Symbiotic nitrogen fixation and interspecific transfer by *Caragana microphylla* in a temperate grassland with ^{15}N dilution technique

Shengnan Ouyang^a, Yuqiang Tian^{a,*}, Qianyuan Liu^b, Li Zhang^b, Yuan Sun^c,
Xingliang Xu^{b,*}, Yinghui Liu^d

^a Center for Human-Environment System Sustainability (CHESS), State Key Laboratory of Earth Surface Processes and Resource Ecology, Beijing Normal University, Haidian District, Beijing 100875, China

^b Key Laboratory of Ecosystem Network Observation and Modeling, Institute of Geographic Sciences and Natural Resources Research, Chinese Academy of Sciences, Beijing 100101, China

^c School of Life Sciences, Yunnan University, NO. 2, Cuihu, Kunming 650091, China

^d College of Resources Science and Technology, Beijing Normal University, Beijing 100875, China

ARTICLE INFO

Article history:

Received 31 July 2015

Received in revised form 8 February 2016

Accepted 10 August 2016

Available online 1 September 2016

Keywords:

Caragana microphylla

Nitrogen fixation

Nitrogen transfer

Temperate grassland

^{15}N dilution technique

ABSTRACT

Quantification of symbiotic nitrogen (N) fixation by legume and its N transfer to the neighboring plants is an important prerequisite to understand the N cycling in the N-limited grasslands. The objective of this study is to explore the symbiotic N_2 -fixation by a legume shrub (*Caragana microphylla*) and its N transfer to coexisting non-legumes with the ^{15}N dilution technique. A paired experimental design (legume vs. non-legume quadrats) was used. A small amount (0.03 g N m^{-2}) of ^{15}N -labeled KNO_3 (99.5% ^{15}N enrichment) was evenly distributed in these paired labeled quadrats, with only water additions as the control. One month after the ^{15}N addition, aboveground parts of *C. microphylla* and four non-legumes of reference plants (i.e. *Cleistogenes squarrosa*, *Artemisia frigida*, *Artemisia capillaris* and *Leymus chinensis*) in both labeled and controlled quadrats were harvested. Symbiotic N_2 -fixation and transfer to non-legumes were estimated based on the aboveground biomass and its ^{15}N in these plant species. The N derived from the atmosphere (%Nd_f) of *C. microphylla* was estimated to range from 84 to 95%. According to the aboveground biomass of *C. microphylla*, symbiotic N_2 -fixation was estimated to be $4.3 \text{ g m}^{-2} \text{ yr}^{-1}$ in the mixed *C. microphylla* community in the temperate grassland of northern China. The amount of N transfer from *C. microphylla* to their coexisting plants varied with plant species. About half of above N in *C. squarrosa* derived from *C. microphylla*, while *L. chinensis* showed almost no N derived from *C. microphylla*. Both *A. frigida* and *A. capillaris* showed about 36% and 37% of N derived from *C. microphylla*, respectively. Totally, about 17% of N fixed in the aboveground part of *C. microphylla* was transferred to its associated species. Considering the amount of N fixed and transferred by *C. microphylla*, we conclude that *C. microphylla* plays an important role in the N cycling of temperate grasslands in northern China.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Nitrogen (N) is an important nutrient limiting plant growth in most terrestrial ecosystems (Vitousek and Howarth, 1991; Lebauer and Treseder, 2008). Legume-rhizobium symbiosis can provide about 65% bioavailable N in the biosphere (Peoples et al., 1995). Therefore, biological N_2 -fixation by legumes is much important for maintenance of ecosystem functions and restoration of degraded grasslands (Murray and Clements, 1998; Cusack et al., 2009).

In addition to N fixation, legumes can also transfer a part of N they fixed to coexisting non-legumes (Murray et al., 2008; Issah et al., 2015). Nitrogen transfer mainly occurs via three pathways: i.e. mycorrhizal networks between legume and non-legume roots (Jalonen et al., 2009), direct uptake of N compounds exuded by legume roots (Ta et al., 1986; Jiang and Zhai 2000), or direct uptake of mineralized N derived from dead legume nodules and senescing root materials (Butler et al., 1959; Pirhofer-Walzl et al., 2012). Using the ^{15}N isotope techniques (Sierra and Daudin, 2010; Carlsson and Huss-Danell, 2014; Chalk et al., 2014), some studies showed that up to 30% of the N fixed by legumes can be transferred to non-legumes (Hauggaard-Nielsen et al., 2001; Peoples et al., 2002; Herridge et al., 2008). This indicates that in the N-limited

* Corresponding authors.

E-mail addresses: tyq@bnu.edu.cn (Y. Tian), xuxingli@hotmail.com (X. Xu).

soils where the legumes grow, coexisting non-legume species can benefit from symbiotic N₂-fixation (Høgh-Jensen and Schjoerring, 2000; Sierra and Nygren, 2006).

Additionally, legumes can conserve soil and water, provide a variety of products and improve the soil fertility in terrestrial ecosystems (Li et al., 2002; Niu and Jiang, 2004; Li et al., 2010). *Caragana microphylla*, a legume shrub, widely distributes in temperate grasslands of northern China. About 5 million hm² is covered by *C. microphylla*, corresponding to 6.5% of the total grassland area of China (Zhou, 1990). Due to overgrazing in recent decades, a large area of temperate grasslands in northern China are experiencing degradation (Gao et al., 2004; Niu and Jiang, 2004; Yan et al., 2014). Many studies have observed that *C. microphylla* often occurs in degraded grasslands (Xiong et al., 2003; Xiong and Han, 2006), and thrives in low N fertility soils (Pollock and Svendsen, 2008; Zhang et al., 2011) because they can utilize N from the atmosphere through associated rhizobium (Moukoudi et al., 2012). In these grasslands, *C. microphylla* can form a “fertility island” due to its biological N fixation and increased litter inputs (Schlesinger and Pilmanis, 1998; Chu et al., 2005; Xiong and Han, 2006). It has a potential to transfer its fixed N to accompanied plants and improve their N status (Xiong et al., 2003; Bai et al., 2004).

With the shrub expansion, *C. microphylla* could affect the productivity and diversity of associated plants because of its symbiotic N₂-fixation. However, its N fixation and N transfer to associated non-legumes still remains unknown in these temperate grasslands. Clarifying these questions would be helpful for better understanding the role of *C. microphylla* in the N cycling of temperate grasslands. We hypothesize: 1) symbiotic N₂-fixation by *C. microphylla* is significant on an annual scale in temperate grasslands; 2) the amount of N transferred to its associated species is species-specific and thereafter dependent upon their associated species. To test two hypotheses above, we chose *C. microphylla* shrubs growing together with four non-legumes (refer to as “mixed”) and the same species growing without legumes (refer to as “pure”) for *in situ* ¹⁵N labelling experiment in the temperate grassland in North of China.

2. Materials and methods

2.1. Study site

The field site lies in the southern margin of the farming-pastoral ecotone, Taibus Banner in Inner Mongolia of northern China (41°35′50″N, 114°51′45″E, 1500 m above sea level). The site is characterized by a semi-arid continental climate. In the past 35 years, the mean annual temperature and precipitation were 2.1 °C and 384 mm, respectively. The precipitation was concentrated in growing seasons (from the end of May to September), occupying 65% of the mean annual precipitation (Xu et al., 2014). The dominant plant species are *Caragana microphylla*, *Artemisia capillaris*, *Artemisia frigida*, *Cleistogenes squarrosa*, *Stipa krylovii*, *Neopallasia pectinata*, *Allium polyrhizum* and *Leymus chinensis*

(Table 1). *A. capillaris*, *A. frigida*, *C. squarrosa* and *L. chinensis* had the largest important values (IV) in the sampling plots (Table 1) were chosen as target species. Soils are chestnut soil (Chinese Soil Taxonomy Research Group, 2001), corresponding to Calcic Orthic Aridisols according to USDA Soil Taxonomy (Soil Survey Staff, 1987). The soil contains organic matter 2.7%, total N 0.14%, with C/N ratio of 11.2 and soil pH 8.17. In the past 10 years since fenced by local government, these grasslands have been harvested at the end of October each year and used as winter forgrass for sheep, dairy cows or other livestock.

A big plot (400 m × 50 m) uniform in cover (0.88 ± 0.02) and species composition (7 ± 0.3 species m⁻²) (Table 1) was selected for this experiment. A paired experimental design (shrub vs. non-shrub quadrats) was used. The experiment had a completely randomized block design. In the plots, eight paired shrub and non-shrub quadrats (2 m × 2 m) were established. Each shrub quadrat (as “mixed”) included the similar size of the legume *C. microphylla* and at least coexisting with four non-legume species (i.e. *C. squarrosa*, *A. frigida*, *A. capillaris* and *L. chinensis*). Each non-shrub quadrat (as “pure”) included the same four non-legume species. The distance between paired quadrats was at least above 20 m, while it was 5 m for quadrats within a paired quadrats. Among these paired quadrats, five used for ¹⁵N labeling and the rest of three used for spraying water as control. Totally, this experiment included eight paired quadrats (i.e. sixteen quadrats).

¹⁵N labelling was done on a sunny day, 1st August of 2013. K¹⁵NO₃ (99.5% ¹⁵N enrichment) was dissolved with distilled water in a large sprayer and was sprayed randomly on five of eight paired quadrats, while the rest three paired quadrats were only sprayed with the same amount of distilled water as the control. To make no fertilization effect on plant growth, the N amount for ¹⁵N labeling was 0.03 g m⁻². After the application of ¹⁵N tracer, additional amount of distilled water was sprayed to prevent ¹⁵N retained on the plant leaf surface. The total amount of added water was equal to about 3 mm of precipitation.

2.2. Sampling and measurements

An assumption of the ¹⁵N dilution technique is that the reference plants (i.e., non-legumes) and legumes would take up the same N source from soil regardless of whether they grew alone or with a legume (Chalk et al., 2014). In this study, four non-legumes, i.e. *A. capillaris*, *A. frigida*, *C. squarrosa* and *L. chinensis*, growing with *C. microphylla*, were selected as reference plants. All reference plants and legumes are perennial, *A. frigida* and *A. capillaris* belong to Compositae showing similar phenology with *C. microphylla*, and *C. squarrosa* and *L. chinensis* belong to Graminoids.

One month after ¹⁵N labelling, aboveground part of *C. microphylla* and reference plants (i.e., *C. squarrosa*, *A. frigida*, *A. capillaris* and *L. chinensis*) in both labeled and controlled quadrats were harvested. They were dried for 48 h at 65 °C after washed carefully by distilled water. All aboveground plant materials were ground to a fine powder by a ball mill (MM200, Fa. Retsch, Haan, Germany) for measurements of N content (%) and ¹⁵N/¹⁴N ratios.

Table 1
The Cover, frequency, height and important value of plant species in the temperate grasslands.

Species	Cover	Frequency	Height (cm)	IV	Order of IV
<i>Cleistogenes squarrosa</i>	0.33 ± 0.18	0.93	10.38 ± 3.78	0.102	1
<i>Artemisia frigida</i>	0.31 ± 0.22	0.80	20.18 ± 7.00	0.098	2
<i>Artemisia capillaris</i>	0.23 ± 0.19	0.90	27.28 ± 5.59	0.096	3
<i>Leymus chinensis</i>	0.14 ± 0.12	0.78	21.02 ± 5.09	0.071	4
<i>Stipa krylovii</i>	0.08 ± 0.08	0.50	44.65 ± 14.15	0.064	5
<i>Neopallasia pectinata</i>	0.17 ± 0.26	0.15	33.20 ± 8.02	0.053	6
<i>Allium polyrhizum</i>	0.07 ± 0.05	0.65	17.77 ± 4.62	0.053	7

Three additional quadrats close to the sampling quadrats were selected to estimate the aboveground and belowground biomass of *C. microphylla* and reference plants. All aboveground biomass were clipped close to the soil surface and classified by species. All belowground biomass were obtained by digging up to 15 cm depth for reference plants and 1 m depth for *C. microphylla*. Five cores (0–15 cm depth) of composite soil samples in each quadrat were collected from four corners and the center of each quadrat with a soil probe (2.8 cm in diameter). The fresh soil samples were processed with 2 mm mesh to remove coarse fragments and for measuring soil basic characteristics.

About 2 mg of plant materials were weighed into tin capsules to analyze $^{15}\text{N}/^{14}\text{N}$ by continuous-flow gas isotope ratio mass spectrometry (MAT253, Finnigan MAT, Bremen, Germany), coupled by ConFlo III device (Finnigan MAT, Bremen, Germany) to an elemental analyzer (EA 1112, CE Instruments, Milan, Italy). Atmospheric N_2 was used as standard. The standard deviation of repeated measurements of laboratory standards was $\pm 0.15\%$. Soil organic matter was measured with wet oxidation by acid dichromate solution described by Kalembasa and Jenkinson (1973). Total N was determined by Kjeldahl digestion with a salicylic acidification (Pruden et al., 1984). Soil pH was measured by a glass electrode with a 1:2.5 soil to water ratio.

2.3. Calculations

The N derived from the atmosphere (%Ndfa) for *C. microphylla* was calculated by the following equation (Vallis et al., 1967):

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

where APE was calculated as atom% ^{15}N difference between the same plant species from ^{15}N -labelled and controlled quadrats. $\text{APE}_{\text{leg(m)}}$ indicates APE for *C. microphylla*, $\text{APE}_{\text{reference(m)}}$ indicates APE for reference plants in the mixed quadrats.

The amount of N fixed by *C. microphylla* (N_{fixed} , g m^{-2}) was calculated by the following equation (Busse, 2000):

$$N_{\text{fixed}} = \% \text{Ndfa} \times \text{Biomass}_{\text{leg}} \times N_{\text{leg}} \quad (2)$$

where the %Ndfa indicates the N derived from the atmosphere for *C. microphylla*, the $\text{Biomass}_{\text{leg}}$ (g m^{-2}) indicates the aboveground biomass of *C. microphylla*, N_{leg} (%) indicates N content in aboveground part of *C. microphylla*.

The percent of non-legume N in a mixed quadrat transferred from *C. microphylla* ($\%N_{\text{transfer}}$) was calculated by the following equation (Chalk, 1985):

$$\%N_{\text{transfer}} = \left(1 - \frac{\text{APE}_{\text{reference(m)}}}{\text{APE}_{\text{reference(p)}}} \right) \times 100 \quad (3)$$

where $\text{APE}_{\text{reference(m)}}$ represents APE for non-legumes in the mixed quadrats, $\text{APE}_{\text{reference(p)}}$ represents APE for non-legume species in the pure quadrats without *C. microphylla*.

The amount of N transferred from *C. microphylla* to non-legumes (N_{transfer} , g m^{-2}) was calculated as the following equation (Chalk, 1985):

$$N_{\text{transfer}} = \%N_{\text{transfer}} \times \text{Biomass}_{\text{non-leg}} \times N_{\text{non-leg}} \quad (4)$$

where $\%N_{\text{transfer}}$ refers to the percent of non-legume N in mixed quadrats transferred from *C. microphylla*, $\text{Biomass}_{\text{non-leg}}$ (g m^{-2}) refers to the aboveground biomass of non-legumes, and $N_{\text{non-leg}}$ (%) refers to the N content in aboveground part of non-legumes.

The standard errors (SE) of means are presented in Figures and Tables as a variability parameter. One-way analysis of variance (ANOVA) was carried out with LSD test to determine effects of

Fig. 1. Biomass and root to shoot ratio of the four non-legumes and legume in a temperate grassland of North China, and error bars show means $\pm$ SE ($n=3$). Different lowercase and capital letters represent significant difference at $p < 0.05$ among aboveground biomass and belowground biomass of the five plant species, respectively. The values in the histogram represent the root to shoot ratio of each plant species. CS, *Cleistogenes squarrosa*; AF, *Artemisia frigida*; AC, *Artemisia capillaris*; LC, *Leymus chinensis*; CM, *Caragana microphylla*.

plant species on aboveground and belowground biomass, effects of legume on N content and $\delta^{15}\text{N}$ of non-legumes in the mixed quadrats of natural condition, the %Ndfa and N transfer from symbiotic N_2 -fixation by legume. Paired t -test was used to analyze the difference in APE between legume and reference plants in the mixed quadrats. All the tests were run by using SPSS 20.0 software package (SPSS Inc. Chicago, IL, USA). Significance was considered at $p < 0.05$.

3. Results

3.1. Biomass and root to shoot ratio in the temperate grassland

Total aboveground biomass for all plants averaged $284 \pm 44 \text{ g m}^{-2}$ in the mixed *C. microphylla* community (Fig. 1, Table 2). Aboveground biomass of *C. microphylla* was about $168 \pm 38 \text{ g m}^{-2}$, about 59% of total aboveground biomass in the mixed legume community. The aboveground biomass of *A. capillaris* and *A. frigida* was higher than that of *C. squarrosa* and *L. chinensis* (Fig. 1, Table 2).

Total belowground biomass of all plants in the mixed *C. microphylla* community was about $1063 \pm 117 \text{ g m}^{-2}$ (Fig. 1), and *C. microphylla* contributed about 90% of total belowground biomass. The belowground biomass of the four non-legumes differed significantly ($F_{(3,8)} = 46.12$, $p < 0.05$). That of *A. frigida* was higher than that of the other three non-legumes ($F_{(3,8)} = 46.12$, $p < 0.05$), and *C. squarrosa* had the minimum belowground biomass (Fig. 1, Table 2).

Root to shoot ratio of *C. microphylla* (5.68 ± 1.1) was significantly higher than that of the four non-legumes (Fig. 1, $F_{(4,10)} = 20.459$, $p < 0.05$). For the four non-legumes, root to shoot ratios of *C.*

Table 2

The APE, N content and aboveground biomass of plants in the temperate grasslands of North China.

Species	APE (%)		N content (%)		Aboveground Biomass (g m ⁻²)
	Pure	Mixed	Pure	Mixed	
<i>Cleistogenes squarrosa</i>	0.335 ± 0.048	0.156 ± 0.014	1.20 ± 0.08	1.64 ± 0.10	21.85 ± 1.63
<i>Artemisia frigida</i>	0.337 ± 0.074	0.210 ± 0.005	1.82 ± 0.05	1.92 ± 0.21	36.88 ± 1.72
<i>Artemisia capillaris</i>	0.525 ± 0.071	0.336 ± 0.046	1.37 ± 0.05	1.93 ± 0.06	37.01 ± 2.18
<i>Leymus chinensis</i>	0.104 ± 0.017	0.144 ± 0.025	1.24 ± 0.05	2.09 ± 0.06	20.56 ± 0.92
<i>Caragana microphylla</i>	-	0.018 ± 0.002	-	2.83 ± 0.16	167.69 ± 38.13

Pure indicates the reference plants growing without legume; Mixed indicates the reference plants growing with legume; - indicates no data.

Fig. 2. Nitrogen content and $\delta^{15}\text{N}$ value in aboveground part of non-legumes in natural condition (no ^{15}N labelling) in a temperate grassland of North China. Mean $\pm$ SE ($n = 3$) are presented. The left and right panels represent the N content and $\delta^{15}\text{N}$ of the four non-legumes respectively. Lowercase and capital letters represent significant differences at $p < 0.05$ among four non-legumes when they growing with legume and without legume, respectively. CS, *Cleistogenes squarrosa*; AF, *Artemisia frigida*; AC, *Artemisia capillaris*; LC, *Leymus chinensis*. The asterisk represents the significant difference at $p < 0.05$, when the same non-legume grow with and without legume.

squarrosa, *A. capillaris* and *A. frigida* are less than one, while that of *L. chinensis* is larger than one (Fig. 1).

3.2. Nitrogen content and $\delta^{15}\text{N}$ in aboveground part of *C. microphylla* and non-legumes

Aboveground N content of non-legumes growing with the legume was obviously higher than those growing without the legume except for *A. frigida* (Fig. 2A, Table 2, $F_{(1,22)} = 18.08$, $p < 0.05$). Similar pattern of higher N content was found for the four reference plants when growing with legume than growing alone. N content of *A. frigida* was higher than the other three non-legumes (Table 2). In the *C. microphylla* community, the N stock in the aboveground biomass was estimated to be 7.73 g m^{-2} , while that of belowground root biomass in 1 m depth was estimated to be 35 g m^{-2} .

Aboveground $\delta^{15}\text{N}$ values of the four non-legumes growing with *C. microphylla* were similar (Fig. 2B). When they grow alone, *A. frigida* was significantly higher than the other three non-legumes ($F_{(3,8)} = 14.8$, $p < 0.05$, Fig. 2B). The aboveground $\delta^{15}\text{N}$ of *A. frigida* ($F_{(1,4)} = 19.885$, $p < 0.05$) and *A. capillaris* ($F_{(1,4)} = 34.287$, $p < 0.05$) differed greatly when they grow with or without *C. microphylla* (Fig. 2B).

3.3. APE in aboveground part of non-legumes and N derived from the atmosphere

The APE for reference plants in aboveground part was distinctly higher than that of *C. microphylla* (0.21% vs 0.02% , $t = 10.4$, $p < 0.001$, Fig. 3). *A. capillaris* exhibited significantly higher APE (0.34 ± 0.05) than the other three reference plants ($F_{(3,16)} = 9.80$, $p < 0.05$). In

contrast, significant difference was found in APE between *C. squarrosa*, *L. chinensis* and *A. frigida* (Fig. 3, $F_{(2,12)} = 3.237$, $p > 0.05$).

The %Ndfa of *C. microphylla* was estimated to range from 84 to 95% (averaged 90%). Based on the aboveground biomass and its N content, the amount of N fixed by *C. microphylla* was conservatively estimated to be about $4.3 \text{ g N m}^{-2} \text{ yr}^{-1}$.

Fig. 3. The APE in aboveground part of legume and reference plants in ^{15}N labeling quadrats in a temperate grassland of North China. Mean $\pm$ SE ($n = 5$) are presented. Different letters represent significant differences at $p < 0.05$ among five plants in the temperate grassland. CS, *Cleistogenes squarrosa*; AF, *Artemisia frigida*; AC, *Artemisia capillaris*; LC, *Leymus chinensis*; CM, *Caragana microphylla*.

Fig. 4. Percentage of non-legume N in aboveground part transferred from legume in the temperate grassland. Mean $\pm$ SE ($n=5$) are presented. Different letters represent significant differences at $p < 0.05$ between the four non-legumes in the temperate grassland. CS, *Cleistogenes squarrosa*; AF, *Artemisia frigida*; AC, *Artemisia capillaris*; LC, *Leymus chinensis*.

3.4. Nitrogen in aboveground part of non-legumes transferred from *C. microphylla*

The aboveground N amount in the non-legumes transferred from *C. microphylla* varied as different non-legume species growing with *C. microphylla* (Fig. 4). About half amount of aboveground N for *C. squarrosa* derived from *C. microphylla*, but there is no significant difference among *A. frigida*, *A. capillaris* and *C. squarrosa* ($F_{(2,12)} = 0.009$, $p > 0.05$). By comparison, *L. chinensis* almost did not obtains any N from its associated *C. microphylla* (Fig. 4).

4. Discussion

With ^{15}N dilution approach, we quantified the amount of N fixed by legume shrub of *C. microphylla* as well as its N transfer to coexisting non-legume species in the temperate grassland of northern China. We found that the %Ndfa of *C. microphylla* was about 90%, falling within estimated ranges (85–92) by previous observations in similar temperate grasslands (Yang et al., 2011). Given the %Ndfa reflects the proportion of N derived from the atmosphere through biological N fixation by legumes (Vallis et al., 1967; Temperton et al., 2007; Carlsson et al., 2009), our estimate indicate that the shrub legume mainly relies on its symbiotic fixation of atmospheric N. The choice of reference plants could influence the estimate accuracy of %Ndfa (Rennie et al., 1982; Holdensen et al., 2007), but a recent study proved the reference plants growing together with legumes can provide a more relatively reliable quantification of N_2 -fixation (Carlsson and Huss-Danell, 2014). To reduce the uncertainties in estimate of the %Ndfa, we totally chose four non-legumes as reference plants.

It is well known that legumes are more competitive in the N-limited ecosystems than non-legumes due to their symbiotic N_2 fixation, e.g. %Ndfa values for legumes growing in soils of low N availability can be up to 90% (Turner and Bergersen, 1983; Ledgard et al., 1987). Such atmospheric N_2 fixation provides advantage for the legumes in N-limited habitats, because to some degree it reduces the competition for soil inorganic N between legumes and other non-legume species. Further, legumes improve the quality and quantity of soil nutrients through providing extra N in temperate grasslands (Murray and Clements, 1988; Paynel et al., 2001). Due to its N fixation, *C. microphylla* shrubs even could grow in bare soils and regulate population dynamics of herbaceous plants at individual shrub scale in temperate grasslands (Koyama

et al., 2015; Guan et al., 2015). Therefore, they are often widely used as a pioneer species in vegetation reestablishment in the degraded grasslands (Guan et al., 2015). Our results partly confirmed that *C. microphylla* would be more easily survive in degraded grasslands.

The amount of N fixed by *C. microphylla* was estimated to be about 4.3 g N m^{-2} , similar with those estimated by Issah et al. (2014) for *Caragana* (4.12 g m^{-2}) in Canada with the same technique. Despite the similar %Ndfa, the N fixed by *C. microphylla* is much larger than that of other legumes in temperate *Leymus* steppes (Li et al., 2002; Yang et al., 2011). This is mainly ascribed to the higher biomass of *C. microphylla* shrub. However, our estimate was less than that (15.5 g m^{-2}) of white clover (*Trifolium repens*) growing in mixture with ryegrass (*Lolium perenne*) (Nutman, 1971). Possible explanation is that the potential of N fixation by legumes (Sanford et al., 1995; Carlsson and Huss-Danell, 2003) maybe differs between species, and the higher temperature and soil moisture for white clover could lead to a higher total N yield in the grass-clover mixture (Sanford et al., 1995). Growing in the semi-arid area of northern China, *C. microphylla* has a well-developed root system and associated with an increase in %Ndfa compared to white clover (Ledgard et al., 1987). This may possibly due to the special characteristics of legume plants (e.g. with mycorrhizal symbiotic systems). We inferred that the N fixed by *C. microphylla* in our studied temperate grassland would account for 75% of the gap of N demand and supply with the same method used by Yang et al. (2011). This indicated that biological N_2 -fixation of *C. microphylla* plays a more important role in N dynamics in N-limited grasslands. Shearer and Kohl (1986) suggested that biological N fixation by legumes ranged from 4 to $40 \text{ g N m}^{-2} \text{ yr}^{-1}$, and could achieve 46% of the total N accumulation of all the aboveground plant biomass. Biological N fixation by *C. microphylla* can contribute 56% of the total N stock in the grasslands. This indicates that *C. microphylla* is an important plant species in maintaining ecosystem functions, supporting our first hypothesis that symbiotic N_2 -fixation by *C. microphylla* is important on an annual scale in temperate grasslands. Especially due to the removal of aboveground biomass by overgrazing, the plants are often limited by the soil available N in temperate grasslands (Yan et al., 2014).

Symbiotic N fixation provides an important N source for those coexisting non-legumes (Pirhofer-Walzl et al., 2012). A fraction of N fixed by legumes can be transferred to their associated grasses (Ledgard and Steele, 1992; Sierra and Daudin, 2010; Makarov et al., 2011; Carlsson and Huss-Danell, 2014). For example, Ledgard (1991) found that 22% of N fixed by white clover was transferred to associated plants. Brophy et al. (1987) showed that 68% of N in *Phalaris arundinacea* derived from *Medicago sativa* and obtained 17% of N fixed by *M. sativa*. In this study, half of *C. squarrosa* aboveground N derived from *C. microphylla*. The legume *C. microphylla* transferred approximately 4.4%, 6.2% and 6.0% of its aboveground fixed N to *C. squarrosa*, *A. frigida* and *A. capillaris*, respectively. *L. chinensis* did not show N acquisition from *C. microphylla*. This reflects that N transferred from legumes to non-legumes varies in non-legume species, confirming our second hypothesis that the amount of N transferred to its associated species could be species-specific and mostly depended upon their associated species. Coexisting non-legume species can acquire N fixed by legumes mainly through mycorrhizal networks between their roots and legume roots (Jalonen et al., 2009), by absorption of mineralized N derived from dead root nodules (Butler et al., 1959; Pirhofer-Walzl et al., 2011), or by uptake of N compounds exuded directly by legume roots (Ta et al., 1986; Jiang and Zhai 2000). Therefore, the difference in N transfer could be ascribed to the root characteristics and regrowth strategies of plant species (Temperton et al., 2007; Pirhofer-Walzl et al., 2012), the distance between legumes and non-legumes (Issah et al., 2015) and the N status in soil (Murray et al., 2008). The root architectures and regrowth

strategies of non-legume species (Pirhofer-Walzl et al., 2012) partly explain the reason for the difference in their N content and $\delta^{15}\text{N}$ when they growing with the legume shrubs and growing alone. Given that only about 17% of fixation N from *C. microphylla* was transferred to associated species, this indicates that most N fixed by the legume was used for themselves' growth and production. Nonetheless, non-legumes can benefit from their associated *C. microphylla*, and the survival of *C. microphylla* in degraded grasslands is very important for their coexisting non-legumes.

The N transferred to non-legumes in this study was assessed only based on the ^{15}N in the aboveground plant parts. This actually underestimated the amount of N fixed by the shrub legume and its N transfer to non-legumes. Further studies should be done to clarify the magnitude on intact plant individuals and the potential effect derived from shrub ages.

5. Conclusion

Symbiotic N_2 -fixation and transfer to non-legumes was estimated based on the aboveground biomass and its ^{15}N . The % Ndfa of *C. microphylla* in temperate grasslands was about 90%. Total N fixation in aboveground part of *C. microphylla* community was estimated to be about $4.3 \text{ g N m}^{-2} \text{ yr}^{-1}$. About half of N in aboveground part of *C. squarrosa* derived from *C. microphylla*, while *L. chinensis* almost did not benefit from *C. microphylla*. Totally, about 17% of N fixed by *C. microphylla* was transferred to its associated species. Since *C. microphylla* is widespread and predominant in the arid and semi-arid area of northern China, their symbiotic N_2 -fixation makes more significant contributions to the N economy of temperate grassland ecosystems. These findings indicate that *C. microphylla* is an important plant species that can strongly modify the N cycling due to its N fixation and transfer in temperate grasslands in North China.

Acknowledgements

This research was supported by the National Key Basic Research Program of China (Grant No. 2014CB138803), and the National Key Research and Development Program of China (Grant No. 2016YFC0500502), Fundamental Research Funds for the Central Universities (Grant No. 2014KJJC01), as well as National Natural Science Foundation of China (31470560, 31570468 and 31270500).

References

- Bai, Y.F., Han, X.G., Wu, J.G., Chen, Z.Z., Li, L.H., 2004. Ecosystem stability and compensatory effects in the Inner Mongolia grassland. *Nature* 431, 181–184.
- Brophy, L.S., Heichel, G.H., Russelle, M.P., 1987. Nitrogen transfer from forage legumes to grass in a systematic planting design. *Crop. Science* 27, 753–758.
- Busse, M.D., 2000. Suitability and use of the ^{15}N -isotope dilution method to estimate nitrogen fixation by actinorhizal shrubs. *For. Ecol. Manag.* 136 (1–3), 85–95.
- Butler, G.W., Greenwood, R.M., Soper, K., 1959. Effects of shading and defoliation on the turnover of root and nodule tissue of plants of *Trifolium repens*, *Trifolium pratense*, and *Lotus uliginosus*. *N. Z. J. Agric. Res.* 2 (3), 415–426.
- Carlsson, G., Huss-Danell, K., 2003. Nitrogen fixation in perennial forage legumes in the field. *Plant Soil* 253 (2), 353–372.
- Carlsson, G., Huss-Danell, K., 2014. Does nitrogen transfer between plants confound ^{15}N -based quantifications of N_2 fixation? *Plant Soil* 374 (1–2), 345–358.
- Carlsson, G., Palmberg, C., Jumpponen, A., Scherer-Lorenzen, M., Höglberg, P., Huss-Danell, K., 2009. N_2 fixation in three perennial *Trifolium* species in experimental grasslands of varied plant species richness and composition. *Plant Ecol.* 205 (1), 87–104.
- Chalk, P.M., Peoples, M.B., McNeill, A.M., Boddey, R.M., Unkovich, M.J., Gardener, M. J., Silva, C.F., Chen, D.L., 2014. Methodologies for estimating nitrogen transfer between legumes and companion species in agro-ecosystems: a review of ^{15}N -enriched techniques. *Soil Biol. Biochem.* 73, 10–21.
- Chalk, P.M., 1985. Estimation of N_2 fixation by isotope dilution: an appraisal of techniques involving ^{15}N enrichment and their application. *Soil Biol. Biochem.* 17 (4), 389–410.
- Chinese Academy of Sciences, 2001. The Chinese Soil Taxonomic Classification Retrieval, third edition Press of University of Science and Technology of China, Hefei.
- Chu, Y., Yang, H.L., Zhu, X.W., Dong, M., 2005. Spatial heterogeneity of *Caragana microphylla* shrub communities in the Otindag Sandland. *Acta Ecol. Sin.* 25 (12), 3294–3300.
- Cusack, D.F., Silver, W., McDowell, W.H., 2009. Biological nitrogen fixation in two tropical forests: ecosystem-level patterns and effects of nitrogen fertilization. *Ecosystems* 12 (8), 1299–1315.
- Gao, Y.Z., Han, X.G., Wang, S.P., 2004. The effects of grazing on grassland soils. *Acta Ecol. Sin.* 24 (4), 790–797.
- Guan, P.T., Zhang, X.K., Yu, J., Ma, N.N., Liang, W.J., 2015. Variation of soil nematode community composition with increasing sand-fixation year of *Caragana microphylla*: bioindication for desertification restoration. *Ecol. Eng.* 81, 93–101.
- Høgh-Jensen, H., Schjoerring, J., 2000. Below-ground nitrogen transfer between different grassland species: direct quantification by ^{15}N leaf feeding compared with indirect dilution of soil ^{15}N . *Plant Soil* 227 (1–2), 171–183.
- Hauggaard-Nielsen, H., Ambus, P., Jensen, E.S., 2001. Interspecific competition, N use and interference with weeds in pea-barley intercropping. *Field Crops Res.* 70 (2), 101–109.
- Herridge, D.F., Peoples, M.B., Boddey, R.M., 2008. Global inputs of biological nitrogen fixation in agricultural systems. *Plant Soil* 311 (1–2), 1–18.
- Holdensen, L., Hauggaard-Nielsen, H., Jensen, E.S., 2007. Short-range spatial variability of soil ^{15}N natural abundance-effects on symbiotic N_2 -fixation estimates in pea. *Plant Soil* 298 (1–2), 265–272.
- Issah, G., Kimaro, A.A., Kort, J., Knight, J.D., 2014. Quantifying biological nitrogen fixation of agroforestry shrub species using ^{15}N dilution techniques under greenhouse conditions. *Agrofor. Syst.* 88 (4), 607–617.
- Issah, G., Kimaro, A.A., Kort, J., Knight, J.D., 2015. Nitrogen transfer to forage crops from a *Caragana* shelterbelt. *Forest* 6 (6), 1922–1932.
- Jalonen, R., Nygren, P., Sierra, J., 2009. Root exudates of a legume tree as a nitrogen source for a tropical fodder grass. *Nutr. Cycl. Agroecosyst.* 85 (2), 203–213.
- Jiang, S.N., Zhai, M.P., 2000. Nitrogen transfer between N_2 -fixing plant and non- N_2 -fixing plant. *J. For. Res.* 11, 75–80.
- Kalembasa, S.J., Jenkinson, D.S., 1973. A comparative study of titrimetric and gravimetric methods for the determination of organic carbon in soil. *J. Sci. Food Agric.* 24 (9), 1085–1090.
- Koyama, A., Sasaki, T., Jamsran, U., Okuro, T., 2015. Shrub cover regulates population dynamics of herbaceous plants at individual shrub scale on the Mongolian steppe. *J. Veg. Sci.* 26 (3), 441–451.
- Lebauer, D.S., Treseder, K.K., 2008. Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89 (2), 371–379.
- Ledgard, S.F., Steele, K.W., 1992. Biological nitrogen fixation in mixed legume/grass pastures. *Plant Soil* 141 (1–2), 137–153.
- Ledgard, S.F., Brier, G.J., Littler, R.A., 1987. Legume production and nitrogen fixation in hill pasture communities. *N. Z. J. Agric. Res.* 30 (4), 413–442.
- Ledgard, S.F., 1991. Transfer of fixed nitrogen from white clover to associated grasses in swards grazed by dairy cows, estimated using ^{15}N methods. *Plant Soil* 131 (2), 215–223.
- Li, X.Z., Zhang, S.M., Xiong, X.R., 2002. Spatial variation of plant biomass and soil chemical element contents induced by *Catagana microphylla*. *Acta Prarm. Sin.* 11 (1), 1–3.
- Li, X.H., Jiang, D.M., Luo, Y.M., 2010. Soil fertile islands of shrub canopy and impacts on vegetation in chronosequence of *Caragana microphylla*. *J. Liaon. Technol. Univ. (Nat. Sci.)* 29 (2), 336–339.
- Makarov, M.I., Malysheva, T.I., Ermak, A.A., Onipchenko, V.G., Stepanov, A.L., Menyailo, O.V., 2011. Symbiotic nitrogen fixation in the alpine community of a lichen heath of the Northwestern Caucasus Region (the Teberda Reserve). *Eur. Soil Sci.* 44 (12), 1381–1388.
- Moukoui, J., Farrell, R.E., van Rees, K.J.C., Hynes, R.K., Belanger, N., 2012. Growth and nitrogen dynamics of juvenile short rotation intensive cultures of pure and mixed *Salix miyabeana* and *Caragana arborescens*. *Biol. Res.* 5, 719–732.
- Murray, P.J., Clements, R.O., 1998. Transfer of nitrogen between clover and wheat: effect of root herbivory. *Eur. J. Soil Biol.* 34 (1), 25–30.
- Murray, U., Herridge, D., Peoples, M., Cadisch, G., Boddey, B., Giller, K., Alves, B., Chalk, P., 2008. Measuring plant-associated nitrogen fixation in agricultural systems. *ACIAR Monograph No. 132*, 163–217.
- Niu, S.L., Jiang, G.M., 2004. The importance of legume in China grassland ecosystem and advances in physiology and ecology studies. *Chin. Bull. Bot.* 21 (1), 9–18.
- Nutman, P.S., 1971. Perspectives in biological nitrogen fixation. *ScienceProgress (Oxford)* 59, 55–74.
- Paynel, F., Murray, P.J., Cliquet, J.B., 2001. Root exudates: a pathway for short-term N transfer from clover and ryegrass. *Plant Soil* 229 (2), 235–243.
- Peoples, M.B., Herridge, D.F., Ladha, J.K., 1995. Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production? *Plant Soil* 174 (1–2), 3–28.
- Peoples, M.B., Boddey, R.M., Herridge, D.F., 2002. Quantification of nitrogen fixation. In: Leigh, G.J. (Ed.), *Nitrogen Fixation at the Millennium*. Elsevier, Amsterdam, pp. 357–389.
- Pirhofer-Walzl, K., Rasmussen, J., Høgh-Jensen, H., Eriksen, J., Søgaard, K., Rasmussen, J., 2012. Nitrogen transfer from forage legumes to nine neighboring plants in a multi-species grassland. *Plant Soil* 350 (1), 71–84.
- Pollock, T., Svendsen, E., 2008. Trees and Shrubs for Agroforestry on the Prairies: Adapted Species Available Through the Prairie Shelterbelt Program. Agriculture and Agri-Food Canada Indian Head, SK, 31.

- Pruden, G., Powlson, D.S., Jenkinson, D.S., 1984. The measurement of ^{15}N in soil and plant material. *Fert. Res.* 6 (3), 205–218.
- Rennie, R.J., Dubetz, S., Bole, J.B., Muendel, H.H., 1982. Denitrogen fixation measured by ^{15}N isotope dilution in two Canadian soybean cultivars. *Agro. J.* 74, 729–730.
- Sanford, P., Pate, J.S., Unkovitch, M.J., Thompson, A.N., 1995. Nitrogen fixation in grazed and ungrazed subterranean clover pasture in south west Australia assessed by the ^{15}N natural abundance technique. *Aust. J. Agric. Res.* 46, 1427–1443.
- Schlesinger, W.H., Pilmanis, A.M., 1998. Plant-soil interactions in deserts. *Biogeochemistry* 42, 169–187.
- Shearer, G., Kohl, D.H., 1986. N_2 -fixation in field settings-estimation based on natural ^{15}N abundance. *Aust. J. Plant Physiol.* 13 (6), 699–756.
- Sierra, J., Daudin, D., 2010. Limited ^{15}N transfer from stem-labeled leguminous trees to associated grass in an agroforestry system. *Eur. J. Agron.* 32 (3), 240–242.
- Sierra, J., Nygren, P., 2006. Transfer of N fixed by a legume tree to the associated grass in a tropical silvopastoral system. *Soil Biol. Biochem.* 38 (7), 1893–1903.
- Soil Survey Staff, 1987. Keys to soil taxonomy, Management Support Service. third edition Cornell University, Ithaca, New York.
- Ta, T.C., Macdowall, F.D.H., Faris, M.A., 1986. Excretion of nitrogen assimilated from N_2 fixed by nodulated roots of alfalfa (*Medicago sativa*). *Can. J. Bot.* 64, 2063–2067.
- Temperton, V.M., Mwangi, P.N., Scherer-Lorenzen, M., Schmid, B., Buchmann, N., 2007. Positive interactions between nitrogen-fixing legumes and four different neighbouring species in a biodiversity experiment. *Oecologia* 151 (2), 190–205.
- Turner, G.L., Bergersen, F.J., 1983. Natural abundance of ^{15}N in root nodules of soybean, lupin, subterranean clover and lucerne. *Soil Biol. Biochem.* 15 (5), 525–530.
- Vallis, I., Haydock, K.P., Ross, P.J., Henzell, E.F., 1967. Isotopic studies on the uptake of nitrogen by pasture plants. III. The uptake of small additions of ^{15}N -labelled fertilizer by *Rhodes grass Townsville lucerne*. *Aust. J. Agric. Res.* 18, 865–887.
- Vitousek, P.M., Howarth, R.W., 1991. Nitrogen limitation on land and in the sea: how can it occur? *Biogeochemistry* 13 (2), 87–115.
- Xiong, X.G., Han, X.G., 2006. Resoruces islands and its roles in the thicketization of grasslands and thicketization grasslands. *Acta Prarm. Sin.* 15 (1), 9–14.
- Xiong, X.G., Han, X.G., Bai, Y.F., Pan, Q.M., 2003. Increased distribution of *Caragana microphylla* in rangelands and its causes and consequences in Xilin River Basin. *Acta Pharm. Sin.* 12 (3), 57–62.
- Xu, L., Gao, Q., Wang, Y.L., 2014. Species richness within a six-year slope enclosure in a temperate grassland and its relationship with aboveground biomass. *Ecol. Environ. Sci.* 23 (3), 398–405.
- Yan, Z.Q., Qi, Y.C., Dong, Y.S., Peng, Q., Sun, L.J., Jia, J.Q., Cao, C.C., Guo, S.F., He, Y.L., 2014. Nitrogen cycling in grassland ecosystem in response to climate change and human activities. *Acta Pharm. Sin.* 23 (6), 279–292.
- Yang, B.J., Qiao, N., Xu, X.L., Ouyang, H., 2011. Symbiotic nitrogen fixation by legumes in two Chinese grasslands estimated with the ^{15}N dilution technique. *Nutr. Cycl. Agroecosyst.* 91 (1), 91–98.
- Zhang, L., Wu, D.X., Shi, H.Q., Zhang, C.J., Zhan, X.Y., Zhou, S.X., 2011. Effects of elevated CO_2 and N addition on growth and N_2 fixation of a legume subshrub (*Caragana microphylla* Lam.) in temperate grassland in China. *PLoS One* 6 (10).
- Zhou, D.W., 1990. *Caragana microphylla* shrub grassland in Inner Mongolia. *Inner Mongolian Prat.* 03, 17–19.