different treatments was MS2 > MS1 > MS3 > S > M > FL, which revealed that the minimum soil nitrogen content was under FL (0.64g/kg), and the maximum soil nitrogen content was recorded under MS2 (1.69 g/kg) (Table 3 and Figure 5). However, under MS2, the spatial distribution of soil total nitrogen in soybean rows was higher when compared with maize rows (Table 3 and Figure 5). The results showed that the maximum total soil nitrogen content (2.47 g kg^{-1}) was recorded in MS2, while the minimum total soil nitrogen content (0.55 g kg^{-1}) was recorded in S or FL, and total nitrogen in MS2 increased by 164.06% in contrast with FL (Table 3).

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TABLE 2 Soil organic matter content under different planting patterns of maize and soybean in 2018–2020.

Treatment[a]	Number of samples	Content range (g/kg)	Average (g/kg)	Coefficient of variation (%)
MS1	45	15.33–36.54	27.02 ± 7.98ab[b]	29.53
MS2	45	15.51–39.72	29.19 ± 9.36a	32.06
MS3	27	14.79–34.19	25.07 ± 5.74abc	22.91
M	18	17.68–24.65	20.42 ± 2.45c	12.02
S	18	14.79–27.89	22.07 ± 3.95bc	17.90
FL	9	8.71–11.46	10.19 ± 0.71d	6.97

[a]MS1, MS2, MS3, M, S, and FL represent the continuous planting of maize/soybean relay strip intercropping, planting of maize/soybean relay strip intercropping in rotation, traditional maize/soybean intercropping, sole maize planting, sole soybean planting, and fallow land, respectively.
[b]Values followed by a different letter within the same column are significantly different at $p < 0.01$.

Under all planting patterns, the spatial distribution of soil total nitrogen content in maize rows was lower as compared with soybean rows; however, the average maximum and minimum total nitrogen and organic matter content was almost identical under various planting patterns. Therefore, it could be speculated that organic matter and total nitrogen had a strong correlation with each other. The results showed that the MS2 planting pattern was most beneficial for soil total nitrogen accumulation.

The correlation analysis revealed that there was moderate variation in soil total nitrogen under MS1, MS2, MS3, M, and S, and weak variation in FL; the most significant variation was observed under MS2 ($R^2 = 0.96$).

3.3 Correlation between soil organic matter and total nitrogen

Based on the results of soil organic matter and total nitrogen content in the six treated soils, the correlation equation and coefficient of determination between them could be obtained from Table 4 and Figure 6. The relationship between soil organic matter (x) and total nitrogen (y) was unevenly linear. Six linear regression equations for soil organic matter and total nitrogen, and their coefficient of determinations were obtained (Table 4). The regression equation of the entire test area was $y = 0.06x - 0.08$, and the coefficient of determination was $R^2 = 0.87$ (Table 4). Furthermore, the correlation between all treatments (planting patterns) for soil organic matter and total nitrogen was significant, where MS1, MS2, MS3, and FL were closer to 1. At the same time, the soil C:N ratio was significantly different among all treatments ($p < 0.01$), and the highest C:N ratio (25.22) was recorded in MS2 (Table 4). These results indicated that the correlation between soil organic matter and total nitrogen in different planting methods was significant and positively correlated.

4 Discussion

4.1 Variations in soil organic matter content

The soil organic matter content is mainly influenced by land-use management (Demir and Ersoy, 2020; Mishra et al., 2020; Zhao et al., 2020), especially the management of different vegetation in the soil (Welegedara et al., 2020). The percentage of organic matter in shrub soil, grassland, and forest soil, within 1 m depth, was 33%, 42%, and 50%, respectively, which were significantly correlated with the type of vegetation (Eghdami et al., 2019; Yeasmin et al., 2020). Similarly, in this study, there were differences in soil organic matter content between maize and soybean planting patterns. During the 3 years, the average SOM of each treatment order was MS2 > MS1 > MS3 > S > M > FL (Table 2 and Figure 4). This phenomenon might

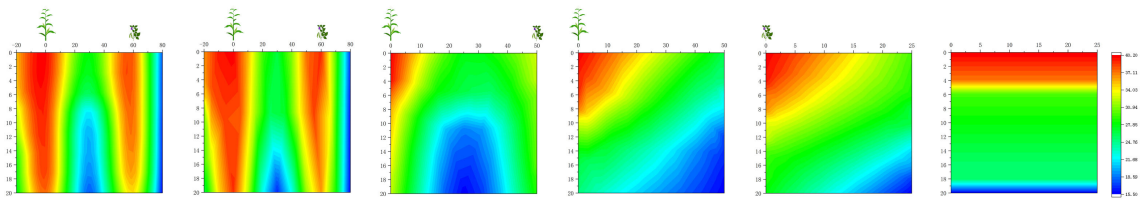

FIGURE 4
Spatial distribution of soil organic matter under different planting patterns of maize and soybean. MS1, MS2, MS3, M, S, and FL represent the continuous planting of maize/soybean relay strip intercropping, planting of maize/soybean relay strip intercropping in rotation, traditional maize/soybean intercropping, sole maize planting, sole soybean planting, and fallow land, respectively.

TABLE 3 Total nitrogen content under different planting arrangements of maize and soybean in 2018–2020.

Treatment[a]	Number of samples	Content range (g/kg)	Average (g/kg)	Coefficient of variation (%)
MS1	45	0.96–2.46	1.48 ± 0.37a[b]	25.05
MS2	45	0.87–2.47	1.69 ± 0.53a	31.35
MS3	27	0.69–1.81	1.23 ± 0.29b	23.73
M	18	0.62–0.99	0.78 ± 0.13cd	16.07
S	18	0.55–1.21	0.98 ± 0.17c	17.21
FL	9	0.55–0.71	0.64 ± 0.04e	6.35

[a]MS1, MS2, MS3, M, S, and FL represent the continuous planting of maize/soybean relay strip intercropping, planting of maize/soybean relay strip intercropping in rotation, traditional maize/soybean intercropping, sole maize planting, sole soybean planting, and fallow land, respectively.
[b]Values followed by a different letter within the same column are significantly different at $p < 0.01$.

be associated with different planting patterns and crop residues of maize and soybean in the field, thereby contributing toward enhanced soil organic matter accumulation in MS2. Different land-use patterns lead to different soil cultivation, soil physical or chemical properties, and soil fertility. These variations directly affect the decomposition and transformation of soil organic matter in different soils (Lai et al., 2016; Segura et al., 2019; Liu et al., 2020). Furthermore, increased organic matter contents under different treatments in this study indicated that soil organic matter played a vital role in soil fertility under the strip relay intercropping system. Our results are consistent with the study of King et al. (2020).

4.2 Variations in soil total nitrogen content

The soil total nitrogen content reflects the soil potential capacity to provide nutrients for vegetation, which, together with soil organic matter and its dynamic balance, constitutes an essential index of soil fertility (Marco et al., 2011; Alhaj et al., 2019; Dai et al., 2020; Li et al., 2020; Meng, 2020). Nitrogen is considered to be blood for crop growth and development because its absorption and utilization can promote crop growth and increase the crop yield (Hua et al., 2020). Nitrogen competition is significantly higher among crop species; however, in the cereal/legumes intercropping system, it was decreased due to the nitrogen fixation mechanism of legumes, contributing toward increased nitrogen availability for absorption and utilization by cereals. Ta and Faris (1987) found that clover increased nitrogen absorption and utilization by 25% in cereals under intercropping. Broadbent (1981) reported that white clover improved the nitrogen absorption and utilization by 80% in ryegrass under intercropping. Du et al. (2019) and Raza et al. (2019) found that in the maize and soybean intercropping system, the nitrogen uptake of maize was increased by 17%–21%, which was mainly attributed to soybean nitrogen fixation. Similarly, this study demonstrated that soil total nitrogen content in maize/soybean strip intercropping was higher than sole cropping (Table 3 and Figure 5). This might be related to soybean nitrogen fixation and increased total organic matter. It was found that soil nitrogen content and spatial distribution were significantly different between intercropping and monoculture, $p < 0.01$. The content and spatial distribution of soil nitrogen in the contour maps were reflected by the grading color, and the difference was obvious. Soil total nitrogen was the highest (2.47 g·kg^{-1}) in MS2 treatment and the lowest (0.55 g·kg^{-1}) in S and FL treatment (Table 3 and Figure 5). Furthermore, this study showed that when legumes and non-legumes were intercropped, legumes' nitrogen fixation could benefit the nitrogen absorption and uptake in non-legumes, thus promoting the growth and development of non-leguminous crops. However, the amount of nitrogen fixation depends on the different legume and non-legume intercropping combinations and different crop varieties, planting patterns, and growth habits of various crops

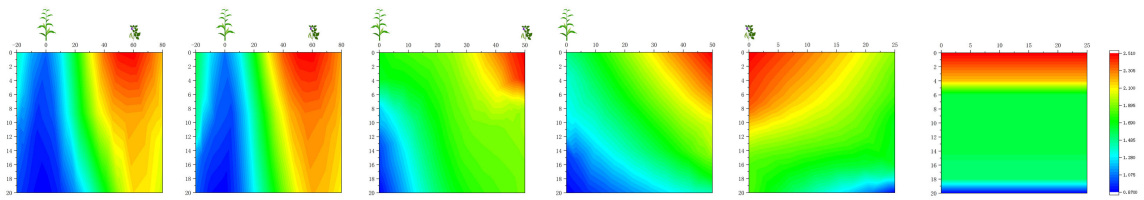

FIGURE 5
Spatial distribution of soil total nitrogen content under different planting patterns of maize and soybean. MS1, MS2, MS3, M, S, and FL represent the continuous planting of maize/soybean relay strip intercropping, planting of maize/soybean relay strip intercropping in rotation, traditional maize/soybean intercropping, sole maize planting, sole soybean planting, and fallow land, respectively.

TABLE 4 Soil organic matter and total nitrogen correlation.

Treatment[a]	Sample number	Linear regression	Coefficient of determination (R^2)	Significance[b]	C:N
Whole test area	162	$y = 0.06x–0.08$	0.87	$p < 0.01$	19.21 ± 0.95c
MS1	45	$y = 0.05x+0.25$	0.96	$p < 0.01$	21.75 ± 1.07b
MS2	45	$y = 0.06x+0.07$	0.96	$p < 0.01$	25.22 ± 0.88a
MS3	27	$y = 0.05x–0.01$	0.94	$p < 0.01$	19.48 ± 1.00c
M	18	$y = 0.04x–0.01$	0.58	$p < 0.01$	17.30 ± 1.04d
S	18	$y = 0.03x+0.25$	0.60	$p < 0.01$	16.19 ± 0.87d
FL	9	$y = 0.06x+0.07$	0.97	$p < 0.01$	15.30 ± 0.94d

[a]Whole test area, MS1, MS2, MS3, M, S, and FL represent the various experiment treatments (MS1+MS2+ MS3+ M+S+ FL), continuous planting of maize/soybean relay strip intercropping, planting of maize/soybean relay strip intercropping in rotation, traditional maize/soybean intercropping, sole maize planting, sole soybean planting, and fallow land, respectively.
[b]Content followed by a different letter within the same column are significantly different at $p < 0.01$.

(Gabriela et al., 2021). The biological nitrogen fixation of legumes can not only improve the nitrogen absorption and utilization of non-legumes, but can also promote growth and development, reduce crop dependence on non-renewable resources, and increase land equivalent ratio and land interest rate (Zhou et al., 2017; Chen et al., 2019). Similarly, this study demonstrated that maize/soybean relay strip intercropping has a positive effect on soil total nitrogen contents.

4.3 Relationship between soil organic matter and soil total nitrogen content

The soil C:N ratio not only plays a vital role in soil organic matter decomposition but is also an essential factor of soil quality evaluation, as it determines the organic matter effectiveness that improves the soil structure, enhances the carbon fixation, and increases the soil potential as a "source/sink" of atmospheric CO_2 and nitrogen regulation (Bewket and Stroosnijder, 2003; Hagedorn et al., 2010). It is generally believed that during the initial stage of mineralization, organic matter having a C:N ratio > 30 cannot produce nitrogen. If organic matter has a C:N ratio < 15 at the beginning of mineralization, the amount of adequate nitrogen will exceed microorganism assimilation in the soil, thereby making it possible for plants to obtain adequate nitrogen from organic matter mineralization (Sun et al., 2020; Veronika et al., 2020).

Our results showed that the variation coefficient of organic matter and total nitrogen content in each treatment was moderate (Table 4). This might be due to the difference between organic matter and total nitrogen content. Furthermore, this could also be influenced by (1) the contrast of parent material and soil texture at the experimental site; (2) the particularity of remote terrain in the

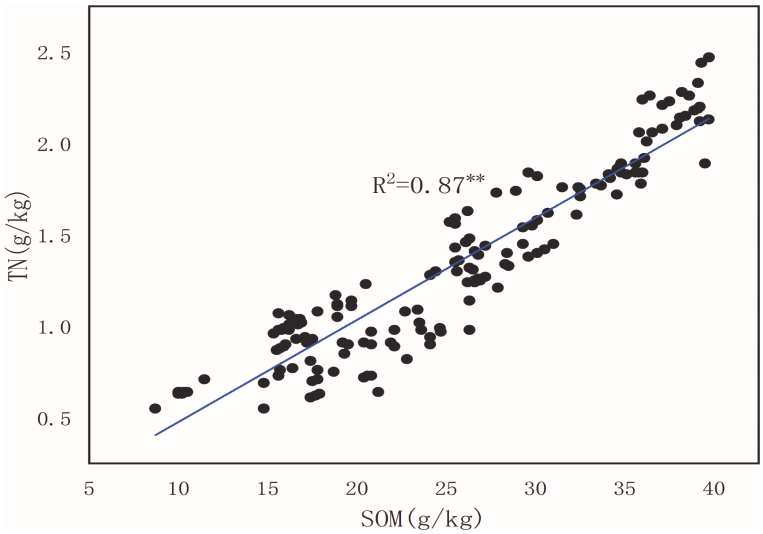

FIGURE 6
Soil organic matter (*x*) and soil total nitrogen (*y*) relationship.

field; (3) impact of crop vegetation; and (4) climatic factors. Meanwhile, the contents of organic matter and total nitrogen in soil samples were compared, and it was found that the spatial difference of organic matter and total nitrogen was significant, and there was a significant positive correlation between organic matter and total nitrogen (Table 4). The results of the present study will serve as a guideline for rational fertilization in agricultural production, thereby contributing toward appropriate fertilizer usage, improved utilization rate of nutrients, and enhanced crop yield from low and medium soils.

5 Conclusions

Taken together, the findings of the current study elucidated the effect of different planting pattern arrangements on soil organic matter and soil nitrogen content under the maize/soybean strip relay intercropping system. It was found that different planting patterns of maize and soybean strip relay intercropping significantly affected soil organic matter and total nitrogen content in soil. The findings revealed that the highest soil organic matter and total nitrogen content was recorded in MS2, while the lowest soil organic matter and total nitrogen content was recorded in FL. Furthermore, under MS2, the spatial distribution of soil organic matter was higher in both maize and soybean crop rows as compared with other cropping patterns, whereas the soil total nitrogen was higher in soybean rows as compared with maize in all other treatments. However, correlation analysis of the treatments showed variations in organic matter content. Moreover, it is recommended that MS2 is a better planting pattern for the strip relay intercropping system, which can increase the spatial distribution of soil organic matter and total nitrogen, thereby improving the soil fertility, C:N ratio, and crop production.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Author contributions

XT and WY conceived and designed the research. MH, KC, JX, MA, and AS conducted the experiments. XT and KC evaluated the data. JX and MA provided different chemical reagents and experimental material. Paper writing was completed by XT, WY, and MH. KC reviewed and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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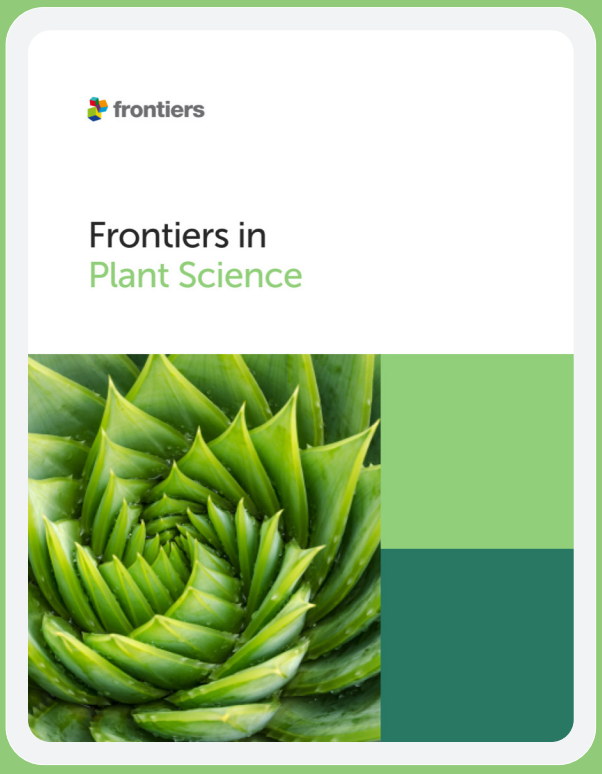

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