Greater Rhizosphere Effects on the Production and Retention of NH_4^+ Than on Those of NO_3^- in Alpine Coniferous Forests

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Abstract

While multiple evidences have demonstrated that root-derived carbon (C) can profoundly regulate mineral nitrogen (N) cycling, it is still not elucidated whether tree roots differentially modulate the production and retention of ammonium (NH_4^+) versus nitrate (NO_3^-) through rhizosphere effect (RE). Using the ^{15}N isotope labeling technique, we investigated how plant roots regulate the production and retention of NH_4^+ and NO_3^- via rhizosphere processes, and thus affect soil N availability in soils of two alpine coniferous forests. Synchronously, the activities of enzymes associated with N cycling and soil physicochemical properties in the rhizosphere and bulk soils were measured to explain the underlying mechanism. The results showed that roots induced greater positive REs of gross mineralization, microbial NH_4^+ immobilization, and dissimilatory nitrate reduction to ammonium (DNRA) to improve rhizosphere NH_4^+ availability. These positive REs can be attributed to the higher microbial biomass C and N and higher activities of N cycling-associated enzymes fueled by root-derived C. In contrast, the REs on NO_3^- production were negative, corresponding to the higher soil C:N ratio and higher microbial NH_4^+ immobilization in the rhizosphere soil than in the bulk soil, which led to relatively low NO_3^- availability in the rhizosphere. Collectively, our study provides field-based empirical evidence that plant roots can stimulate NH_4^+ production and immobilization, whereas they can limit NO_3^- production to achieve a high rhizosphere NH_4^+ supply in alpine coniferous forests. These findings provide comprehensive insights into how plants sustain their nutrition and growth by soil microbial N processes in their rhizosphere.

Introduction

Nitrogen (N) is the fundamental element limiting productivity in terrestrial ecosystems (Lebauer and Treseder, 2008). Most plants rely primarily on inorganic N forms (i.e., ammonium (NH_4^+) and nitrate (NO_3^-)); however, inorganic N generally comprises less than 10% of the total soil N pool (Hill et al., 2011, Socci and Templer 2011). In the soils of temperate/boreal coniferous forests and tropical rainforests, NH_4^+ availability is commonly higher than NO_3^- availability, companying with lagging nitrification (Davidson et al., 1992; Zhang et al., 2011; Fang et al., 2011; Wang et al., 2018). Similarly, the ratio of NH_4^+ to NO_3^- in the rhizosphere is higher than that in the bulk soils (Koranda et al., 2011; Lin et al., 2018; Cui et al., 2019). However, how plants regulate soil N transformation processes by soil microbes to cause the higher ratio of NH_4^+ to NO_3^- in the rhizosphere than in the bulk soil remains unclear.

It is generally recognized that the relative availability of NH_4^+ and NO_3^- in soil is governed by microbial N transformation, including the processes of mineral N production (i.e., gross mineralization and gross nitrification) and retention (i.e., microbial immobilization of NH_4^+ and NO_3^- and dissimilatory nitrate reduction to ammonium (DNRA)) (Corre et al., 2007). In general, the rates of these processes are controlled by a series of abiotic and biotic factors, including soil physiochemical characteristics (e.g., available carbon (C), C:N ratio, pH, and cation exchange capacity (CEC)) and microbial properties (e.g., microbial biomass, microbial C:N ratio, microbial community composition and enzyme activities) (Baldos et al., 2015; Ribbons et al., 2016; Wang et al., 2018). For instance, gross mineralization is positively correlated with higher microbial biomass, soil C availability and dissolved organic N (DON), whereas a high soil C:N ratio can exert a negative effect on gross

mineralization and nitrification depending on substrate quality (Booth et al., 2005; Li et al., 2019, 2020). Given the large spatial-temporal variations in the physiochemical and biological characteristics between the rhizosphere and bulk soils induced by root morphology, rhizodeposition and the formation of symbionts, large variations in gross N cycling processes are expected between the rhizosphere and bulk soils. However, most previous studies investigating gross N cycling in soil considered mineral soil as integral and did not distinguish between the rhizosphere and bulk soils (Zeng et al., 2014; Baldos et al., 2015; Masses et al., 2016). Elucidating the rhizosphere effect (RE) on the production and retention of mineral N is critical to understanding how plants enhance their nutrient acquisition and retain N in the plant-soil system (Corre et al., 2010).

Plant roots supplying C to decomposers and mycorrhizal fungi in exchange for N (i.e., the “C cost/N benefit”), is an important evolutionary strategy for plants to cope with N limitation (Herman et al., 2006; Jones et al., 2009). The interaction of roots and rhizosphere microbes can differentially affect the availability of NH_4^+ and NO_3^- in the rhizosphere by driving specific gross N-cycling processes. For example, benefiting from root-derived C, rhizosphere microbes increase investment in the synthesis of N-cycling enzymes, resulting in high gross mineralization in the rhizosphere (i.e., the rhizosphere priming effect, RPE; Dijkstra et al., 2013; Cheng et al., 2014). Other studies have shown that plants can produce and release nitrification inhibitors to inhibit gross nitrification, which limits NO_3^- production in the rhizosphere (Subbarao et al., 2015). Roots can also affect denitrification directly by inhibiting the activity of denitrifiers by releasing root exudates or indirectly by creating an anaerobic environment, and altering C and N availability in the rhizosphere (Philippot et al., 2013; Bardon et al., 2014). While the influence of roots on rhizosphere N cycling has previously been studied, to date these studies have largely focused on only one or relatively few processes within the N cycle (Dijkstra et al., 2009; Bengtson et al., 2012; Zhu et al., 2014; Cheng et al., 2014). The lack of empirical evidence of the discrepancies in gross N cycling rates between the rhizosphere and bulk soils restricts the establishment of a plant trait-based framework for linking plant N acquisition strategies to microbial N cycling processes (Moreau et al., 2019).

Temperate and boreal coniferous forests store a large proportion of the global terrestrial C in aboveground biomass and soil, and the primary productivity of these forests is limited by N (Clemmensen et al., 2013; Du et al., 2020). As a component of typical temperate coniferous forests, the predominant inorganic N pool in alpine coniferous forests is usually NH_4^+, which accounts for 18-49% of the dissolved N pool in the topsoil (Zhang et al., 2017; Cui et al., 2019). In this study, we aimed to investigate how plants improve mineral N supply by governing gross N transformation processes in the alpine coniferous forests located on the eastern Qinghai-Tibet Plateau (QTP). We used the ^{15}N isotope labeling and pool dilution technique to characterize gross N transformation processes in the rhizosphere and bulk soils. Furthermore, the RE on gross N transformations and soil N availability were quantified to analyze whether and how alpine coniferous tree roots induce differences in NH_4^+ and NO_3^- supply capacities. Basing on our previous studies showing that alpine coniferous trees have high root-derived C input and a higher absorption for NH_4^+ than for NO_3^- (Zhang et al., 2018, 2019), we hypothesized that roots would induced positive rhizosphere effect on the production and retention of NH_4^+ by stimulating high microbial biomass and N-transforming enzyme activities, but limit the production and retention of NO_3^- (i.e., relative low positive RE, or even negative RE). Addressing this hypothesis provides a new insight into how alpine coniferous species sustain their nutrition and growth in N-poor habitats by regulating different N production and retention processes in rhizosphere.

Methods And Materials

Site description and soil characteristics

The field research was conducted at the Miyaluo Experimental Forests on the eastern QTP in Lixian County, Sichuan Province, China (31° 35'N, 102° 35'E, and 3150 m a.s.l.). The mean annual temperature is 8.9°C; July is the warmest month (12.6 °C) and January is the coldest month (-8°C). The mean annual precipitation ranges from 600 to 1100 mm; the highest precipitation frequency and amount occur from May to September. According to the local Forestry Bureau, large areas of natural forests were deforested for agriculture from the 1950s to 1980s. Subsequently, plantations were established and currently cover one million hectares in western Sichuan Province, China, accounting for approximately 50% of the forest area in this region. The current study was conducted in a 70-year-old planted forest dominated by dragon spruce (*Picea. asperata*; hereafter called "the plantation") and in a 200-year-old natural forest dominated by spruce (*Picea. asperata*) and fir (*Abies faxoniana*) (hereafter called "the natural forest"). The understory of the spruce plantation is less dense and is dominated by the herbaceous plants *Festuca ovina*, *Deyeuxia arundinacea* and *Carex capillifoemia*. The understory of the spruce-fir forest is dominated by *Acer mono, Lonicera spp., and Betula albosinensis* with occurrences of the herbaceous plants *Anemone rivularis* and *Carex capilliformis*. The soils at both sites are Cambic Umbrisols according to the IUSS Working Group (2007).

Soil sampling

Soil sampling was conducted at the height of the warm and rainy season (July) in 2017. From each forest type, soil samples were collected from three grids (approximately 4 m × 4 m) that were randomly placed in a representative 100 m × 100 m plot. In each grid, the O horizon was removed, and five subsamples were then taken close to the targeted species (*P. asperata*) using a large diameter soil core (15 cm diameter, 0-15 cm depth) to obtain as many fine roots of *P. asperata* as possible. This depth was chosen because it had the largest fine root biomass at these sites (Liu et al., 2008). In the field, five subsamples were pooled together and the living roots of *P. asperata* were empirically identified by features such as morphology, color, and elasticity. Rhizosphere soil was operationally defined as soil adhering to the roots of *P. asperata* and was carefully collected using forceps and brushes, while the remaining soil was considered bulk soil (*sensu* Phillip & Fahey, 2006). Soil samples (rhizosphere and bulk soil) in each grid were sieved (2 mm), and homogenized in situ and then split into three parts. One part was used immediately after sieving to determine gross N cycling in situ. The second part was immediately placed in an ice box, transported to the laboratory and stored at -4°C for analysis of microbial biomass and enzymatic activity. The third part was air dried for analysis of soil properties.

The measurement of gross N production and retention

Potential rates of gross N mineralization and nitrification were measured using the ^{15}N pool dilution techniques (Hart et al., 1994). For the ^{15}N labeling experiments, 50 g of the rhizosphere or bulk soil was weighted into a black cylindrical plastic jar with a wide mouth (4.7 cm diameter × 10.2 cm height). A total of four jars per soil fraction (rhizosphere or bulk soil) for each grid were prepared. Two were injected with $(^{15}NH_4^+)_2SO_4$ solution, and the other two were injected with $K^{15}NO_3$ solution. The concentrations of the labeled solution were determined based on measurement of the background concentrations of NH_4^+ and NO_3^- in the soils.

Specifically, each jar received five 0.5 mL injections of the solutions (50 atom% ^{15}N) that contained 90 μg NH_4^+ mL^{-1} or 45 μg NO_3^- mL^{-1}, equivalent to an average rate of 4.5 μg NH_4^+ g^{-1} soil or 2.25 μg NO_3^- g^{-1} soil. The injected NH_4^+ was 40-62% (rhizosphere and bulk soils in the plantation) and 29-38% (rhizosphere and bulk soils in the plantation) of the initial soil NH_4^+ concentration. The injected NO_3^- was 40-50% (rhizosphere and bulk soils in the plantation) and 31-36% (rhizosphere and bulk soils in the plantation) of the initial NO_3^- concentration. One jar from each labeled pair was collected and the soil was mixed in a plastic bag and subsamples (15 g) were extracted with 2 M KCl solution (solution:soil = 5:1) 10 min after ^{15}N injection (T_0) (Silver et al., 2005; Henneron et al., 2020). The other jar of the labeled pair was incubated for 1 day (T_1) in situ and subsamples (15 g) were extracted with 2 M KCl solution (solution:soil = 5:1). Simultaneously, we also used the $^{15}NH_4^+$- and $^{15}NO_3^-$-labeled samples to assess microbial NH_4^+ immobilization (IA) and microbial NO_3^- immobilization rates (IN), respectively (as measures of microbial N retention). Approximately 10 g of the T_1 $^{15}NH_4^+$- and $^{15}NO_3^-$-labeled samples was fumigated with $CHCl_3$ for 48h, and the corresponding subsamples were incubated without $CHCl_3$ fumigation and then extracted with 0.5 M K_2SO_4 (approximately a 5:1 ratio of solution to soil) (Davison et al., 1991). For the extracts of T_1 samples, extractable organic N and ^{15}N enrichment were determined using persulfate digestion as described by Corre et al. (2007). The concentrations of NH_4^+ and NO_3^- were determined on a continuous-flow analyzer (Skalar, Breda, Netherlands). Part of the extracts was frozen immediately and kept frozen during transport by air to Nanjing for ^{15}N analyses at the School of Geography Sciences, Nanjing Normal University. For isotope analysis, NH_4^+ and NO_3^- were separated following the same ^{15}N diffusion procedures described by Zhang et al. (2013). The isotopic compositions of NH_4^+ and NO_3^- were determined using an automated C/N analyzer coupled to an isotope ratio mass spectrometer (Europa Scientific Integra, UK).

The gross rates of N mineralization, nitrification, and microbial NH_4^+ and NO_3^- immobilization were calculated to characterize the production and retention of NH_4^+ and NO_3^- following the equations given by Kirkham and Bartholomew (1954) and Davidson et al. (1991). Rates of DNRA, also a measure of microbial NO_3^- retention, were calculated from the $^{15}NO_3^-$-labeled samples following the same calculations used by Silver et al. (2001, 2005). The formulas are listed in the Appendix A.

To allow an overall comparison between two soil compartments and to study to what extent mineral N immobilization plays an important role in N retention, different ratios were calculated as described by Vervaet et al. (2004). The ratio of microbial NH_4^+ immobilization to gross mineralization (IA:GM) or microbial NO_3^- immobilization to gross nitrification (IN:GN) was calculated to evaluate the extent to which microbes contribute to retaining the newly produced NH_4^+ or NO_3^- (Geisseler et al., 2010). The ratio of gross nitrification to gross mineralization (i.e., relative nitrification, GN:GM) was calculated to indicate which type of N form (NH_4^+ or NO_3^-) dominated the N cycling (Gilliam et al., 2001). High rates of relative nitrification suggest that N cycling in soils is mostly dominated by NO_3^- rather than NH_4^+ and vice versa.

Microbial biomass C and N and microbial nutrient limitation

Soil microbial biomass C and N were determined from the unlabeled soil samples using the $CHCl_3$ fumigation and 0.5 M K_2SO4 solution extraction method (Brookes et al., 1985; Vance et al., 1989). Organic C and total N in the extracts were determined simultaneously on a TOC/TN analyzer (Multi-N/C 2100, Analytik Jena AG, Germany). Microbial biomass C and N were calculated based on the differences in extractable organic C and total N between the fumigated and nonfumigated samples, correcting for unrecovered biomass using a factor of 0.45 (Jenkinson, 2004). The stoichiometric imbalance ratios between microbes and their resources in rhizosphere and in the bulk soils were calculated as the ratio of SOC:N to microbial biomass C:N, which reflect the relative extent to microbial nutrient limitation of rhizosphere compared to the bulk soil (Mooshammer et al., 2014). An increasing stoichiometric imbalance indicates increasing microbial N limitation, and vice versa, a decreasing stoichiometric imbalance indicates increasing microbial C limitation.

Enzyme activity assays

The potential activity of six enzymes involved in soil N cycling in the rhizosphere and bulk soil, including proteases, leucine aminopeptidase (LAP), β-1,4-N-acetylglucosaminidase (NAG), phenol oxidase (POX), peroxidase(PER) and nitrate reductase (NIR), was assayed. The specific information for each enzyme is shown in Table A.1. Simply speaking, the activities of NAG and LAP enzyme were measured with 4-methyl-umbelliferyl substrate conjugate using microplate fluorometic assay with 365 nm excitation and 450 nm emission filters on Varioskan Flash multiplate reader (Thermo Scientific, USA), and the activities of PPO and POX enzyme were measured spectrophotometrically as absorbance at 460 nm using $_L$-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate (Saiya-Cork et al., 2002). We assayed potential proteases activity using trichloroacetic acid solution to terminate the activity following the method modified from Brzostek et al. (2012). The concentration of amino acids in the incubated and initial subsamples was quantified using the o-phthaldialdehyde and β-mercaptoethanol (OPAME) method with glycine as the standard (Jones et al., 2002). Protease activity was calculated from the difference in amino acid concentration between the incubated and initial samples. NIR activity was measured the sulphamic acid-naphthalamine colorimetric method with the absorbance reading at 543 nm after incubating in dark and anaerobic conditions for 24 h using KNO_3 as the substrate (Schinner et al., 1996). The detailed processes of enzyme activity analysis are shown in the Appendix A.

Other supporting parameters

In this study, a series of soil physicochemical characteristics (including the concentration of soil organic C (SOC), total N (TN), dissolved organic C (DOC), NH_4^+, NO_3^-, pH, CEC, and base saturation (BS)) were measured to analyze whether these factors control the gross rates of mineral N production and retention. Subsamples of rhizosphere and bulk soils were ground to pass through a 0.15 μm sieve, and then the SOC and TN concentrations were analyzed using a CN analyzer (Vario MACRO, Elementar Analysensysteme GmbH, Hanau, Germany). The soil pH was measured with a pH meter (Meltler-Toledo Instruments Co., Ltd., Shanghai, China) using a 2.5:1 soil to H_2O extract. The CEC was determined from air-dried soils (passed through a 2 mm sieve) by percolating with unbuffered 1 M NH_4Cl, followed by analysis of the percolates for exchangeable element concentrations (Ca, Mg, K, Na, Fe, Al and Mn) using inductively coupled plasma optical emission spectroscopy (ICP-OES; Optima 5300 DV, Perkin Elmer). BS was calculated as the percentage of exchangeable base cations relative to the CEC. The concentrations of different N forms in the rhizosphere and bulk soils were also measured to evaluate the differences in soil N availability between the two soil fractions. The NH_4^+ and NO_3^-

were extracted with 2 M KCl solution (solution:soil = 5:1) and then determined on a continuous-flow analyzer (Skalar, Breda, Netherlands). The soil organic N (SON) was calculated as the difference between the total N concentration and inorganic N content (SON = TN – ($[NH_4^+]$ + $[NO_3^-]$)) (Staelens et al., 2011; Masses et al., 2016).

Statistical analysis

The normality and equality of variance were tested using the Kolmogorov-Smirnov *D* and the Levene statistic for each parameter in the rhizosphere and bulk soils of the plantation and the natural forest. Log transformation was conducted when the parameters showed heterogeneous variance. Considering that many previous studies have compared difference in soil N cycling between forest types, we focused on illuminating how plants regulate rhizosphere gross N transformations to improve soil N availability in alpine forest ecosystems with regardless of the differences between plantation and natural forest in this study. Therefore, paired Student's *t*-tests were conducted to examine the differences in soil physicochemical characteristics, N-cycling rates (GM, GN, IA, IM and DNRA) and microbial properties (microbial biomass and N-cycling enzyme activities) between the rhizosphere and the bulk soil at each site. The RE were calculated as the percentage difference of the N-cycling rates (GM, GN, IA, IM and DNRA) and N-pool sizes between the rhizosphere and bulk soil to assess whether rhizosphere and bulk soils differed in N supply capacities (Phillips and Fahey, 2006). To assess the influence of soil physicochemical and microbial properties on soil N cycling rates in the rhizosphere and bulk soil, Pearson correlation tests were conducted to identify relationships between gross mineral N transformation rates, soil variables, and microbial variables. Means and standard errors (SE) of the four replicates for each soil compartment in each forest type are reported as measures of central tendency and dispersion. Differences are considered statistically significant when *p*-values <0.05, unless otherwise stated. All analyses were conducted using SAS 8.0 for Windows (SAS Institute Inc., Cary NC, USA). Graphic illustrations were generated using Origin 8.0 software (Origin Lab Corporation, Northampton, MA, USA).

Results

NH_4^+ production and retention in the rhizosphere and bulk soils

The rates of NH_4^+ production and retention were significantly higher in the rhizosphere than in the bulk soil at the two coniferous sites. Specifically, the gross rates of mineralization and microbial NH_4^+ immobilization in the rhizosphere were 66% and 109% higher, respectively, than those in the bulk soil in the plantation (P = 0.02 for GM and P = 0.04 for IA) (Fig. 1a, c). Similarly in the natural forest, the gross rates of mineralization and microbial NH_4^+ immobilization in the rhizosphere were 32% and 98% higher, respectively, than those in the bulk soil (P = 0.03 for GM and P = 0.05 for IA) (Fig. 1a, c).

NO_3^- production and retention in the rhizosphere and bulk soils

The differences in NO_3^- production and retention rates between rhizosphere and bulk soils vary with specific processes. For the production and retention of NO_3^-, gross nitrification in the rhizosphere was 32% and 24% lower than that in the bulk soil in the plantation and the natural forest, respectively (P = 0.02 in the plantation and P = 0.04 in the natural forest) (Fig. 1b). The difference in microbial NO_3^- immobilization rates between the

rhizosphere and bulk soils was not significant at the two coniferous sites (Fig. 1d). The DNRA rates in the rhizosphere were 106% (P = 0.007) and 164% (P = 0.02) higher than those in the bulk soil of the plantation and natural forest, respectively (Fig. 1e). In addition, the REs on gross mineralization, nitrification and microbial NH_4^+ immobilization were more pronounced in the plantation than in the natural forest, whereas the rhizosphere effect on DNRA was the opposite.

Ratios of specific gross N cycling rates

The GN:GM ratios in the rhizosphere (0.32 ± 0.01 in the plantation and 0.39 ± 0.02 in the natural forest) were lower than those in the bulk soil (0.78 ± 0.07 in the plantation and 0.72 ± 0.14 in the natural forest) (P < 0.01; Fig. 2a), suggesting that N cycling in the rhizosphere was dominated by NH_4^+ and resulted in higher NH_4^+ availability, whereas N cycling in the bulk soil was dominated by NO_3^-. The IA:GM ratio in the rhizosphere (0.68 ± 0.02) was higher than that in the bulk soil (0.54 ± 0.11) in the plantation (P = 0.02), while the IA:GM ratio in the rhizosphere (0.75 ± 0.05) was also higher than that in the bulk soil (0.53 ± 0.07) in the natural forest (P = 0.03) (Fig. 2b). The IN:GN ratio in the rhizosphere (0.76 ± 0.10) was higher than that in the bulk soil (0.49 ± 0.12) in the plantation (P = 0.02), and a similar pattern was found in the natural forest (P = 0.02) (Fig. 2c). The high IA:GM and IN:GN ratios in the rhizosphere compared to those in the bulk soil indicated that microbes in the rhizosphere have a higher capacity to retain the mineral N produced by gross mineralization (NH_4^+) and nitrification (NO_3^-) than microbes in the bulk soil.

Relationships between abiotic and biotic factors and gross N cycling

Most of the soil physicochemical characteristics (e.g., SOC, TN, C:N ratio and NH_4^+) were significantly higher in the rhizosphere than in the bulk soil in both the plantation and natural forest (P < 0.05; Table 1), whereas the significance of the differences in other parameters (such as DOC, NO_3^-, SON, pH and CEC) between the rhizosphere and bulk soil varied with forest type (only significant in one site, or neither, Table 1). The soil microbial biomass C, N, microbial C:N ratio and stoichiometric C:N imbalances were higher in the rhizosphere than in the bulk soil (all P < 0.05, Fig. 3b), while the differences in microbial C:N ratio between the rhizosphere and bulk soil were not significant in two sites (all P > 0.05) (Fig. 3c). Similar to the pattern of the microbial biomass, enzyme activities in rhizosphere soil were generally higher than those in the bulk soil in the plantation and the natural forest, except for LAP activity in both sites, PER activity in the plantation, and NIR activity in the natural forest (Table 2).

Correlation analysis showed that the rates of mineral N production (GM and GN) and retention (IA, IN and DNRA), to some extent, were closely correlated with soil physiochemical properties, microbial biomass, and N-cycling-associated enzyme activities (Fig. A.1). In the spruce plantation, gross N mineralization was positively correlated with SOC, TN, DOC, SOC:TN ratio, microbial biomass C, and the activities of proteases and NAG (R = 0.82 to 0.96, all P < 0.05), i.e., the higher value of these parameters in the rhizosphere soil than in the bulk soil, the higher the rates of gross N mineralization were in the former. Microbial NH_4^+ immobilization was positively correlated with SOC, TN, DOC, DON, protease activity, CEC and gross mineralization (R = 0.84 to 0.98, all P < 0.05). Gross nitrification was negatively correlated with SOCT:N ratio, DOC, protease activity, and microbial NH_4^+ immobilization (R = -0.81 to -0.90, all P < 0.05). DNRA was positively correlated with DOC (R = 0.85, P =

0.03) and NIR (R = 0.83, P = 0.04) (Fig. A.1a). In the spruce-fir forest, gross mineralization was positively correlated with SOC, TN, DOC, DON, microbial biomass C and N, proteases and oxidases (R = 0.84 to 0.98, all P < 0.05). Microbial NH_4^+ immobilization was significantly positively correlated with SOC, TN, DON, MBC, MBN and gross mineralization rates (R = 0.82 to 0.98, all P < 0.05). Gross nitrification was negatively correlated with SOC:TN ratio, microbial biomass C and N, and microbial NH_4^+ immobilization (R = -0.79 to -0.85, P < 0.05) (Fig. A.1b).

Discussion

Rhizosphere effects on NH_4^+ production and retention

Our results showed that the RE on gross N mineralization and microbial NH_4^+ immobilization (Fig. 4a, b), and the NH_4^+ production and microbial NH_4^+ immobilization in the rhizosphere soil were closely coupled (i.e., higher IA:GM ratio, Fig. 2a) compared with the bulk soil. This result supports our first hypothesis that NH_4^+ production and retention would be higher in the rhizosphere than in the bulk soil. The rates of gross mineralization and microbial NH_4^+ immobilization in the rhizosphere are within the range of rates in the rhizosphere of temperature and boreal coniferous forests (4.49-12.96 mg N kg^{-1} d^{-1}, Landi et al., 2006; Holz et al., 2016; Phillips et al., 2011), while the corresponding rates in the bulk soil are comparable to those in the bulk soil of other coniferous forests (1.1-9.20 mg N kg^{-1} d^{-1}, Corre et al., 2007; Staelens et al., 2011; Masse et al., 2016; Zhang et al., 2013). Closely coupled mineral N production and microbial N immobilization have also been reported in rhizosphere soil (Landi et al., 2006; Holz et al., 2016) and bulk soil of boreal/temperate forests and high elevation tropical mountain rainforests (Cheng et al., 2011, Baldos et al., 2015), suggesting that rapid immobilization of mineralized N by microorganisms is a common characteristic in N limiting ecosystems and is beneficial for NH_4^+ retention in plant-soil systems (Carmosini et al., 2002). Given that gross N mineralization and microbial NH_4^+ immobilization are two primary microbial processes to modulate other N-transformation processes and then affect soil NH_4^+ availability, we will focus on the comparison of gross mineralization and microbial NH_4^+ immobilization rates between rhizosphere and bulk soil to further illustrate how roots regulate NH_4^+ production and retention by microbes.

In the respective of NH_4^+ production, the positive RE on gross mineralization can be attributed to the difference in major C source for microbes (i.e., rhizosdeposites in the rhizosphere vs. litters and SOM in the bulk soil). Rhizosdeposites, including low molecular mass compounds (i.e., sugars, amino acids and organic acids), polymerized sugar, root border cells and dead root cap cells, are used by soil microbes as labile C sources and pronouncedly increase the abundance, activity, and growth of microbes in the rhizosphere (Blagodatskaya et al, 2010). Another study conducted at the same time showed that the magnitude of root-derived C was 47.5 ± 9.3 g C m^{-2} yr^{-1} in the spruce plantation and 80.8 ± 13.6 g C m^{-2} yr^{-1} in the fir-spruce natural forest, respectively, in the growing season (Zhang et al., 2018), which supported our results that the rhizosphere harbored higher microbial biomass than the bulk soil due to root-derived C (Fig. 3a). When rhizosphere microbes assimilate root-derived labile C, they need to increase the production of extracellular enzymes to mine the additional N from soil organic matter (SOM) (i.e., the microbial N-mining hypothesis, Koranda et al., 2011; Dijkstra et al., 2013). Similar results were found in our study sites showing that the activities of protease, NAG and POX were

higher in the rhizosphere than in the bulk soil (Table 2). Such positive REs on soil C availability, microbial biomass, N-cycling enzyme activity, gross mineralization, and NH_4^+ availability can be found in other studies (Phillips et al., 2011; Zhu et al., 2014; Li et al., 2018). Additionally, the higher SON concentration in the rhizosphere than in the bulk soil could result in the difference in gross mineralization between soil fractions (Table 1).

Compared with the bulk soil, the rhizosphere showed higher NH_4^+ retention in microbial biomass (i.e., higher RE on microbial NH_4^+ immobilization) (Fig, 4). One possible reason for the higher microbial NH_4^+ immobilization in the rhizosphere than bulk soil was that microbial N limitation was more intensive in the rhizosphere relative to the bulk soil due to root-derived C and plant N uptake (the N-limiting hypothesis; Kuzyakov, 2002; Drake et al., 2013). Mooshammer et al. (2014) suggested that higher stoichiometric C:N imbalance between microbes and their resources indicated stronger microbial N limitation, and thus more N immobilization into microbial biomass. Our results showed that stoichiometric C:N imbalance was higher in the rhizosphere than in the bulk soil (Fig. 3d), suggesting that microbial N limitation was stronger in the rhizosphere than in the bulk soil. In this case, the newly produced NH_4^+ was rapidly immobilized by rhizosphere microbes to alleviate N limitation, which was supported by the higher microbial biomass N in the rhizosphere relative to the bulk soil (Fig. 3b). Furthermore, we found that the CEC as well as the TN were higher in the rhizosphere than in the bulk soil (Table 2), suggesting that the chemical N retention mechanism (i.e., NH_4^+ absorbed onto soil exchange sites) could contribute to higher NH_4^+ retention in the rhizosphere soil compared with the bulk soil.

Rhizosphere effects on the NO_3^- production and retention

The negative RE on gross nitrification and lower GN:GM ratios in the rhizosphere suggest that the rhizosphere can enhance NH_4^+ supply capacity by inhibiting gross nitrification in addition to accelerating gross mineralization, which supports our second hypothesis. It has been reported that N-limited ecosystems generally exhibit low nitrification rates and low GN:GM ratios; Gross nitrification rates range from 0.04 to 5 mg N g^{-1} d^{-1}, and the GN:GM ratios range from 7% to 52% in the mineral soil of temperate and boreal forests (Veraet et al., 2004; Zhang et al., 2013; Staelens et al., 2011; Masse et al., 2016).

Although the substrate (i.e., NH_4^+) availability for nitrifiers was higher in the rhizosphere, the gross nitrification rate was lower in the rhizosphere than in the bulk soil (Fig 1b), similar to other studies (Herman et al., 2006; Koranda et al., 2011). We propose two possible reasons for the negative RE on gross nitrification. One reason is that the uptake of NH_4^+ by fine roots and root-derived C accelerates microbial N demand (higher NH_4^+ immobilization) in the rhizosphere, which induces fierce competition among plants, nitrifiers and other microbes for NH_4^+ (Kuzyakov and Xu, 2013). Fisk et al. (2015) indicated that the addition of synthetic root exudates, to some extent, caused a decrease in gross nitrification ($P < 0.05$). Holz et al (2016) also found that gross nitrification was significantly increased by 64% after root removal, corresponding to a significant decline in NH_4^+ immobilization. The second reason may be related to the higher SOC:N ratio in the rhizosphere than in the bulk soil (Table 2). We found that gross nitrification was negatively correlated with the soil C:N ratio in the plantation and the natural forest ($R = -0.92$ and -0.94, both $P < 0.01$, Table S2). This result was consistent with the previous finding that a high SOC:TN ratio would lead to low gross nitrification and then prevent nitrous

oxide emission and NO_3^- leaching loss (Midgley & Phillips, 2013). Although we did not focus on nitrification inhibitors in this study, the important of these root-released substances in inhibiting rhizosphere nitrification has been widely studied and recognized in agrosystems (Subbarao et al., 2007; 2015). Therefore, more attention should be pay to the development of novel techniques for identifying the substances released by roots to inhibit nitrification in forest ecosystems in future.

DNRA provides an alternative pathway of NH_4^+ production in addition to mineralization and has been measured in bulk soil in numerous studies (Staelens et al., 2011; Rütting et al., 2011); however, there is a lack of evidence in the rhizosphere of forest ecosystems. In the current study, DNRA rates fell within the range of values reported in temperate forests (0.004 ± 0.001 to 0.448 ± 0.024 mg $kg^{-1}d^{-1}$) (Rütting et al., 2011). Notably, we first observed that DNRA rates were higher in the rhizosphere soil than the bulk soil in alpine coniferous forest ecosystems (Fig. 1e). The DOC:NO_3^- ratio and O_2 status have been identified as the most important factors regulating DNRA in soil (Philippot et al., 2013). Previous studies reported that organic C availability regulates the population of the bacteria community involved in DNRA, and the addition of carbohydrates (e.g., glucose and mannitol) can stimulate the DNRA rate (Tiedje, 1988, Morley and Bahhs, 2010). Tiedje (1988) found that DNRA increases as the ratio of dissolved organic C to extractable NO_3^- (DOC:NO_3^- ratio) increases, and significant DNRA occurred only at a DOC:NO_3^- ratio above 12 (Yin et al., 1998). In the current study, the DOC:NO_3^- ratio in the rhizosphere (51 ± 4) was higher than that in the bulk soil (30 ± 4) in the plantation (P =0.04). A similar pattern was found in the natural forest, showing that the DOC:NO_3^- ratio was 91 ± 16 in the rhizosphere and 71 ± 4 in the bulk soil (P = 0.05). We argue that the higher labile C availability driven by root-derived C and/or the higher NIR activity (at least for the plantation) in the rhizosphere contributed to the higher DNRA in the rhizosphere compared to the bulk soil. Moreover, given that plant roots consume oxygen and thereby create an anaerobic environment of the rhizosphere (Hinsinger et al., 2009), DNRA is likely favored in the rhizosphere. Collectively, the lower GN:GM ratio, the higher ratios of IA:GM and IN:GN and the higher DNRA in the rhizosphere compared to the bulk soil in these alpine forests indicate that N cycling in the rhizosphere is relatively conservative. Therefore, the risk of nitrous oxide emission through nitrification and NO_3^- leaching is low, allowing more mineral N to be retained in the plant-soil system (de Vries and Bardgett, 2016).

Conclusion

By using the ^{15}N isotope labeling technique, we isolated rhizosphere and bulk soil to evaluate rhizosphere effects on specific processes of gross N transformation in two subalpine coniferous forests. We found that plant roots differentially modulate the N production and retention processes to improve N nutrition in rhizosphere in these subalpine coniferous forests by (1) stimulating microbes to increase the production and immobilization of NH_4^+, and by (2) limiting microbial processes that lead to N losses (i.e., nitrification), or enhancing the transformation of NO_3^- to NH_4^+ (i.e., DNRA). Our results imply that roots stimulate mineralization and retention in the rhizosphere while inhibited/reduced nitrification is a key component of nutrient acquisition strategies for coniferous trees to satisfy their N demand and sustain their productivity in N-limited habitats. We fully acknowledge that the present results on rhizosphere N transformation processes deriving from a small size of sampling should be considered with caution. Nevertheless, our results robustly indicate the importance of rhizosphere processes in the mechanistic links between plant nutrient acquisition strategy and soil nutrient

cycling, and highlight the needed to long-term research focusing on the interactions between root, soil microbes, and soil in forest ecosystems.

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Code availability: Not applicable

Authors’ contributions: XMZ and HJY conceived the study, analysed the data, and wrote the manuscript; XMZ and DYL performed sampling in the field and measurements in the laboratory work. All authors contributed to the presentation and interpretation of results.

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Tables

Table 1 Soil physicochemical characteristics in the top 0-15 depth of the mineral layer in the plantation (the spruce plantation) and the natural forest (the spruce-fir forest). Soil samples were collected in July, 2017. Values are mean (SE), n = 4. Different lowercase letters denoted significant differences (P< 0.05) in soil physicochemical characteristics between the rhizosphere and bulk soil of two coniferous sites. SOC: soil organic carbon, TN: soil total nitrogen, SOC:TN: the ratio of SOC to TN, DOC: dissolved organic carbon, SON: soil organic nitrogen, CEC: cation exchange capacity, BS: base saturation.

Forest type	Soil fractions	SOC (g C kg^{-1})	TN (g N kg^{-1})	SOC:TN	DOC (mg C kg^{-1})	NH_4^+ (mg N kg^{-1})	NO_3^- (mg N kg^{-1})	SON (g N kg^{-1})	pH
Spruce plantation	Rhizopshere	28.69 (0.74) a	1.81 (0.05) a	15.85 (0.41) a	232 (14) a	11.20 (0.74) a	3.52 (0.07) a	1.80 (0.05) a	6.12 (0.23) a
	Bulk soil	23.30 (0.94) b	1.79 (0.13) a	13.01 (0.15) b	159 (20) b	7.30 (0.30) b	5.63 (0.80) b	1.77 (0.13) a	6.22 (0.17) a
Spruce-fir forest	Rhizosphere	81.78 (0.19) a	5.57 (0.06) a	14.68 (0.29) a	578 (19) a	15.33 (0.36) a	4.28 (0.41) a	5.55 (0.06) a	5.50 (0.13) a
	Bulk soil	70.26 (0.40) b	5.21 (0.09) a	13.48 (0.59) b	511 (77) a	11.66 (0.66) b	7.15 (0.81) a	5.19 (0.09) a	5.78 (0.21) a
		CEC （mmol kg^{-1}）							BS (%)
		K	Ca	Na	Mg	Mn	Al	Fe	
Spruce plantation	Rhizopshere	7.29 (0.20) a	126 (13) a	1.36 (0.23) a	10.2 (1.7) a	0.55 (0.06) a	1.00 (0.11) a	0.33 (0.01) a	98.71 (0.10) a
	Bulk soil	4.24 (0.61) b	82 (4) b	0.90 (0.13) a	7.1 (1.4) b	0.42 (0.02) b	0.78 (0.10) b	0.25 (0.03) a	98.47(0.14) a
Spruce-fir forest	Rhizosphere	3.93 (0.71) a	75 (7) a	0.21 (0.00) a	5.0 (0.8) a	1.26 (0.08) a	0.20 (0.04) a	0.06 (0.01) a	98.21 (0.16) a
	Bulk soil	2.13 (0.37) b	53 (2) b	0.21 (0.00) a	3.7 (0.5) b	0.65 (0.03) b	0.15 (0.00) a	0.04 (0.00) a	98.59 (0.08) a

Table 2 The activities of N-cycling associated enzyme in the rhizosphere and bulk soils of the plantation and the natural forest. Values are mean (SE), n = 4. NAG: β-1,4-N-acetylglucosaminidase; LAP: leucine aminopeptidase; PER: peroxidase; POX: phenol oxidase; NIR: nitrate reductase.

Forest type	Soil compartment	Protease	NAG	LAP	PER	POX	NIR
		μg AA-N ·[g dry soil]$^{-1}$· h^{-1}	μmol·[g dry soil]$^{-1}$·h^{-1}				μg NO_2-N·[g dry soil]$^{-1}$·h^{-1}
Spruce plantation	Rhizosphere	5.58 (0.50) a	1.81 (0.02) a	0.25 (0.01) a	1.02 (0.06) a	1.30 (0.15) a	0.26 (0.07) a
	Bulk soil	1.83 (0.28) b	0.66 (0.10) b	0.27 (0.03) a	0.71 (0.17) a	0.89 (0.03) b	0.05 (0.01) b
Spruce-fir forest	Rhizosphere	8.34 (0.46) a	1.24 (0.09) a	0.25 (0.02) a	1.34 (0.14) a	1.55 (0.14) a	0.02 (0.00) a
	Bulk soil	5.38 (0.53) b	0.81 (0.08) b	0.26 (0.2) a	0.83 (0.15) b	1.09 (0.51) b	0.03 (0.00) a

Figures

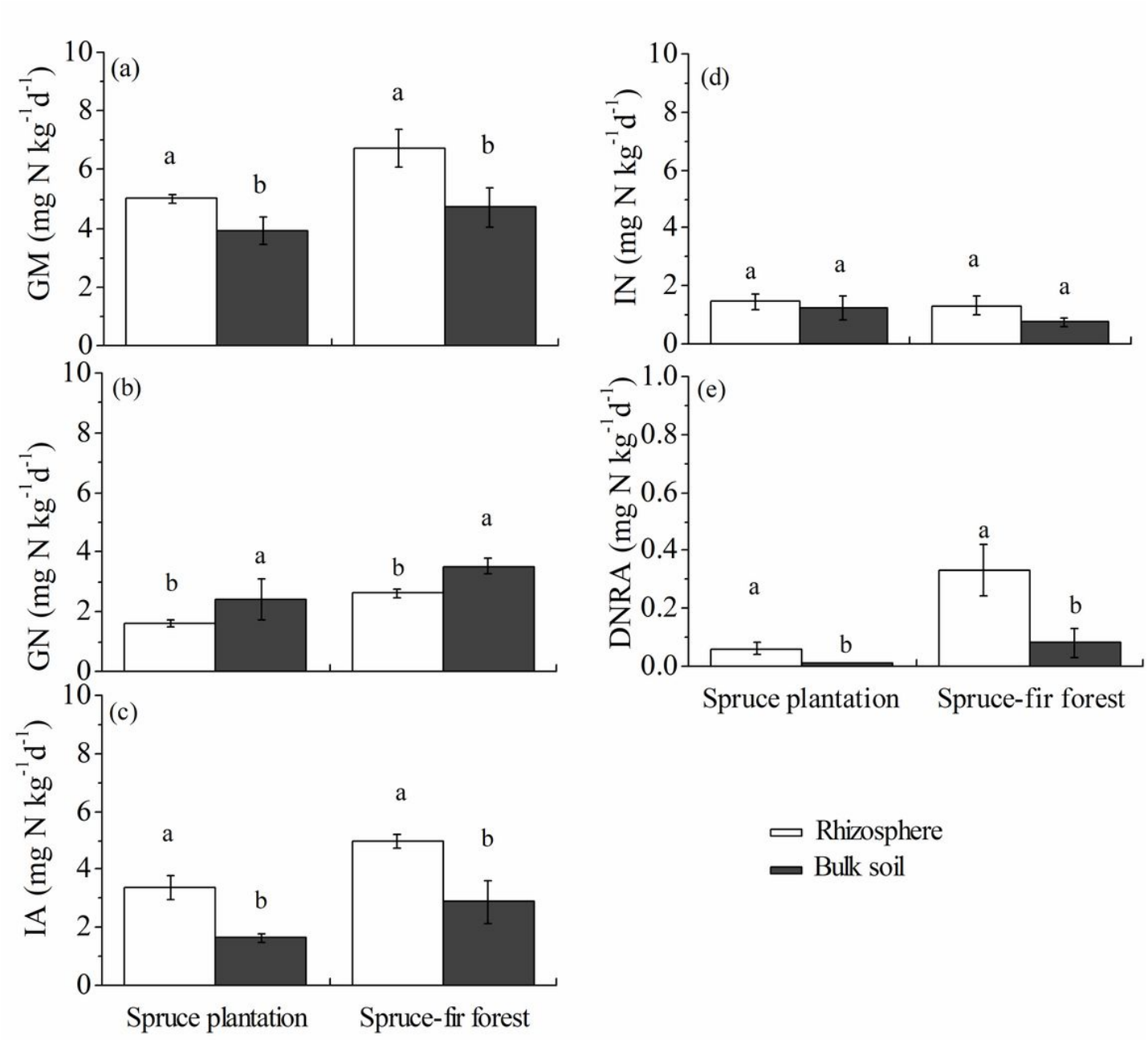

Figure 1

Gross rates of mineral N transformation in the rhizosphere and the bulk soils in the spruce plantation and the spruce-fir forest on the eastern Qinghai-Tibet plateaus. Values are mean (SE), n = 4. Different lowercase letters denoted significant differences (P< 0.05) in N transformation rates between the rhizosphere and bulk soils. (a) GM: gross mineralization, (b) GN: gross nitrification, (c) IA: microbial NH4+ immobilization, (d) IN: microbial NO3- immobilization, (e) DNRA: dissimilatory nitrate reduction to ammonium.

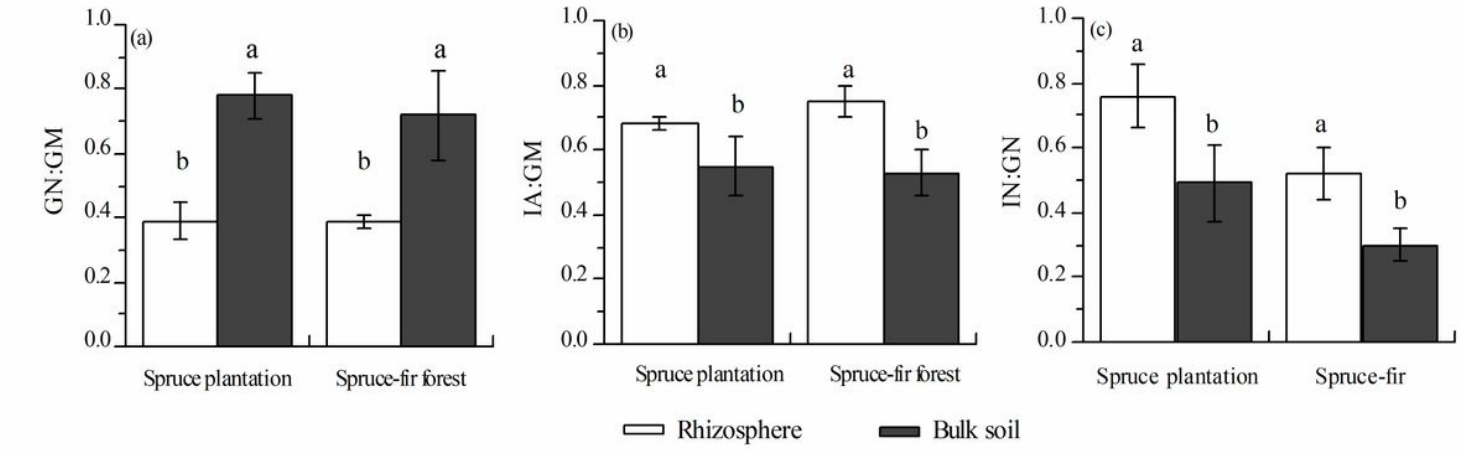

Figure 2

The ratio of gross nitrification to mineralization (relative nitrification; GN:GM) (a), the ratio of microbial NH4+ immobilization to gross mineralization (IA:GM) (b) and the ratio of microbial NO3- immobilization to gross mineralization (IN:GN) (c) in the rhizosphere and bulk soils in the spruce plantation and the spruce-fir plantation. Values are mean (SE), n = 4. Different lowercase letters denoted significant differences (P< 0.05) in two ratios between the rhizosphere and bulk soils.

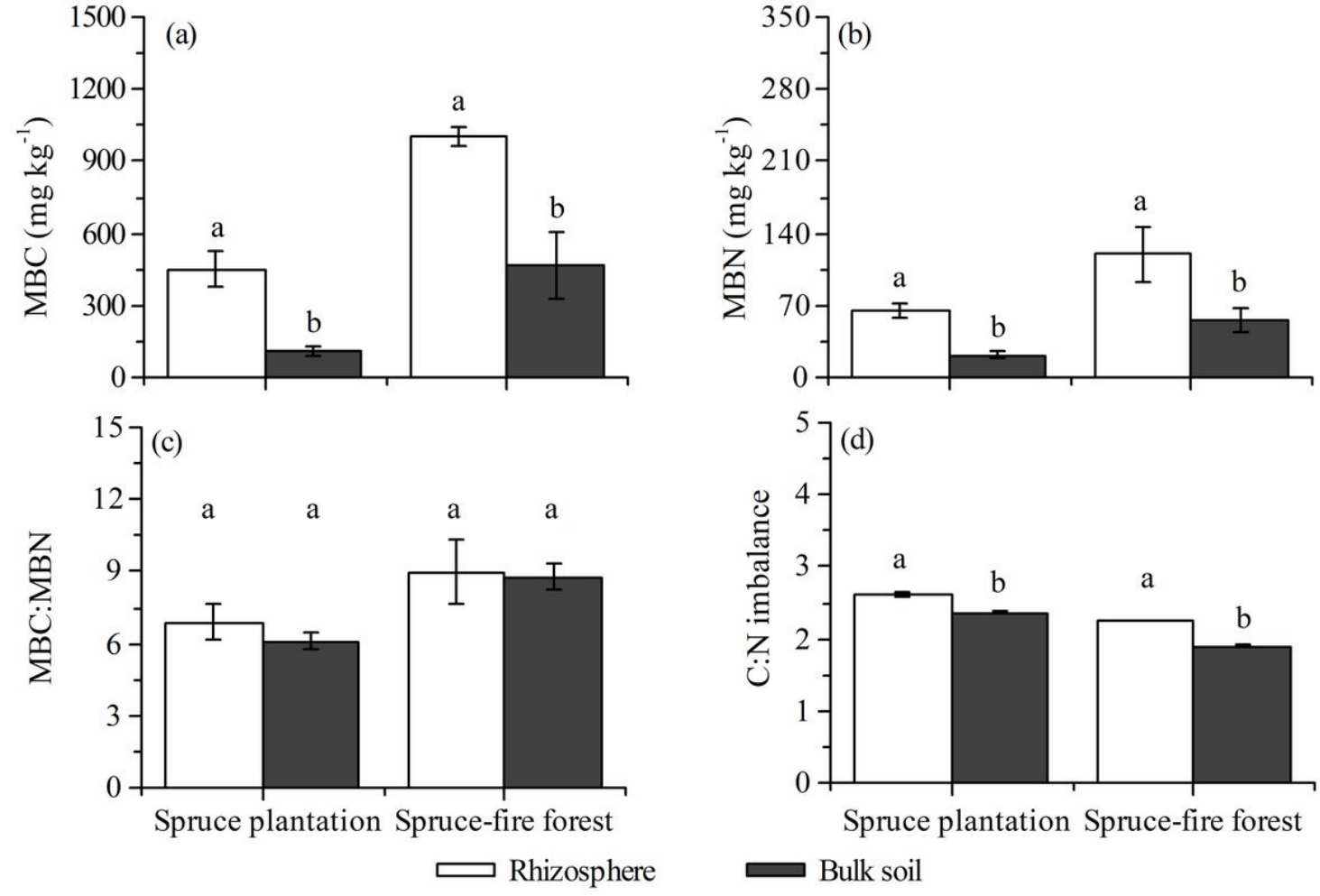

Figure 3

Microbial biomass C (MBC) (a), N (MBN) (b), C:N ratio (c) and the stoichiometric imbalance of C: N between microbes and their resource (calculated as the ratio of SOC:N to microbial biomass C:N) (d) in the rhizosphere and bulk soils of the spruce plantation and the spruce-fir forest on the eastern Qinghai-Tibet plateau. Values are mean (SE), n = 4. Different lowercase letters denoted significant differences (P< 0.05) between the rhizosphere and bulk soils.

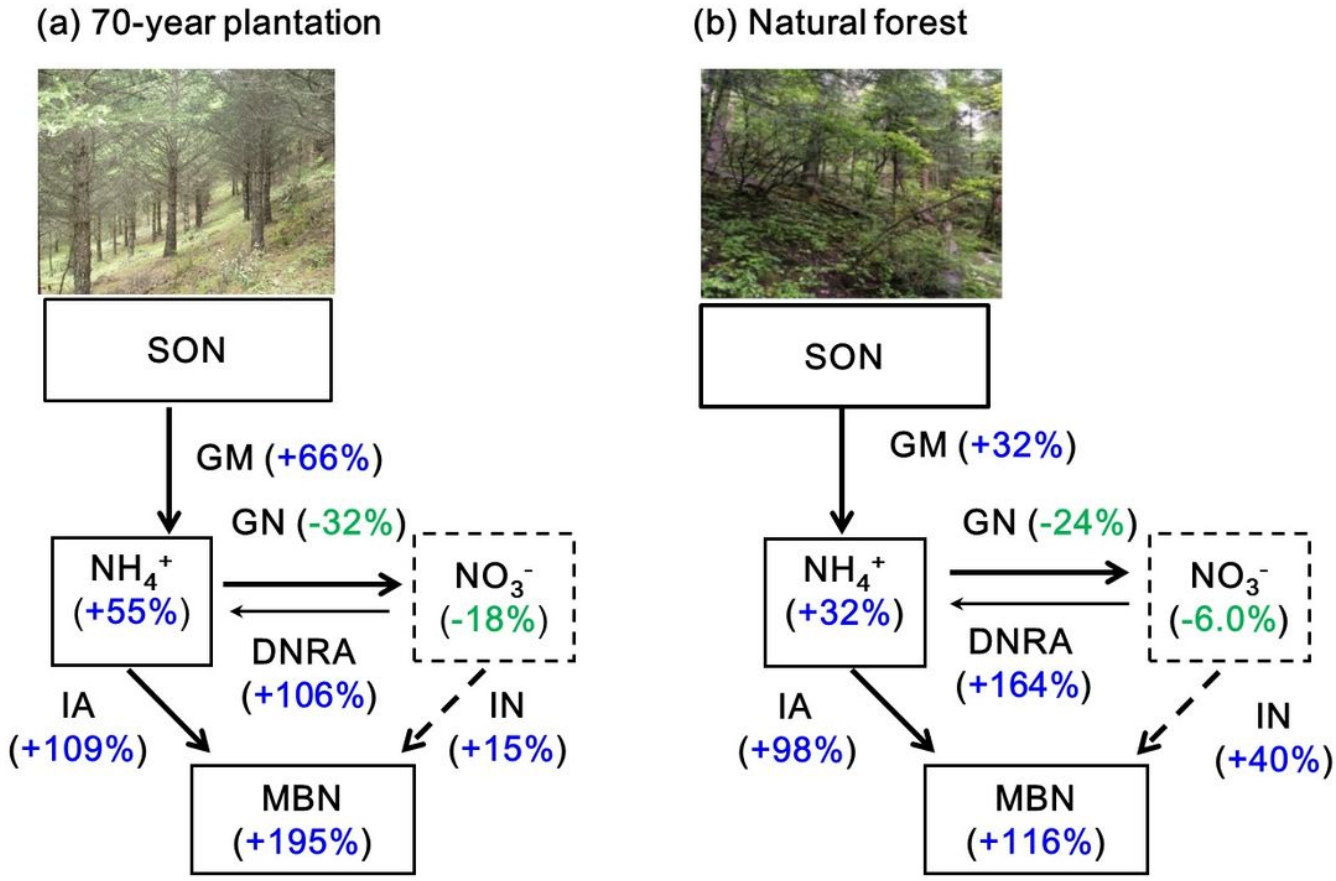

Figure 4

Rhizosphere effects on gross N production and retention in soils of the alpine coniferous plantation (a) and natural forest (b) in eastern Qinghai-Tibet plateau. The squares represent various N pools, and the arrows represent various N fluxes. The solid line represents that the rhizosphere effect was significant; while the dotted line represents that the rhizosphere effect was not significant. The blue digital denotes the positive rhizosphere effect, while the green digital denotes the negative effect. NH4+ production and retention: gross mineralization (GM) and microbial NH4+ immobilization (IA); NO3- production and retention: gross nitrification (GN), microbial NO3- immobilization (IN) and dissimilatory nitrate reduction to ammonium (DNRA). SON: soil organic nitrogen; MBN: microbial biomass nitrogen.

Supplementary Files

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- Appendix.docx