Nutr Cycl Agroecosyst (2011) 91:91–98
DOI 10.1007/s10705-011-9448-y

ORIGINAL ARTICLE

Symbiotic nitrogen fixation by legumes in two Chinese grasslands estimated with the ^{15}N dilution technique

Baijie Yang · Na Qiao · Xingliang Xu · Hua Ouyang

Received: 11 January 2011 / Accepted: 22 July 2011 / Published online: 31 July 2011
© Springer Science+Business Media B.V. 2011

Abstract Symbiotic nitrogen (N) fixation by legumes was investigated using the ^{15}N dilution technique in two Chinese grasslands: one in the north-eastern Tibetan Plateau and the other in Inner Mongolia in China. A small amount (0.03 g N m^{-2}) of ^{15}N labelled $(NH_4)_2SO_4$ fertilizer was evenly distributed in two soils. One month after the ^{15}N addition, four legumes (*Astragalus* sp., *Gueldenstaedtia diversifolia*, *Oxytropis ochrocephala* and *Trigonella ruthenica*) in the alpine meadow and two legumes (*Thermopsis lanceolata* and *Melissitus ruthenica*) in the temperate steppe were collected. Several non-legume plant species were harvested as the reference. Above-ground biomass of legumes ranged from 8 to 24 g m^{-2} in the alpine meadow and from 11 to 35 g m^{-2} in the temperate steppe. The reference plants showed distinctly higher ^{15}N atom% excess than legumes (0.08% vs. 0.02% in the alpine meadow, 0.10% vs. 0.02% in the temperate steppe). The N derived from atmosphere (%Ndfa) ranged from 50 to 90% N in the alpine meadow, while it ranged from 85 to 92% in the temperate steppe. Based on the legume above-ground biomass, total symbiotic N_2-fixation rate was estimated to be 1.00 g N m^{-2} year^{-1} in the alpine meadow and 1.15 g N m^{-2} year^{-1} in the temperate steppe. These N inputs by legumes can account for 9% of the gap between the N demand and the seasonal N release by mineralization in the alpine *Kobresia* grassland and 20% in the temperate *Leymus* grassland, respectively. Considering additional contribution of the root biomass, we suggest that biological N_2-fixation by legumes plays an important role in the cycling of N in both *Kobresia* and *Leymus* grasslands on an annual scale.

Keywords ^{15}N tracer · Alpine meadow · Temperate steppe · *Kobresia humilis* · *Leymus chinensis*

B. Yang · X. Xu (✉) · H. Ouyang
Key Laboratory of Ecosystem Network Observation and Modelling, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, 11A Datun Road, Chaoyang District, Beijing 100101, People's Republic of China
e-mail: xuxingl@hotmail.com

B. Yang · N. Qiao
Graduate University of Chinese Academy of Sciences, Beijing 100049, People's Republic of China

N. Qiao
Forest Ecosystem Research Center, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, 88 Xuefu Road, Kunming 650223, Yunnan, People's Republic of China

Introduction

Nitrogen (N) is an important nutrient which limits plant growth in terrestrial ecosystems worldwide (Chapin et al. 1986). In natural grasslands, the symbiotic N_2-fixation by legumes has been suggested to play an important role in N input because of lack of artificial fertilizers (Cusack et al. 2009). N_2-fixation by legumes varies in different ecosystems with changes in environmental factors and plant species composition (Vitousek et al. 2002).

In both alpine and temperate grasslands, many legume species are often observed, and some that are not edible for livestock (e.g. *Thermopsis lanceolata*) remain in the field after grazing. These legumes might partially offset the imbalance between the N demand by plants and the N release by mineralization. However, many researchers argue that biological N_2-fixation by legumes may make only a small contribution by to these grasslands in cold environments (Holzmann and Haselwandter 1988; Arnone 1999), where temperatures fall below the optimum range of 20–35°C for nitrogenase activity (Dart and Day 1971). Therefore, quantifying the contribution of biological N_2-fixation by these non-edible legumes will be helpful to understand the role of N_2-fixation by legumes in these grasslands.

Low temperature and low soil moisture lead to high soil organic matter (SOM) accumulation by suppressing mineralization of SOM. Consequently, plant growth in these grasslands is often limited by available N supplies (Haselwandter et al. 1983; Song et al. 2007) because a seasonal demand for N by grasses often exceeds estimated seasonal release by mineralization (Körner 2003; Cao et al. 2004).

Although several studies have investigated biological N_2-fixation by legumes in alpine meadows and temperate steppes (Holzmann and Haselwandter 1988; Bowman et al. 1996; Li et al. 2002), so far few studies have been conducted to quantify the contribution of biological N_2-fixation by legumes in alpine meadows in the Tibetan Plateau and in temperate steppes in Inner Mongolia.

The Tibetan Plateau covers more than 2.5 million km^2 with an average altitude of over 4,000 m a.s.l. Approximately 35% of its area is dominated by alpine meadows (Zheng 2000). Typical temperate steppes in Northern China, covering 0.4 million km^2, are an important part of Eurasian steppes (Chen and Wang 2000). Soil temperature at 20 cm depth in July, the hottest month of the year, averaged 11.4°C in the alpine meadows (Li et al. 2004) whereas it averaged 17°C in the temperate steppes (Gao et al. 2004). These temperatures are lower than suggested optimum temperature of nitrogenase activity (Dart and Day 1971). We therefore put forward two hypotheses: (1) the contribution of symbiotic N_2-fixation by legumes is small on an annual scale because of low temperature in these grasslands; (2) symbiotic N_2-fixation is greater in temperate steppes than in alpine meadows because of higher soil temperature in temperate steppes than in alpine meadows during the growth seasons.

To test the hypotheses above, we investigated the contribution of symbiotic N_2-fixation by legumes in the two grasslands using the ^{15}N isotope dilution technique because of its advantage to measure biological N_2-fixation by legumes in situ (Chalk 1991; Carranca et al. 1999). The principal assumption of the ^{15}N dilution technique is that the ^{15}N enrichment of the reference plants accurately reflects the ^{15}N enrichment of the soil N taken up by legumes.

Materials and methods

Description of experimental sites

The alpine meadow site was located at the Haibei Alpine Meadow Ecosystem Station, Chinese Academy of Sciences, Qinghai Province (37° 36′ 60″ N, 101° 19′ 14″ E, 3,215 m a.s.l.). The area is characterized by a typical alpine climate with the annual temperature and precipitation averaging −1.7°C and 600 mm, respectively. Average temperature during the growing season (from May to September) was 7.2°C and 79% of precipitation occurred between May and September (Li et al. 2004). Dominant species are *Kobresia humilis* Serg., *Stipa aliena* Keng., *Poa* sp., *Festuca ovina* Linn., *Gentiana aristata* Maxim., *Gentiana straminea* Maxim., *Saussurea superba* Anth., and *Gueldenstaedtia diversifolia* Maxim. Legume species in the alpine meadow are *Astragalus* sp., *Gueldenstaedtia diversifolia*, *Oxytropis ochrocephala* and *Trigonella ruthenica*. The soil is classified as Mat-Gryic Cambisol corresponding to Gelic Cambisol (WRB 1998).

The temperate steppe site was located at Hulunbeier Grassland Ecosystem Observation and Research Station in Inner Mongolia (49° 22′ 42″–49° 22′ 92″ N, 120° 01′ 54″–120° 02′ 32″ E, 620–630 m a.s.l.). It is characterized by semi-arid climate. The annual air temperature averaged 0.5°C and annual precipitation averaged 330 mm. Average temperature during the growing season was 15.8°C and most of the precipitation was concentrated from May to August (Meng et al. 2009). Dominant species are *Leymus chinensis* (Trin.) Tzvel., *Festuca ovina* Linn., *Artemisia tanacetifolia* Linn., *Pulsatilla turczaninovii* Krylov et Serg., *Artemisia dracunculus* Linn., *Koeleria cristata* (Linn.) Pers. Leguminous species included *Thermopsis lanceolata* and *Melissitus ruthenica*. The soil is classified as chestnut soil (Zhao 1998), corresponding to Haplic Kastanozem. Some characteristics of the two soil types are shown in Table 1. These soil samples (0–10 cm depth) were collected in a distance of around 20 m from the study plots. Both grasslands were excluded from grazing.

Eight 1×1 m^2 plots were randomly set up in either of the two grasslands. Labeling was done in August, 2007 (air average temperature −0.4°C and rainfall 510 mm) in the alpine meadow, whereas it was done in August, 2010 (air average temperature 1.5°C and rainfall 341 mm) in the temperate steppe. $(^{15}NH_4)_2SO_4$ (98.4% ^{15}N enrichment) was dissolved in water and sprayed on four plots, while the others were sprayed with water as the control. The amount of N was applied at a rate of 0.03 g N m^{-2}. This is far less than the level of available N in the soil and so cannot affect the soil processes or stimulate plant growth. To avoid the tracer being absorbed by the leaf surface and to ensure uniform fertilizer distribution, an additional volume of distilled water was sprayed after the tracer application. The total amount of water used equaled 3 mm of precipitation. In addition to the irrigation, higher soil water content in alpine meadows and a small amount of rain on the temperate steppe while the tracer was sprayed could reinforce the effect of penetration of labeled N into the soil by gravitational water.

Table 1 Characteristics of the top 10 cm (Ah) of soil in a *Kobresia humilis* meadow and a *Leymus chinensis* steppe

	Kobresia humilis meadow[a]	*Leymus chinenisis* steppe
pH	8.0 ± 0.2	6.7 ± 0.1
C:N ratio	12.7 ± 0.3	9.6 ± 0.1
Organic C content (%)	7.36 ± 0.42	3.56 ± 0.08
Total soil N (%)	0.58 ± 0.08	0.37 ± 0.01

Means ± 1SE of 12 replicates are presented

[a] From Xu et al. (2011a)

Sampling and calculations

An important assumption of the ^{15}N dilution technique is that the reference plants accumulate N with the same ^{15}N enrichment that is derived from the soil by the legumes. Given the difficulties in testing the assumption, we selected several reference plants, i.e. *Saussurea superba*, *Gentiana straminea*, *Kobresia humilis* and *Stipa aliena* in the alpine meadow and *Leymus chinensis* and *Thalictrum aquilegifolium* in the temperate steppe. All legume and reference plants were perennial. The reference plants *Saussurea superba*, *Gentiana straminea* and *Thalictrum aquilegifolium* showed similar phenology with legumes, normally reaching their maximum N contents in August and September and exhibited similar rooting depth and architecture. Although graminoids (*Stipa aliena*, *Leymus chinensis*) possibly recovered more labeled N than the legume, we still used them as reference plants to estimate and show the variation of N_2-fixation by the neighboring legumes.

Three plots (25×25 cm) close to those used for estimation of biological N_2-fixation were selected for above-ground biomass measurements in the alpine grassland. Three soil cores (2.8 cm in diameter) were collected from the 0–15 cm layer to estimate root biomass (Xu et al. 2011b). In the temperate grassland, three plots (30×30 cm) were used to estimate above-ground biomass and three soil cores (3.8 cm in diameter) were collected from the top soil for root biomass measurements.

One month after labeling, all legumes (*Astragalus* sp., *G. diversifolia*, *O. ochrocephala* and *T. ruthenica* in the alpine meadow; *T. lanceolata* and *M. ruthenica* in the temperate steppe) and several non-legume reference plants (*S. superba*, *G. straminea*, *K. humilis* and *S. aliena* in the alpine meadow; *L. chinensis* and *T. aquilegifolium* in the temperate steppe) in both treated and control plots were harvested, dried for 48 h at 60°C and weighed for above-ground biomass. All the plant materials were ground to a fine power using a ball mill (MM200, Fa. Retsch, Haan, Germany). Aliquots (2 mg) were weighed into tin capsules for analyzing total N, C and ^{15}N atom% by a isotope ratio mass spectrometer (Mat 253, Finnigan MAT, Bremen, Germany), coupled by ConFlo III device (Finnigan MAT, Bremen, Germany) to an elemental analyser (EA 1112, CE Instruments, Milan, Italy). Atmospheric

N_2 was used as reference. Stable isotope abundances are reported as

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} - 1\right) \times 1{,}000‰ \quad (1)$$

where R is the ratio of $^{15}N/^{14}N$ of either the sample or standard. The standard deviation of repeated measurements of laboratory standards was ±0.15‰.

Atom% excess ^{15}N (APE) was calculated as the atom% ^{15}N difference between the same plant species from ^{15}N-treated and from control plots. We used the Eq. 2 (McAuliffe et al. 1958) to calculate N derived from atmosphere (%Ndfa) for individual legume species. Calculations were carried out using APE of the individual legume and average APE of the reference plants of same site. Non-graminoids were used as reference plants alone and together with graminoids.

$$\%\text{Ndfa} = \left(1 - \frac{\text{APE}_{\text{legume}}}{\text{APE}_{\text{reference}}}\right) \times 100 \quad (2)$$

The quantity of N fixed by legumes (N fixed, g N m^{-2}) was calculated as the following equation (Busse 2000):

$$\text{N fixed} = (\%\ \text{Ndfa}) \times (\text{biomass}) \times (\text{Nconc.}) \quad (3)$$

The standard errors of means are presented in figures and tables as a variability parameter. One-way analysis of variance (ANOVA) was carried out with Bonferroni test to determine effects of plant species on APE, %Ndfa and N fixed. Paired *t* test was used to analyze the difference in APE between legumes and the reference plants. All the tests were run using SPSS 18.0 software package (SPSS Inc. Chicago, IL, USA).

Results

Biomass and its N accumulation in two grasslands

Above-ground biomass of legumes ranged from 8 to 24 g m^{-2} in the alpine *K. humilis* dominated meadow and from 11 to 35 g m^{-2} in the temperate *L. chinensis* steppe (Fig. 1). *G. diversifolia* exceeded the biomass production of the other three legumes that showed similar values in the alpine meadow. The above-ground biomass of the two legumes in the temperate steppe significantly differed between each other. Total leguminous above-ground biomass was 48.45 ± 4.4 g m^{-2} in the alpine meadow and 45.9 ± 14.2 g m^{-2} in the temperate steppe.

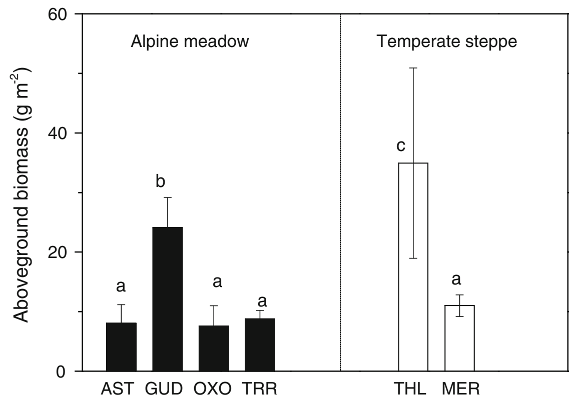

Fig. 1 Above-ground biomass of legumes in a *Kobresia humilis* alpine meadow and a *Leymus chinensis* steppe. Mean ± 1SE of four replicates are presented. *Different letters* indicate significant differences at $P < 0.05$ between plant species across the two grasslands. AST, *Astragalus* sp.; GUD, *Gueldenstaedtia diversifolia*; OXO, *Oxytropis ochrocephala*; TRR, *Trigonella ruthenica* in the alpine meadow. THL, *Thermopsis lanceolata*; MER, *Melissitus ruthenica* in the temperate steppe

Total above-ground biomass averaged 309.8 ± 10.3 g m^{-2} in the alpine meadow in 2007 and 295.4 ± 19.8 g m^{-2} in the temperate steppe in 2010, respectively. Legumes contributed about 15.7% to total above-ground biomass in the alpine meadow and 15.6% in the temperate steppe. Total belowground biomass in the upper 15 cm layer in the alpine meadow (1,494.2 ± 22.7 g m^{-2}) was significantly higher than in the temperate steppe (585.4 ± 43.7 g m^{-2}).

Based on N concentration in plant tissues, N accumulation in above-ground biomass was estimated at 6.85 ± 0.23 g N m^{-2} in the alpine grassland and 2.84 ± 0.19 g N m^{-2} in the temperate grassland. N accumulation in the belowground part was amounted to 20.17 ± 0.31 g N m^{-2} in the alpine grassland and 4.68 ± 0.35 g N m^{-2} in the temperate grassland.

^{15}N atom% excess

^{15}N atom% excess (APE) of different plant species varied as the reference plants showed distinctly higher APE than legumes in both grasslands (t = −3.784, $P = 0.004$ for the *K. humilis* meadow; t = −5.260, $P = 0.003$ for the *L. chinensis* steppe). Among legumes, *G. diversifolia* and *T. ruthenica* showed higher APE than *Astragalus* sp. and *O. ochrocephala* in the alpine meadow and were not significantly different from each other. The legumes in the temperate steppe, *T. lanceolata* and *M. ruthenica*, showed similar APE (Fig. 2). The reference plants in the alpine meadow exhibited significantly differing APE, ranging from 0.10% of *S. superba* to 0.03% of *S. aliena*. In contrast,there was no significant difference between the two reference plants in the temperate steppe.

N derived from the atmospheric N_2

The N derived from the atmospheric N_2 (%Ndfa) of the legumes was estimated to range from 50.4% to 89.7% in the alpine meadow while it ranged from 85.3% to 92.2% in the temperate steppe (Fig. 3). %Ndfa of *Astragalus* sp. and *O. ochrocephala* significantly exceeded that of *G. diversifolia* and *T. ruthenica*. In the temperate steppe, the two legumes showed similar %Ndfa, which on average was higher than that of the legumes in the alpine meadow.

Symbiotic N_2-fixation was lowest for *T. ruthenica* with a value of 0.17 g N m^{-2} and highest for

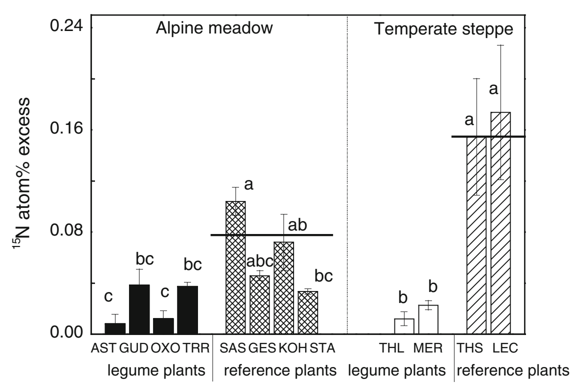

Fig. 2 ^{15}N atom% excess of legumes and reference plants in a *Kobresia humilis* alpine meadow and a *Leymus chinensis* steppe. Mean ± 1SE of four replicates are presented. *Different letters* indicate significant differences at $P < 0.05$ between plant species across the two grasslands. AST, *Astragalus* sp.; GUD, *Gueldenstaedtia diversifolia*; OXO, *Oxytropis ochrocephala*; TRR, *Trigonella ruthenica*; SAS, *Saussurea superba*; GES, *Gentiana straminea*. KOH, *Kobresia humilis*; STA, *Stipa aliena* in the alpine meadow. THL, *Thermopsis lanceolata*; MER, *Melissitus ruthenica*; THS, *Thermopsis squarrosum*; LEC, *Leymus chinensis* in the temperate steppe. The *bold horizontal lines* represent the average value of ^{15}N atom% excess of non-Graminoids reference plants

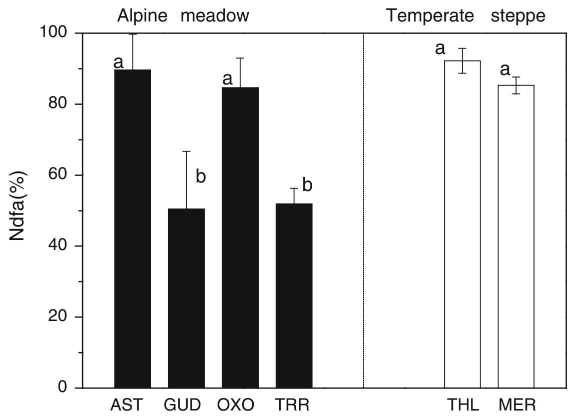

Fig. 3 Percentage of legume N derived from the atmosphere (%Ndfa) in a *Kobresia humilis* alpine meadow and a *Leymus chinensis* steppe. Mean ± 1SE of four replicates are presented. *Different letters* indicate significant differences at $P < 0.05$ between plant species across the two grasslands. AST, *Astragalus* sp.; OXO, *Oxytropis ochrocephala*; GUD, *Gueldenstaedtia diversifolia*; TRR, *Trigonella ruthenica* in the alpine meadow. THL, *Thermopsis lanceolata*; MER, *Melissitus ruthenica* in the temperate steppe

G. diversifolia with a value of 0.36 g N m^{-2} in the alpine meadow (Table 2). But for *T. lanceolata* (0.95 g N m^{-2}) in the temperate steppe showed superior values among the species, the difference among all other species were not statistically important. The total N input by leguminous plants was similar in the two grasslands (1.00 g N m^{-2} in the alpine meadow vs. 1.15 g N m^{-2} in the temperate steppe, Table 2).

Discussion

The %Ndfa value denotes the portion of N derived from the atmosphere in legumes. Körner (2003) suggested that in the alpine zone the Ndfa% was small compared with the temperate zone, because the leguminous plants in alpine areas are inhibited by lower temperature in alpine regions (Heimann and Reichstein 2008). Nonetheless, the %Ndfa values were estimated to range from 70% to 100% for *Trifolium* at 3,650 m a.s.l. on Niwot Ridge (Bowman et al. 1996), and varied from 59% to 90% for nine leguminous species in the Swiss Alps along an altitude gradient from 900 to 2,600 m a.s.l. (Jacot et al. 2000a). Our estimates of 50–90% for the legumes in both grasslands are thus within the latter

Table 2 Nitrogen fixation by legume plants (g N m^{-2}) in a *Kobresia humilis* meadow and a *Leymus chinensis* steppe

	Kobresia humilis meadow	*Leymus chinensis* steppe
Astragalus sp.	0.25[b]	
Gueldenstaedtia diversifolia	0.36[b]	
Oxytropis ochrocephala	0.21[b]	
Trigonella ruthenica	0.17[b]	
Thermopsis lanceolata		0.95[a]
Melissitus ruthenica		0.20[b]
SUM	1.00*	1.15*

Different letters indicate significant differences at the $P < 0.05$ level between plant species across the two grasslands. The asterisk indicates no significant difference in N_2-fixation between two grasslands

range (Fig. 3). Despite lower soil temperature in the alpine meadow that measured 11.4°C versus 17°C in the temperate steppe during the hottest month, two of alpine legume species (*Astragalus* sp. and *O. ochrocephala*) still showed comparable %Ndfa values. This indicates that the legume capacity to fix atmospheric N_2 vary in a species-specific way in alpine meadows. But the hypothesis that higher soil temperature favors nitrogenase activity is supported by our results showing that, on the average, the legumes in the temperature steppe were more efficient in N_2-fixation.

Previous studies have shown (Wojciehowski and Heimbrook 1984; Holzmann and Haselwandter 1988) that in alpine meadows, symbiotic N_2-fixation by legumes was lower than that in temperate grassland. In this study, *T. lanceolata* in the temperate steppe fixed significantly more amounts of N than the other legumes due to its highest %Ndfa and highest biomass. Despite richer composition of leguminous species in the alpine grassland, the total N_2-fixation by these legumes (on the basis of above-ground biomass) was not significantly different from that in the temperate grassland (1.00 vs. 1.15 g N m^{-2}, respectively). Therefore, our hypothesis that N_2-fixation by legumes in the temperate steppe is greater than in the alpine meadow was not fully supported by our data.

When ^{15}N dilution technique is used, the choice of reference plants often affects estimation of N_2-fixation. In this study, including graminoids and non-graminoids as the reference, we found that the use of graminoid plants significantly decreased the Ndfa% values in the *Kobresia humilis* meadow (t = 4.35, $P = 0.001$) while significantly increased the Ndfa% values in a *Leymus chinensis* steppe (t = −4.78, $P = 0.002$). However, this effect was minor (1.00 vs. 0.91 in the alpine grassland and 1.15 vs. 1.16 in the temperate grassland). Considering a possible transfer of fixed N from a legume to a reference plant, our estimates of N_2-fixation is conservative. Nonetheless, symbiotic N_2-fixation estimated in this study is much higher than reported earlier for a similar temperate steppe (0.15 g N m^{-2}, Li et al. 2002) and alpine grasslands (0.49 g N m^{-2}, Bowman et al. 1996) despite similar values of %Ndfa. This is mainly ascribed to the higher biomass in our studied sites. In contrast, with similar %Ndfa values, the N_2-fixation by legumes amounted to 2.6 g N m^{-2}, also only on the above-ground biomass basis, in Swiss Alps (Jacot et al. 2000b), two times higher than our estimation. A possible explanation is that the lower altitude of the sites in the Swiss Alps gives rise to higher temperature and leads to a high net primary production and thus a high N input by legumes. Furthermore, in this study excluding the two grasslands from grazing for several years might have decreased the N output. As a result, the necessity for N inputs was also low. The N_2-fixation by legumes in this study by far exceeded the N_2-fixation by soil crust (3–8 mg N m^{-2}) in Inner Mongolia (Holst et al. 2009), while it is comparable to the N input by precipitation (0.72–1 g N m^{-2}) and the N_2-fixation by azotobacter (0.5 g N m^{-2}) in alpine meadows (Zhang and Cao 1999).

The role of symbiotic N_2-fixation in these grasslands can be assessed by the balance of the seasonal N demand by grasses and the seasonal N release by mineralization of SOM. In the alpine meadow, the seasonal N accumulation was 27 g N m^{-2} as estimated by Cao et al. (2004), who reported that the seasonal N release by mineralization was around 16 g N m^{-2} $year^{-1}$. In the temperate steppe, N accumulation totaled 7.5 g N m^{-2}, while net mineralization was reported to be 12 mg m^{-2} day^{-1} (Yang et al. 2005). Assuming that growing season totals 150 days, the seasonal N release by mineralization would be about 1.8 g N m^{-2}. Because both studied grasslands were not grazed, the N demand can be regarded as the conservative seasonal N demand by grasses. The gap between the N demand and the N supply is about 5.7 g N m^{-2} $year^{-1}$ in the temperate grassland and 11.0 g N m^{-2} $year^{-1}$ in the alpine grassland. The N input by legumes estimated based on the above-ground biomass, accounts for 9% of the gap in the alpine *Kobresia* meadow and 20% in the temperate *Leymus* steppe. The small contribution of legumes to the alpine *Kobresia* meadow could be related to the low soil temperature during growing seasons which limits the rhizobium-legume symbiosis. Besides aboveground parts, roots also contribute to N input. Chen and Wang (2000) and Xu et al. (2004) approximated that one third of the roots in alpine meadows were replaced in 1 year and observed a 50% root turnover in temperate steppes. Assuming a similar root to shoot ratio for legumes as the whole plant community, the N_2-fixation in the legume roots amounts to 0.72 and 0.32 g N m^{-2} in the alpine meadow and the temperate steppe, respectively. As a result, the legumes contributed at least 16% to fill in the gap between N demand and supply in the alpine grassland and 26% in the temperate grassland. This indicates that, not as presupposed previously, the contribution of biological N_2-fixation by legumes is important on an annual scale in alpine and temperate grasslands. The legume litter is easy to decompose by soil microorganisms and provides plant-available N for plants (Ledgard and Steele 1992; Mulder et al. 2002). This invalidates our hypothesis that symbiotic N_2-fixation by legumes makes small contribution to alpine and temperate grasslands because of low temperature.

Conclusion

The %Ndfa values of legume plants in both the alpine *Kobresia* meadow and the temperate *Leymus* steppe were comparable to those observed in other alpine and temperate grasslands. The total N_2-fixation in aboveground parts was estimated to be 1.00 g N m^{-2} in the alpine *Kobresia* meadow and 1.15 g N m^{-2} in the temperate *Leymus* steppe, respectively. Despite these low amounts of N fixed, N_2-fixation by legumes could contribute 9% (alpine *Kobresia* grassland) and 20% (temperate *Leymus* grassland) to reduce the gap between the seasonal demand and release of N in the two grasslands. We suggest that symbiotic N_2-fixation by legumes is important for plant N-nutrition in ungrazed alpine *Kobresia* and temperate *Leymus* grassland on an annual scale.

Acknowledgments We are greatly grateful to Yakov Kuzyakov for his helpful comments on an earlier version of the manuscript and to Markisha Berrien for the language improvement. We also thank two anonymous reviewers for their helpful comments to greatly improve our manuscript. This study was supported by National Basic Research Program of China (Grant No. 2010CB833501) and the National Natural Science Foundation of China (Grant No. 30870424 & 41071209).

References

Arnone JA III (1999) Symbiotic N_2 fixation in a high alpine grassland: effects of four growing seasons of elevated CO_2. Funct Ecol 13:383–387

Bowman WD, Schardt JC, Schmidt SK (1996) Symbiotic N_2 fixation in alpine tundra: ecosystem input and variation in fixation rates among communities. Oecologia 108: 345–350

Busse MD (2000) Suitability and use of the ^{15}N-isotope dilution method to estimate nitrogen fixation by actinorhizal shrubs. For Ecol Manag 136:85–95

Cao GM, Wu Q, Li D, Hu QW, Li YM, Wang X (2004) Effects of nitrogen supply and demand status of soil and herbage system on vegetation succession and grassland degradation in alpine meadow. Chin J Ecol 23:25–28

Carranca C, Varennes A, Rolston DE (1999) Biological nitrogen fixation estimated by ^{15}N dilution, natural ^{15}N abundance, and N difference techniques in a subterranean clover-grass sward under Mediterranean condition. Eur J Agron 10:81–89

Chalk PM (1991) The contribution of associative and symbiotic nitrogen fixation to the nitrogen nutrition of non-legumes. Plant Soil 132:29–39

Chapin FS, Vitousek PM, Vancleve K (1986) The nature of nutrient limitation in plant-communities. Am Nat 127:48–58

Chen ZZ, Wang SP (eds) (2000) Chinese typical grassland ecosystem. Science Press, Beijing

Cusack DF, Silver W, McDowell WH (2009) Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. Ecosystems 12:1299–1315

Dart PJ, Day JM (1971) Effects of incubation temperature and oxygen-tension on nitrogenase activity of legume root nodules. Plant Soil 35:167–184

Gao CX, Su LJ, Song JH, Gong CN, Cheng W (2004) Relationship between distribution of frozen earth and soil temperature in northeast of Inner Mongolia. Meteorol J Inner Mongolia 19–22

Haselwandter K, Hofmann A, Holzmann HP, Read DJ (1983) Availability of nitrogen and phosphorus in the nival zone of the Alps. Oecologia 57:266–269

Heimann M, Reichstein M (2008) Terrestrial ecosystem carbon dynamics and climate feedbacks. Nature 451:289–292

Holst J, Butterbach-Bahl K, Liu CY, Zheng XH, Kaiser AJ, Schnitzler JP, Zechmeister-Boltenstern S, Bruggemann N (2009) Dinitrogen fixation by biological soil crusts in an Inner Mongolian steppe. Biol Fert Soils 45:679–690

Holzmann HP, Haselwandter K (1988) Contribution of nitrogen fixation to nitrogen nutrition in an alpine sedge community (*Caricetum curvulae*). Oecologia 76:298–302

Jacot KA, Lüscher A, Nösberger J, Hartwig UA (2000a) Symbiotic N_2 fixation of various legume species along an altitudinal gradient in the Swiss Alps. Soil Bio Biochem 32:1043–1052

Jacot KA, Lüscher A, Nösberger J, Hartwig UA (2000b) The relative contribution of symbiotic N_2 fixation and other nitrogen sources to grassland ecosystems along an altitudinal gradient in the Alps. Plant Soil 225:201–211

Körner C (2003) Alpine plant life-functional plant ecology of high mountain ecosystems. Springer, New York

Ledgard SF, Steele KW (1992) Biological nitrogen-fixation in mixed legume grass pastures. Plant Soil 141:137–153

Li YZ, Redmann RE, Zhu TC, Li JD (2002) Nitrogen fixation in *Leymus chinensis* grassland in Northeast China. Acta Agrestia Sin 10:164–166

Li YN, Zhao XQ, Cao GM, Zhao L, Wang QX (2004) Analyses on climates and vegetation productivity background at Haibei alpine meadow ecosystem research station. Plateau Meteorol 23:558–567

McAuliffe C, Chamblee DS, Uribe-Arango H, Woodhouse WW (1958) Influence of inorganic nitrogen on nitrogen fixation by legumes as revealed by ^{15}N. Agron J 50:334–337

Meng X, Li X, Xin X, Zhou R (2009) Study on community characteristics and a diversity under different grazing intensity on *Leymus chinensis (Trin.) Tzvel.* meadow steppe of Hulunbeier. Acta Agrestia Sin 17:239–244

Mulder CPH, Jumpponen A, Hogberg P, Huss-Danell K (2002) How plant diversity and legumes affect nitrogen dynamics in experimental grassland communities. Oecologia 133:412–421

WRB (1998) World reference base for soil resources. FAO/ISRIC/ISSS, Rome

Song MH, Xu XL, Hu QW, Tian YQ, Ouyang H, Zhou CP (2007) Interactions of plant species mediated plant competition for inorganic nitrogen with soil microorganisms in an alpine meadow. Plant Soil 297:127–137

Vitousek PM, Cassman K, Cleveland C, Crews T, Field CB, Grimm NB, Howarth RW, Marino R, Martinelli L, Rastetter EB, Sprent JI (eds) (2002) Towards an ecological understanding of biological nitrogen fixation. Biogeochemistry (Kluwer Academic Publishers)

Wojciehowski MF, Heimbrook ME (1984) Dinitrogen fixation in alpine tundra, Niwot Ridge, Front Range, Colorado, U.S.A. Arctic Alpine Res 16:1–10

Xu XL, Ouyang H, Cao GM, Pei ZY, Zhou CP (2004) Nitrogen deposition and carbon sequestration in alpine meadows. Biogeochemistry 71:353–369

Xu XL, Ouyang H, Cao GM, Richter A, Wanek W, Kuzyakov Y (2011a) Dominant plant species shift their nitrogen uptake patterns in response to nutrient enrichment caused by a fungal fairy in an alpine meadow. Plant Soil 341:495–504

Xu XL, Wanek W, Cao GM, Richter A, Song MH, Zhou CP, Ouyang H, Kuzyakov (2011b) Effects of nitrogen addition on forliar N:P ratio and δ^{15}N in an alpine meadow on the Tibetan Plateau. J Plant Nutr Soil Sci (submitted)

Yang XH, Dong YS, Qi YC, Geng YB, Liu LX (2005) Soil net nitrogen mineralization in an *aneulolepidium chinensis* grassland, Inner Mongolia. Progr Geogr 24:30–37

Zhang JX, Cao GM (1999) The nitrogen cycle in an alpine meadow ecosystem. Acta Ecol Sin 19:509–513

Zhao QG (1998) Chinese soil resources. Nanjing university press, Nanjing

Zheng D (2000) Mountain geoecology and sustainable development of the Tibetan plateau. Kluwer, Dordrecht