Effect of *Epichloë gansuensis* Endophyte on the Nitrogen Metabolism, Nitrogen Use Efficiency, and Stoichiometry of *Achnatherum inebrians* under Nitrogen Limitation

Jianfeng Wang,[†,‡] Zhibiao Nan,*[,†,‡] Michael J. Christensen,[§] Xingxu Zhang,[†,‡] Pei Tian,[†,‡] Zhixin Zhang,[†,‡] Xueli Niu,[†,‡] Peng Gao,[†] Tao Chen,[†,‡] and Lixia Ma[†,‡]

[†]The State Key Laboratory of Grassland Agro-ecosystems, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730020, Gansu, China

[‡]Key Laboratory of Grassland Livestock Industry Innovation, Ministry of Agriculture, China, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou 730020, Gansu China

[§]Retired scientist of AgResearch, Grasslands Research Centre, Private Bag 11-008, Palmerston North 4442, New Zealand

ABSTRACT: The systemic fungal endophyte of the grass *Achnatherum inebrians*, *Epichloë gansuensis*, has important roles in enhancing resistance to biotic and abiotic stresses. In this work, we first evaluated the effects of *E. gansuensis* on nitrogen metabolism, nitrogen use efficiency, and stoichiometry of *A. inebrians* under varying nitrogen concentrations. The results demonstrated that *E. gansuensis* significantly improved the growth of *A. inebrians* under low nitrogen conditions. The fresh and dry weights, nitrogen reductase, nitrite reductase, and glutamine synthetase activity, NO_3^-, NH_4^+, N, and P content, and also the total N accumulation, N utilization efficiency, and N uptake efficiency were all higher in leaves of *A. inebrians* with *E. ganusensis* (E+) plants than *A. inebrians* plants without this endophyte (E−) under low nitrogen availability. In conclusion, *E. gansuensis* has positive effects on improving the growth of *A. inebrians* under low-nitrogen conditions by modulating the enzymes of nitrogen metabolism and enhancing nitrogen use efficiency.

KEYWORDS: *Achnatherum inebrians, Epichloë endophyte, low-nitrogen, nitrogen metabolism, nitrogen use efficiency, stoichiometry*

■ INTRODUCTION

Nitrogen (N) is an essential macronutrient for plant growth and development, and so low N is one of the most important factors limiting plant growth and productivity in both natural and agricultural environments.[1] To enhance crop yields, substantial amounts of N fertilizers are applied. However, plants uptake less than half of the N fertilizers applied, with the great majority lost to the atmosphere or dissolved in groundwater.[2] Extensive studies have been performed on plant biomass, nitrate uptake, and root architecture to link these factors with growth under low-nitrogen conditions.[3,4] Nevertheless, there is a shortage of information about the fine-tuning of plant nutrient content under low-nitrogen conditions. Understanding the changes in grass growth in low-nitrogen conditions can point to the direction for improving the utilization of N.[5,6]

Leaf and root stoichiometry, especially for carbon (C), nitrogen (N), and phosphorus (P), play a key role in analyzing the composition, structure, and function of a plant community and ecological system.[7−9] The ratio of C:N:P is an important index to reflect plant growth states.[10] C:N:P stoichiometry has been widely applied in diverse ecological processes and successfully incorporated to explain many phenomena at all levels of biology.[11,12] It has been reported that C:N and C:P were important indicators for reflecting the health of plants and their growth status, and a negative correlation between plant growth rates and ratios of C:N and C:P was developed.[13,14] Through analyzing N and P content and the corresponding ratio of N:P, the limiting elements of organism growth,

development, and reproductive growth stages can be effectively identified.[15] A change in the N:P ratio is thought to be related to nutrient limitations during the plant growth process and that it might alter the competitiveness of the species depending on its growth rate and lifestyle.[16]

A wide range of microorganisms can assist plants to take up nutrients from the soil. These include mycorrhizal fungi, in particular arbuscular mycorrhizal fungi that assist in the uptake of P from soil.[17] Recently, a study reported that magnesium fertilizer induced an increase of symbiotic microorganisms and improved forage growth and quality.[18] Further, a previous study showed that endophytic microbes improved plant growth and zinc accumulation in grains of rice (*Oryza sativa* L.).[19] It was also reported that *Bacillus amyloliquefaciens* GB03 improved growth and metabolite accumulation in *Codonopsis pilosula* (Franch.) Nannf.[20] Another class of microorganisms that enhance the uptake of nutrients by host plants are the fungal endophytes belonging to the genus *Epichloë*. *E. coenophila* in tall fescue (*Festuca arundinacea*) that affected growth and mineral uptake and transport and efficiency ratios.[21] Similarly, the fungal endophyte *E. festucae* altered the nutrient content of *F. rubra* host plants, and endophyte-infected (E+) plants contained more P (62%) and N (19%) in their shoots than endophyte-free (E−) plants.[22] In addition, this fungal

endophyte altered the nutrient content of *F. rubra* host plants regardless of water availability.[22] Recently, it had been reported that E+ *Hordeum brevisubulatum* plants had higher N and P content than E− plants under salt stress.[7] To the best of our knowledge, the roles of *Epichloë* endophytes on C, N, and P stoichiometry of host grasses are little understood.

Many cool-season grasses of the subfamily Pooideae are host of these fungal endophytes of the genus *Epichloë*, the asexual members of which were previously classified as *Neotyphodium* spp.[23] The associations between grasses and *Epichloë* endophytes are considered in general to be mutualistic.[24] Their presence can significantly increase plant tolerance to environmental stresses such as drought,[25] salt,[7] heat,[26] heavy metal,[27] insects,[28] diseases[29] and waterlogging.[30] These fungi have become so adapted to this internal plant habitat that they are never found anywhere else in nature. Key points about the association between the endophytic fungi and their host grasses are that the hyphae are intercellular, their growth is fully synchronized with that of the grass, and all tissues of the host plants are colonized except for the roots.[31] In view of such a close association between the host grass and fungal endophyte, it is perhaps not surprising that the uptake and utilization of ions and their subsequent storage are enhanced. With these complex functions, fungal endophytes can affect the economic value of forage production in natural rangelands and sown pastures, and deserve further study to explore possible applications.

To investigate the role that an *Epichloë* endophyte can have on the C:N:P stoichiometry of a host grass, we utilized the grass *Achnatherum inebrians*. This grass, commonly called drunken horse grass from the effects it has on animals that attempt to eat it,[32] is found throughout the vast arid to semiarid grasslands of northwest China.[32,33] The conditions where it grows are harsh, and the soil is nutrient poor. Nearly all plants growing in these grasslands are infected with a species of *Epichloë*. We utilized *E. gansuensis* infected (E+) and endophyte-free (E−) *A. inebrians* for this study. We first evaluated the role of *E. gansuensis* endophyte on growth of *A. inebrians* plants under low nitrogen conditions. We considered it likely that the presence of the endophyte would enhance nitrogen uptake and thus alter the ratios of C:N, C:P, and N:P compared within endophyte-free plants under low-nitrogen growth conditions.

The aim of this study is to gain new understanding of effects from the presence of the mutualistic systemic seed-borne fungal endophyte *E gansuensis* on *A. inenbrians* plants growing in a low-available nitrogen environment as E+ plants of this species are becoming abundant in the low-fertility arid/semiarid grassland of northwest China. The grasslands of northwest China are not alone in being overgrazed, resulting in ecosystem degeneration with a resulting low soil fertility including N content, and the presence of an *Epichloë* endophyte may enable host grasses to thrive under these conditions. This study will complement other studies that have shown that the presence of *E. gansuensis* confers advantages in some other abiotic stresses as well as biotic stresses. With the proven value of *Epichloë* endophytes in livestock grazing based on perennial ryegrass (*Lolium* perenne) and tall fescue, further research into the mechanisms that serve to enhance host grasses when growing in stress conditions that may occur in natural grasslands and sown pastures is justified. The effect of the presence of an *Epichloë* endophyte on the nitrogen metabolism and nitrogen-use efficiency of host plants under low nitrogen environment is a factor that has not yet been investigated. We propose that the presence of an *Epichloë* endophyte will provide a benefit to plants growing in low availability nitrogen of soil, and the evidence will be obtained through the use of a pot-based study with plants being exposed to the low levels of nitrogen.

■ MATERIALS AND METHODS

Plant Growth Conditions and Nitrogen Treatments. We collected seed from *E. gansuensis* infected (E+) and endophyte-free (E−) *A. inebrians* plants that were grown in an experimental field of the College of Pasture Agriculture Science and Technology, Yuzhong campus of Lanzhou University in 2013. Seed samples from each plant of 2013 were maintained at 4 °C. We used seed originating from a single E+ and E− plant to reduce variability within our plant material at the start of the study. In September 2015, seeds from the 200 E+ and from the 200 E− *A. inebrians* were collected, bulked as E+ or E−, and stored at a constant 4 °C for the present study. A pot experiment was performed from 2 June to 15 October 2016 in the greenhouse of the College of Pastoral Agriculture Science and Pastoral Agriculture Science and Technology, Yuzhong campus of Lanzhou University (104°39′ E, 35°89′ N, altitude 1653 m). Before planting, 30 seeds were selected randomly from the E+ and E− seeds and were used to confirm the infection status of the seeds used to establish this part of the study. The result showed that all 30 E+ seeds were endophyte-infected, and the 30 E− seeds were all endophyte-free. On June 2, healthy-looking and well-filled seeds were sown in 120 pots (60 pots for E+ plants and 60 pots for E− plants), with 6 seeds per pot, filled with vermiculite (75 g) that had been sterilized in an oven at 150 °C for 3 h. After germination, each pot (10 cm in lower diameter ×18.5 cm in upper diameter ×19.5 cm in height) was thinned to three uniform seedlings. These pots were placed in two sets of trays, each containing 6 pots. All trays were irrigated with distilled water every 7 days. Following the appearance of the second fully expanded leaf, 1/2 strength Hoagland's solution was applied to the pots every 7 days. These trays were assigned at random to a position within a constant temperature greenhouse (temperature: 26 ± 2 °C, moisture: 42 ± 2%). After 45 days, we selected 36 pots of E+ plants and 36 pots of E− plants with uniform growth for use in the nitrogen concentration study. Twelve pots of E+ plants and 12 pots of E− plants were treated with one of these three modified 1/2 strength Hoagland nutrient solutions that contained 0.1 mM, 1 mM, and 7.5 mM nitrogen. 150 mL of 1/2 strength Hoagland's solution containing these three different nitrogen concentrations was applied to the appropriate treatment pots every 7 days. The infection status of each seedling in the study was confirmed by microscopic examination of leaf sheath pieces stained with aniline blue. Each N concentration treatment was applied to both E+ and E− plants, and each treatment included 12 replications.

Dry Weight and Enzymatic Activity Determination. After receiving the different nitrogen concentration treatments for 90 days, all the plants, from all the treatments, were carefully removed from the growth medium and washed with distilled water before being manually separated into root and leaf lamina parts. To determine leaf lamina and root dry weights, one plant of each pot of every treatment was oven-dried at 80 °C until a constant weight was reached. To determine enzymatic activity, leaves (excluding the pseudostems) and roots from the remainder of plants of each treatment were separately collected and were immediately frozen in liquid nitrogen and stored at −80 °C.

Determination of NO_3^- Content. NO_3^- content was determined by the colorimetric method utilizing salicylic acid according to Cataldo et al.,[34] with some modifications. In brief, 0.3 g of leaves and roots, respectively, were placed in 5 mL of H_2O for the extraction of NO_3^- and boiled for 30 min. After centrifuging at 13,000g for 15 min, 0.2 mL of the supernatants were pipetted into 50-mL Erlenmeyer flasks and mixed thoroughly with 0.8 mL of 5% (w/v) salicylic acid in concentrated H_2SO_4. After 20 min at room temperature, 19 mL of 2 N NaOH was added slowly into the flasks. Samples were cooled to room temperature, and NO_3^- contents were determined at 410 nm.
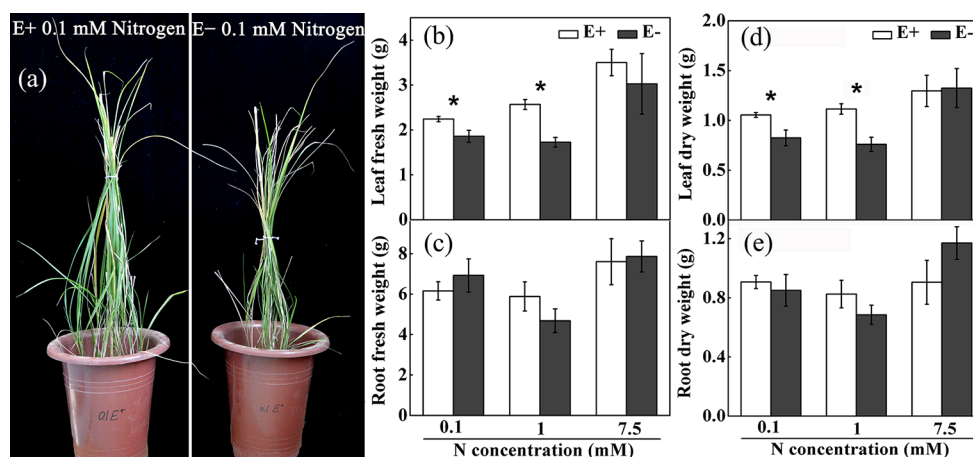
Figure 1. Phenotype of E+ and E− *Achnatherum inebrians* plants under 0.1 mM nitrogen for 90 days (a). Leaf fresh weight (b), root fresh weight (c), leaf dry weight (d), and root dry weight (e) of *A. inebrians* plants with and without endophyte under different nitrogen concentration treatments. Data presented are the means ± SE of four independent biological replicates. Asterisk (*) means significant difference ($P < 0.05$) between E+ and E− plants.

Determination of NH_4^+ Content. The homogenate was centrifuged at 25000g (2 °C) for 10 min, and the supernatant was transferred to 500-mL 0.45 mm polysulphone centrifugation filters (Micro VectraSpin; Whatman Ltd., Maidstone, UK) and spun at 5000g (2 °C) for 5 min. NH_4^+ content was determined by the ninhydrin colorimetric method according to Rosen,[35] with some modifications. In brief, 0.3 g of leaves and roots, respectively, were placed in 5 mL of 10% acetate for the extraction of NH_4^+, later made up to 50 mL with distilled water, following which they were filtered. One milliliter of each supernatant was pipetted into 10-mL centrifuge tubes and mixed thoroughly with 3 mL of ninhydrin reagent (1.1 g of ninhydrin was placed in a beaker, 15 mL of propanol was added, shaken, and dissolved, and then 30 mL of butyl alcohol and 60 mL of glycol were added, mixed, following which 9 mL of acetate buffer pH 5.4 was added and the solution mixed) and 0.1 mL of 1% ascorbic acid and boiled for 15 min. After samples were cooled, 4 mL of ethanol was added into centrifuge tubes. NH_4^+ content was determined at 580 nm.

Determination of C, N, and P Content. Total organic C content was determined with the oil bath-K_2CrO_7 titration method (oxidization with dichromate in the presence of H_2SO_4, heated at 180 °C for 5 min and titrated with $FeSO_4$).[36] Total N and P contents in the plant samples were assayed by flow injection analysis (FIAstar 5000 Analyzer, FOSS, Sweden), using H_2SO_4 for digestion for 2 h in 420 °C, and K_2SO_4 and $CuSO_4·5H_2O$ (K_2SO_4:$CuSO_4·5H_2O$ = 10:1) as the catalyzer. Data on C, N, and P content from the samples were then used to calculate the C:N, C:P, and N:P ratios of the leaves and roots, expressed as mass ratios. Additionally, total N accumulation (TNA), nitrogen utilization efficiency (NUtE), and nitrogen uptake efficiency (NUpE) were calculated as follows: (1) total N accumulation (TNA) was calculated as the N concentration × total plant dry weight (mg N);[37] (2) nitrogen utilization efficiency (NUtE) was calculated as the total plant dry weight divided by N concentration (g^2 TDW mg^{-1} N);[38] (3) the nitrogen uptake efficiency (NUpE) was calculated as TNA divided by root dry weight (mg N g^{-1} RDW).[39]

Nitrate Reductase Activity Assay. Nitrate reductase (NR) activity was determined according to the method described by Du et al.,[40] with some modifications. In brief, about 0.3 g of leaves and roots, respectively, were ground in an extract buffer containing 100 mM Hepes-KOH (pH 7.5), 3% PVPP, 1 mM EDTA, and 7 mM cysteine, and the homogenate was centrifuged at 10,000g for 10 min at 4 °C. An extract of 1 mL was incubated in a 5 mL reaction mixture buffer [50 mM Hepes-KOH (pH 7.5), 100 μM NADH, 2 mM EDTA, and 5 mMKNO_3]. For the negative control, another batch of the identical NR reaction mixture was added with 500 μL of 30% trichloroacetic acid to denature NR activity. NR reaction mixtures and negative controls were incubated at 25 °C for 30 min in the dark. Nitrate reduction in the NR reaction mixtures was stopped by the addition of

500 μL of 30% trichloroacetic acid. After the addition of 2 mL of 1% sulphanilamide and 2 mL of 0.02% N-(1-naphtyl)-ethylene-diamine, dihydrochloride nitrite in the NR reaction mixtures and negative controls were determined with a spectrophotometer at 540 nm.

Nitrite Reductase Activity Assay. Nitrite reductase (NiR) activity was determined according to the method described by Datta and Sharma,[41] with some modifications. In brief, about 0.3 g of leaves and roots, respectively, were homogenized in 3.8 mL of 50 mM potassium phosphate (pH 8.8) buffer consisting of 1 mM EDTA, 25 mM cystein, and 3% (w/v) bovine serum albumin (BSA), and the homogenate was centrifuged at 13,000g for 15 min at 4 °C. The assay mixture consisted of 1.4 mL of 100 mM potassium phosphate buffer (pH 8.8), 100 μL of 5 mM KNO_2, 200 μL of enzyme extract, and 100 μL of methyl viologen (20 mg/mL). The volume was made up to 1.8 mL with distilled water. To start the assay, 200 μL of sodium dithionite (25 mg/mL in 200 mM $NaHCO_3$ solution) was added and incubated for 30 min at 30 °C. At the end of the incubation period, 100 μL of the assay mixture was added to 6 mL of water and vortexed immediately to oxidize the dithionite. The amount of nitrite used by nitrite reductase was estimated by adding 1 mL of sulfanilamide [1% (w/v) in 3 N HCl] and 1 mL of 0.05% (w/v) NED solution, and the absorbance was read at 540 nm.

Glutamine Synthetase Activity Assay. Glutamine synthetase (GS) activity was determined according to the method described by Oaks et al.[42] with some modifications. In brief, about 0.3 g of leaves and roots, respectively, were ground in the extract buffer containing 50 mM Tris-HCl (pH 8.0), 2 mM $MgSO_4$, 2 mM DTT, and 400 mM sucrose, and the homogenate was centrifuged at 13,000g for 20 min at 4 °C. The extract of 0.7 mL was incubated in a 1.6 mL reaction mixture buffer [100 mM Tris-HCl (pH 7.4), 2 mM EGTA, 80 mM $MgSO_4$, 20 mM glutamate, 80 mM hydroxylamine, and 20 mM cysteine], and then this was added to 0.7 mL 40 mM ATP. GS reaction mixtures were incubated at 37 °C for 30 min after 1 mL of the chromogenic reagent (200 mM TCA, 370 mM $FeCl_3$ and 600 mM HCl) had been added, mixed well, and centrifuged at 7000g for 10 min, and then the GS activity was determined with a spectrophotometer at 540 nm.

Statistical Analysis. Data analyses were performed with SPSS version 17.0 (SPSS, Inc., Chicago, IL). Two-way ANOVA was used to determine the effects of the different N treatments and endophyte on C, N, P content, ratios of C:N, C:P and N:P, as well as TNA, NUtE, and NUpE. The significance of difference between E+ and E− plants in all of the parameters was carried out by independent t tests. Statistical significance was defined at the 95% confidence level. Means are reported with their standard errors.
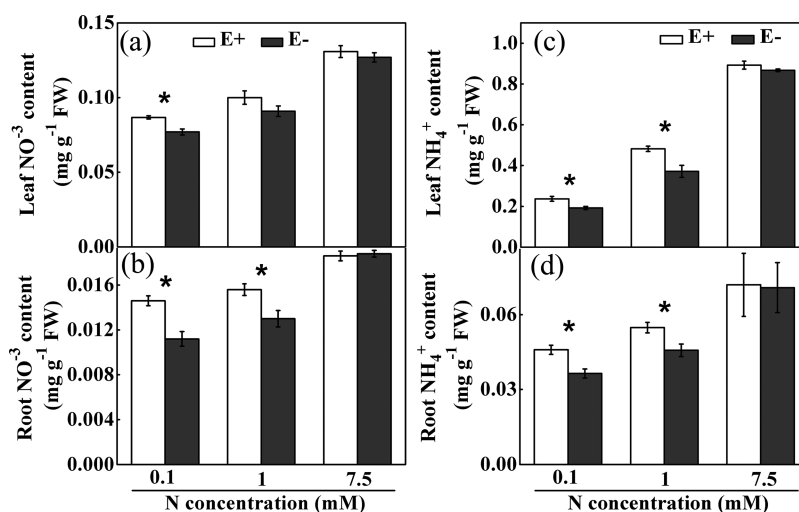
Figure 2. NO_3^- and NH_4^+ content of leaves and roots of E+ and E− plants under different nitrogen concentration treatments. NO_3^- content of leaves (a) and roots (b), NH_4^+ content of leaves (c) and roots (d) of E+ and E− plants. Data presented are the means ± SE of three independent biological replicates. Asterisk (*) means significant difference ($P < 0.05$) between E+ and E− plants.

■ RESULTS

Fresh and Dry Weight of Leaves and Roots of E+ And E− Plants under Low-Nitrogen Conditions. After treatment with the three concentrations of external nitrogen for 90 days, differences in the phenotypes of the E+ and E− plants under 0.1 mM N concentration were evident to the naked eye (Figure 1a). The results indicated that compared with the 7.5 mM nitrogen treatment, the fresh and dry weight of leaves and roots of *A. inebrians* decreased under the 0.1 mM and 1 mM nitrogen concentrations (Figure 1). The fresh and dry weight of roots of E+ and E− *A. inebrians* plants showed no significant differences under 0.1 mM, 1 mM, and 7.5 mM nitrogen conditions (Figure 1c and e) and no significant differences in fresh and dry weight of leaves under the 7.5 mM N treatment (Figure 1b and d). However, compared with 7.5 mM nitrogen, the availability of just 0.1 and 1 mM nitrogen had significant effects on the fresh and dry weight of leaves between E+ and E− plants, with E+ plants having larger fresh and dry leaf weights than E− plants (Figure 1b and d). For example, with the 0.1 mM nitrogen treatment, the fresh weight of leaves of E+ plants was 2.24 ± 0.058 g, but the fresh weight of the leaves of E− plants was only 1.86 ± 0.132 g. The dry weight of leaves of E+ plants was 1.06 ± 0.024 g, but the dry weight of leaves of E− plants was 0.83 ± 0.079 g. With the 1 mM nitrogen treatment, the fresh weight of leaves of E+ plants was 2.57 ± 0.110 g, but the fresh weight of the leaves of E− plants was only 1.73 ± 0.106 g; the dry weight of leaves of E+ plants was 1.12 ± 0.053 g, but the dry weight of leaves of E− plants was 0.76 ± 0.072 g. However, there were no significant differences between the fresh and dry weights of the roots of E+ plants and the fresh and dry weights of the roots of E− plants under 0.1, 1, and 7.5 mM N concentrations (Figure 1c and e).

NO_3^- and NH_4^+ Content of Leaves and Roots. Low nitrogen reduced NO_3^- content in the leaves and roots of E+ and E− plants, and the NO_3^- content of leaves and roots of *A. inebrians* increased as the nitrogen concentration increased (Figure 2). Further, our results showed that the NO_3^- content of leaves and roots of E+ plants was higher than that of E− plants counterparts under the 0.1 mM nitrogen concentration (Figure 2), but the NO_3^- content of leaves and roots had no significant difference between E+ plant and E− plant under the

7.5 mM nitrogen concentration (Figure 2). For example, the NO_3^- content of leaves of E+ plants was increased by 12.6% compared with E− plants in the presence of the 0.1 mM nitrogen concentration (Figure 2a). Similarly, the NO_3^- content of roots of E+ plants was increased by 30.4% and 20.0% compared with E− plants in the presence of the 0.1 and 1 mM nitrogen concentration, respectively (Figure 2b). Taken together, these results indicated that the endophyte could increase NO_3^- contents accumulation under low nitrogen conditions. Obviously, the endophyte affected the activity of key enzymes of nitrogen metabolism during low nitrogen adaption. Hence, we examined the NH_4^+ content in leaves and roots of the E+ and E− plants. Our data showed that the NH_4^+ content of leaves and roots of *A. inebrians* increased as the nitrogen concentration increased (Figure 2), but leaves and roots of E+ plants had higher NH_4^+ content than E− plants counterparts under the 0.1 and 1 mM nitrogen concentrations (Figure 2c and d). Further, our results showed that the NH_4^+ content of leaves of E+ plants was increased by 23.3% and 29.8% compared with E− plants under the 0.1 and 1 mM nitrogen concentrations, respectively (Figure 2c). Similarly, the NH_4^+ content of roots of E+ plants was increased by 26.1% and 19.9% compared with E− plants under the 0.1 and 1 mM nitrogen concentrations, respectively (Figure 2d). However, the NH_4^+ content of the leaves and roots had no significant difference under the 7.5 mM nitrogen concentration (Figure 2).

NR Activity in Leaves and Roots. The endophyte-infection caused a significant impact on the response of the NR activity to low nitrogen treatments, and endophyte-containing plants had higher NR activity compared to their noninfected counterparts. For example, the NR activity of leaves of E+ plants was increased by 22.4% and 11.1% compared with E− plants under the 0.1 and 1 mM nitrogen concentrations, respectively (Figure 3a). Similarly, the NR activity of roots of E+ plants was increased by 8.1% and 10.7% compared with E− plants under the 0.1 and 1 mM nitrogen concentrations, respectively. But NR activities in leaves and roots were not different under the 7.5 mM nitrogen concentration (Figure 3a and b).

NiR Activity in Leaves and Roots. There were nitrogen effects on the NiR activity in both the leaves and roots samples.
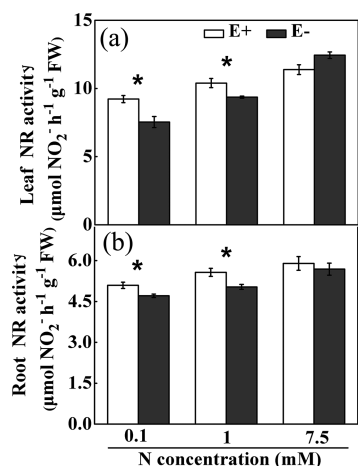
4025

Figure 3. NR activity in leaves (a) and roots (b) of *Achnatherum inebrians* with and without endophyte under different nitrogen concentration treatments. Data presented are the means ± SE of three independent biological replicates. Asterisk (*) means significant difference ($P < 0.05$) between E+ and E− plants.

The NiR activity of E+ and E− plants declined as the nitrogen concentrations decreased (Figure 4). However, E+ plants had
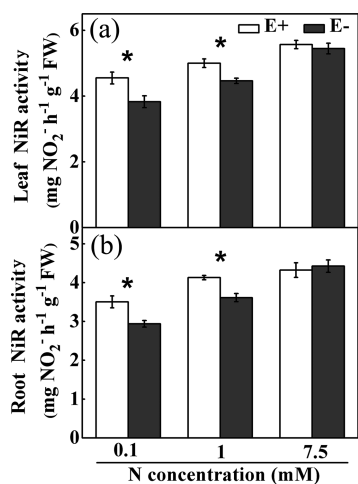

Figure 4. NiR activity in leaves (a) and roots (b) of *Achnatherum inebrians* with and without endophyte under different nitrogen concentration treatments. Data presented are the means ± SE of three independent biological replicates. Asterisk (*) means significant difference ($P < 0.05$) between E+ and E− plants.

higher NiR activity in leaves and roots compared to their noninfected counterparts under the 0.1 and 1 mM nitrogen concentrations (Figure 4a and b). For example, the NiR activity of leaves of E+ plants was increased by 18.9% and 12.0% compared with E− plants in the presence of the 0.1 and 1 mM nitrogen concentrations, respectively (Figure 4a). A similar trend of NiR activity change was present in roots, and the NiR activity of roots of E+ plants was increased by 19.2% and 14.3% compared with E− plants in the presence of 0.1 and 1 mM nitrogen concentrations, respectively (Figure 4b). But there was no obvious difference in NiR activity in leaves and roots under the 7.5 mM nitrogen concentration (Figure 4a and b).

GS Activity in Leaves and Roots. The GS activity in leaves and roots of the E+ and E− plants was significantly influenced by the presence of 0.1 and 1 mM nitrogen compared

with 7.5 mM nitrogen. Further, the GS activity of the leaves and roots of E+ plants was higher than in E− plants counterparts under the 0.1 mM nitrogen concentration (Figure 5). The GS
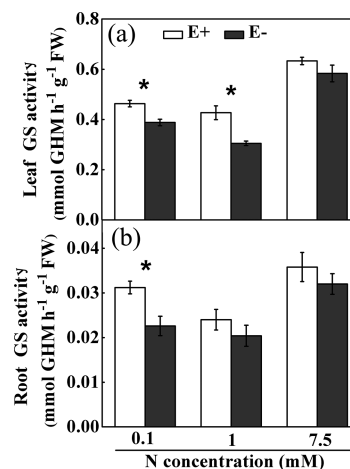

Figure 5. GS activity in leaves (a) and roots (b) of *Achnatherum inebrians* with and without endophyte under different nitrogen concentration treatments. Data presented are the means ± SE of three independent biological replicates. Asterisk (*) means significant difference ($P < 0.05$) between E+ and E− plants.

activity of E+ leaves was higher compared to their noninfected counterparts under the 1 mM nitrogen concentration. Further, the GS activity in leaves of E+ plants was increased by 19.4% and 39.9% compared with E− plants under the 0.1 and 1 mM nitrogen concentrations, respectively (Figure 5a). There was a similar trend of change of GS activity in roots, and the GS activity of roots of E+ plants was increased by 38.1% compared with E− plants under the 0.1 mM nitrogen (Figure 5b). However, the GS activity of leaves and roots had no significant difference under the 7.5 mM nitrogen concentration (Figure 5).

C, N, and P Content. There were significant low-nitrogen treatment effects on the organic C content in leaf samples: the C content increased as the nitrogen concentration increased (Figure 6a), but there were no significant effects on C content in root samples (Figure 6d). The endophyte × nitrogen concentrations interaction did not have an influence on the C content in either the leaves ($P = 0.148$) or roots ($P = 0.145$, Table 1). The N content increased in both the leaves and roots as nitrogen concentration increased, and infected plants had a greater content of N in leaves and roots than E− plants under the 0.1 mM and 1 mM nitrogen concentrations (Figure 6b and e). However, nitrogen content was not significantly different between the E+ and E− plants under the 7.5 mM nitrogen concentration, with both the leaves and roots (Figure 6b and e). The presence of the endophyte in plants had a significant impact on the nitrogen content of roots ($P = 0.031$, Table 1). However, the endophyte × nitrogen concentrations interaction had no obvious differences in leaves ($P = 0.231$) and roots ($P = 0.063$, Table 1). Compared with the 7.5 mM nitrogen concentration, the P content significantly decreased under the 0.1 mM and 1 mM nitrogen concentrations (Figure 6c and f), but E+ plants had a greater content of P in leaves and roots than E− plants under the 0.1 mM nitrogen (Figure 6c and f), and E+ plants had higher P content in roots than E− plants under 1 mM nitrogen (Figure 6f). The presence of endophyte in plants had a significant impact on the response of the P content of roots ($P < 0.001$, Table 1), but for P content,
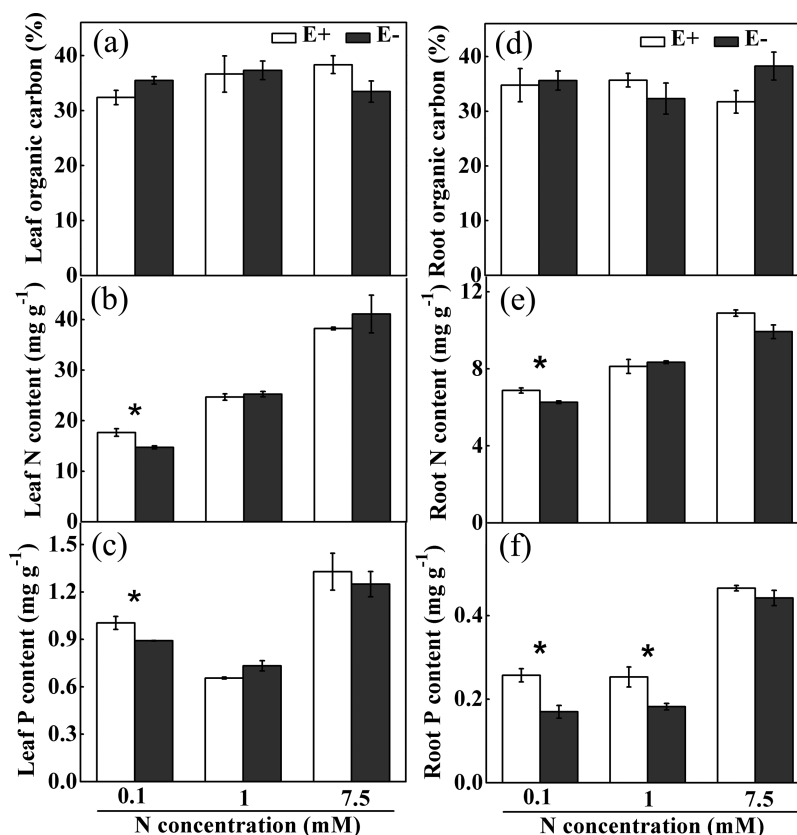
Figure 6. C, N, and P content in leaves (a, b, c) and roots (d, e, f) of *Achnatherum inebrians* with and without endophyte under different nitrogen concentration treatments. Data presented are the means ± SE of three independent biological replicates. Asterisk (*) means significant difference (*P* < 0.05) between E+ and E− plants.

Table 1. Results of Two-Way ANOVA for the Effects of Endophyte (E) and Nitrogen Concentrations (N) on C, N, and P Content in the Leaves and Roots of *Achnatherum inebrians*

	Treatments	df	C content		N content		P content	
			F-value	*P*	F-value	*P*	F-value	*P*
Leaf	E	1	0.055	0.818	0.017	0.899	0.580	0.461
	N	2	1.282	0.313	109.380	<0.001	46.985	<0.001
	E * N	2	2.249	0.148	1.661	0.231	1.351	0.296
Root	E	1	0.485	0.500	5.928	0.031	22.300	<0.001
	N	2	0.151	0.862	142.332	<0.001	152.897	<0.001
	E * N	2	2.273	0.145	3.515	0.063	2.216	0.152

endophyte × nitrogen concentrations interaction had no obvious differences in leaves (*P* = 0.296) and roots (*P* = 0.152, Table 1).

Nitrogen Use Efficiency. E+ plants had higher total nitrogen accumulation (TNA) than E− plants under the 0.1 mM and 1 mM nitrogen concentrations (Figure 7a), and E+ plants had higher nitrogen utilization efficiency (NUtE) and nitrogen uptake efficiency (NUpE) than E− plants under the 1 mM and 0.1 mM nitrogen concentrations, respectively (Figures 7b and c). Further, the endophyte × nitrogen concentrations interaction was not significantly different in TNA (*P* = 0.256) and NUpE (*P* = 0.982, Table 2), but the endophyte × nitrogen concentration interaction was different in NUtE (*P* = 0.023, Table 2). Also, endophyte-infection caused a significant difference on NUtE (*P* = 0.002, Table 2).

Ratios of C:N, C:P, and N:P. The ratio of C:N declined as the nitrogen concentration increased in both the leaves and roots of *A. inebrians* (Figure 8a and d). Further, our results

showed that E+ plants had lower C:N ratios in leaves than in E− plants under the 0.1 mM nitrogen concentration (Figure 8a). However, with the 1 mM and 7.5 mM nitrogen concentrations, there was no significant difference in the C:N ratios between the leaves and root of E+ and E− plants (Figure 8a and d). For the C:N ratios, our data showed that endophyte × nitrogen concentration interaction was significantly different in leaves (*P* = 0.01, Table 3), but not significantly different in roots (*P* = 0.132, Table 3). The C:P ratios had differences between the leaves of E+ and E− plants under the 0.1 mM nitrogen concentration, but there were no differences under the 1 mM and 7.5 nitrogen concentrations. Further, the data showed no differences between the roots of E+ and E− plants under the 0.1 mM, 1 mM, and 7.5 mM nitrogen concentrations (Figure 8b and e). Results of two-way ANOVA for the effects of endophyte and nitrogen concentrations showed that C:P ratios had no significant difference in leaves (*P* = 0.197) and roots (*P* = 0.173, Table 3), but the endophyte-infection had a
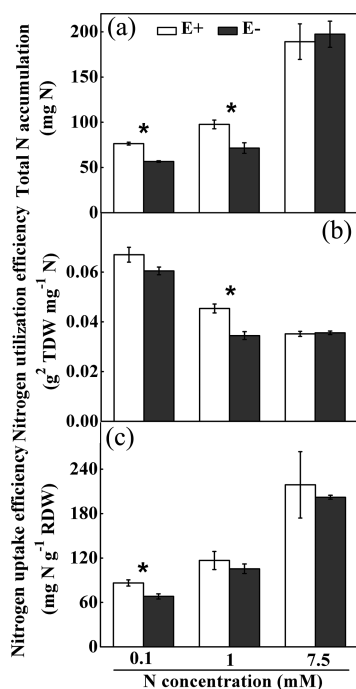
Figure 7. Total N accumulation (a), nitrogen utilization efficiency (b), and nitrogen uptake efficiency (c) of *Achnatherum inebrians* with and without endophyte under different nitrogen concentration treatments. Data presented are the means ± SE of three independent biological replicates. Asterisk (*) means significant difference (*P* < 0.05) between E+ and E− plants.

more significant impact on the response of the C:P ratios in roots (*P* = 0.004, Table 3). The ratios of N:P in the leaves and roots of *A. inebrians* had a different pattern under the 0.1 mM nitrogen concentration, and E+ plants had higher N:P ratios in leaves than E− plants under this nitrogen concentration (Figure 8c). However, E+ plants had lower N:P ratios in roots than E− plants under the 0.1 mM and 1 mM nitrogen concentrations (Figure 8f). The N:P ratios were not different in E+ and E− plants under the 7.5 mM nitrogen concentration, for both leaves and roots (Figure 8c and f). For N:P ratios, our data showed that endophyte × nitrogen concentration interactions were significantly different in roots (*P* = 0.016, Table 3), but not significantly different in leaves (*P* = 0.069, Table 3). However, endophyte-infection caused a significant difference in the N:P ratios of roots (*P* = 0.001, Table 3).

DISCUSSION

This study has revealed that the presence of the *E. gansuensis* in *A. inebrians* enhanced the plant growth, the activity of enzymes involved in nitrogen uptake and utilization, the content of N and P, and the nutrient content, and it favorably altered the C:N, C:P, and N:P rations when plants were growing under

low N availability. These effects were generally revealed in both the leaves and roots. The positive enhancement of these factors was confirmed by the enhanced fresh and dry weights of leaves under low-nitrogen conditions. The effect of the endophyte on these factors, including growth, was absent or minimal when plants were growing under high nitrogen availability.

The presence of the endophyte resulted in increased growth compared with E− plants, particularly in the growth of leaves. With roots, there were no significant differences in the fresh and dry weights between E+ and E− plants under low and high available nitrogen. A previous study showed that plant growth-promoting rhizobacteria strain *Bacillus amyloliquefaciens* NJN-6-enriched bio-organic fertilizer promoted the growth of banana plants.[43] The enhanced growth resulting from the presence of the endophyte at the lowest concentration of available nitrogen indicates that the roots have enhanced ability to uptake nitrogen and make it available to the shoot apex region and then to the leaves. This increased N update ability occurs even though hyphae of the endophyte are not in the roots. Nitrogen uptake is the first crucial step in the process of nitrogen utilization, and without this enhancement it seems unlikely that there would be distinct differences in N metabolism of E+ and E− plants as observed in this study. Our results showed E+ leaves and roots with access to the low nitrogen concentrations had higher NO_3^- content than E− plants. Previous studies have also shown the different available nitrogen concentration directly influenced the NO_3^- content of tissues.[44] NO_3^- is a key molecule to nitrogen metabolism, supporting the synthesis of plant N compounds, and NO_3^- content is regulated by NR. Our results showed that the presence of the endophyte increased NR activity compared with E− plants under low nitrogen conditions. Low nitrogen availability has been reported to decrease NR activity in tissues.[44] This was also found in our study; however, the presence of the endophyte alleviated the decrease of NR activity in plants growing in the lowest concentration of nitrogen. Further, NO_2^- is reduced to NH_4^+ by nitrite reductase. NiR activity is modulated under the different nitrogen concentrations.[45] Similarly, our results showed that the leaves and roots of E+ plants had higher NiR activity than E− plants counterparts under the 0.1 and 1 mM nitrogen concentrations. Thus, the presence of the endophyte also reduced the decrease of NiR activity when the host grass was growing under low nitrogen conditions. Our study has shown that the changes in NR and NiR activity are instigated by the presence of the endophyte in plants growing under low nitrogen conditions and this will lead to enhanced utilization of nitrogen in the plants and hence plant growth. The C:N, C:P, and N:P ratios are all of key importance in plant growth and health. In this study we found that when plants were growing in the lowest levels of nitrogen, the plants with endophyte have the most favorable ratios of the three primary elements.

Table 2. Results of Two-Way ANOVA for the Effects of Endophyte (E) and Nitrogen Concentrations (N) on TNA, NER, NUtE, and NUpE in *Achnatherum inebrians*

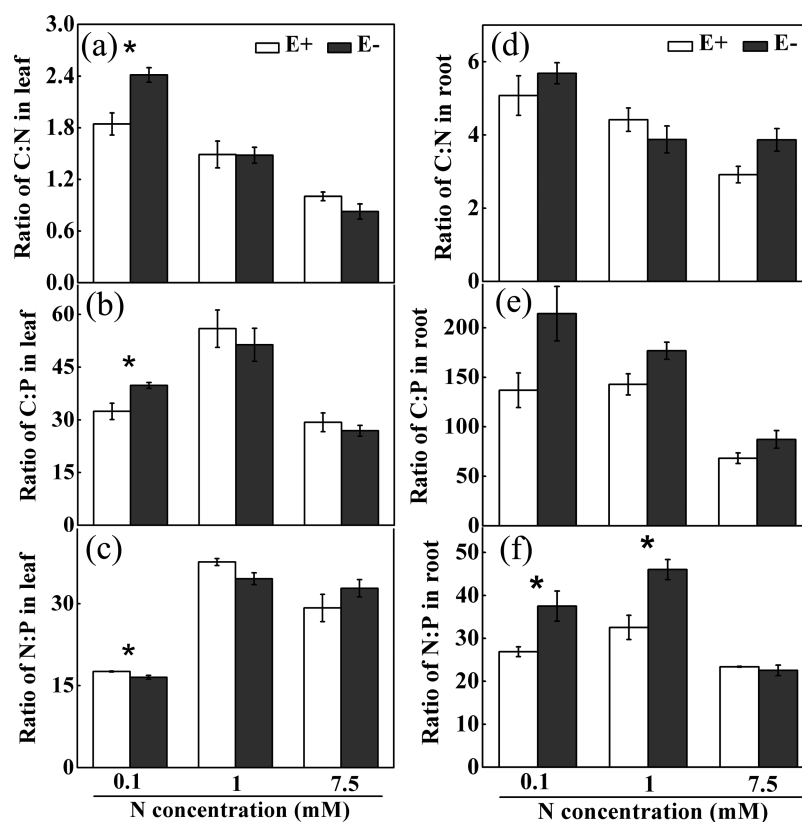| Treatments	df	TNA		NUtE		NUpE	
		F-value	P	F-value	P	F-value	P
E	1	2.162	0.167	15.543	0.002	0.945	0.350
N	2	85.991	<0.001	149.512	<0.001	25.713	<0.001
E * N	2	1.531	0.256	5.278	0.023	0.018	0.982

Figure 8. C:N, C:P, and N:P ratios in leaves (a, b, c) and roots (d, e, f) of *Achnatherum inebrians* with and without endophyte under different nitrogen concentration treatments. Data presented are the means ± SE of three independent biological replicates. Asterisk (*) means significant difference ($P < 0.05$) between E+ and E− plants.

Table 3. Results of Two-Way ANOVA for the Effects of Endophyte (E) and Nitrogen Concentrations (N) on Ratios of C:N, C:P, and N:P in Leaves and Roots of *Achnatherum inebrians*

			C:N		C:P		N:P	
	Treatments	df	F-value	P	F-value	P	F-value	P
Leaf	E	1	2.248	0.160	0.002	0.964	0.028	0.870
	N	2	66.767	<0.001	31.230	<0.001	111.349	<0.001
	E * N	2	6.945	0.010	1.863	0.197	3.358	0.069
Root	E	1	1.363	0.266	12.544	0.004	18.806	0.001
	N	2	15.953	<0.001	24.485	<0.001	27.936	<0.001
	E * N	2	2.405	0.132	2.038	0.173	6.005	0.016

Key to the growth of plants is the conversion of inorganic nitrogen into amino acids, and one enzyme involved in this process is glutamine synthatase. The enzyme also plays an important role in the process of reactivating nitrogen[46] and helping plants to adapt to low nitrogen conditions.[47] Our study found that the presence of the endophyte in plants growing under the lowest concentration of nitrogen had higher GS activity than E− plants.

Our study showed that the endophyte enhanced nitrogen accumulation under low nitrogen growth conditions. The presence of the endophyte modified the process of NH_4^+ content and nitrogen accumulation, a process that was directly related to nitrogen concentration.[44] The whole process of nitrogen uptake and utilization is clearly more complex than what we have investigated in this study. However, our findings have shown that the presence of the endophyte plays an important role for modulating the activity of key enzymes of nitrogen metabolism, enabling the host plant to adapt to low nitrogen conditions.

High growth rates of plants require high availability of N metabolites.[37] The presence of the endophyte altered the N and P content of *A. inebrians* plants but not the C content, with the leaves and roots of E+ plants having a higher N and P content than those of E− plants under the 0.1 mM nitrogen concentration. According to the growth rate hypothesis proposed by many researchers,[13,14] the decrease in fresh and dry weight of leaves of *A. inebrians* under 0.1 mM and 1 mM compared with the 7.5 mM nitrogen concentration might be partly explained by the decline in the C:N and C:P ratios. However, the decrease in the growth under the low-nitrogen conditions may be a function of limited P availability. In this study, leaves and roots of E+ plants had a higher P content than E− plants counterparts under the 0.1 mM nitrogen concentration and this may provide one explanation for the enhanced growth of E+ plants under low N conditions. The findings of this study show that the lowest rate of nitrogen used in our trial had significant impacts on the N and P stoichiometry of leaves and roots of *A. inebrians*.

The availability of nutrients, including N, to roots of plants as happened in our trial is different from what would happen under field conditions. In our study, only the concentration of N was varied while all treatments had the same high availability of the other nutrients from the use of the half strength Hoagland's solution. In the field it is likely that the roots would be exposed to a general low level of all nutrients, but N levels could greatly increase locally from the urine of animals. However, our study still is valuable as it has shown that the presence of the endophyte increases the ability of a plant to acquire and utilize N. The physiological and molecular steps involved in NO_3^- uptake and assimilation can be used to identify traits that are important for N use efficiency (NUE). It is generally believed that development of genetic varieties with improved NUE is essential for sustainable agriculture.[48] Whatever the crop, be it root, leaf, fruit, or seed, the method to measure NUE usually depends on calculating the plant biomass production per unit of applied N.[1,49] In the present study, our results indicated that E+ plants had higher TNA and NUpE than E− plants under the 0.1 mM nitrogen concentration. Taken together, these results suggest that *E. gansuensis* can increase NUE of host grasses. The natural sites of growth of this grass are in the grasslands of northwest China. The growing conditions in these ecosystems are harsh with low water and nutrient availability. The findings of this study indicated that the presence of the endophyte enables *A. inebrians* host plants to grow better when exposed to low available nitrogen.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: zhibiao@lzu.edu.cn.
ORCID
Jianfeng Wang: 0000-0002-5990-8166

■ ACKNOWLEDGMENTS

■ REFERENCES

(1) Xu, G.; Fan, X.; Miller, A. J. Plant nitrogen assimilation and use efficiency. *Annu. Rev. Plant Biol.* **2012**, *63*, 153−182.

(2) Heaton, T. H. Isotopic studies of nitrogen pollution in the hydrosphere and atmosphere: a review. *Chemical Geology: Isotope Geoscience Section* **1986**, *59*, 87−102.

(3) Drew, M.; Saker, L. Nutrient supply and the growth of the seminal root system in barley: II. Localized, compensatory increases in lateral root growth and rates of nitrate uptake when nitrate supply is restricted to only part of the root system. *J. Exp. Bot.* **1975**, *26*, 79−90.

(4) Lea, P. J.; Azevedo, R. A. Nitrogen use efficiency. 1. Uptake of nitrogen from the soil. *Ann. Appl. Biol.* **2006**, *149*, 243−247.

(5) Hooper, P.; Zhou, Y.; Coventry, D. R.; McDonald, G. K. Use of Nitrogen Fertilizer in a Targeted Way to Improve Grain Yield, Quality, and Nitrogen Use Efficiency. *Agronomy Journal* **2015**, *107*, 903−915.

(6) Ergon, Å.; Kirwan, L.; Bleken, M. A.; Skjelvåg, A. O.; Collins, R. P.; Rognli, O. A. Species interactions in a grassland mixture under low nitrogen fertilization and two cutting frequencies: 1. dry-matter yield and dynamics of species composition. *Grass Forage Sci.* **2016**, *71*, 667−682.

(7) Song, M.; Chai, Q.; Li, X.; Yao, X.; Li, C.; Christensen, M. J.; Nan, Z. An asexual *Epichloë* endophyte modifies the nutrient stoichiometry of wild barley (*Hordeum brevisubulatum*) under salt stress. *Plant Soil* **2015**, *387*, 153−165.

(8) Han, W.; Fang, J.; Guo, D.; Zhang, Y. Leaf nitrogen and phosphorus stoichiometry across 753 terrestrial plant species in China. *New Phytol.* **2005**, *168*, 377−385.

(9) Gao, Y.; Yu, G.; He, N. Equilibration of the terrestrial water, nitrogen, and carbon cycles: advocating a health threshold for carbon storage. *Ecological engineering* **2013**, *57*, 366−374.

(10) Elser, J.; Sterner, R.; Gorokhova, E. a.; Fagan, W.; Markow, T.; Cotner, J.; Harrison, J.; Hobbie, S.; Odell, G.; Weider, L. Biological stoichiometry from genes to ecosystems. *Ecology Letters* **2000**, *3*, 540−550.

(11) Sterner, R. W.; Elser, J. J. *Ecological stoichiometry: the biology of elements from molecules to the biosphere*; Princeton University Press: Princeton, 2002.

(12) Ågren, G. I.; Weih, M. Plant stoichiometry at different scales: element concentration patterns reflect environment more than genotype. *New Phytol.* **2012**, *194*, 944−952.

(13) Elser, J.; O'brien, W.; Dobberfuhl, D.; Dowling, T. The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. *J. Evol. Biol.* **2000**, *13*, 845−853.

(14) Hessen, D. O.; Jensen, T. C.; Kyle, M.; Elser, J. J. RNA responses to N- and P-limitation; reciprocal regulation of stoichiometry and growth rate in *Brachionus*. *Functional Ecology* **2007**, *21*, 956−962.

(15) Wu, T.-G.; Yu, M.-K.; Geoff Wang, G.; Dong, Y.; Cheng, X.-R. Leaf nitrogen and phosphorus stoichiometry across forty-two woody species in Southeast China. *Biochem. Syst. Ecol.* **2012**, *44*, 255−263.

(16) Elser, J. J.; Dobberfuhl, D. R.; MacKay, N. A.; Schampel, J. H. Organism size, life history, and N: P stoichiometry toward a unified view of cellular and ecosystem processes. *BioScience* **1996**, *46*, 674−684.

(17) Bucher, M. Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. *New Phytol.* **2007**, *173*, 11−26.

(18) Chen, J.; Li, Y.; Wen, S.; Rosanoff, A.; Yang, G.; Sun, X. Magnesium fertilizer-induced increase of symbiotic microorganisms improves forage growth and quality. *J. Agric. Food Chem.* **2017**, *65*, 3253−3258.

(19) Wang, Y.; Yang, X.; Zhang, X.; Dong, L.; Zhang, J.; Wei, Y.; Feng, Y.; Lu, L. Improved plant growth and Zn accumulation in grains of rice (*Oryza sativa* L.) by inoculation of endophytic microbes isolated from a Zn hyperaccumulator, *Sedum alfredii* H. *J. Agric. Food Chem.* **2014**, *62*, 1783−1791.

(20) Zhao, Q.; Wu, Y.-N.; Fan, Q.; Han, Q.-Q.; Paré, P. W.; Xu, R.; Wang, Y.-Q.; Wang, S.-M.; Zhang, J.-L. Improved Growth and Metabolite Accumulation in *Codonopsis pilosula* (Franch.) Nannf. by Inoculation of *Bacillus amyloliquefaciens* GB03. *J. Agric. Food Chem.* **2016**, *64*, 8103−8108.

(21) Rahman, M.; Saiga, S. Endophytic fungi (*Neotyphodium coenophialum*) affect the growth and mineral uptake, transport and efficiency ratios in tall fescue (*Festuca arundinacea*). *Plant Soil* **2005**, *272*, 163−171.

(22) Vázquez-de-Aldana, B. R.; García-Ciudad, A.; García-Criado, B.; Vicente-Tavera, S.; Zabalgogeazcoa, I. Fungal Endophyte (*Epichloë festucae*) Alters the Nutrient Content of Festuca rubra Regardless of Water Availability. *PLoS One* **2013**, *8*, e84539.

(23) Leuchtmann, A.; Bacon, C. W.; Schardl, C. L.; White, J. F., Jr; Tadych, M. Nomenclatural realignment of Neotyphodium species with genus Epichloë. *Mycologia* **2014**, *106*, 202−215.

(24) Müller, C. B.; Krauss, J. Symbiosis between grasses and asexual fungal endophytes. *Curr. Opin. Plant Biol.* **2005**, *8*, 450−456.

(25) Kuldau, G.; Bacon, C. Clavicipitaceous endophytes: their ability to enhance resistance of grasses to multiple stresses. *Biol. Control* **2008**, *46*, 57−71.

(26) Redman, R. S.; Sheehan, K. B.; Stout, R. G.; Rodriguez, R. J.; Henson, J. M. Thermotolerance generated by plant/fungal symbiosis. *Science* **2002**, *298*, 1581−1581.

(27) Zhang, X.; Li, C.; Nan, Z. Effects of cadmium stress on growth and anti-oxidative systems in Achnatherum inebrians symbiotic with *Neotyphodium* gansuense. *J. Hazard. Mater.* **2010**, *175*, 703−709.

(28) Hennessy, L. M.; Popay, A. J.; Finch, S. C.; Clearwater, M. J.; Cave, V. M. Temperature and plant genotype alter alkaloid concentrations in ryegrass infected with an *Epichloë* endophyte and this affects an insect herbivore. *Front. Plant Sci.* **2016**, *7*, 1−10.

(29) Xia, C.; Zhang, X.; Christensen, M. J.; Nan, Z.; Li, C. *Epichloë* endophyte affects the ability of powdery mildew (*Blumeria graminis*) to colonise drunken horse grass (*Achnatherum inebrians*). *Fungal Ecology* **2015**, *16*, 26−33.

(30) Song, M.; Li, X.; Saikkonen, K.; Li, C.; Nan, Z. An asexual *Epichloë* endophyte enhances waterlogging tolerance of *Hordeum brevisubulatum*. *Fungal Ecology* **2015**, *13*, 44−52.

(31) Christensen, M. J.; Bennett, R. J.; Ansari, H. A.; Koga, H.; Johnson, R. D.; Bryan, G. T.; Simpson, W. R.; Koolaard, J. P.; Nickless, E. M.; Voisey, C. R. *Epichloë* endophytes grow by intercalary hyphal extension in elongating grass leaves. *Fungal Genet. Biol.* **2008**, *45*, 84−93.

(32) Shi, Z. *Important poisonous plants of China grassland*; China Agriculture Press: Beijing, 1997; Vol. *166*, p 176.

(33) Nan, Z.; Li, C. In *Neotyphodium in native grasses in China and observations on endophyte/host interactions*, Proceedings of the 4th international neotyphodium-grass interactions symposium, Soest, 2000; pp 41−50.

(34) Cataldo, D.; Maroon, M.; Schrader, L.; Youngs, V. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid 1. *Commun. Soil Sci. Plant Anal.* **1975**, *6*, 71−80.

(35) Rosen, H. A modified ninhydrin colorimetric analysis for amino acids. *Arch. Biochem. Biophys.* **1957**, *67*, 10−15.

(36) Sparks, D.; Bartels, J. *Pt. 3: Chemical methods*; Soil Science Society of America: Madison [etc.], 1996.

(37) Lawlor, D. W. Carbon and nitrogen assimilation in relation to yield: mechanisms are the key to understanding production systems. *Journal of Experimental Botany* **2002**, *53*, 773−787.

(38) Siddiqi, M. Y.; Glass, A. D. Utilization index: a modified approach to the estimation and comparison of nutrient utilization efficiency in plants. *J. Plant Nutr.* **1981**, *4*, 289−302.

(39) Elliott, G. C.; Læuchli, A. Phosphorus efficiency and phosphate-iron interaction in maize. *Agronomy Journal* **1985**, *77*, 399−403.

(40) Du, S.; Zhang, Y.; Lin, X.; Wang, Y. U. E.; Tang, C. Regulation of nitrate reductase by nitric oxide in Chinese cabbage pakchoi (*Brassica chinensis* L.). *Plant, Cell Environ.* **2008**, *31*, 195−204.

(41) Datta, R.; Sharma, R. Temporal and spatial regulation of nitrate reductase and nitrite reductase in greening maize leaves. *Plant Sci.* **1999**, *144*, 77−83.

(42) Oaks, A.; Stulen, I.; Jones, K.; Winspear, M. J.; Misra, S.; Boesel, I. L. Enzymes of nitrogen assimilation in maize roots. *Planta* **1980**, *148*, 477−484.

(43) Yuan, J.; Ruan, Y.; Wang, B.; Zhang, J.; Waseem, R.; Huang, Q.; Shen, Q. Plant Growth-Promoting Rhizobacteria Strain *Bacillus amyloliquefaciens* NJN-6-Enriched Bio-organic Fertilizer Suppressed Fusarium Wilt and Promoted the Growth of Banana Plants. *J. Agric. Food Chem.* **2013**, *61*, 3774−3780.

(44) Luo, J.; Li, H.; Liu, T.; Polle, A.; Peng, C.; Luo, Z.-B. Nitrogen metabolism of two contrasting poplar species during acclimation to limiting nitrogen availability. *J. Exp. Bot.* **2013**, *64*, 4207−4224.

(45) Balotf, S.; Kavoosi, G.; Kholdebarin, B. Nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase expression and activity in response to different nitrogen sources in nitrogen-starved wheat seedlings. *Biotechnol. Appl. Biochem.* **2016**, *63*, 220−229.

(46) Miflin, B. J.; Habash, D. Z. The role of glutamine synthetase and glutamate dehydrogenase in nitrogen assimilation and possibilities for improvement in the nitrogen utilization of crops. *J. Exp. Bot.* **2002**, *53*, 979−987.

(47) Fuentes, S. I.; Allen, D. J.; Ortiz-Lopez, A.; Hernández, G. Over-expression of cytosolic glutamine synthetase increases photosynthesis and growth at low nitrogen concentrations. *J. Exp. Bot.* **2001**, *52*, 1071−1081.

(48) Haegele, J. W.; Cook, K. A.; Nichols, D. M.; Below, F. E. Changes in Nitrogen Use Traits Associated with Genetic Improvement for Grain Yield of Maize Hybrids Released in Different Decades. *Crop Science* **2013**, *53*, 1256−1268.

(49) Good, A. G.; Shrawat, A. K.; Muench, D. G. Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? *Trends Plant Sci.* **2004**, *9*, 597−605.