Nitrogen addition results in *Medicago sativa* switching nitrogen sources

Yinliu Wang[a,b], Muqier Hasi[a,b], Dongdong Bu[a,b,c], Ang Li[a], Jianguo Xue[a], Changhui Wang[a], Qiuying Tian[a], Guoxiang Niu[a,b], Qianqian Geng[a,b], Lixin Wang[d] and Jianhui Huang[a,b,*]

[a]*State Key Laboratory of Vegetation and Environmental Change, Institute of Botany, the Chinese Academy of Sciences, Beijing, China;* [b]*College of Resources and Environment, University of Chinese Academy of Sciences, Beijing, China;* [c]*Beijing Micro & Macro Science and Technology Co. Ltd., Beijing, China;* [d]*Department of Earth Sciences, Indiana University Purdue University Indianapolis, Indianapolis, IN, USA*

***Corresponding author**. E-mail: jhhuang@ibcas.ac.cn

Abstract

Background: Nitrogen (N) addition may have strong impacts on legume growth and their biological N fixation (BNF), but how legume N acquisition sources respond to N inputs have yet to be comprehensively assessed.

Aims: We quantified the effects of N addition on the growth and BNF of *Medicago sativa* and to assess the response of legume N acquisition to N addition.

Methods: We grew M. sativa in the greenhouse with NH_4NO_3 added at rates of 0, 2, 5, 10, 20, 50 g N m^{-2} yr^{-1}, and analysed the variables that were relative to growth and N

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fixation, such as N concentration, biomass, $\delta^{15}N$ values, nodule number, the percentage of plant N derived from symbiotic fixation of atmospheric N_2 (Ndfa%).

Results: Nitrogen addition had marginal effects on accumulative plant biomass production (total biomass was 20.36 ± 1.57 and 22.26 ± 2.08 g pot^{-1} for the N0 and N50 treatment, respectively) and foliar N concentration (4.62 ± 0.21% and 4.89 ± 0.09% for the same treatments). The $\delta^{15}N$ value of the leaves increased with increasing added N, while Ndfa% decreased. The number of nodules formed also decreased with N addition while the nitrogenase (*nifH*) gene copies per unit nodule mass was not significantly different with N addition.

Conclusions: These findings indicate that increasing mineral N availability decreases symbiotic investment into BNF, mainly by reducing nodule formation; this was found to have no significant impact on plant growth because the plant changes its N source from BNF-N to mineral N derived from the soil.

Keywords: biological N fixation; biomass; N concentration; $\delta^{15}N$; nodulation; stable isotope

1. Introduction

Nitrogen (N) is an essential element, required for growth and maintenance of all plants (Vitousek and Howarth 1991; Elser et al. 2007; LeBauer and Treseder 2008). Biological nitrogen fixation (BNF), characteristic of some free living or symbiotic N fixers, is a dominant pathway of new N input into terrestrial ecosystems (Vitousek et al. 2013). Over recent decades, the increasing atmospheric N deposition caused by intensifying anthropogenic activities also become increasingly important N sources for ecosystems (Vitousek et al. 1997; Galloway et al. 2004, 2008). BNF has a high energy cost and appears to be sensitive to exogenous N inputs (Gutschick 1981; Zheng et al. 2019, 2020). Thus, understanding how exogenous N inputs affects the growth of N-fixing plants, especially the ability to fix atmospheric N_2, is needed to better predict the contribution of BNF to the N cycle in terrestrial ecosystems, considering the increasing N inputs in the future.

Legumes are an important plant functional group of terrestrial vegetation worldwide and appear to be provided with a competitive advantage in the N-limited ecosystems because of their ability of symbiotic BNF (Rogers et al. 2009; Vitousek et al. 2013). However, under which circumstances BNF benefits legumes is largely related to soil N availability, which provides a less energy-demanding N source for plants (Menge et al. 2017; Regus et al. 2017). Previous work has demonstrated at both regional and global scales that N inputs could increase legume biomass (Skogen et al. 2011; Barneze et al. 2020) and suppress BNF (Batterman et al. 2013a; Zheng et al. 2019, 2020). Additionally, increasing soil N availability is accompanied with decreasing nodulation, which, in turn,

affects species composition and diversity of N-fixing bacteria (Wang et al. 2017a). Meanwhile, some other studies found no response, minor response or even positive response of BNF, to the improved soil N availability (Binkley et al. 2003; Drake 2011). Regarding the different response patterns to increasing soil N availability, two distinct strategies have been proposed, i.e., the facultative N-fixing and obligate N-fixing strategy (Barron et al. 2011; Menge et al. 2014, 2015; Menge and Chazdon 2016). Facultative N-fixers can adjust their BNF depending on the exogenous N availability. In contrast, obligate N-fixers generally keep active N fixation regardless of soil N conditions. For this reason, the facultative strategy is considered more adoptive than the obligate one (Menge et al. 2009).

In addition to increasing soil N availability, there are some other potential mechanisms that could explain how N inputs affect legume BNF. First, N inputs could cause leaching loss of phosphorus (P), thereby lead to N fixation constraint, as P is used for parts of nitrogenase and cell metabolism (Batterman et al. 2013b; Zheng et al. 2016). Second, N inputs causes soil acidification, which can change the microbial community and affect the host-rhizobia symbiosis via decreasing rhizobia colonization and nitrogenase activity (Caetanoanolles et al. 1989; Graham 1992; Lu et al. 2014; Ferreira et al. 2016). Third, N inputs also incur indirectly negative effects on BNF via molybdenum (Mo) limitation (a key component of nitrogenase) (Vitousek and Howarth 1991; Wurzburger et al. 2012).

Since the 1990s, N deposition has increased dramatically throughout China from 9.4 kg N ha^{-1} yr^{-1} in 1980 to 20.6 kg N ha^{-1} yr^{-1} in 2018 (Yu et al. 2019; Wen et al. 2020) and

N fertilizer consumption in China is highest in the world (FAOSTAT, 2010). As an important plant functional group in terrestrial ecosystems worldwide, legumes have played critical roles in the functioning of N cycling and provisioning of ecosystem services. However, how they respond to a less costly mineral N source derived from atmospheric sources is unclear yet. Here we report a greenhouse experiment on the effects of N addition (covered both N deposition and current N fertilization amount) on the growth and BNF of a leguminous plant with application of stable isotope and qPCR analysis, combined with some conventional plant morphological and ecological measurements. Our target species *Medicago sativa* L. is a widely cultivated pasture species and a common N-fixing plant with wide natural distribution in the grasslands of northern China. The objectives of this study were to quantify how the growth and N fixation capacity would respond to N inputs and if *M. sativa* would adjust its N acquisition source. We expected that *M. sativa* would (1) increase its biomass after N addition; (2) change its N source from BNF to the less costly soil mineral N source (i.e., with a facultative strategy) with increasing N addition, and would suppress its N fixation altogether; and (3) reduce investment into nodulation after N addition, therefore would be decrease in number of nodules formed and in the abundance of rhizobia sustained in the nodules.

Materials and methods

Experimental design

Our experiment was carried out in the greenhouse at the Institute of Botany, the Chinese Academy of Sciences. The temperature in the greenhouse during experiment was maintained at 25±5℃ and the relative humidity was ca. 20%. Commercially available alfalfa seeds of the variety Longmu 801 (*Medicago sativa* L. cv. Longmu 801) were obtained and the seeds were surface sterilized with 98% sulfuric acid before the seeds were germinated in a glass culture dish. After germination, seedlings with similar size and healthy appearance were transplanted into pots (H 25 cm × D 20 cm) with soil media. The soil, classified as Haplic Calcisol (IUSS Working Group WRB, 2014), was collected from a typical steppe grassland in Xilingol, Inner Mongolia, China, where *M. sativa* has been cultivated as forage for many years (Deng et al. 2014; McNeill et al. 2021). The soil was completely homogenised prior to planting. Soil physical-chemical properties were shown in Table 1. To ensure sufficient supply of nutrients, we added P_2O_5 (in the form of $Ca(H_2PO_4)_2$, 0.41 g/pot), K (K_2SO_4, 0.56 g/pot) and Mg ($MgSO_4$, 2.05 g/pot) to the soil. We also applied 20 ml of a modified N-free Hoagland's nutrient solution every two months to all plants throughout the experiment to provide the necessary nutrients (McNickle et al. 2013).

After growing 14 d in the greenhouse, liquid inoculum containing ~10^8 cell ml^{-1} of *Sinorhizobium meliloti* strain AFS32 was injected in the soil around the roots of the plants at a rate of 5 ml per plant to ensure nodule formation (Elkherbawy et al. 1989; Elnesairy et al. 2005). Two weeks after inoculation, the plants were fertilised with N at

the following rates: equivalent of annual rates of 0 (control), 2, 5, 10, 20, 50 g N m^{-2} yr^{-1} as NH_4NO_3, designated as N0, N2, N5, N10, N20 and N50, respectively. The background rate of N deposition in the study area was 1.8 g N m^{-2} $year^{-1}$. Our N addition levels involved the ambient N deposition rate, the threshold of biomass production response (ca. 10 g N m^{-2} $year^{-1}$), and a high N fertilization rate for the artificial hay production pastures (Li et al. 2015; Xu et al. 2015). Each level of N addition treatment had six replicate potted plants, totalling 36 pots in the experiment. As *M. sativa* is a perennial plant, we ran our experiment for about 16 months, from April 2017 to July 2018, with the N treatment repeated at the end of April 2018. All plants were watered equally as needed. Pots were rotated by 45° each day and were rearranged randomly weekly. During the first year of the experiment (and before the second N addition) the plant were clipped back to ground level four times (at the end of July 2017, September 2017, December 2017 and March 2018). The plants were harvested at the end of July 2018.

Plant biomass

Plant heights were measured before the harvest in the end of July 2018, and the average plant height of each treatment was calculated at the end of the experiment. To determine above-ground biomass (AGB), each plant was cut at the soil surface and dried at 105℃ for 15 min and then at 65℃ to a constant weight. We also collected the belowground biomass (BGB). Root systems were separated from soil and cleaned with distilled water, and the axial root length was recorded. The roots were dried at 65℃ for 48 h and weighed. Total biomass was calculated as the sum of AGB and BGB. Healthy

green leaves were sampled from all individuals in the last harvest for analyzing $\delta^{15}N$ and nutrient concentration. Soil samples from each pot were collected to measure soil pH, and concentration of NH_4^+ and NO_3^-.

Foliar N and P concentration

Leaf samples were ground in a Mixer Mill MM400 (Retsch Technology, Haan, Germany). Total N concentration of leaves was analysed with a Vario El cube CHNS elemental analyzer (Elementar Analysensysteme, Germany). Leaf P was determined by inductive coupled plasma emission spectrometer (ICP-OES; ICAP6300; Thermo Electric, West Chester, Pennsylvania, USA) after being digested with a mixture of nitric acid and hydrogen peroxide.

Foliar ^{15}N determination

The N source used by the plants was estimated by the ^{15}N natural abundance method (Shearer et al. 1983; Boddey et al. 2000). The small difference in ^{15}N abundance between samples and the air are usually expressed as $\delta^{15}N$ which is calculated with the following equation (Eq. 1):

$$\delta^{15}N\ (‰) = \frac{1000\times(\text{atom\%}^{15}\text{N sample-atom\%}^{15}\text{N standard})}{\text{atom\%}^{15}\text{N standard}} \quad \text{(Eq. 1)}$$

The standard is atmospheric N_2 (0.3663 atom% ^{15}N). Because N_2 fixation uses the N from atmospheric N_2 and there is almost no fractionation during N fixation, plants that benefit from symbiotic relationships with N-fixing microbes show a $\delta^{15}N$ value closer to that of the standard atmospheric N_2. In general, soil N is usually more abundant in ^{15}N than atmospheric N_2 because of the fractionation during the soil N transformation processes, thus non-N_2-fixing plants that take N source from soil are expected to have

more abundant ^{15}N value than N_2-fixing plants that take N source from atmosphere (Shearer et al. 1978; Shearer and Kohl 1986). The $\delta^{15}N$ were measured by an isotopic-ratio mass spectrometers (IRMS; Thermo Finnigan MAT DELTA^{plus} XP; Thermo Scientific, Germany).

N fixation

The percentage of plant N derived from symbiotic fixation of atmospheric N_2 (i.e., Ndfa%) was calculated by the following formula (Eq. 2) (Sanford et al. 1994; Unkovich et al. 1994; Skogen et al. 2011):

$$\text{Ndfa\%} = 100\times \frac{\delta^{15}N_{\text{reference plant}} - \delta^{15}N_{\text{sample}}}{\delta^{15}N_{\text{reference plant}} - B} \qquad \text{(Eq. 2)}$$

where $\delta^{15}N_{\text{reference plant}}$ is the $\delta^{15}N$ of non-N_2-fixing plant selected to match the study legume closely in terms of absorbing the soil sources of N, $\delta^{15}N_{\text{sample}}$ is the $\delta^{15}N$ for the target N_2-fixing plant in the particular treatment, and B is the $\delta^{15}N$ of N_2-fixing growing solely with N-free nutrient media. In this study the reference plant was *Leymus chinensis* (Poaceae), planted and treated the same way as *M. sativa*; we used a B value of -0.68‰ from published literature (Unkovich et al. 2008). The standard error of the Ndfa% was calculated with the following equation (Eq. 3) (Shearer et al. 1983; Shearer and Kohl 1986):

$$SE^2 = \frac{(\delta^{15}N_{\text{sample}} - B)^2(SE_{\delta^{15}N\text{reference plant}})^2}{(\delta^{15}N_{\text{reference plant}} - B)^4} + \frac{(SE_{\delta^{15}N\text{sample}})^2}{(\delta^{15}N_{\text{reference plant}} - B)^2} + \frac{(\delta^{15}N_{\text{reference plan}} - \delta^{15}N_{\text{sample}})^2(SE_B)^2}{(\delta^{15}N_{\text{reference plant}} - B)^4} \qquad \text{(Eq. 3)}$$

Nodule collection and genetic analysis

The *nifH* gene abundance in the nodules was measured using quantitative polymerase chain reaction (qPCR). Nodule samples were collected during root biomass measurement. The number of nodules from each plant was recorded and at least 10

nodules were kept for further genetic analyses. The collected nodules were surface sterilised with ethanol (Weese et al. 2015) and stored in 5 mL centrifuge tubes containing silica gel for cryopreservation before being sent to Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) for qPCR. Two primers were used, *nifHF* (5′-AAAGGYGGWATCGGYAARTCCACCAC-3′) and *nifHR* (5′-TTGTTSGCSGCRTACATSGCCATCAT-3′) (Rosch et al. 2002). Quantitative PCR was performed using the ChamQ SYBR Color qPCR Master Mix (2X) (Vazyme Biotech Co., Ltd., Nanjing, China) and LineGene 9600 Plus Real-Time PCR detection system (Bioer Technology Co., Ltd, Hangzhou, China). PCR reactions were conducted under the following conditions: an initial incubation at 95 °C for 5 min, followed by 40 cycles at 95 °C for 5 s, 56 °C for 30 s, and 72 °C for 40 s. Each sample was used in triplicates, and the average values were used for quantification. Relative gene expression levels were calculated using the ΔCt method according to Schmittgen and Livak (2008), and the results were expressed as bacterial colonies per gram of nodule.

Soil pH and inorganic N

Fresh soil samples were sieved using a 2-mm sieve. Air-dried soil samples, 10g each, were placed in 50mL CO_2-free deionized water and and left to stand for 30 min after being stirred to measure pH (Hanna PH211; Hanna Instruments, Padova, Italy). A sub-sample was stored at 4℃ for inorganic N analysis. Nitrate (NO_3^--N) and ammonium (NH_4^+-N) were extracted with 2 M KCl and were measured with an AA3 continuous flow-analyzer (Bran+Luebbe, Germany).

Statistical analyses

Data were log-transformed to achieve homoscedasticity and normality where necessary. Regression analyses were made to analyse the relationship between Ndfa% and N addition levels. One - way ANOVA with a Duncan test was carried out to determine the effects of N addition levels on the rest of the variables. Data were analyzed in R 3.6.3 (R Core Team 2020) statistically significant differences were determined at a significance level set at $p \leqslant 0.05$.

Results

Plant growth

The average height of plants at harvest was not related to the applied level of N addition; root length at the final harvest was significantly different among N addition rates (Figure 1a, b). The axial root length increased with N addition up to N10 but decreased when N addition reached the level of N20. Nitrogen addition had no significant effect on AGB, BGB and total biomass (Table 2). There was no significant effect of N addition on the root : shoot ratio, although there was a decreasing trend with increasing N addition (Figure S1).

Foliar N and P concentration

N fertilisation had no significant effect on either on leaf N or P concentration (Figure 2). The lowest mean (±SE) foliar N concentration was 4.3% (± 0.1%) at 10 g N m^{-2} yr^{-1}, while the highest was 4.9% (± 0.1%) at 50 g N m^{-2} yr^{-1}. Mean (±SE) P concentration ranged from 3.63 mg g^{-1} (± 0.17 mg g^{-1}) to 3.99 mg g^{-1} (± 0.34 mg g^{-1}). Correspondingly, there were no significant differences in the N:P among treatments ($P > 0.05$).

$\delta^{15}N$ and %N derived from atmospheric N_2-fixation

We examined the $\delta^{15}N$ and Ndfa% to explore if *M. sativa* indeed used the added N. The $\delta^{15}N$ value of the leaves increased with increasing rates of N addition (Figure 3). At the N0 and N2 addition levels, the $\delta^{15}N$ values were negative at -0.42‰ (± 0.14‰) and -0.52‰ (± 0.15‰), respectively. The $\delta^{15}N$ values turned to positive with further increase of additional N and they were significantly different among addition levels ($P < 0.001$, $F = 34.90$).

When N addition was low (N0 and N2), *M. sativa* obtained most of their N from N_2 fixation at Ndfa% of 87% (± 7%) and 91% (± 8%), respectively; further increase in N addition rates decreased sharply the proportion of atmospheric N in the leaf tissues (Figure 4). The value reached a low of 3.7 ± 11%) in the N20 treatment and the Ndfa% value was negative (recorded as 0) in the N50 treatment.

Nodule formation and nifH gene copies

Given the changes in $\delta^{15}N$ and Ndfa% in the N treatments, we investigated how the symbionts and corresponding microorganisms would respond to the N addition. Overall, the average number of nodules per plant decreased with N addition (Figure. 5, $P = 0.001$, $F = 6.86$). Nodule formation was completely absent in the N50 treatment.

We determined the *nifH* gene copies per unit mass of nodules to represent the *Rhizobium* quantity in nodules. N fertilisation resulted in no significant differences in the quantity of *nifH* among treatments (Figure 6); in the N50 *nifH* could not be as no nodules were formed.

Discussion

N addition did not change N leaf N concent and biomass production

Previous studies have indicated that N inputs can increase the biomass production of grasslands where non-leguminous plants dominate (LeBauer and Treseder 2008; Xia and Wan 2008; Tian et al. 2016). However, our results indicate that the N addition largely had no significant effects on the biomass production of *M. sativa* and thus our results do not support our first hypothesis. We are aware that our results obtained from a greenhouse experiment using amenable temperature and irrigation in limited pot size require careful interpretation and extrapolation to plant growth in the field, especially in dryland ecosystems. Although, similar results have been reported previously whereby mineral N applications did not affect the biomass and N concentration of grain legumes (Lee et al. 2003; Guinet et al. 2018; Pampana et al. 2018).

The statistically non-significant effects of N addition on the biomass production of *M. sativa* may closely correlate with no significant changes in the leaf N and P concentration, which corroborated biomass production as most N in leaves were used to produce enzymes such as rubiscos for photosynthesis carbon assimilation. The results of foliar N and P concentration also indicate that *M. sativa* shows relatively strong stoichiometric homoeostasis, with the foliar N and P concentration and their ratios being stable along the N supply gradient. This stoichiometric homoeostasis appears to be stronger than that reported in non-leguminous plant species in semiarid grasslands (Yu et al. 2010). It may also implicate that legumes can have high stoichiometric homoeostasis because of their capacity to capture N by the BNF. Our results on the responses of *M. sativa* foliar N concentration to the exogenous N input were largely consistent with previous field studies (Menge and Hedin 2009) and with the findings of

a meta-analysis (Xia and Wan 2008). The results of the alfalfa biomass production and foliar N concentration indicate that alfalfa can keep itself N-unlimited by a shift of N source from the biologically fixed N to the soil available N.

Our results also showed that high N supply not only suppresses N fixation but might even damage growth. *M. sativa* increased the length of axial roots when N addition was low but decreased the length and increased the diameter of roots when N addition increased, resulting in largely no significant change in BGB. Moreover, the axial root tips turned black and damaged in the N50 treatment. All the above results indicate that lower dose of N addition could promote root development in terms of root axial length while higher levels of N supply might cause acidity-induced toxicity to root growth (Mills and Jones 1979; Voisin et al. 2002).

N addition inhibited N fixation

Our results showed that alfalfa shifted its N source from the biologically fixed N gradually to fertiliser N, which supported our second hypothesis. The leaf $\delta^{15}N$ values increased with N addition indicating that alfalfa reduced BNF gradually, which was also corroborated by changes in the Ndfa% results (Figures. 3 and 4). The Ndfa% became 0 when N addition reached N50, which indicated that the N absorbed by alfalfa was exclusively from fertilized source and the BNF was completely stopped. These results were also consistent with Skogen et al.(2011) and Guinet et al.(2018). The increasing tendency of leaf $\delta^{15}N$ and decreasing tendency of Ndfa% after N addition related to the level of N addition illustrated the downregulation of BNF, which indicated that *M. sativa* is a facultative N fixer (Liu et al. 2016). Facultative N fixers adjust N fixation

rates to match change in soil N supply (Barron et al. 2011; Wurzburger and Hedin 2016).

Previous studies have pointed out that BNF may be constrained by P availability especially in the P-poor soils (Binkley et al. 2003; Batterman et al. 2013b; Ament et al. 2018). However, in this study we added sufficient P to the matrix at the beginning of the experiment. Our results of the foliar P concentration showed no significant difference among different levels of N addition indicating that the alfalfa in our experiment had sufficient uptake of P irrespective of N addition. Other micronutrients that could affect BNF were also provided equally at beginning of the experiment. Thus, the availability of P and other micronutrients could not be the limiting factor for changes in the BNF of alfalfa, and we consider that the changes in the BNF in our study were primarily caused by additional N input.

N addition decreased alfalfa nodulation

Compared with the N from the biological fixation, fertiliser N provides a less costly N source for legumes which will offset the growth benefits from rhizobial nodulation for host plants (Patriarca et al. 2002; Batterman et al. 2013b; Regus et al. 2015). As a result, it is most likely for legumes, especially those with a facultative N strategy, to reduce their investment in nodule formation and to decrease symbiotic N_2 fixation where N is freely available in the soil. Our results revealed that nodulation was significantly lower when N addition reached agricultural application level at N10 and was completely inhibited when N addition reached the level of N50, which was largely consistent with our third hypothesis. However, it was uncertain whether plants reduce nodule formation in response to a collapse of cost-benefit relation in the symbiosis or

other factors such as soil acidification caused by N addition. A previous study showed that the accumulation of nitrate in the soil was the primary factor that caused the decrease of nodule formation (Tanner and Anderson 1964). In this study, soil pH did not show significant change with N addition below N20, but some decrease was found at the level of N50. The unchanged pH under low N addition might relate to the increased uptake by plants while more considerable N in excess of plant demand under the highest N addition was still retained in the soil and converted to nitrate (significant higher NO_3^- concentration with N50 treatment, Figure. S3a) by nitrification process which could cause acidification. Nevertheless, the accumulation of nitrate in soil and the NO_3^-:NH_4^+ ratio were significantly higher at the end of the experiment when N addition reached N20 and N50. Therefore, the remarkable accumulation of nitrate and accompanying soil pH decrease under higher N addition might be the direct reason for the decrease in nodule formation in this study. Soil nitrate have long been considered to cause a series of impacts on the biological N fixation of legumes related to rhizobia infection, nitrogenase activity, and nodulation (Streeter 1988), all relevant plastic responses are seemingly genetically controlled (Murray et al. 2017).

As we inoculated the *M. sativa* plants with rhizobium bacteria at start of the experiment, we only determined the *nifH* gene copies but not the diversity of rhizobia in the nodules to compare the abundance of rhizobium under different rates of N addition. We did not find a decreasing tendency in *nifH* gene copies, while a previous study reported that the number of of the *nifH* gene copies was positively correlated with pH and negatively with inorganic N (ammonium and nitrate) concentration was high

(Pereira e Silva et al. 2013). In our study, the insignificant changes in *nifH* gene copies before the highest N addition rate might relate to that neither soil pH decrease nor soil nitrate concentration increase (Figure. S3) after N addition (except at the highest N addition rate). Previous studies have also shown that influences of N addition on plant growth, nodulation and *nifH* gene abundance may vary with the N addition levels (Wang et al. 2017b), the type of N fertiliser (Cui et al. 2017), and genotypes of legume species (Guinet et al. 2018; Pampana et al. 2018). Thus, our results from this study indicate that *M. sativa* decreases BNF primarily by reducing nodulation which probably results from soil inorganic N accumulation especially with high N addition (Figure. S4).

Conclusions

Our study showed that N addition did not enhance foliar N concentration and biomass production in *M. sativa* grown experimentally, however *M. sativa* changed its N source, which shifted gradually from biologically fixed N to available mineral soil N. Our results indicate that *M. sativa* is a typical facultative strategy N-fixing plant, i.e., it switches on BNF when N mineral is below the need for plant growth, but completely shut off BNF when available N for plant growth is sufficient. Increasing N supply by fertilization decreased the BNF primarily by reducing nodulation, which is likely a resultant of significant accumulation of soil inorganic N. Our results suggest that increasing N deposition will have fundamental impacts for crop genetic diversity and crop improvement breeding programs because efficient symbionts may not be necessary anymore for legumes under the predicted increasing N deposition. Furthermore, we demonstrate that there is a limit for exogenous N fertilization that

controls a facultative strategy of alfalfa (switch from BNF to soil increasing available N from N addition) and consequently impacts its function as an economic plant and as part of healthy ecosystem functioning.

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Notes on contributors

Yinliu Wang is a Ph.D. student, interested in the abiotic and biotic controls of biological nitrogen fixation in legumes.

Muqier Hasi is a Ph.D. student who works on ecosystem CO_2 exchange in grasslands.

Dongdong Bu is a Master's student, interested in the restoration of degraded grasslands.

Ang Li is an assistant professor, interested in ecosystem services and political ecology.

Jianguo Xue is an engineer. He is interested in grassland vegetation restoration.

Changhui Wang is an associate professor, with an interest in the nitrogen cycle, especially soil nitrogen mineralization.

Qiuying Tian is an ecophysiologist.

Guoxiang Niu is a Ph.D student, interested in soil carbon and microbial community composition.

Qianqian Geng is a Master's student in ecology.

Lixin Wang is an associate professor and his research focuses on the interactions

between water, vegetation and soil nutrients in various ecosystems.

Jianhui Huang is an professor, interested in research and development of the comprehensive approaches and experimental demonstration on adaptive ecosystem management.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Table 1 Initial physical-chemical properties of the soil used in this study.

Soil properties	**Value obtained**
Total C (%)	0.88 (±0.02)
Total N (%)	0.08 (±0.001)
Available P (mg/kg)	16.48 (±5.48)
Exchangeable K (mg/kg)	387.80 (±2.55)
Exchangeable Ca (mg/kg)	1093.90 (±4.10)
Exchangeable Mg (mg/kg)	19.59 (±0.21)
pH	7.65 (±0.10)

Table 2 Alfalfa biomass (mean ± SE) under different N addition treatments (n = 6). AGB represents the aboveground biomass, BGB the belowground biomass.

N levels ($g\ m^{-2}\ yr^{-1}$)	AGB (g)	BGB (g)	Total Biomass (g)
0	9.84 (±0.75)	10.52 (±1.19)	20.36 (±1.57)
2	11.27 (±0.92)	11.33 (±1.04)	22.60 (±1.56)
5	10.77 (±0.69)	8.49 (±1.12)	19.26 (±0.95)
10	10.66 (±0.48)	11.03 (±1.36)	21.69 (±1.60)
20	11.70 (±1.08)	9.69 (±1.46)	21.40 (±1.50)
50	11.72 (±0.94)	10.55 (±1.79)	22.26 (±2.08)

Figure captions

Figure. 1 Average height (a) and axial root length (b) of alfalfa at increasing N addition levels. Bars represented as the geometric mean ± SE (n = 6). Different letters above the bars indicate significant differences among treatments (α = 0.05).

Figure. 2 Foliar nitrogen (N) and phosphorus (P) concentration and their ratios (inset) at increasing N addition levels (mean ± SE, n = 6).

Figure. 3 The $\delta^{15}N$ values (mean ± SE) of alfalfa leaves at increasing N addition levels (n = 6). Different letters indicate significant differences among treatments (α = 0.05).

Figure. 4 The relationship between the percentage of plant N derived from atmospheric N_2 (Ndfa%) and N addition levels (values are mean ± SE, n = 6). Error bars are sometimes hidden behind symbols.

Figure. 5 The average number of nodules formed per plant at increasing N addition levels (n = 6). Bars represented as the geometric mean ± SE. Different letters above the bars indicate significant differences among the N addition treatments (α = 0.05). No nodule was formed at N50 treatment.

Figure. 6 The quantity of *nifH* copies with per unit nodule at increasing N addition levels (n = 3). Bars represented as the geometric mean ± SE. Different letters above the bars indicate significant differences among the N treatments (α = 0.05). Data was not determined at N50 treatment as no nodule was collected.

Figure. 7 The integrative response of alfalfa to the increasing N addition

Figure. 1

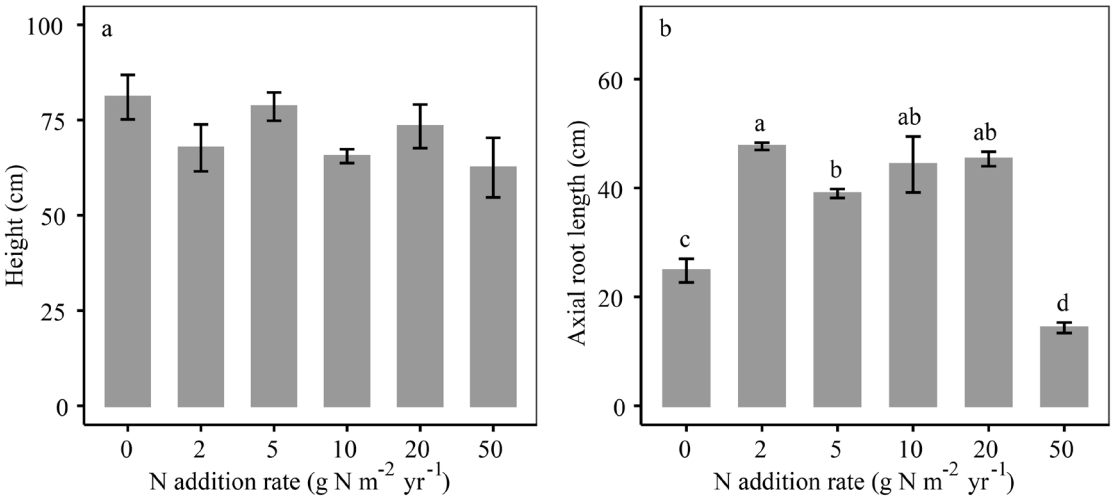

a
Height (cm)
100
75
50
25
0
0
2
5
10
20
50
N addition rate (g N m-2 yr-1)
b
Axial root length (cm)
60
40
20
0
c
a
b
ab
ab
d
0
2
5
10
20
50
N addition rate (g N m-2 yr-1)

Figure. 2

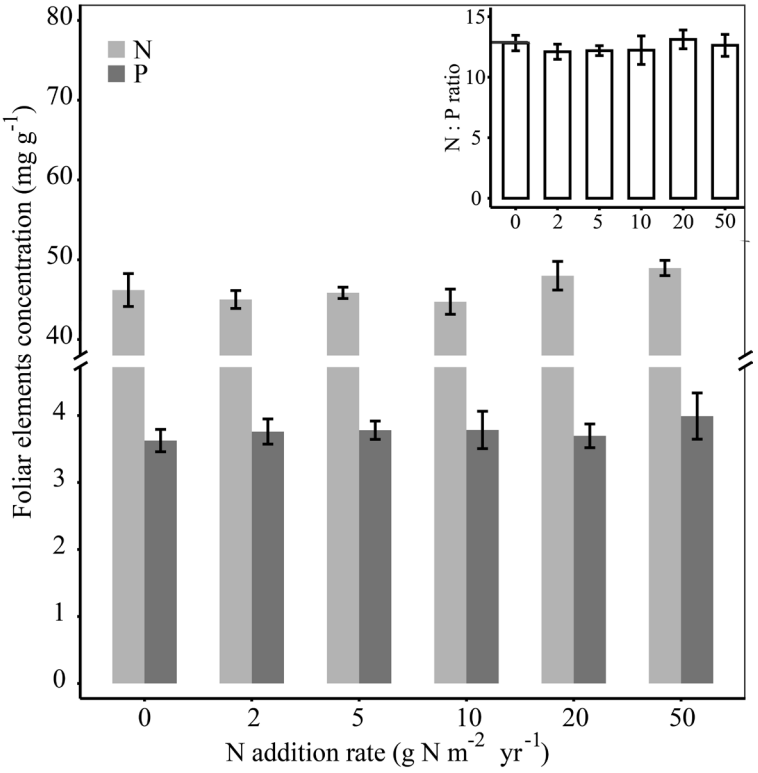

N
P
Foliar elements concentration (mg g-1)
N : P ratio
N addition rate (g N m-2 yr-1)
0
2
5
10
20
50

Figure. 3

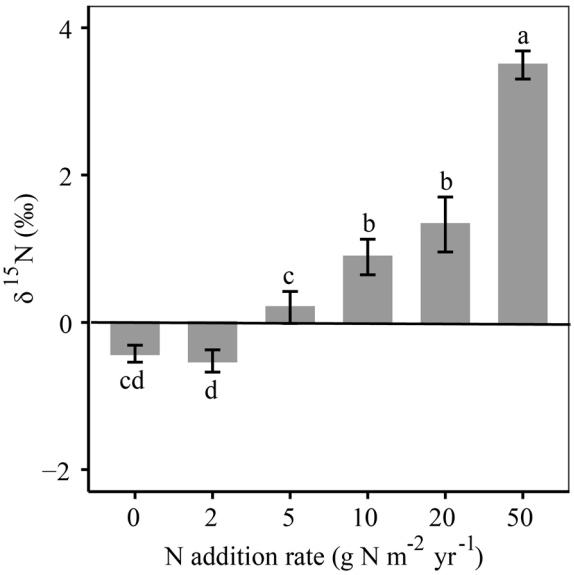


Figure. 4

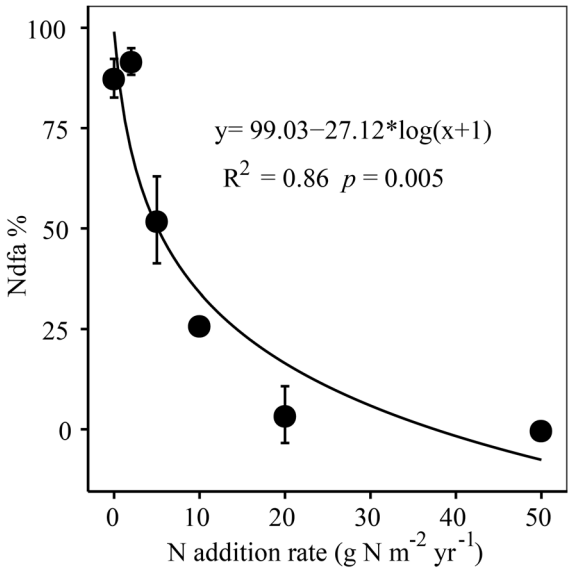

100
75
50
25
0
Ndfa %
y= 99.03−27.12*log(x+1)
R2 = 0.86 p = 0.005
0
10
20
30
40
50
N addition rate (g N m-2 yr-1)

Figure. 5

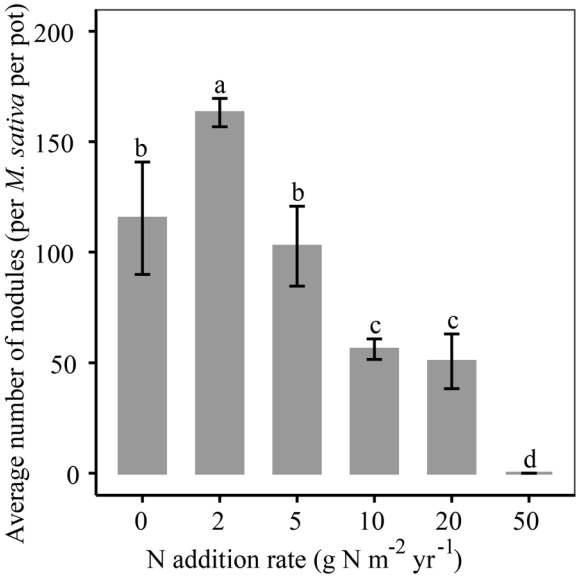

Average number of nodules (per M. sativa per pot)
200
150
100
50
0
a
b
b
c
c
d
0
2
5
10
20
50
N addition rate (g N m-2 yr-1)

Figure. 6

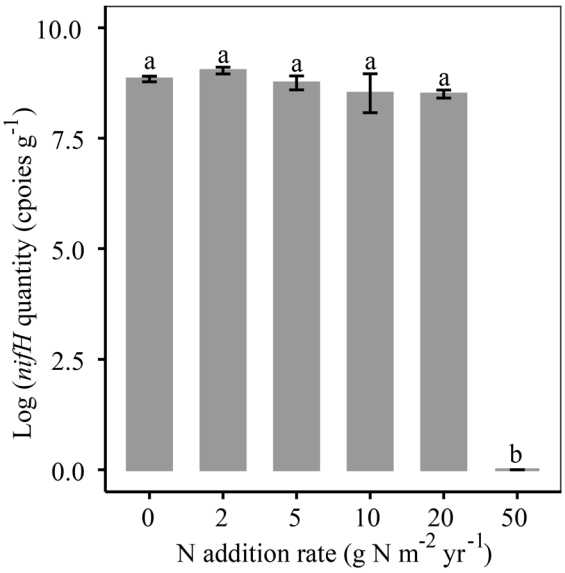
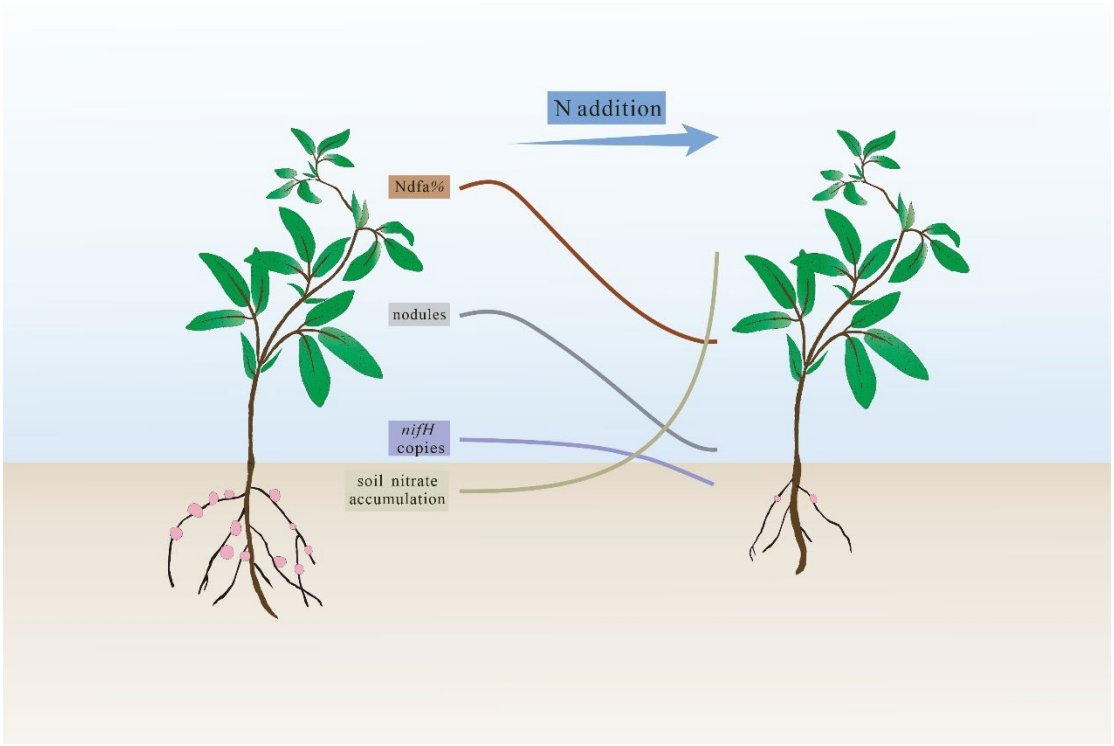

10.0
7.5
5.0
2.5
0.0
Log (nifH quantity (cpoies g-1)
a
a
a
a
a
b
0
2
5
10
20
50
N addition rate (g N m-2 yr-1)

Figure. 7


N addition
Ndfa%
nodules
nifH copies
soil nitrate accumulation

Supplementary information

Fig. S1. Changes in the root:shoot ratio at increasing N addition levels. The error bars represent the 1 SE (α = 0.510).

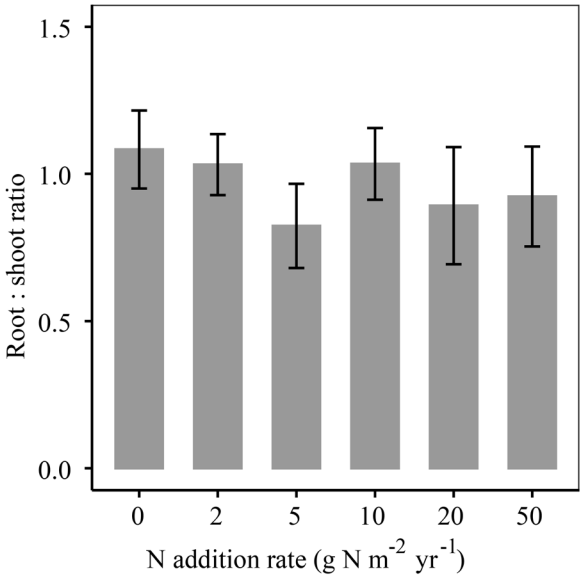


Fig. S2 Relationship between foliar N concentration of alfalfa and soil inorganic N concentrations, and the calculated value of stoichiometric homeostasis (*H*) based on the relationship.

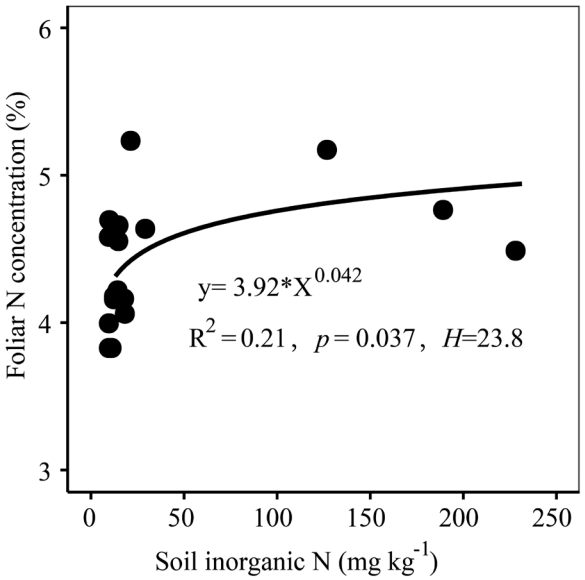


Fig. S3. Changes in the soil inorganic N concentration (a), NO_3^- : NH_4^+ ratio (b) and changes in the soil pH value (c) as a function of increasing N addition levels. Errors bars represent 1 SE. In (a) different lowercase and capital letters denote significant difference ($\alpha < 0.05$) in NH_4^+-N and NO_3^--N among N addition treatments, respectively. In (b) and (c) different letters above columns indicate the significant difference among treatments ($\alpha < 0.05$).

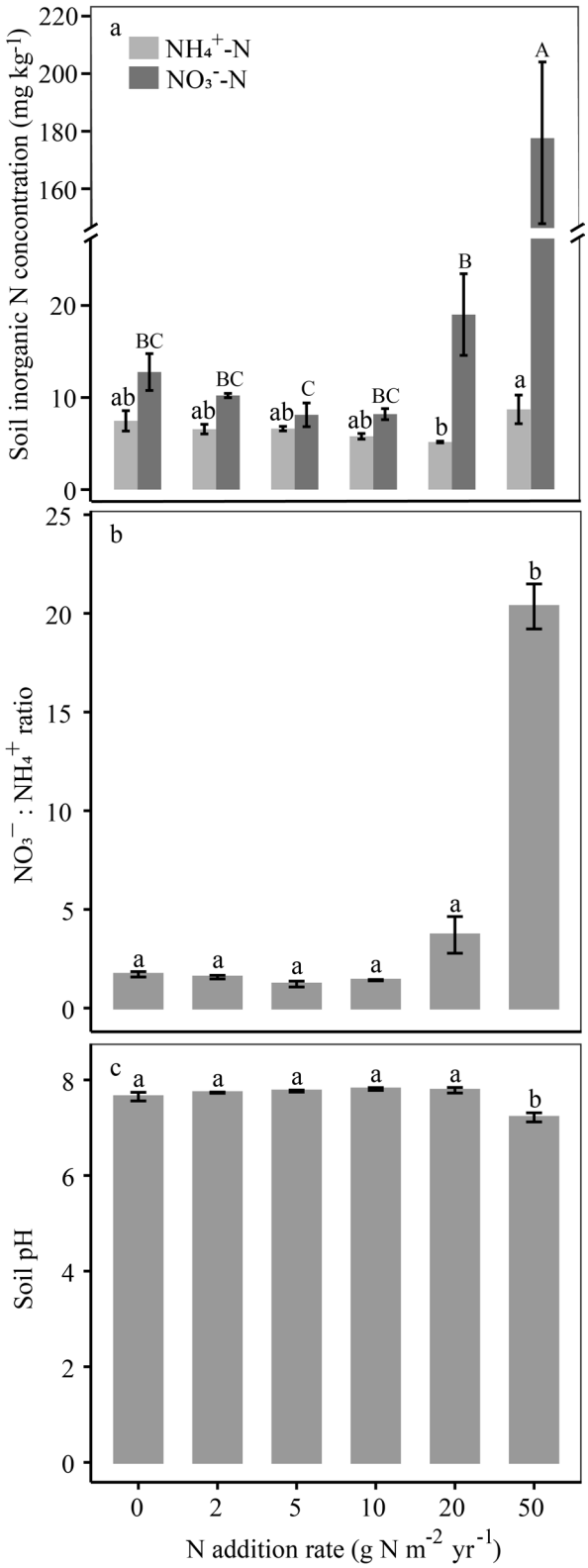

a
NH_4^+-N
NO_3^--N
Soil inorganic N concentration (mg kg^{-1})
220
200
180
160
20
10
0
ab
BC
ab
BC
ab
C
ab
BC
b
B
a
A
b
NO_3^- : NH_4^+ ratio
25
20
15
10
5
0
a
a
a
a
a
b
c
Soil pH
8
6
4
2
0
a
a
a
a
a
b
0
2
5
10
20
50
N addition rate (g N m^{-2} yr^{-1})

Fig. S4. The average number of nodules under different soil nitrate concentration. Errors bars represent 1 SE.