

Nutrient limitation of alpine plants: Implications from leaf N : P stoichiometry and leaf $\delta^{15}\text{N}$

Xingliang Xu^{1*}, Wolfgang Wanek², Caiping Zhou¹, Andreas Richter², Minghua Song¹, Guangmin Cao³, Hua Ouyang¹, and Yakov Kuzyakov^{1,4,5}

¹ Key Laboratory of Ecosystem Network Observation and Modeling, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, 11A Datun Road, Chaoyang District, 100101 Beijing, China

² Department of Microbiology and Ecosystem Science, Terrestrial Ecosystem Research, University of Vienna, Althanstrasse 14, A-1090 Wien, Austria

³ Northwest Institute of Plateau Biology, Chinese Academy of Sciences, NO. 23 Xinning Road, 810008 Xining, China

⁴ Department of Soil Science of Temperate Ecosystems, University of Göttingen, 37077 Göttingen, Germany

⁵ Department of Agricultural Soil Science, University of Göttingen, 37077 Göttingen, Germany

Abstract

Nitrogen (N) deposition can affect grassland ecosystems by altering biomass production, plant species composition and abundance. Therefore, a better understanding of the response of dominant plant species to N input is a prerequisite for accurate prediction of future changes and interactions within plant communities. We evaluated the response of seven dominant plant species on the Tibetan Plateau to N input at two levels: individual species and plant functional group. This was achieved by assessing leaf N : P stoichiometry, leaf $\delta^{15}\text{N}$ and biomass production for the plant functional groups. Seven dominant plant species—three legumes, two forbs, one grass, one sedge—were analyzed for N, P, and $\delta^{15}\text{N}$ 2 years after fertilization with one of the three N forms: NO_3^- , NH_4^+ , or NH_4NO_3 at four application rates (0, 7.5, 30, and 150 kg N ha⁻¹ y⁻¹). On the basis of biomass production and leaf N : P ratios, we concluded that grasses were limited by available N or co-limited by available P. Unlike for grasses, leaf N : P and biomass production were not suitable indicators of N limitation for legumes and forbs in alpine meadows. The poor performance of legumes under high N fertilization was mainly due to strong competition with grasses. The total above-ground biomass was not increased by N fertilization. However, species composition shifted to more productive grasses. A significant negative correlation between leaf N : P and leaf $\delta^{15}\text{N}$ indicated that the two forbs *Gentiana straminea* and *Saussurea superba* switched from N deficiency to P limitation (e.g., N excess) due to N fertilization. These findings imply that alpine meadows will be more dominated by grasses under increased atmospheric N deposition.

Key words: ammonium / plant functional groups / nitrate / nitrogen cycling / alpine meadow

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1 Introduction

Alpine meadows are among the most important types of ecosystems on the Tibetan Plateau, covering approximately 35% of the plateau area (Cao et al., 2004a). Although a large amount of nitrogen (N) is stored in alpine meadow soils, plant growth has frequently been reported to be co-limited by the availability of N and phosphorus (P) (Zhou, 2001). A previous study on alpine meadows showed that N mineralization is dominated by microbial immobilization during the growth season (Song et al., 2007). Additionally, these meadows in the eastern Tibetan Plateau experience N deposition of 7–10 kg N ha⁻¹ y⁻¹ through rainfall (Zuo et al., 1986; Lü and Tian, 2007). N deposition by rainfall can alleviate N limitation of plant growth during the growth season in alpine meadows. Previous studies showed that dominant plant species in Tibetan alpine meadows differ in N acquisition strategies (Xu et al., 2011) and require different amounts of N (Cao et al., 2004b). This suggests that N input may have the potential to affect species composition and abundance in alpine meadows.

Therefore, a better understanding of the responses of dominant plant species to N input is essential to accurately predict future changes in the plant community composition and the productivity in alpine meadows. However, little is known about how alpine plants respond to N deposition at the species level in these meadows.

From freshwater to terrestrial ecosystems, plant N : P stoichiometry has widely been used as an indicator to identify the nutrients that limit plant growth (Güsewell, 2004; Güsewell and Verhoeven, 2006; Elser et al., 2007, 2009; Ågren, 2008). Numerous studies have suggested that biomass N : P ratios of less than 14 (mass basis) indicate N limitation, and that ratios greater than 16 indicate P limitation in terrestrial plants (Koerselman and Meuleman, 1996; Aerts and Champin, 2000). On the basis of a meta-analysis, Güsewell (2004) suggested that the N : P ratio indicating N limitation was lowered to 10 and the limit for P limitation was raised to 20; for ratios



* Correspondence: X. Xu; e-mail: xuxingl@hotmail.com

between 10 and 20, plant growth may be co-limited by N and P. However, several studies have suggested that species-specific leaf N : P ratios do not reflect the type of nutrient limitation at the local scale (von Oheimb et al., 2010).

^{15}N fractionation due to discrimination against the isotopically heavier ^{15}N substrates can occur during N uptake and assimilation by plant tissues (e.g., enzymatic NH_4^+ or NO_3^- assimilation; Evans, 2001). Plant ^{15}N fractionation strongly depends on the balance between plant N demand and N supply (Evans, 2001; Wanek and Zotz, 2011). That is, leaf $\delta^{15}\text{N}$ in N-limited environments is expected to reflect the isotopic signature of soil inorganic N and plant to show weak ^{15}N fractionation because most of the available N is assimilated and recycled in these plants (Evans, 2001). When conditions change from those of N limitation to N excess (or P limitation), ^{15}N fractionation increases during plant N utilization, leading to a decrease in plant $\delta^{15}\text{N}$ (McKee et al., 2002; Wanek and Zotz, 2011). Therefore, plant $\delta^{15}\text{N}$ has the potential to reflect N limitation in terrestrial ecosystems.

Recently, a few studies have investigated nutrient limitation by using leaf $\delta^{15}\text{N}$ in combination with measurements of plant N : P stoichiometry in wetlands (Clarkson et al., 2005; Inglett and Ramesh, 2006; Troxler, 2007), mangroves (McKee et al., 2002), and tropical forest canopies (Wanek and Zotz, 2011). A significant negative correlation between leaf $\delta^{15}\text{N}$ and N : P ratio was observed, reflecting the tendency of P limitation to increase plant ^{15}N fractionation and that of N limitation to decrease plant ^{15}N fractionation. Nonetheless, some plant species do not show clear variation in leaf $\delta^{15}\text{N}$ in relation to leaf N : P ratios. This was ascribed to the species-specific differences in nutrient requirements and nutrient acquisition mechanisms (Clarkson et al., 2005; Inglett and Ramesh, 2006). In Tibetan alpine meadows it remains unclear how leaf $\delta^{15}\text{N}$ of dominant plant species changes with leaf N : P ratios.

A fertilization experiment showed that NH_4^+ additions increased the biomass of vascular plants, while NO_3^- additions had no effect (Verhoeven et al., 2011), suggesting that

both N forms may have different effects on plant growth. In alpine meadows, dominant plant species showed different preferences for NO_3^- and NH_4^+ (Xu et al., 2003, 2011). However, the ways in which these species respond to the two N forms are still not well understood. Considering that most previous studies focused on nutrient limitation (i.e., N and P limitation) of plant growth at the ecosystem level (Elser et al., 2007; LeBauer and Treseder, 2008), in this study, we evaluated nutrient limitation on plant growth at two levels: plant species and plant functional group. We aimed to determine whether dominant plant species in an alpine meadow are N or P-limited and how they respond to N additions.

2 Material and methods

2.1 Study site

The experiment was carried out at the Haibei Alpine Meadow Ecosystem Station of the Chinese Academy of Sciences, Qinghai Province (lat 37°36'60"N, long 101°19'14"E, 3,215 m above sea level). The area is located in the low alpine meadow zone, characterized by a typical alpine meadow climate (Zhou, 2001). The 25-year means for temperature and precipitation are -1.7°C and 600 mm, respectively. Dominant plant species include *Kobresia humilis* Serg., *Stipa aliena* Keng., *Poa* sp., *Festuca ovina* Linn., *Gentiana aristata* Maxim., *Gentiana straminea* Maxim., *Saussurea superba* Anth., and *Guedenstaedia diversifolia* Maxim. (Zhou, 2001). The soil is classified as Mat-Cryic Cambisol (Chinese Soil Taxonomy Research Group, 1995; Table 1), corresponding to Gelic Cambisol (WRB, 1998). The soil Na^+ content in all fertilization treatments with NaNO_3 was not significantly different from that of the control (Table 1).

2.2 Experimental design and treatments

In 2005, three blocks were established in a typical *K. humilis* meadow, in a location that was homogenous in cover and species composition. In each block, 10 plots (2 m × 2 m)

Table 1: Soil properties in the top 10 cm depth 2 years after N addition. Means (\pm SE) of three replicates are presented. Stars indicate that treatments are different to the control at $P < 5\%$ level.

Treatments	N addition / kg N ha ⁻¹	Soil organic carbon / %	Total nitrogen / %	P / mg g ⁻¹	Na / mg g ⁻¹	Soil $\delta^{15}\text{N}$ / ‰	C : N	N : P
Control	0	7.06 \pm 0.37	0.55 \pm 0.03	0.86 \pm 0.03	13.37 \pm 0.11	3.95 \pm 0.09	12.78 \pm 0.16	6.46 \pm 0.65
NO_3^-	7.5	7.00 \pm 0.13	0.56 \pm 0.02	0.87 \pm 0.01	13.17 \pm 0.05	3.96 \pm 0.09	12.44 \pm 0.23	6.46 \pm 0.15
	30	7.60 \pm 0.28	0.61 \pm 0.01	0.84 \pm 0.02	13.62 \pm 0.13	4.08 \pm 0.53	12.47 \pm 0.16	7.23 \pm 0.32
	150	7.34 \pm 0.14	0.59 \pm 0.02	0.85 \pm 0.06	13.32 \pm 0.14	3.44 \pm 0.26	12.57 \pm 0.19	6.93 \pm 0.42
NH_4^+	7.5	7.15 \pm 0.04	0.59 \pm 0.01	0.83 \pm 0.02	13.69 \pm 0.16	6.50 \pm 0.1*	12.11 \pm 0.02*	7.11 \pm 0.14
	30	6.83 \pm 0.27	0.55 \pm 0.02	0.84 \pm 0.04	13.28 \pm 0.33	6.00 \pm 0.8*	12.36 \pm 0.02	6.58 \pm 0.27
	150	7.14 \pm 0.22	0.58 \pm 0.02	0.85 \pm 0.01	13.33 \pm 0.23	4.72 \pm 0.11	12.40 \pm 0.11	6.75 \pm 0.26
NH_4NO_3	7.5	6.73 \pm 0.15	0.52 \pm 0.01	0.81 \pm 0.05	13.45 \pm 0.21	3.81 \pm 0.20	13.06 \pm 0.17	6.44 \pm 0.47
	30	7.01 \pm 0.11	0.53 \pm 0.01	0.74 \pm 0.00*	12.19 \pm 0.99	3.55 \pm 0.12	13.19 \pm 0.17	7.40 \pm 0.87
	150	6.70 \pm 0.30	0.54 \pm 0.03	0.85 \pm 0.02	13.26 \pm 0.20	3.93 \pm 0.26	12.42 \pm 0.19	6.36 \pm 0.33

were arranged. In total, 30 plots were established. Because over 95% of roots are located in the upper 15 cm of soil in this type of meadow (Zhou, 1982), plots were separated from the surrounding areas by using iron sheets inserted 30 cm into the soil, with 1 m-wide buffer zones on all sides of each plot. Three types of N fertilizers, *i.e.*, NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3 , were added to 27 plots, providing three treatments: NO_3^- , NH_4^+ , and NH_4NO_3 , respectively. The fertilizers were applied at rates of 7.5, 30, and 150 kg N $\text{ha}^{-1} \text{y}^{-1}$, respectively. The remaining three plots that did not receive any fertilizer were used as controls. The $\delta^{15}\text{N}$ values of NaNO_3 , $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3 application were +11.63, -1.76, and +3.88‰, respectively. Because most precipitation occurs in July and August (Zuo *et al.*, 1987), N fertilizer was added twice every year: once in July and once in August, with half the total amount added on each occasion. Fertilizer application began in July 2005. N fertilizers were dissolved in distilled water and sprayed on the plots. After N addition, the vegetation was carefully rinsed by spraying with water to remove fertilizer drops from the leaf surfaces. The total volume of water applied equated to approximately 3 mm of rainfall. The control treatment involved spraying with the equivalent volume of water.

2.3 Sampling and analyses

Two years after N addition, seven dominant plant species (Table 2) were selected from these 30 plots: one sedge, one grass, three leguminous species, and two forbs. In early July 2007, leaves were collected from at least five individuals of each plant species per plot and pooled by plot to yield a total of 210 leaf samples. Leaves were dried at 75°C for 48 h. Above-ground biomass was estimated by harvesting plants from a 25 cm × 25 cm area in each plot, while root biomass was measured using soil cores. Five soil cores (3.8 cm diameter) to a depth of 15 cm were collected from each plot and combined to form one composite soil sample per plot. Soil samples were transferred to the laboratory immediately after collection. Living roots were carefully removed from these soil samples and washed with water and then dried at 75°C for biomass measurement. The samples were sieved to < 2 mm, and 20 g fresh soil was dried at 75°C for 48 h. Dried plant and soil materials were ground to a fine powder using a ball mill (MM200, Retsch, Haan, Germany).

Table 2: Basic characteristics of seven dominant plant species in an alpine meadow at Hebei experimental station.

Plant species	Family	Functional type
<i>Kobresia humilis</i>	Cyperaceae	grass
<i>Elymus nutans</i>	Gramineae	grass
<i>Gueldenstaedtia diversifolia</i>	Leguminosae	legume
<i>Trigonella ruthenica</i>	Leguminosae	legume
<i>Oxytropis ochrocephala</i>	Leguminosae	legume
<i>Saussurea superba</i>	Asteraceae	forb
<i>Gentiana straminea</i>	Gentianaceae	forb

Leaf and soil samples were weighed into tin capsules and analyzed for total N, C, and $\delta^{15}\text{N}$ by continuous-flow isotope-ratio mass spectrometry (CF-IRMS) using an elemental analyzer (EA 1110; CE Instruments, Milan, Italy) and a ConFlo II device (Finnigan MAT, Bremen, Germany) connected to a gas isotope ratio mass spectrometer (DeltaPLUS, Finnigan MAT, Bremen, Germany). The abundance of ^{15}N in plant samples is expressed in δ units, which denote the deviation in ‰ of the sample $^{15}\text{N} : ^{14}\text{N}$ ratio from that in the atmospheric N_2 . Atmospheric N_2 is used as the reference standard for N isotopic analyses and has a $\delta^{15}\text{N}$ value defined as 0‰. The standard deviation of repeated measurements of laboratory standards was $\pm 0.15\%$.

Because a high Na^+ concentration in soil might stress plant growth and NaNO_3 was used as a fertilizer, the Na^+ content of plant leaves and soils was measured. Molybdenum (Mo) is an important component of the enzyme nitrogenase, and low levels of plant-available Mo may limit biological N_2 fixation in legumes, and thus affect the $\delta^{15}\text{N}$ values of legumes. Therefore, the Mo contents of soils were also analyzed. The P and Mo contents in leaf tissues were measured using optical emission spectrometry (Optima 5300DV; PerkinElmer, Shelton, USA) after nitric-perchloric acid digestion (Parkinson and Allen, 1975; Grimshaw, 1987).

2.4 Statistical analysis

The standard errors of the means are presented in figures and tables. Dunnett's test was used to compare the effects of N fertilizer application on soil properties, plant biomass and leaf N : P of plant functional groups with the control treatment. A one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test was used to compare effects of different fertilization rates on leaf $\delta^{15}\text{N}$ and N : P ratios of different plant species. Regression analysis was conducted for leaf $\delta^{15}\text{N}$ versus leaf N : P ratios, and for above-ground biomass versus N fertilization rates and leaf N : P ratios of dominant plant species. All differences were tested for significance at $P < 5\%$ by using the SPSS 16.0 software package (SPSS Inc., Chicago, IL, USA).

3 Results

3.1 Above and below-ground biomass

To assess whether responses of leaf N : P ratios to N fertilizer application reflect N limitation, we evaluated the total above-ground biomass of two functional groups: legumes (high N : P) and non-legumes (low N : P). The above-ground biomass of non-legumes increased with increasing N fertilization rate under both NH_4^+ ($R^2 = 0.37$, $P < 5\%$) and NO_3^- ($R^2 = 0.57$, $P < 5\%$) application, but a similar correlation was not observed with NH_4NO_3 fertilization. Similar patterns were observed for the correlation between above-ground biomass of non-legumes and leaf N : P (NH_4^+ application: $R^2 = 0.36$, $P < 5\%$; NO_3^- application: $R^2 = 0.53$, $P < 5\%$). As expected, the above-ground biomass of legumes was markedly reduced by increasing fertilizer application ($P < 5\%$, Table 3), with the exception of NH_4^+ application at 30 kg N $\text{ha}^{-1} \text{y}^{-1}$.

Table 3: Above-ground plant biomass of three functional groups and total below-ground biomass 2 years after N addition. Means (\pm SE) of three replicates are presented. Stars indicate that treatments are different to the control at $P < 5\%$ level.

N form	N addition / kg N ha ⁻¹	Above-ground parts / g m ⁻²			Below-ground parts / g m ⁻²
		Legumes	Grasses	Forbs	
Control	0	67.2 \pm 13.3	155.8 \pm 16.7	86.9 \pm 17.3	1494.2 \pm 22.7
NO ₃ ⁻	7.5	29.8 \pm 3.2*	123.0 \pm 13.4	132.1 \pm 25.0	1406.0 \pm 138.2
	30	28.5 \pm 5.8*	122.5 \pm 24.8	94.9 \pm 3.3	1824.0 \pm 308.3
	150	3.2 \pm 1.5*	261.7 \pm 45.4*	119.9 \pm 10.2	1452.4 \pm 122.0
NH ₄ ⁺	7.5	35.5 \pm 0.0*	112.7 \pm 12.3	85.5 \pm 9.5	1505.7 \pm 122.6
	30	51.8 \pm 8.6	141.2 \pm 10.7	145.8 \pm 37.1*	1503.5 \pm 207.8
	150	13.0 \pm 0.4*	212.0 \pm 2.6*	104.5 \pm 17.4	1674.8 \pm 212.7
NH ₄ NO ₃	7.5	22.3 \pm 7.7*	153.6 \pm 21.4	131.7 \pm 15.1	1356.9 \pm 170.2
	30	38.1 \pm 6.7*	176.5 \pm 17.6	138.2 \pm 17.8	1932.8 \pm 253.0
	150	11.7 \pm 3.0*	213.9 \pm 19.3	71.8 \pm 8.6	1303.9 \pm 372.1

Grasses responded significantly positively to the highest N rates, but not to NH₄NO₃ application. Forbs showed maximal biomass at low/intermediate rates, with biomass increasing significantly only at intermediate NH₄⁺ levels (Table 3). Total root biomass values were not significantly different from those of the control plots. The total above-ground biomass ranged between 245 and 385 g m⁻², but was not affected by N form or fertilizer rate ($P > 5\%$, data not shown). Above-ground: below-ground biomass ratios also varied between 0.13 and 0.27, indicating that root biomass was four to eight times higher than shoot biomass in this meadow. The ratios were not affected by N form or fertilizer rate ($P > 5\%$, data not shown).

3.2 Leaf N : P ratios

In the control plots, the leaf N : P ratios of all seven dominant plant species averaged 14.8 ± 0.7 . Unfertilized grasses and forbs showed similar N : P ratios (11.8 and 12.8), whereas

legumes exhibited significantly higher N : P ratios (18.0). The responses of leaf N : P to N addition varied according to plant functional group and fertilizer rate. The highest fertilizer rates of NH₄⁺, NO₃⁻, and NH₄NO₃ significantly increased the leaf N : P ratios of grasses and forbs, whereas increased leaf N : P ratios of legumes were detected at the two lower NH₄⁺ fertilizer rates (Table 4).

The leaf N : P ratios of individual species responded differently to N fertilizer rate and N form (Fig. 1). In *E. nutans* and *S. superba*, leaf N : P increased significantly at high N fertilizer rates. In *G. diversifolia*, the difference in leaf N : P between the control and fertilized plants was only significant at 30 kg N ha⁻¹ y⁻¹. Nitrate application significantly increased the leaf N : P of *K. humilis* (from 13.2 to 16.6; $P < 5\%$) and *G. diversifolia* (from 18.5 to 19.9; $P < 5\%$). The legume *M. ruthenica* and the grasses and forbs responded strongly to high NH₄⁺ and NO₃⁻ fertilizer rates.

Table 4: Leaf N : P ratios of three functional plant groups (legumes, grasses and forbs) 2 years after N additions. Means (\pm SE) of six to nine replicates are presented. Different letters for each column indicate significant differences between the treatment and the control at $P < 5\%$ level.

N form	N addition / kg N ha ⁻¹	N : P ratios		
		Legumes	Grasses	Forbs
Control	0	18.0 \pm 0.6	12.8 \pm 0.7	11.8 \pm 0.6
NO ₃ ⁻	7.5	17.6 \pm 0.6	14.2 \pm 1.2	13.9 \pm 0.3
	30	19.0 \pm 0.8	14.0 \pm 1.0	13.9 \pm 0.3
	150	18.7 \pm 0.8	17.8 \pm 1.1*	17.9 \pm 1.4*
NH ₄ ⁺	7.5	20.4 \pm 0.6*	13.5 \pm 1.3	12.9 \pm 0.7
	30	19.9 \pm 0.6*	13.7 \pm 0.9	13.4 \pm 0.4
	150	19.4 \pm 0.6	17.3 \pm 0.3*	16.8 \pm 0.6*
NH ₄ NO ₃	7.5	17.6 \pm 0.3	12.9 \pm 0.5	13.5 \pm 0.2
	30	17.8 \pm 0.5	13.0 \pm 0.8	12.2 \pm 0.4
	150	17.3 \pm 0.5	16.4 \pm 0.5*	17.4 \pm 1.2*

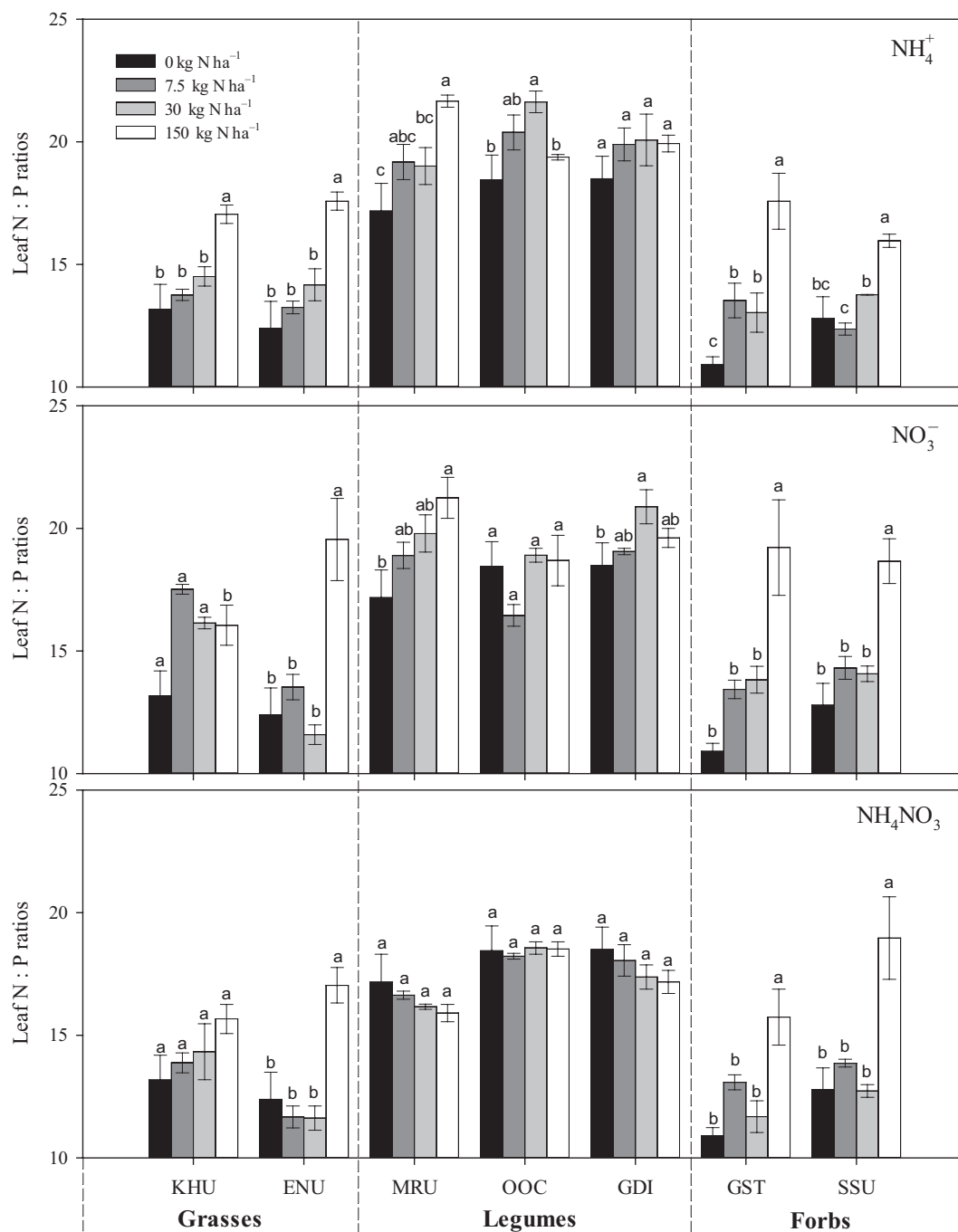


Figure 1: Leaf N : P ratios of seven dominant species in the fertilized *Kobresia humilis* meadow 2 years after N fertilization as: NH_4^+ , NO_3^- , and NH_4NO_3 . Values are means (\pm SE) of three replicates. Different letters indicate significant differences at $P < 5\%$ level between dominant plant species. KHU: *Kobresia humilis*, ENU: *Elymus nutans*, MRU: *Medicago ruthenica*, OOC: *Oxytropis ochrocephala*, GDI: *Gueldenstaedtia diversifolia*, GST: *Gentiana straminea*, and SSU: *Saussurea superba*.

3.3 Leaf $\delta^{15}\text{N}$ and Mo content

Of the seven species, *G. straminea* was the only plant showing positive $\delta^{15}\text{N}$ values. Although the $\delta^{15}\text{N}$ value of NH_4^+ fertilizer was negative (-1.8‰), the leaf $\delta^{15}\text{N}$ of *G. straminea* was not significantly influenced by NH_4^+ application. *E. nutans* was significantly ^{15}N -depleted at a low NH_4^+ application rate compared to the control, but was ^{15}N -enriched at the highest NH_4^+ application rate (Fig. 2). Similar to NH_4^+ application,

increasing the NH_4NO_3 rate did not alter the leaf $\delta^{15}\text{N}$ values of *K. humilis*, *S. superba*, and *O. ochrocephala*. *G. straminea* exhibited positive leaf $\delta^{15}\text{N}$ values at all N rates (Fig. 2).

Nitrate fertilizer had a positive $\delta^{15}\text{N}$ value ($+11.6\text{‰}$), but none of the seven species developed leaf $\delta^{15}\text{N}$ similar to the fertilizer $\delta^{15}\text{N}$ value. In contrast, all fertilized plants became ^{15}N -depleted compared to the control, *i.e.*, the $\delta^{15}\text{N}$ values decreased (Fig. 2). In particular, the leaf $\delta^{15}\text{N}$ of *K. humilis*

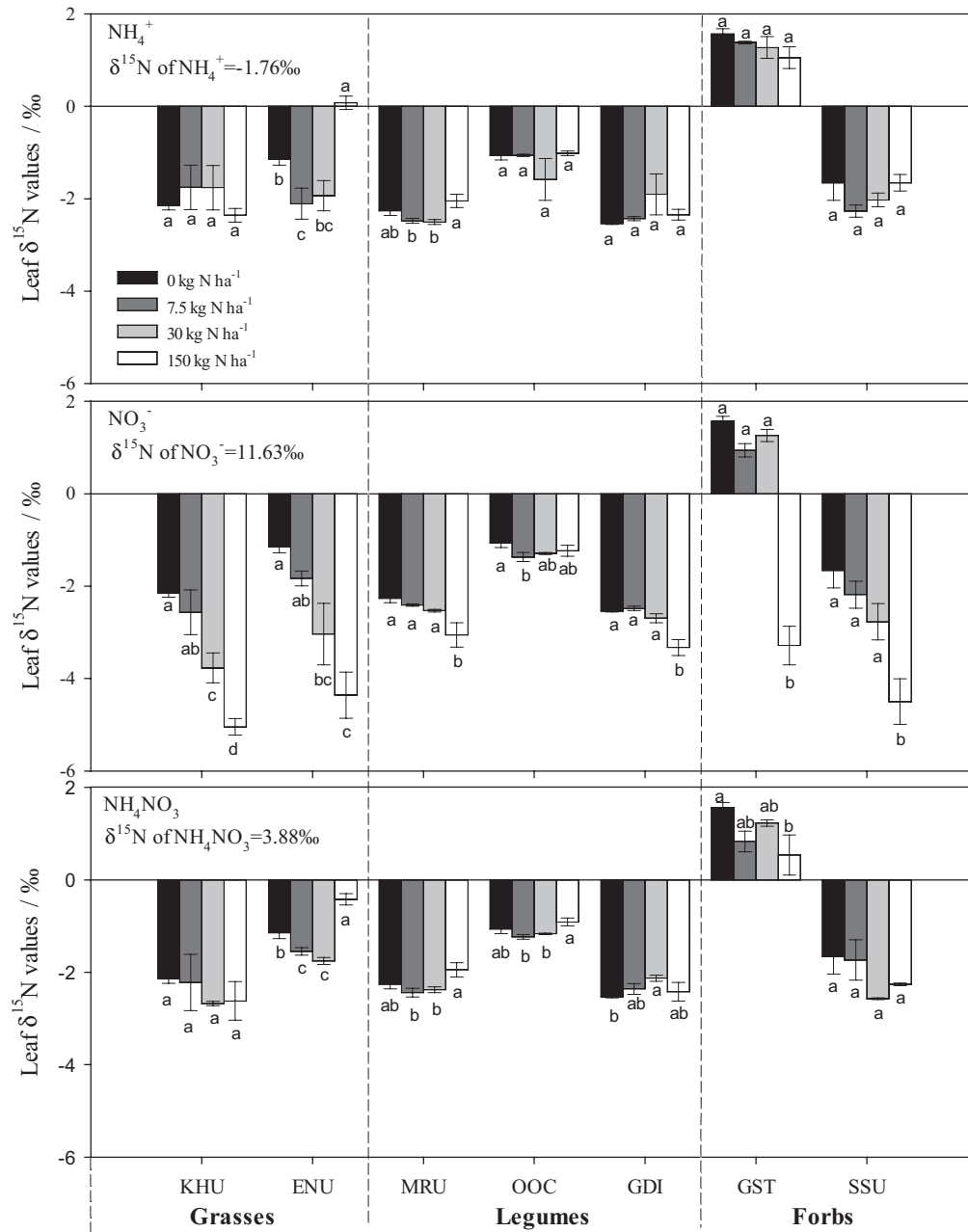


Figure 2: Leaf $\delta^{15}\text{N}$ of seven dominant species in the fertilized *Kobresia humilis* meadow 2 years after N additions as: NH_4^+ , NO_3^- , and NH_4NO_3 . Values are means (\pm SE) of three replicates. Different letters for each species indicate significant differences at $P < 5\%$ level between N fertilization rates. KHU: *Kobresia humilis*, ENU: *Elymus nutans*, MRU: *Medicago ruthenica*, OOC: *Oxytropis ochrocephala*, GDI: *Gueldenstaedtia diversifolia*, GST: *Gentiana straminea*, and SSU: *Saussurea superba*.

and *E. nutans* decreased significantly with increasing NO_3^- rates. The leaf $\delta^{15}\text{N}$ values of *S. superba*, *M. ruthenica*, and *G. diversifolia* significantly decreased only at the highest NO_3^- input levels (Fig. 2).

The Mo concentration in soil varied from 0.2 to 2.7 $\mu\text{g g}^{-1}$, whereas the leaf Mo concentration of the seven plant species ranged from $0.47 \pm 0.11 \mu\text{g g}^{-1}$ (*G. straminea*) to $2.51 \pm 0.19 \mu\text{g g}^{-1}$ (*G. diversifolia*) (Fig. 3). For legumes, leaf $\delta^{15}\text{N}$ decreased exponentially with increasing leaf Mo concentration ($R^2 = 0.51$, $P < 0.01\%$; Fig. 3).

3.4 Correlations between leaf $\delta^{15}\text{N}$ and leaf N : P ratios

Correlations between leaf $\delta^{15}\text{N}$ and N : P ratios depended on species and the N form added (Table 5). There was a significant negative correlation between leaf $\delta^{15}\text{N}$ and leaf N : P for the forb *G. straminea* under both NO_3^- and NH_4NO_3 application. The correlation between leaf $\delta^{15}\text{N}$ and N : P ratios for the forb *S. superba* was significantly negative under NO_3^- application, but was significantly positive under NH_4^+ fertilization. The grass *E. nutans* showed a positive correlation

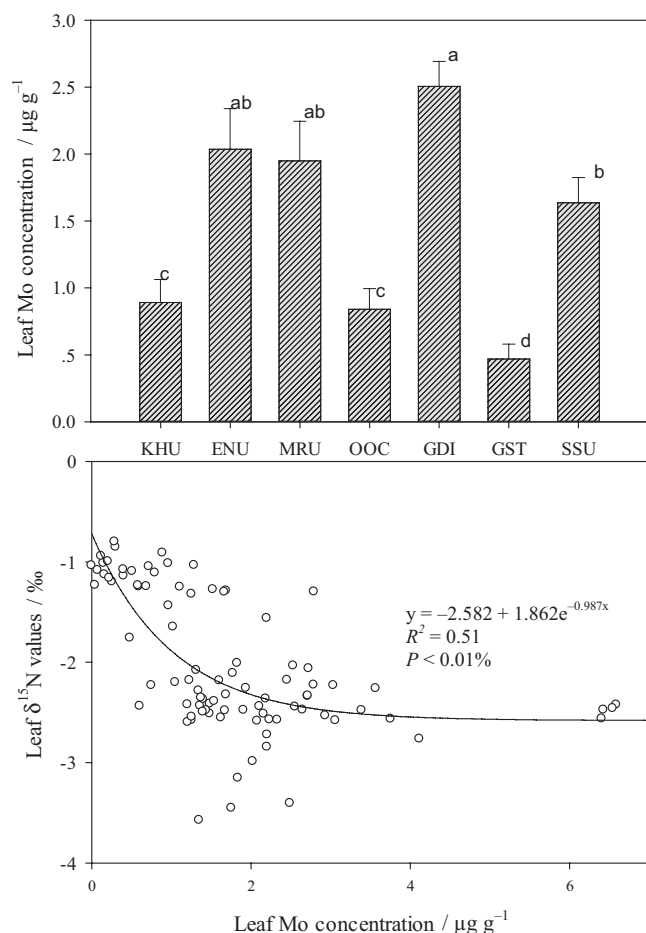


Figure 3: Leaf Mo concentrations (upper panel) of seven dominant species and correlations between leaf Mo concentrations and leaf $\delta^{15}\text{N}$ of three legumes (lower panel) in the fertilized *Kobresia humilis* meadow 2 years after N additions as: NH_4^+ , NO_3^- , and NH_4NO_3 . Values in the upper panel are means (\pm SE) of three replicates. Different letters for each species indicate significant differences at $P < 5\%$ level among species. KHU: *Kobresia humilis*, ENU: *Elymus nutans*, MRU: *Medicago ruthenica*, OOC: *Oxytropis ochrocephala*, GDI: *Gueldenstaedtia diversifolia*, GST: *Gentiana straminea*, and SSU: *Saussurea superba*.

between leaf $\delta^{15}\text{N}$ and N : P ratios under both NH_4^+ and NH_4NO_3 application. Of the three legumes, only the $\delta^{15}\text{N}$ of *O. ochrocephala* was positively correlated with leaf N : P ratios under NO_3^- fertilization (Table 5).

4 Discussion

4.1 Nutrient limitation at the plant functional group level

Our results show that leaf N : P ratios differ significantly among plant functional groups (*i.e.*, grasses, forbs, and legumes) in alpine meadows, and the values we obtained are within the range reported previously (Güsewell, 2004). The leaf N : P ratios of grasses in this study were low (12.8) and comparable to those of subarctic grasses (13.2), but considerably lower than the global average for grasses (17.8)

(Güsewell, 2004). This indicates that grasses growing at high altitudes or in cold climates may be more N-limited than those growing in warmer climates due to the slow mineralization of soil organic matter caused by low temperature (Zhou, 2001; Song *et al.*, 2007) as well as lower N_2 fixation by legumes. Positive responses in the above-ground biomass for grasses to NH_4^+ and NO_3^- application (Table 3) confirm that they are limited or co-limited by available N and P in alpine meadows. Surprisingly, however, insignificant positive responses were observed for the NH_4NO_3 application. This could be due to the fact that NO_3^- uptake by grasses is inhibited by the presence of NH_4^+ when NH_4NO_3 is applied (Kronzucker *et al.*, 1999).

Forbs also had low leaf N : P ratios, and the maximal N addition rate significantly increased their leaf N : P ratio. However, significant positive responses in the above-ground biomass to N application were not observed except at moderate NH_4^+ addition. According to these results, it seems that forbs were not limited by available N in alpine meadows. However, their ^{15}N data showed a significant negative correlation with leaf N : P ratio, indicating that these forbs switched from N deficiency to N excess (*e.g.*, P limitation) due to N application. The difference in the responses of grasses and forbs can be ascribed to their different traits, *i.e.*, grasses prefer to grow in an environment with better nutrient conditions, while native forbs are well adapted to low-nutrient conditions (Xu *et al.*, 2011). Therefore, N limitation of the forbs in this alpine meadow was masked by strong competition with grasses, which depressed the growth of forbs under high N application.

Compared with forbs and grasses, legumes showed the highest N : P ratios (around 18–20), similar to those observed by He *et al.* (2008) in the Chinese grassland biomes. In this study, legumes showed rather invariant leaf N : P ratios with N rate, and legume leaf N : P ratio increased only at low NH_4^+ rate. However, their above-ground biomass was markedly reduced by the maximal N addition (Table 3). The legumes are adapted to low N conditions because they fix atmospheric N_2 via symbiotic rhizobia (Yang *et al.*, 2011). However, they lose their advantage and are gradually outcompeted by grasses at high N level. This indicates that leaf N : P ratio and biomass production are not suitable indicators of N limitation for legumes as a functional group.

The above-ground biomass of grasses was increased by high N application, whereas that of legumes was strongly reduced (Table 3), leading to an insignificant change in the total above-ground biomass. This phenomenon was observed consistently over 6 years at the same site (Song *et al.*, 2012). The absence of response to N application for the ecosystem as a whole could be because of the limitation of other nutrients (*e.g.*, P and magnesium) when N input is substantially high. Although the total above-ground biomass was not significantly increased by N application, shifts in species composition were observed and grasses became more productive (Song *et al.*, 2012). This implies that alpine meadows will most probably become more dominated by grasses under conditions of increased atmospheric N deposition.

Table 5: Correlations between leaf $\delta^{15}\text{N}$ and leaf N : P ratios for seven dominant plant species under NH_4^+ , NO_3^- and NH_4NO_3 application in an alpine meadow.

Species	N form	Direction +/-	Significance P / %	R^2
<i>Kobresia humilis</i>	NH_4^+	(-)	19	
	NO_3^-	0	31	
	NH_4NO_3	0	51	
<i>Elymus nutans</i>	NH_4^+	+	2	0.43
	NO_3^-	(-)	6	
	NH_4NO_3	+	0.02	0.78
<i>Gueldenstaedtia diversifolia</i>	NH_4^+	(+)	11	
	NO_3^-	0	90	
	NH_4NO_3	0	37	
<i>Oxytropis ochrocephala</i>	NH_4^+	0	75	
	NO_3^-	+	< 1	0.50
	NH_4NO_3	0	21	
<i>Medicago ruthenica</i>	NH_4^+	0	50	
	NO_3^-	(-)	7	
	NH_4NO_3	0	55	
<i>Saussurea superba</i>	NH_4^+	+	4	0.37
	NO_3^-	-	3	0.43
	NH_4NO_3	0	92	
<i>Gentiana straminea</i>	NH_4^+	(-)	16	
	NO_3^-	-	< 0.01	0.83
	NH_4NO_3	-	0.2	0.65

4.2 Nutrient limitation at the plant species level

As suggested by previous studies (McKee et al., 2002; Clarkson et al., 2005; Inglett and Ramesh, 2006; Troxler, 2007; Wanek and Zotz, 2011), a strong negative correlation between leaf $\delta^{15}\text{N}$ and N : P ratios provides strong evidence for changes from N to P limitation during plant growth in fertilizer experiments. Upon N application, plants are expected to switch from N deficiency to N excess (e.g., P limitation). This leads to increased leaf N : P ratio and decreased leaf $\delta^{15}\text{N}$. Therefore, plants growing under P-limited conditions should exhibit a negative correlation between leaf $\delta^{15}\text{N}$ and N : P when they are supplied with ample N. This was observed for the forb *G. straminea* and partially (NO_3^- fertilization) for the forb *S. superba*, suggesting that forbs are rather N than P-limited in alpine meadows.

In contrast, significant positive relationships were found between leaf $\delta^{15}\text{N}$ and N : P ratio for the grass *E. nutans* (NH_4^+ and NH_4NO_3 application), the legume *O. ochrocephala* (NO_3^- application), and the forb *S. superba* (NH_4^+ application). The remaining three species did not show significant correlations between leaf $\delta^{15}\text{N}$ and N : P ratio (Table 5). These positive correlations do not support the hypothesis that the five dominant species in alpine meadows were N limited.

The ^{15}N data are beneficial for understanding the effects of nutrient limitation on not only plant growth but also N acquisition patterns. A nonsignificant negative correlation between leaf $\delta^{15}\text{N}$ and leaf N : P of the sedge *K. humilis* indicates weak N limitation of this grass species, masked by slow growth, low N-uptake rates, and high plant internal N recycling (Xu et al., 2004), which reflect a species' low N requirement. Unlike the other six species, *G. straminea* showed positive leaf $\delta^{15}\text{N}$, indicating that its N uptake and assimilation patterns were distinctly different (Evans, 2001). However, its N uptake and assimilation should be further investigated to interpret its $\delta^{15}\text{N}$ pattern. No changes in the leaf $\delta^{15}\text{N}$ of observed legumes after N fertilization suggested that they invariably relied on N_2 fixation by their symbiotic rhizobia and that their N_2 fixation did not change markedly. Strong competition with grasses is the reason for the poor performance of legumes after N application in alpine meadows.

The difference in isotope patterns in leaves between NO_3^- and NH_4^+ treatments may indicate that different mechanisms are responsible for N uptake and metabolism in alpine plants. If NH_4^+ is almost completely assimilated by plant roots, there will be no/low efflux of residual unassimilated NH_4^+ , leading to no substantial ^{15}N fractionation in plants (Evans, 2001). This could explain why leaf N : P, but not leaf $\delta^{15}\text{N}$, increased markedly in response to increasing NH_4^+ fertilizer rate. In contrast, NO_3^- is often only partially assimilated in roots by nitrate

reductase in plant species (Evans, 2001; Gavrichkova and Kuzyakov, 2008, 2010). This results in a significant efflux of ^{15}N -enriched un-assimilated NO_3^- and causes increased plant ^{15}N fractionation in natural ecosystems. Therefore, NO_3^- application might cause strong responses in plant ^{15}N fractionation, but less significant increases in leaf N : P due to a lower preference for NO_3^- uptake among alpine plant species. Additionally, alpine meadows are generally characterized by a closed N cycle, where inorganic N pools become increasingly depleted in ^{15}N , while the organic N pool is gradually enriched in ^{15}N via the soil N cycle. NO_3^- is more ^{15}N -depleted compared to NH_4^+ and organic N due to the mineralization and nitrification processes (Nadelhoffer and Fry, 1994).

Our previous studies have shown that amino acids may be an important N source for alpine meadow plants (Xu et al., 2004, 2006). Therefore, plant species that prefer NH_4^+ /organic N uptake over NO_3^- uptake are expected to have more positive $\delta^{15}\text{N}$ values. In this study, we found that the leaf $\delta^{15}\text{N}$ for an unfertilized, NO_3^- -preferring species (*K. humilis*) was similar to that for an NH_4^+ -preferring species *E. nutans* (Xu et al., 2011). Only the NH_4^+ -preferring species *G. straminea* was significantly ^{15}N -enriched compared to *K. humilis*, and therefore followed the expected pattern. A possible explanation for the aberrant behavior of *E. nutans* is that high arbuscular mycorrhizal colonization of its roots (Xu et al., 2011) helps the roots to acquire and incorporate N from other isotopically distinct soil (organic) sources without ^{15}N fractionation (Hobbie et al., 2000; Hobbie and Hobbie, 2006).

In this study, the $\delta^{15}\text{N}$ values of legumes (-2‰) were slightly lower than those of legumes that completely rely on N_2 fixation, with a range between 0 and -1.8‰ (Körner, 2003). This indicates that legumes strongly but not completely rely on N_2 fixation in alpine meadows. Direct estimation of biological N_2 fixation by legumes at the same site using the ^{15}N dilution approach showed that biological N_2 fixation represented approximately 40% of N assimilation in *M. ruthenica* and *G. diversifolia*, and 81% in *O. ochrocephala* (Yang et al., 2011). The strong but incomplete reliance on N_2 fixation might be associated with low temperatures, which are far below the optimum of 20–35°C for nitrogenase (Dart and Day, 1971). Since Mo is an essential component of nitrogenase, a significant negative correlation was expected and observed between leaf Mo concentration and leaf $\delta^{15}\text{N}$ of the legume species studied ($R^2 = 0.51$, $P < 0.01\%$, Fig. 3). Therefore, reduced plant availability of Mo because of high amounts of CaCO_3 in Tibetan meadow soils (Zhou, 2001) could be another explanation for the observed N_2 fixation accounting for only 40% of N assimilation in the two legumes.

5 Conclusion

Grasses in alpine meadows are N-limited or co-limited by available P on the basis of biomass production and leaf N : P ratios, but biomass production and leaf N:P are not suitable indicators of N limitation for legumes and forbs. Of all seven dominant plant species, only the forbs *G. straminea* and *S. superba* showed a significant negative correlation between leaf N : P ratio and $\delta^{15}\text{N}$. This reflects that the forbs switched

from N deficiency to P limitation (e.g., N excess) due to N application, and indicates that they are N-limited in these alpine meadows. At an ecosystem level, N application did not significantly alter total above-ground biomass, but caused shifts in species composition with grasses being more productive. This suggests that alpine meadows might become more dominated by grasses under conditions of increasing atmospheric N deposition.

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