

A belowground trait-based understanding of nitrogen driven plant diversity loss

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Keywords

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Abstract

Belowground plant traits play important roles in plant diversity loss driven by atmospheric nitrogen (N) deposition. However, the way N enrichment shapes plant microhabitats by patterning belowground traits and finally determines aboveground responses is poorly understood. Here, we investigated the rhizosheath trait of 74 plant species in seven N-addition-simulation experiments across multiple grassland ecosystems in China. We found that rhizosheath formation differed among plant functional groups and contributed to changes in plant community composition induced by N enrichment. Compared with forb species, grass and sedge species exhibited more distinct rhizosheaths; moreover, grasses and sedges expanded their rhizosheaths with increasing N-addition rate which allowed them to colonize belowground habitat and competitively exclude forbs. Grasses also shaped a different microenvironment around their roots compared with forbs by affecting the physicochemical, biological and stress-avoiding properties of their rhizosphere soil. Rhizosheaths act as a “biofilm-like shield” by accumulating of protective compounds, carboxylic anions and polysaccharides, determined by both plants and microorganisms. This enhanced the tolerance of grasses and sedges to stresses induced by N enrichment. Conversely, forbs lacked the protective rhizosheaths which renders their roots sensitive to stresses induced by N enrichment, thus contributing to their disappearance under N-enriched conditions. This study uncovers the processes by which belowground plant traits affect aboveground responses under conditions of nitrogen enrichment, and advances our mechanistic understanding of the contribution of competitive exclusion and environmental tolerance

to plant diversity loss caused by N deposition.

Significance Statement

Atmospheric nitrogen (N) deposition drives plant diversity loss across different grasslands worldwide. Despite several hypotheses being considered to explain this phenomenon, the biological processes and underlying mechanisms have not been fully understood. We discovered that a belowground plant trait determined aboveground responses to N enrichment by shaping soil properties in rhizosphere microhabitats. Grasses and sedges established an ideal microhabitat by forming and expanding rhizosheaths, thus allowing them to competitively exclude forbs and finally survive in stresses caused by N enrichment. The rhizosheath is not only a ‘belowground bridge’ linking plants, soil and microorganisms, but also a ‘belowground switch’ to pattern plant community structure under N enriched environments. These findings enhance our mechanistic understanding of biodiversity loss caused by N enrichment.

Introduction

Biodiversity reduction caused by atmospheric nitrogen (N) deposition is a serious problem for grassland ecosystems worldwide (Galloway et al., 2008; Bobbink et al., 2010; Clark et al., 2019). The hypotheses of competitive exclusion and environmental tolerance are important theories widely considered to explain N deposition-driven plant diversity loss (Grime, 1973; Newman, 1973; Tilman, 1987; Rajaniemi, 2003; Bowman et al., 2008; Hautier et al., 2009; Borer et al., 2014; Farrer & Suding, 2016; DeMalach, 2018). The competitive and tolerant performance of plants depend on their functional traits and the trait plasticity in response to alterations in resources and stress (Lavorel & Garnier, 2002; Suding et al., 2005; Cleland et al., 2008; Ceulemans et al., 2011; Funk et al., 2017). Compared with aboveground plant traits, the contribution of belowground traits to N deposition-caused shifts in plant community composition or diversity decline remains largely unknown (Fargione & Tilman, 2006). Some plant species evolved special belowground traits, such as sand-binding roots, cluster roots and dauciform roots, which allow them to survive in severely nutrient-impooverished environments (Shane et al., 2005; Lambers et al., 2015; Basirat et al., 2019). However, in the context of nutrient enrichment induced by atmospheric N deposition, the significance of root specializations of grassland plants in aboveground community changes has not been experimentally established. Therefore, exploring how N enrichment affects belowground plant traits will provide experimental evidence for mechanisms underlying competitive exclusion and stress tolerance involved in biodiversity loss caused by N enrichment.

In extreme habitats, such as desert, some plant species exhibit sand-binding root specialization with rhizosheath formation (Price, 1911). This root trait has been known for well over a century (Volkens, 1887). The rhizosheath is a belowground microecosystem; it comprises the soil adhering to roots on excavation (Brown et al., 2017). Desert plants develop distinct rhizosheaths, which minimize water loss as an important mechanism to withstand extremely dry soil (Basirat et al., 2019). Rhizosheaths can also enhance nutrient availability in the rhizosphere, thus improving plant functioning in nutrient-poor soil (Haling et al., 2013; Abrahão et al., 2019). In addition to extreme habitats, the physiological functions of rhizosheaths are gradually being discovered in terms of plant adaptation to other stressful environments (Delhaize et al., 2012). Nitrogen deposition often causes a variety of environmental stresses in grassland ecosystems including soil acidification, nutrient deficiency, and metal toxicity, causing plant species loss (Stevens et al., 2004; Crawley et al., 2005; Bowman et al., 2008; Thayer et al., 2008; Tian et al., 2016). Hence, rhizosheaths will alter the tolerance of plants to environmental stresses caused by N deposition, which likely further shapes plant community structure.

The microhabitat characteristics of the rhizosheaths are determined by the interactions among plants, soil, and microorganisms (Kuzyakov & Blagodatskaya, 2015; de Vries et al., 2020). The intensive fluxes of organic compounds from roots into the rhizosphere stimulate microbial activity, and drive changes in microbiome structure and function

which, in turn, facilitate the availability of mineral nutrients by exuding low- and high-molecular-weight substances such as organic acids and viscous exopolysaccharides, commonly known as mucilage (Paterson et al., 2007; Jones et al., 2009; Gunina et al., 2014). These exudates, especially mucilage, not only modify nutrient availability in the rhizosphere, but also improve the mechanical stability of rhizospheres by influencing their hydraulic properties (Read et al., 2003). Therefore, plants with rhizospheres often form associations with soil microorganisms by exchanging greater amounts of substances between their roots and soil, thus allowing them to better cope with harsh environments (Rabbi et al., 2018; Basirat et al., 2019). The most distinct shift of plant communities in grasslands driven by N enrichment is the suppression of forbs and stimulation of grass growth (Clark & Tilman, 2008; Bobbink et al., 2010; Tian et al., 2016). Whether the contrasting responses of forbs and grasses to N enrichment are associated with their rhizosphere properties and whether the rhizospheres of different functional groups in grasslands differ in their responses to N enrichment remains to be experimentally investigated.

Given the important roles of rhizospheres in plant functioning in harsh environments, we hypothesize that plant species with distinct rhizospheres may be advantaged in acclimation to N-enriched environments compared with those with less distinct rhizospheres. Therefore, we compared the characteristics associated with rhizospheres of 74 common plant species across seven grassland ecosystems in China, and evaluated their responses to N enrichment. We further examined the differences in soil

characteristics in the rhizosphere between forbs and grasses, and investigated the effects of N enrichment on physical, chemical and biological properties of rhizosphere. We also assessed the role of rhizosheaths in protecting grasses from N enrichment-induced metal toxicity by externally applying the chemical analogues exuded from grass roots and rhizosphere microorganism to forb rhizosphere soil. Our results identified a novel indicator to reflect plant responses to atmospheric N deposition, and revealed the way N enrichment shapes plant microhabitats and finally affects aboveground responses. Our findings significantly contribute to a mechanistic understanding of the biodiversity reduction in grassland ecosystems globally, driven by atmospheric N deposition.

Results

Linking rhizosheaths to aboveground responses to N enrichment

A number of N-addition experiments that simulated atmospheric N deposition across different types of grasslands including desert steppe, typical steppe, alpine steppe, mountain steppe, and alpine meadow have been established in China (Fig. 1d). We detected a negative response of forb biomass to N enrichment in these grasslands across different sites (Fig. 1a). In contrast, grasses and sedges exhibited positive responses to N enrichment across these grasslands (Fig. 1b, c). To link the changes in aboveground plant biomass to belowground processes, we quantified the rhizosheath size of plant species in these grasslands. In natural habitats, forb species exhibited lack of rhizosheaths, i.e. very little soil adhering to their roots, while sedge and grass species exhibited distinct rhizosheaths (Fig. 2). Under the conditions of N enrichment, the rhizosheath of grasses and sedges in three grassland ecosystems (in Inner Mongolia, Sichuan and Tibet) showed an expanding trend with increasing N-addition rates (Fig. 3a). There was a significant positive correlation between the rhizosheath expansion in sedge and grass species and their aboveground plant biomass changes (Fig. 3b), suggesting the involvement of rhizosheath processes in the aboveground responses of plants to N enrichment.

Nitrogen enrichment-induced differences in functional traits in rhizosphere soil microhabitats between grasses and forbs

In addition to the marked differences in rhizosheath size between forbs and grasses, there were substantial differences in soil properties of their rhizosphere between the

two functional groups at three sites (Fig. 4; Table S4). Soil in the rhizosphere of grasses showed higher soil water content, plant-available phosphorus (P), dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) concentrations than in that of forbs, regardless of experimental sites (Figs 4, S1-3). Nitrogen addition also significantly impacted soil properties in the rhizosphere microhabitat of forbs and grasses (Table S4). Nitrogen enrichment significantly increased water content, plant-available P, DON and DOC concentrations in the rhizosphere soil of grasses, while N enrichment had little effect on these parameters in the rhizosphere of forbs (Fig. 4, S1-3). The decrease in soil pH in the rhizosphere of grasses caused by N enrichment was less than that in the rhizosphere of forbs (Figs. S1-3). There were no significant differences in the concentrations of inorganic N in rhizosphere soil of grasses and forbs (Figs. S1-3). In addition to the abiotic soil parameters, the microbial responses to N enrichment in the rhizosphere of grasses also differed from those of forbs (Figs. 4, S1-3). For example, N addition led to greater decreases in microbial biomass and abundance in the rhizosphere of forbs than in that of grasses including decreases in microbial carbon (MBC), microbial nitrogen (MBN), microbial phosphorus (MBP), bacterial and fungal abundance (Fig. 4; Fig. S1-3). The protective compounds in the rhizosphere secreted by roots and microorganisms such as carboxylic anions, phenolics and mucilaginous compounds, differed in their response to N enrichment (Fig. 4). Nitrogen enrichment significantly increased concentrations of carboxylic anions, phenolics and mucilaginous compounds in the rhizosphere of grasses, whereas the concentrations of these compounds in the rhizosphere of forbs were hardly affected by

N addition (Figs. 4, S1-3).

Correlations between aboveground responses and functional traits in rhizosphere soil microhabitats

We divided the soil functional traits into three categories, i.e. physicochemical (soil nutrient property), biological (microbial feature), and plant-microorganism-co-regulated traits (protective substance). The three categories were, respectively, represented by the first axis (principal component, PC1) of the three traits including six parameters (soil pH, water content, plant-available P concentration, inorganic N concentration, DON and DOC concentrations), five parameters (MBC, MBN, MBP, bacterial abundance and fungal abundance) and three parameters (phenolics, carboxylic anions and mucilaginous compounds) (Fig. S4). PC1 at the three experimental sites (Inner Mongolia, Sichuan and Tibet) explained about 50% of the variables (Fig. S4), and thus PC1 was considered a good representative of the three types of properties in the rhizosphere microhabitat. The physicochemical and biological traits respectively reflected physical and chemical characteristics, and the status of soil microorganisms in the rhizosphere (Fig. S4). The plant-microorganism-co-regulated traits were related to nutrient availability in the rhizosphere and mainly included the complex compounds secreted by roots and microorganisms (Fig. S4). The aboveground biomass of grasses was positively correlated with PC1 representing soil physicochemical traits and PC1 representing plant-microorganism-co-regulated traits, while the aboveground biomass of forbs exhibited negative correlations with these two traits (Fig. 5a). The aboveground biomass of grasses and forbs did not show significant correlations with PC1, which

represented soil biological traits (Fig. 5a).

Ecophysiological roles of the soil traits in rhizosphere microhabitats shaped by rhizosheaths under N enriched condition

To determine the roles of the plant-microorganism-co-regulated traits in the rhizosphere affected by rhizosheaths in aboveground parts of plants in response to N enrichment, we applied oxalate and galacturonic acids to the rhizosphere of forbs to simulate the increases in the protective compounds in the grass rhizosphere. Application of these compounds markedly enhanced tolerance of forb species to metal toxicity caused by N enrichment (Figs 5b; S5, S6). High concentrations of metal ions in the growth medium, especially manganese (Mn^{2+}), inhibit root elongation and photosynthesis of forbs (Tian et al. 2016, Tian et al. 2020), and was associated with a decline in their aboveground and belowground biomass (Fig. S6). Addition of the compounds to the rhizosphere alleviated the inhibitory effect of metal toxicity on forbs, such that it significantly enhanced forb biomass in the presence of toxic concentration of Mn^{2+} (Figs 5b; S6). In contrast with effects on forb species, applying the same concentrations of these compounds to the grass rhizosphere had no significant influence on grass biomass (Fig. 5b). These results suggest that the biochemical properties of the grass microhabitat shaped by their rhizosheaths conferred them tolerance to stresses under conditions of N enrichment.

Discussion

Plant functional traits determine aboveground responses to resource enrichment by affecting plant competitiveness and environmental tolerance (Lavorel & Garnier, 2002; Suding et al., 2005; Cleland et al., 2008; Ceulemans et al., 2011; Funk et al., 2017). Our previous studies revealed that grassland plants showed different nutrient acquisition under conditions of N enrichment associated with changes in root exudates (Tian et al., 2021). In the present study, we systematically studied the belowground traits of 74 plant species in different types of grasslands focusing on the rhizosheath, a combination of plants, soil and microorganisms. Our study is the first attempt to compare rhizosheath traits among grassland plant species in response to N enrichment. We found that grass species, unlike forbs, conglomerated soil particles strongly and bound the soil particles to their root surface, thus exhibiting distinct rhizosheaths (Figs. 2). This root feature confers tolerance of plants to severe environments (Hartnett et al., 2013; Basirat et al., 2019; Abrahão et al., 2019). Here, we found N enrichment led to the rhizosheaths of grass and sedge species expanding (Fig. 3), which likely allowed them to competitively exclude other plant species. The rhizosheath can as an indicator to directly show the sensitivity of grassland plants to N enrichment.

The rhizosheath is a 'belowground bridge' that links plants to soil; in the rhizosheath, various physiological processes are more intense (Paterson et al., 2007; Gunina et al., 2014; Kuzyakov & Blagodatskaya, 2015). Under conditions of N enrichment, forbs and grasses showed differences in their rhizosphere microhabitats. There was a greater

amount of water and nutrients in the rhizosphere of grasses than in that of forbs (Fig. S3-5) which provided a favorable habitat for microorganisms (Paterson et al., 2007; Gunina et al., 2014). Therefore, grass rhizosphere showed greater microbial biomass and abundance than those of forbs (Fig. S1-3). These results indicate that microorganisms together with grass roots form a more favorable association, thus allowing both to function under environmental changes induced by N enrichment.

In extreme habitats, both plant roots and microorganisms release protective compounds into the rhizosphere, such as phenolics, organic acids and mucilage (Kochian et al., 2004; Lamber et al., 2015). Some plants also exude these compounds upon exposure to nutrient deficiency or drought and thus mobilize nutrients or maintain a wetter environment (Oliveira et al., 2015; Lambers et al., 2015). Our previous study showed that the roots of *Artemisia frigida*, a dominant forb species, exudes more oxalate than those of *Stipa krylovii*, a dominant grass species, under condition of N enrichment (Tian et al., 2021). However, in the present study, we found the amount of carboxylic anions in the rhizosphere of grasses was greater than that in the forb rhizosphere (Fig. 4) which was the result of interactions between soil microorganisms and roots. We also found that N enrichment increased concentrations of mucilage in the grass rhizosphere (Fig. 3). Mucilage is a polymeric gel composed of polysaccharides, and a small amount of lipids exuded by both roots and microorganisms (Read et al., 2003). The mucilage not only maintains a higher hydraulic conductivity at the root-soil interface, but also decreases P sorption by soil particles (Gaume et al., 2000). These chemical compounds

provide an ideal microhabitat for grasses favoring their roots to continue absorbing nutrients and water under N enrichment.

Our study illustrates that the exudates released by microbes and plants can alleviate metal stress caused by N enrichment. Addition of these compounds to the soil of forbs significantly increased the tolerance of these forbs to metal toxicity, whereas applying them to grasses was less effective (Fig. 5). This is expected because of the initially higher concentrations of these exudates in the grass rhizosphere. The result that exogenous application of carboxylic anions and mucilage alleviated metal toxicity to plants is consistent with previous reports (Oburger et al., 2009; Klug & Horst, 2010). These compounds chelate metals, thus affecting the rate of metal transfer from the soil to the roots. Mucilage can also adhere to soil particles, increasing their aggregation and the root-soil contact (Carminati et al., 2017). Therefore, with increasing N-addition rates, the concentrations of mucilage increased in the rhizosphere of grasses which likely contributed to more soil particles adhering to their roots and expansion of their rhizosheaths (Fig. 3). The rhizosheath appears to act as a “biofilm-like shield” to protect grasses from various stresses such as drought, mechanical stress, nutrient deficiency, and metal toxicity related to N enrichment (Fig. S7).

In summary, our study provides firm evidence for the ‘competitive exclusion hypothesis’ and ‘environmental tolerance hypothesis’ by focusing on the rhizosheath, a pivotal root feature, in plant diversity loss caused by N enrichment. Grass and sedges roots exploit

a greater belowground space by increasing their root-sheath size under N enrichment, which enhances their belowground competitive capacity. The rhizosheath functions as a “biofilm-like shield” due to greater secretions of protective compounds affected by both plants and microorganisms. These protective compounds in the grass rhizosphere provide grasses with tolerance of nutrient limitation and metal toxicity caused by N enrichment. Conversely, forb roots hardly form rhizosheaths around their roots which makes their roots vulnerable to various stresses, and finally allows their aboveground parts to exhibit negative responses to N enrichment.

Materials and methods

Site description

Seven grasslands on the Eurasian steppes were included in the study, located in five provinces in China (Inner Mongolia, Qinghai, Sichuan, Xinjiang, Tibet). These sites are typical grasslands of the Mongolian Plateau (MP) and Qinghai-Tibetan Plateau (QTP) in China. The N-addition experiments on the MP were carried out in Duolun county, Hulun Buir and Xisu Banner in the Inner Mongolia Autonomous Region. The N-addition experiments on the QTP were carried out in Hongyuan county, Sichuan province, Damxung county, the Tibet Autonomous Region, Sanjiaocheng farm, Qinghai province, and Bayinbuluk alpine, the Xinjiang Uygur Autonomous Region. These sites encompass the major vegetation and soil types with an altitude range from 760 m to 4500 m; the mean annual precipitation ranges from 180 mm to 747 mm, and the mean annual temperature from -4.8°C to 4.3°C (Table S1). The soils at these sites display different nutrient availability and neutrality or alkalinity of soil pH (Table S2). The design and treatment of these N-addition experiment are detailed in Table S3. The plant and soil samples were collected, respectively, in 2016 and 2017 at these sites.

Aboveground biomass measurements and rhizosphere soil sampling

In mid-August, at peak biomass, one quadrat ($1.0\text{ m}\times 1.0\text{ m}$) per plot was used to determine aboveground plant biomass. Plant species were harvested separately by clipping. Plant material was oven dried at 70°C to constant weight. At some sites, we only collected plant data in a subset of plots (N levels in Duolun: 0, 2, 4, 8, $16\text{ g N m}^{-2}\text{ yr}^{-1}$; N levels in Hulun Buir: 0, 3, 5, $10\text{ g N m}^{-2}\text{ yr}^{-1}$; N levels in Xisu Banner: 0, 2, 8,

16 g N m⁻² yr⁻¹; N levels in Qinghai: 0, 2, 8 g N m⁻² yr⁻¹; N levels in Sichuan: 0, 8, 16 g N m⁻² yr⁻¹; N levels in Tibet: 0, 2.5, 5, 10 g N m⁻² yr⁻¹; N levels in Xinjiang: 0, 3, 9 g N m⁻² yr⁻¹).

To explore the differences in belowground microhabitats between dominant forbs and grasses, we used a 3-cm diameter soil corer at the center of one dominant plant at a depth of 0-10 cm to collect the rhizosphere soil. The whole soil block including plants was deposited on paper and the rhizosphere soil was collected according to the method reported by Zhu et al. (2020) and Yu et al. (2020). In Inner Mongolia, Sichuan and Tibet, we selected two forb species inside each plot to collect their rhizosphere soil. To collect more soil, three soil cores were collected for one forb species and then bulked to give one sample for the subsequent analyses. We also selected two grass species to collect their rhizosphere soil by the same method. Soil samples were immediately transported to the laboratory (Institute of Botany, Chinese Academy of Sciences, Beijing), where they were sieved using a 2-mm sieve. One subsample of soil was stored at -80°C for measurements of soil microorganisms, microbial carbon (MBC), microbial nitrogen (MBN), microbial phosphorus (MBP), organic acids, total phenolics, mucilaginous compounds, inorganic N (IN), dissolved organic nitrogen (DON) and dissolved organic carbon (DOC) concentrations. One part of the soil samples was air-dried for determination of soil pH and plant-available phosphorus (AP) concentrations.

Soil parameter analyses

The measurement procedures of soil pH, inorganic N and plant-available P were according to Tian et al. (2016). Soil DOC and DON were analyzed with a TOC analyzer

(Elementar Vario TOC cube, Langensfeld, Germany). For measurement of the concentrations of carboxylic anions, phenolics and mucilaginous compounds in soil, 2.5 g of soil was extracted with 5 mL distilled water at 25°C (200 r/min) for 30 min. After centrifugation (3,000 g, 10 min), the supernatant was filtered with a 0.22 µm aqueous filter and used to determine the concentrations of organic anions, phenols and mucilaginous compounds. Carboxylic anions were measured using a Hypsil C18 column (5 µm, 4.6 mm×250 mm) and High Performance Liquid Chromatography (HPLC, Waters 550, Milford, MA, USA). A solution containing 0.5% (w/v) KH₂PO₄ and 0.1 mM tetrabutylammonium hydrogen sulfate, buffered at pH 2.4 with orthophosphoric acid was the static phase and the mobile phase. The flow rate was 1.0 mL min⁻¹ and the detection wavelength was 210 nm. We measured 11 carboxylic anions including malate, oxalate, citrate, succinate, malonate, lactate, tartrate, fumarate, aconitate, formate and propionate. We found that oxalate was the main carboxylic anion in soil.

Total phenolics were measured with the Folin-Denis assay (Box, 1983). The reaction mixture (2.5 mL) contained 0.5 mL Folin-Ciocalteu phenol reagent (10-fold diluted with distilled water), and 1 mL soil extract. After 5 min, 1 mL saturated Na₂CO₃ solution was added. The absorbance was measured at 760 nm against a blank prepared as described above with distilled water, Folin-Ciocalteu reagent, and Na₂CO₃ solution after 2 hours. The concentrations of total phenolics was calculated using gallic acid as a standard. The concentrations of mucilaginous compounds were determined according to the method reported by Blumenkrantz and Asboe-Hansen (1973). The soil extract (1

mL) was mixed with 1.2 mL sodium tetraborate solution (12.5 mM sodium tetraborate solution-H₂SO₄) into a 10 mL tube. The tubes were refrigerated in crushed ice. The mixture was shaken in a Vortex mixer and the tubes heated in a water bath at 100°C for 5 min, and then 20 µL of *m*-hydroxydiphenyl reagent was added. The tubes were shaken and, within 5 min, the absorbance was measured at 520 using galacturonic acid as a standard.

Soil microbial biomass C, N and P (MBC, MBN, MBP) were measured by fumigation with chloroform and extraction with 0.5 M K₂SO₄. The dissolved organic C and N in extracts was measured with a TOC/TON analyzer (Elementar Vario TOC cube, Langensfeld, Germany) and dissolved P was determined with a continuous flow analyzer (SEAL Analytical GmbH, Norderstedt, Germany).

For the determination of microbial gene abundance, DNA was extracted from 0.25 g of soil using the Soil Genomic DNA kit (CW BIO, Beijing, China). DNA quality and concentration were measured using a SmartSpec Plus spectrophotometer (Bio-RAD, Hercules, USA) and electrophoresis in agarose gels (1% w/v in TAE). Quantitative PCR (qPCR) was used to quantify the gene copy numbers of bacterial 16S rRNA and fungal ITS, respectively, using the primer pairs 515F/907R (515F: 5'-GTGCCAGCMGCCGCGG-3'; 907R: 5'-CCGTCAATTCMTTTRAGTTT-3') and ITS3F/ITS4R (ITS3F: 5'-GCATCGATGAAGAACGCAGC-3'; ITS4R: 5'-TCCTCCGCTTATTGATATGC-3').

Microcosm experiment

Metal toxicity by N enrichment is a key factor to inhibit forb growth, eventually leading

to the loss of forb species from a grassland community (Tian et al., 2016). To test whether the rhizosheath contained compounds that protect grasses from metal toxicity, we simulated the microenvironment in the grass rhizosphere by addition of carboxylic anions and mucilaginous compounds to the growth medium of forbs and grasses, and investigated the responses of grasses and forbs to metal toxicity. We only applied oxalate and galacturonic acids to simulate the increases in putatively protective compounds (PC) released following N enrichment in the grass rhizosheath, because there was no significant difference in concentrations of phenolics in grass and forb rhizosphere under the conditions of N enrichment. We designed four treatments and a control (CK), +Mn (manganese addition), +Mn+PC10, +Mn+PC25 and +Mn+PC50 (PC10, PC25, and PC50 represented the levels of putatively protective compounds being 10, 25, and 50 μM C). The Mn addition gave a concentration similar to that in soil of the N-addition treatments in a field experiment. Each treatment was replicated five times. Seedling pre-cultivation was according to Tian et al. (2016). Shoot biomass was measured after treatments for 40 days.

Data analyses and statistics

The root-sheath size was the ratio of the weight of soil adhering to the root surface to the total weight of roots and soil. The rhizosheath expansion was calculated as the log response ratio: $\ln[\text{mean root-sheath size (N treatment)}/\text{mean root-sheath size (control)}]$. The aboveground response ratios of functional groups to N-addition rates were also calculated as the log response ratio. We used one-way ANOVA to test for differences in root-sheath size of different functional groups. If the data did not have homogeneity of

variance or normal distribution, ANOVA was performed after natural-log transformation of these data. To determine the effects of N addition, plant species and their interaction on soil properties, data were analyzed with linear mixed-effects models (LMM) using the 'lmerTest' function in the 'lme4' package (Kuznetsova et al., 2017) of the R statistical software (R Core Team, 2020). The values of microbial abundance were first log transformed to increase normality to foster comparability of effect sizes. Nitrogen addition rates and plant functional groups were set as fixed effects, and experimental sites were set as random effects. On the base of the LMM, multiple comparisons were conducted to compare the differences in the effects of N addition rates on soil properties using function 'emmeans' in package 'emmeans' (R. i386.3.6.3).

We divided the rhizosphere traits into three categories, physicochemical traits, biological traits, and plant-microorganism-co-regulated traits. Principal component analysis (PCA) was used to determine the principal components of variables to represent these three categories (R. i386 3.6.3). The three traits were, respectively, represented by the first axis (principal component, PC1) of the three traits including six parameters (soil pH, water content, plant-available P concentration, inorganic N concentration, DON and DOC concentrations), five parameters (MBC, MBN, MBP, bacterial abundance and fungal abundance) and three parameters (phenolics, carboxylic anions, and mucilaginous compounds). Principal components retained more than 5% of the variance were used. A nonlinear regression was used to test the significance of the correlation between aboveground responses of functional groups and N-addition rates,

and resistance-agent-addition levels. We performed a linear regression to explore the correlations between aboveground plant responses with rhizosphere traits (PC1 scores). Pearson correlation analysis was used to examine the correlations between changes in aboveground biomass and root-sheath expansion.

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Legends

FIGURE 1 Aboveground responses of grassland plants to nitrogen (N) enrichment. Forbs (a), grasses (b) and sedges (c) in seven grassland ecosystems show contrasting responses to N enrichment. (d) Geographic and vegetation information of grassland ecosystems included in this study. Fitted lines indicate significant change of aboveground biomass to N addition rate (forbs: $r^2=0.386$, $p<0.001$, $n=130$; grasses: $r^2=0.401$, $p<0.001$, $n=100$; sedges: $r^2=0.433$, $p<0.001$, $n=128$).

FIGURE 2 Belowground rhizosphere size of grassland plants in natural habitats. Forbs, sedges and grasses exhibit different rhizosphere sizes in seven grassland ecosystems. The inset shows the average values of the rhizosphere size in forbs, sedges and grasses. Data are means \pm SD. Different letters represent significant difference of rhizosphere sizes among functional groups at $p<0.05$.

FIGURE 3 Correlations between rhizosphere expansion in response to nitrogen (N) enrichment and change in aboveground biomass of grasses and sedges. (a) Rhizosphere expansion of grasses and sedges in response to nitrogen (N) enrichment at three sites. Rhizosphere expansion is expressed as the log response ratio of rhizosphere size to N addition (see Methods). Data are means \pm SE. (b) Change in aboveground biomass of grasses and sedges positively correlated with rhizosphere expansion. Fitted lines represent significant correlations between change in aboveground biomass and rhizosphere expansion in Inner Mongolia (grasses: $r=0.944$, $p<0.001$, $n=25$; sedges: $r=0.870$, $p<0.001$, $n=25$), Tibet (grasses: $r=0.926$, $p<0.001$, $n=20$; sedges: $r=0.819$, $p<0.001$, $n=20$) and Sichuan (grasses: $r=0.938$, $p<0.001$, $n=15$; sedges: $r=0.840$,

$p < 0.001$, $n = 15$).

FIGURE 4 Soil properties in response to nitrogen (N) enrichment shaped by rhizosheaths of grasses and forbs in a range of grassland ecosystems. Property ①: Physicochemical properties of soil in the rhizosheath of forbs and grasses including soil pH, water and nutrient concentrations. Property ②: Biological properties of soil in the rhizosheath of forbs and grasses including microbial abundance and biomass. Property ③: Stress-avoiding properties of soil in rhizosphere of forbs and grasses based on the concentrations of protective compounds including polysaccharides, phenols and carboxylic anions.

FIGURE 5 Relationship between aboveground biomass and soil characteristics in the rhizosphere microhabitats of forbs and grasses. (a) Correlations between aboveground biomass of forbs and grasses and soil properties in their rhizosphere. (b) Addition of protective compounds to simulate the stress-avoiding properties of soil in the grass rhizosphere enhance forb tolerance to N enrichment-induced metal toxicity. Protective compounds mainly include carboxylic anions and polysaccharides. Red circles represent the average value of the control (no metal-ions and no protective compounds). Black squares represent the average value of the metal ion treatment (metal-ion addition, but no protective compounds). Statistics and fitted lines represent significance at $p < 0.05$ of addition of protective compounds.

Supporting Information

Table S1. Geographic, climatic and vegetation information of a range of grassland ecosystems compared in this study.

Table S2. Soil properties of different grassland ecosystems. SOC: soil organic carbon; DIN: dissolved inorganic nitrogen (N); AP: plant-available phosphorus (P); total alkaline ions: exchangeable potassium (K^+), calcium (Ca^{2+}) and magnesium (Mg^{2+}); total metal ions: exchangeable iron (Fe^{3+}), manganese (Mn^{2+}), copper (Cu^{2+}) and zinc (Zn^{2+}).

Table S3. The design and treatments of nitrogen (N)-addition experiments in different grassland ecosystems.

Table S4 The statistical analysis of soil parameters by linear mixed-effects models. N: nitrogen addition level; FG: functional groups (grasses and forbs); Wc: water content; IN: inorganic nitrogen; AP: available phosphorus; DON: dissolved organic nitrogen; DOC: dissolved organic carbon; BA: bacterial abundance; FA: fungal abundance; MBC: microbial carbon; MBN: microbial nitrogen; MBP: microbial phosphorus; MC: mucilaginous compounds (polysaccharides); TP: total phenols; OA: organic acids.

Figure S1 Microhabitat variation in the rhizosphere of grasses and forbs in response to nitrogen (N) addition in Duolun county in Inner Mongolia. Data are means \pm SE. Data are means \pm SE. Different letters represent significant difference of soil parameters among N treatments at $p < 0.05$.

Figure S2 Microhabitat variation in the rhizosphere of grasses and forbs in response to nitrogen (N) addition in Sichuan. Data are means \pm SE. Different letters represent

significant difference of soil parameters among N treatments at $p < 0.05$.

Figure S3 Microhabitat variation in the rhizosphere of grasses and forbs in response to nitrogen (N) addition in Tibet. Data are means \pm SE. Different letters represent significant difference of soil parameters among N treatments at $p < 0.05$.

Figure S4 Principal component analysis of soil properties in the rhizosphere of different functional groups (grasses and forbs).

Figure S5 Effects of addition of protective compounds released under nitrogen (N) enrichment on forb and grass growth. +Mn: manganese addition; +PC: addition of protective compounds; +PC+Mn: addition of both Mn and protective compounds.

Figure S6 Effects of the addition of protective compounds on shoot and root biomass of common forb and grass species. +Mn: manganese addition; +PC: addition of protective compounds; +PC+Mn: addition of both Mn and protective compounds. Data are means \pm SE ($n=5$). Different letters represent significant difference at $p < 0.05$ among treatments.

Figure S7 The link between rhizosphere variation and the aboveground response to nitrogen (N) deposition. Left, low-level N condition; Right, N-enrichment condition. Nitrogen enrichment causes rhizosphere expansion in grasses which shapes a different belowground microhabitat in their rhizosphere from that in forbs. Rhizosphere expansion confer grasses colonize belowground space. Rhizospheres modify soil properties and establish a buffer zone around grass roots by the release of the protective compounds including organic acids (OA) and mucilaginous compounds (MC, i.e. polysaccharides). These protective compounds enhance phosphorus (P) availability and

offset the toxicity of metals (manganese, Mn^{2+} and aluminum, Al^{3+}) by complexing the metal cations, and preventing phosphate sorption. The microhabitat variation accompanying rhizosheath expansion avoids grasses being affected by N enrichment-induced various stresses caused by N enrichment, and allows their aboveground parts to display positive responses to N enrichment.

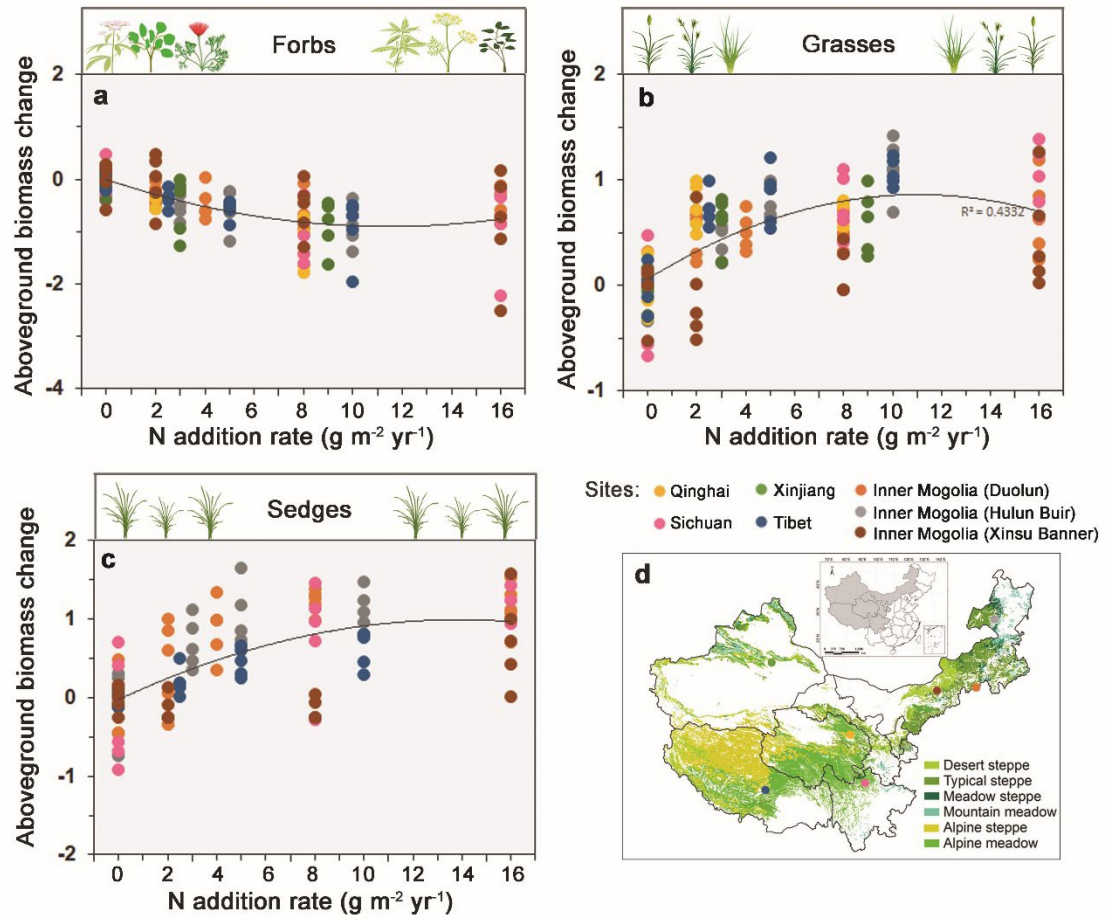


FIGURE 1

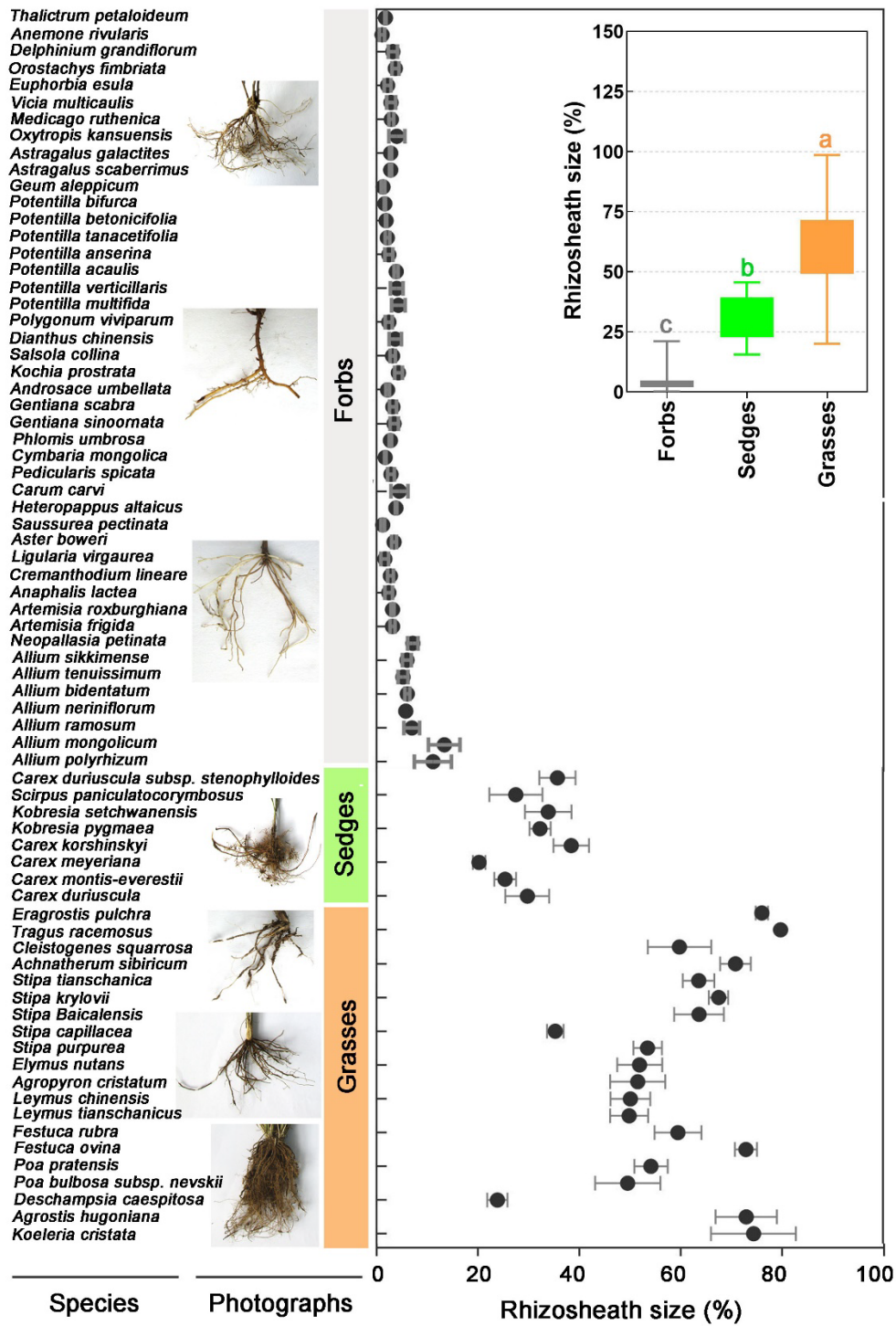


FIGURE 2

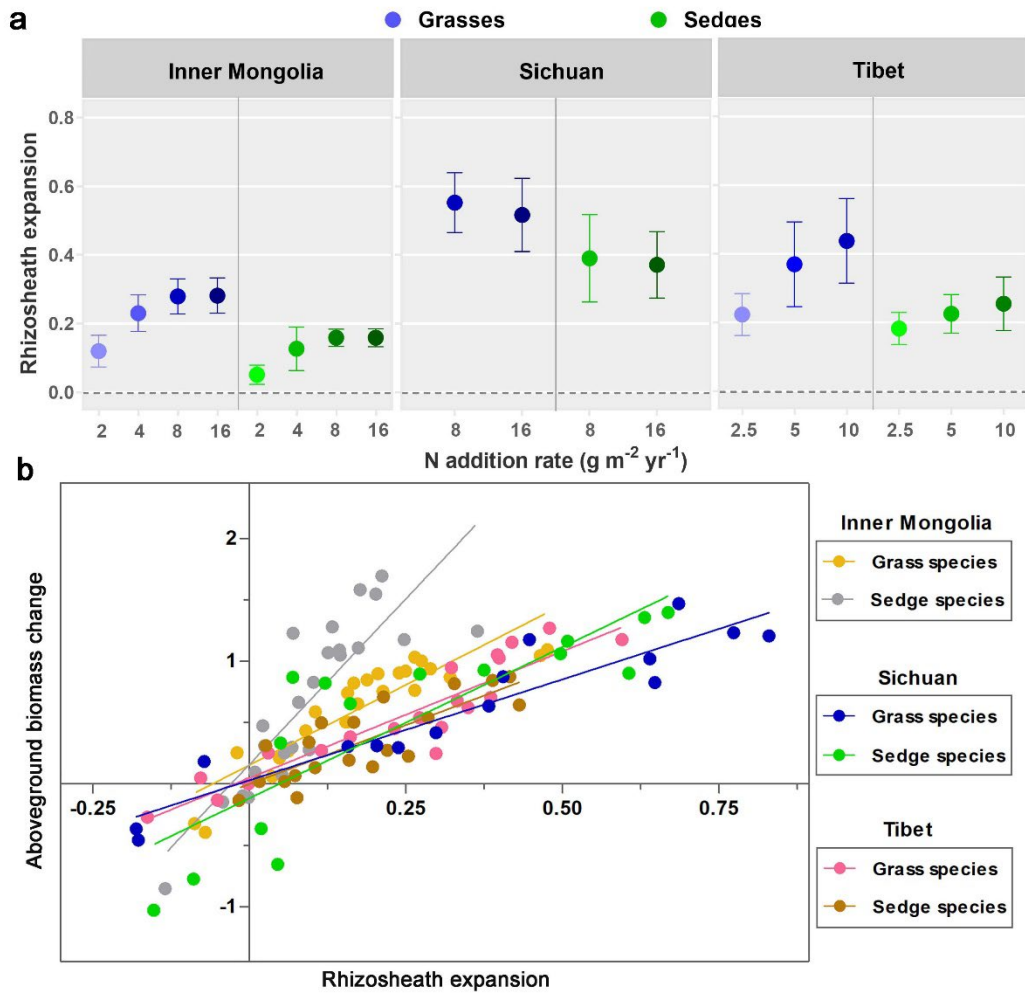


FIGURE 3

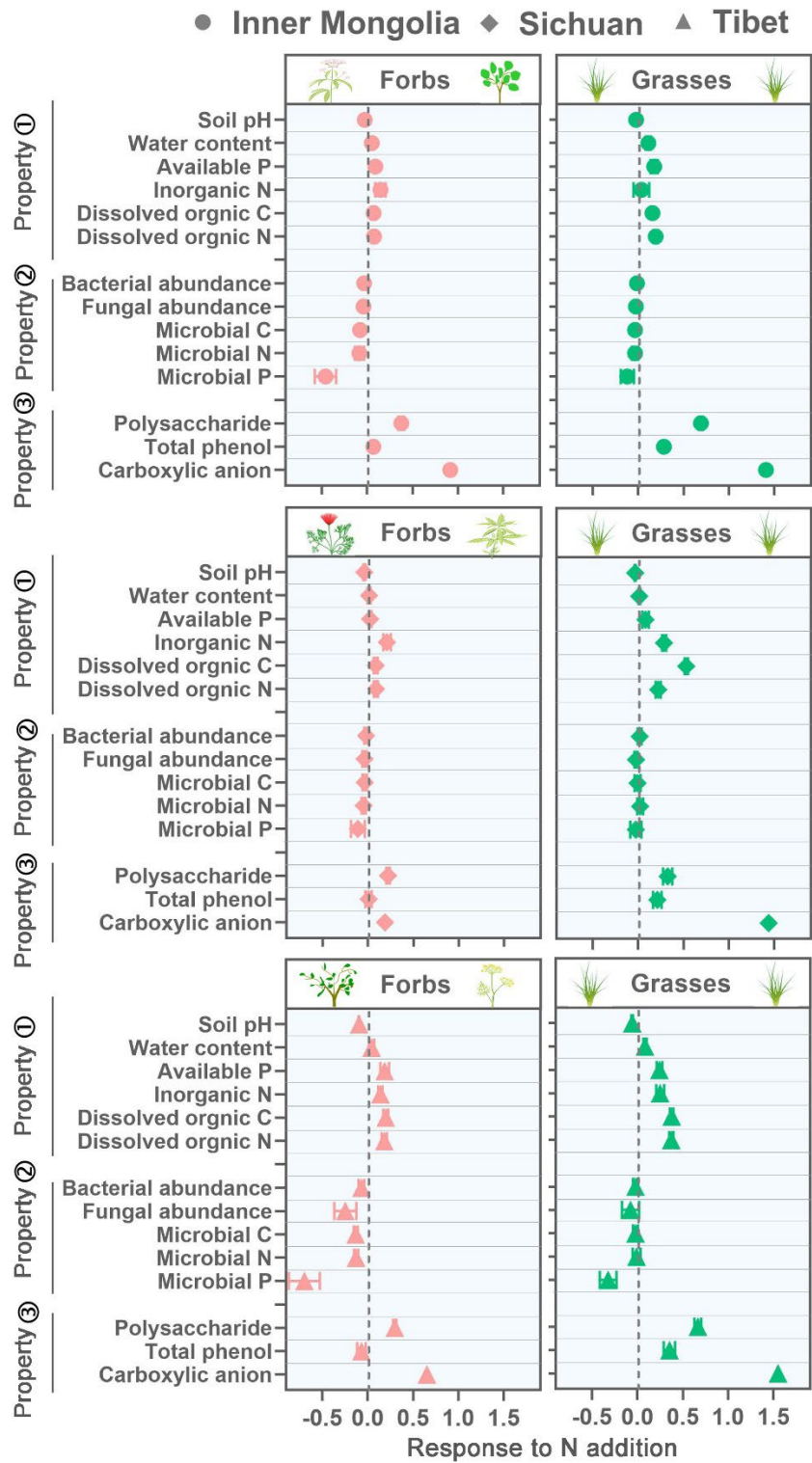


FIGURE 4

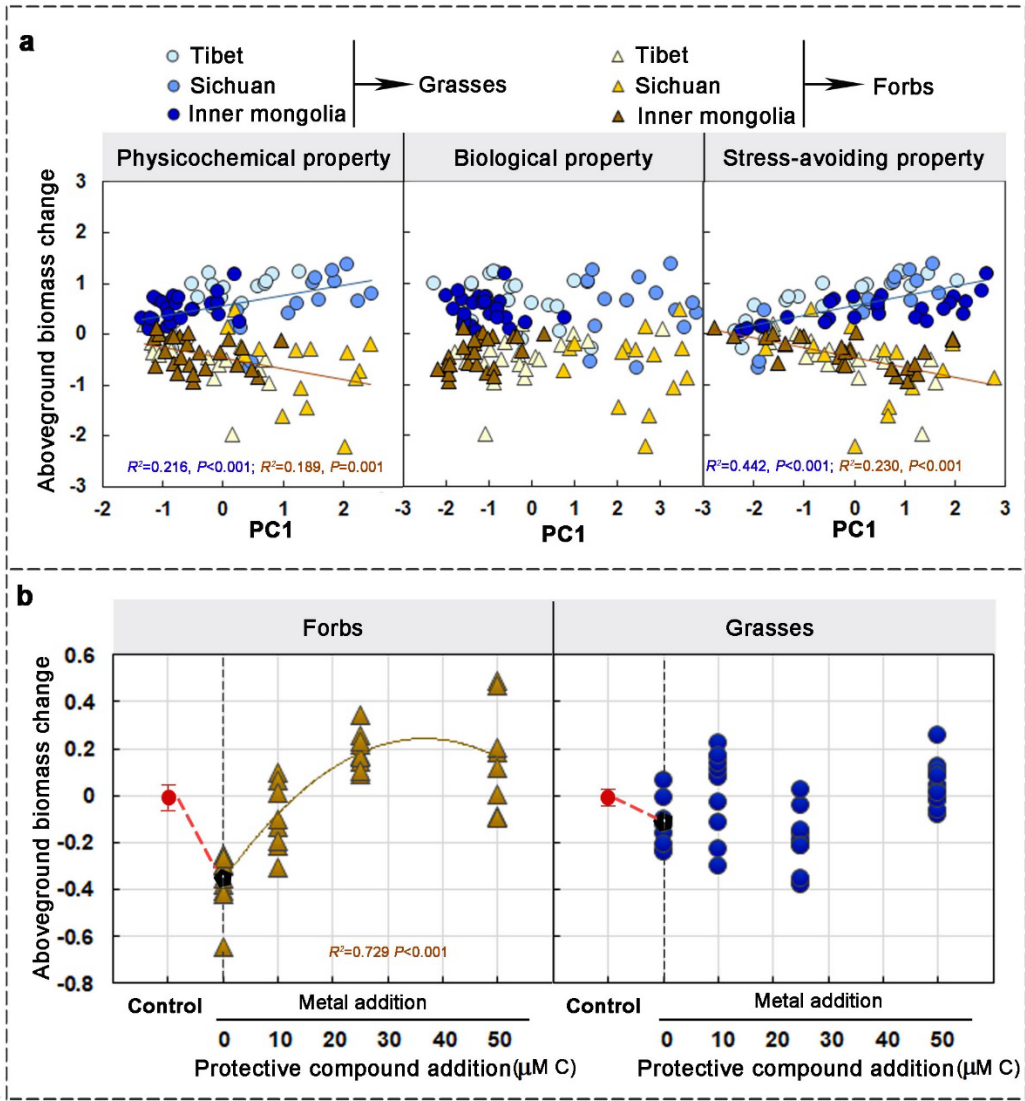


FIGURE 5