Phosphorus does not alleviate the negative effect of nitrogen enrichment on legume performance in an alpine grassland

Fei Ren¹,², Weimin Song³, Litong Chen¹, Zhaorong Mi¹, Zhenhua Zhang¹, Wenyan Zhu¹,², Huakun Zhou¹, Guangmin Cao¹ and Jin-Sheng He¹,³,*

¹ Key Laboratory of Adaptation and Evolution of Plateau Biota, Northwest Institute of Plateau Biology, Chinese Academy of Sciences, 23 Xinning Rd., Xining 810008, People’s Republic of China
² University of Chinese Academy of Sciences, 19A Yuquan Rd., Beijing 100049, China
³ Department of Ecology, College of Urban and Environmental Sciences, and Key Laboratory for Earth Surface Processes of the Ministry of Education, Peking University, 5 Yiheyuan Rd., Beijing 100871, China
*Correspondence address. Department of Ecology, College of Urban and Environmental Sciences, Peking University, 5 Yiheyuan Road, Beijing 100871, China. Tel: +86-10-6275-4404; Fax: +86-10-6275-8684; E-mail: jshe@pku.edu.cn

Abstract

Aims
Nitrogen (N)-fixing legumes, despite being highly phosphorus (P)-demanding, constitute an important plant functional group and play key roles in N-poor ecosystems such as alpine grasslands. However, legume performance, including biomass, abundance and species richness, is expected to change, because anthropogenic activities have drastically increased soil N and P availability worldwide. We conducted a field experiment to assess the effects of N and P addition, alone and in combination, on legume performance in an alpine grassland, and identified and clarified the mechanisms underlying these changes.

Methods
A three year field experiment of N addition (10 g N m⁻² year⁻¹), P addition (5 g P m⁻² year⁻¹), and N + P combined addition (both N and P, same amounts as solo treatments) was conducted in an alpine grassland on the Tibetan Plateau in China from 2011 to 2013. Effects of nutrient addition were assessed at the community level (aboveground net primary production (ANPP), height and light intensity), functional group level (biomass, species richness, relative height, relative coverage and relative density of legumes) and species level (foliar N, P concentration of two legumes).

Important findings
Overall, adding N alone significantly increased ANPP by 20.82%, but adding P alone did not; whereas, addition of N and P together resulted in a large increase in ANPP (+37.03%) than addition of either alone, indicating potential co-limitation of alpine grasslands. In contrast, adding P alone significantly promoted legume performance as measured by 65.22% increase in biomass and 58.45% increase in relative abundance, while adding N alone reduced legume performance as measured by 39.54% decrease in biomass and 50.36% in relative abundance. Combining P and N addition did not mitigate the negative effect of N addition on legume performance and, surprisingly, suppressed legume biomass by 53.14% and relative abundance by 63.51%. N and P addition altered the balance of light competition between grasses and legumes as indicated by the changes in light levels, plant heights and litter accumulation. However, there were no obvious changes in legume species richness in response to N and P within our experimental timeframe. This study provides further evidence of the importance of P as a co-limiting nutrient in alpine grasslands, contrary to the traditional view that N limitation predominates in such regions. The contrasting effects of N and P addition on legume performance provide important insights into potential changes in legume performance in nutrient-limited grasslands following N and P enrichment under climate change, with implications for nutrient management in alpine grasslands.

Keywords: Tibetan Plateau, nutrient addition, soil-nutrient interactions, legume productivity, species richness

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INTRODUCTION

Co-limitation by nutrients, especially by nitrogen (N) and phosphorus (P), is increasingly recognized in terrestrial ecosystems worldwide (Elser et al. 2007; Harpole et al. 2011). Many nutrient manipulation studies have been conducted globally to test the hypothesis that nutrients are co-limiting (e.g. Nutrient Network, see Fay et al. 2015). However, comparable evidences for alpine grasslands is relatively limited. Although the responses of community performance (e.g. biomass, richness and abundance) as a whole to nutrient additions have been well documented in past decades, how plant functional groups respond to nutrient addition remains poorly understood. Plant functional groups may differ in their responses to nutrient addition (Avolio et al. 2014; Isbell et al. 2013; Pennings et al. 2005; Suding et al. 2005). Therefore, it is necessary to understand the effects of nutrient addition on the performance of plant functional groups, especially legumes, which strongly affect N and P availability, but have not been well studied in alpine grasslands.

Legumes can fix N by means of symbionts (Bordeleau and Prévost 1994), and transfer a portion of the fixed N to non-leguminous neighbours (Temperton et al., 2007), thus playing an important role in terrestrial ecosystem functions (Spehn et al. 2002; Vitousek et al. 2002), especially in typically N-limited regions such as alpine grasslands (Jin et al. 2013; Souzdilovskaia et al. 2012; Spehn et al. 2002; Yang et al. 2011a).

Phosphorus availability could greatly affect the productivity and abundance of legumes (Vitousek and Field 1999). On the one hand, legumes require P for growth, root development, and formation of nodules with symbionts (Batterman et al. 2013; Novotny et al. 2007); on the other hand, the symbionts also demand P-rich molecules because of the high energy cost of the nitrogenase reaction (Augusto et al. 2013; Divito and Sadras 2014). However, soil-available P is scarce in most ecosystems (Han et al. 2005), limiting legume performance and abundance.

Increased anthropogenic activities have enhanced availability of N and/or P even in typically nutrient-poor ecosystems such as alpine grasslands (Galloway et al. 2008; Peñuelas et al. 2012; Xu et al. 2014). Nevertheless, studies have still not explicitly test how changes in soil N and P availability would affect legume performance (e.g. production, species richness and abundance).

Previous studies have indicated that N addition suppresses legume performance as measured by lowered production, abundance and richness (Avolio et al. 2014; Isbell et al. 2013) via competition from faster growing grasses for resources, such as light (Bobbink 1991; Hautier et al. 2009; Suding et al. 2005) and water (Bobbink 1991). Furthermore, N addition also suppresses legumes by N-induced soil acidification (Lu et al. 2011). More importantly, because of the higher rate of N deposition than that of P deposition (Peñuelas et al. 2012; Vitousek et al. 2010), increased N availability in soils would shift the intrinsic N-vs.-P-limitation balance (Elser et al. 2009; Peñuelas et al. 2012), resulting in intense competition between plants, and a depletion of soil P, which is critical for legumes because of their high P-demand (Augusto et al. 2013; Silvertown et al. 2006; Spehn et al. 2002).

Despite the limiting nature of P on legume function, few studies have examined how legumes would respond to P enrichment in P-poor regions. Furthermore, we know even less about the interactive effects of N and P enrichment on legume performance. Specifically, it is unclear whether P addition could alleviate the negative effects of N enrichment on legumes when both N and P are added to such potentially N and P co-limited areas. Results from the few existing studies have been equivocal, reporting positive (Grünzweig and Korner 2003), neutral (Avolio et al. 2014) and negative (Sun et al. 2016) interactive effects of N and P additions on legume biomass, and none have directly addressed legume performance in the context of combined N and P enrichment, especially for alpine grasslands on the Tibetan Plateau (but see Sun et al. 2016).

The Tibetan Plateau is an ideal location for examining the effects of N and P on legume performance in alpine grasslands. These grasslands are nutrient-poor due to lower decomposition and mineralization rates, although researchers historically assumed that they are mainly N- but not P-limited because the soil is relative young (Reich and Oleksyn 2004). In this study, we took advantage of these suitable conditions to assess legume performance in response to N and P enrichment. We hypothesized that: (i) N addition suppresses legume performance, while (ii) P addition promotes legume performance, (iii) combining P addition with N addition alleviates the negative effects of N on legumes and (iv) nutrient additions change the species richness of legumes as predicted by the literature.

METHODS

Study site

The study site was an alpine meadow near the Haihe National Field Research Station of Alpine Grassland Ecosystem, Northwest Institute of Plateau Biology, Chinese Academy of Sciences (37°37′N, 101°12′E; altitude: 3250 m), in Qinghai Province. This site has been overgrazed in the past but has been fenced and only grazed during non-growing seasons since 2001. The mean annual temperature is −1.1°C (1981–2010). Mean annual precipitation is 490 mm, of which more than 80% occurs during the growing season from May to September (Wang et al. 2014; Zhao and Zhou 1999). The soil is classified as Gelic Cambisols (FAO classification); soil characteristics are presented in Supplementary Table S1. The vegetation type is a typical Kobresia humilis (C.A.Mey. ex Trautv.) Serg. meadow. The dominant grasses species are Stipa aliena Keng, Elymus nutans Griseb. and Festuca ovina L. The legumes found in the experimental site include Medicago archiducis-nicolai Sirj., Tibetia himalaica (Baker) H.P.Tsui, Oxytropis

Experimental design

In May 2009, we fenced an experimental area of 1 ha to exclude grazing disturbance, and in May 2011, we started a fertilization experiment. A completely randomized design was used with four treatments comprising four replicates each (6 × 6 m per plot, 16 plots in total). The four treatments were as follows: Control (with no nutrient addition); N addition (urea, 10 g m⁻² year⁻¹); P addition (superphosphate, 5 g P m⁻² year⁻¹) and NP addition (both N and P, same amounts as solo treatments). Plots received N and/or P amendments were applied three times each year. The granular fertilizer was divided into three equal parts and was applied by hand broadcasting on 1 June, 1 July and 1 August in 2011 and 2012, and 1 June, 31 June, and 2 August in 2013.

Plant sampling and analysis

In mid-August of each year, a 50 × 50 cm quadrat was randomly selected within each plot for biomass harvest. Above-ground parts of plants were clipped at the ground surface and then sorted by species before dry-weight measurement (drying at 65°C for >48 h). Above-ground net primary production (ANPP) of the community and plant functional groups (grasses, sedges, legumes and forbs) were determined. Standing dead matter and litter in quadrats were also collected and measured as litter stock.

In 2012 and 2013, we estimated pre-harvest plant heights by species in quadrats in each plot, then calculated community height, and the relative height of legume functional group. Individual plant heights were measured using steel tape measures. Community height was the average of species’ heights. Relative height of legumes was calculated as the average height of legumes divided by the community height. Similar data for other functional groups were also collected but are not reported in detail in this paper.

In the 2012 growing season, light transmission through the canopy was measured above and below the canopy (approximately at the soil surface) using a Quantum PAR meter (Apogee MQ-200 PAR Meter, USA) on three sunny days. Light intensity (percentage) under the canopy was calculated (Apogee MQ-200 PAR Meter, USA) on three sunny days. Light intensity (percentage) under the canopy was calculated by linear regression analysis using R. Graphs were created using SigmaPlot (version 12.5, Systat Software, Inc., Point Richmond, CA).

RESULTS

Response at community level

There were significant effects of nutrients, year, and their interactions on ANPP (Table 1), and these effects were mostly similar in the three years, although the trends were generally stronger and more significant in 2011 and 2012 (Fig. 1a). Overall, N addition alone significantly increased ANPP by 20.82%, but P alone did not; N and P combined resulted in larger increases of ANPP (+37.03%) than either N or P alone (Fig. 1a).

Nutrient additions led to an asymmetry changes in plant heights among species and thus community height as ANPP changed. Average across 2012 and 2013, combined NP addition, but not addition of either nutrient alone, significantly increased community height, as indicated by a significant N × P interaction (P = 0.019) (Table 2; Fig. 1b).

Legume performance

Legume biomass was significantly affected by nutrients, year and their interactions (Table 2; Fig. 2a). Legume biomass

<table>
<thead>
<tr>
<th>Source</th>
<th>ANPP Source df</th>
<th>F</th>
<th>P</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>1.33</td>
<td>57.53</td>
<td>&lt;0.001</td>
<td>1.21</td>
<td>33.20</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P</td>
<td>1.33</td>
<td>8.09</td>
<td>0.007</td>
<td>1.21</td>
<td>13.49</td>
<td>0.001</td>
</tr>
<tr>
<td>N × P</td>
<td>1.33</td>
<td>2.959</td>
<td>0.095</td>
<td>1.21</td>
<td>6.50</td>
<td>0.019</td>
</tr>
<tr>
<td>Year</td>
<td>2.33</td>
<td>111.37</td>
<td>&lt;0.001</td>
<td>1.21</td>
<td>46.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>N × year</td>
<td>2.33</td>
<td>12.38</td>
<td>&lt;0.001</td>
<td>1.21</td>
<td>35.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P × year</td>
<td>2.33</td>
<td>0.93</td>
<td>0.046</td>
<td>1.21</td>
<td>0.43</td>
<td>0.522</td>
</tr>
<tr>
<td>N × P × year</td>
<td>2.33</td>
<td>8.372</td>
<td>0.001</td>
<td>1.21</td>
<td>4.30</td>
<td>0.038</td>
</tr>
</tbody>
</table>

Results are from mixed-effects model for repeated-measures ANOVA. Significant differences (P < 0.05) are bolded and italicized. Abbreviations: ANOVA = analysis of variance; df = degree of freedom.
ranged from 4.93 g m$^{-2}$ to 83.56 g m$^{-2}$, contributing to 1.4–28.70% of ANPP. Across the three years, the average legume biomass decreased by 39.54% with N addition alone, but increased by 65.22% with P addition alone. Surprisingly, legume biomass decreased by 53.14% with NP addition, more than the decrease with N addition alone. Accordingly, the legume biomass percentage increased by 69.25% with P addition, but decreased by 50.24% with N addition alone, and by 63.51% with NP addition (Supplementary Table S2 and Fig. S1a).

Nutrient addition did not significantly influence legume height. However, nutrient addition altered the relative height of legumes compared with the mean community height (relative height, hereafter) (Table 2; Fig. 2c). Across 2012 and 2013, N addition significantly decreased the relative height by 21.36%, but P and NP addition did not (Fig. 2c).

Additionally, relative coverage and relative density of legumes followed similar trends as legume biomass and biomass percentage. Across 2012 and 2013, N addition significantly decreased the relative coverage of legumes by 38.73%, P addition significantly increased it by 74.72%, while NP addition significantly decreased it by 51.10% (Supplementary Table S2 and Fig. S1b). Similarly, across 2012 and 2013, N addition significantly decreased relative density of legumes by 52.45%,
P addition significantly increased it by 49.36%, while NP addition significantly decreased it by 61.51% (Supplementary Table S2 and Fig. S1b and c).

Contrary to the significant effects of treatments on legume biomass and height, the average species richness of legumes was 3.6, and no significant species loss was found across all treatment (Table 2; Fig. 2b). Moreover, although legume richness was different across the three years ($F_{2,23} = 3.541, P = 0.040$), no significant interactive effect of nutrient vs. year on richness was found. In addition, N and P additions had neither main effects nor interaction effects.

Nutrient-induced changes in productivity and plant height, altered light penetration in the canopy considerably. In 2012, addition of both nutrients significantly decreased the light intensity in the lower canopy (Fig. 3).

With increase in ANPP after nutrient addition, more standing litter accumulated and created more shade. There was a negative correlation between legume biomass and litter stock (Fig. 4, $R^2 = 0.22, P < 0.001$).

Two common legumes species in all plots, *T. himalaica* and *M. archiducis-nicolai* together constitute approximately 75% of total legume biomass. N addition alone significantly increased foliar N concentration [N] of *T. himalaica*, but had no effect on [N] of *M. archiducis-nicolai* (Fig. 5a and b), indicating that legumes’ [N] response are species-specific. Moreover, the interaction between N and P was significant, indicating that the response of foliar [N] to N addition depended on whether or not the P was co-added. However, N addition alone did not significantly affect the foliar P concentration [P] of either species (Fig. 5c and d). In contrast, changes of [P] in plots with P addition were more...
distinct: for both species, addition of either P alone or NP significantly increased [P], but had no effect on [N].

DISCUSSION

After adding N and P to an alpine grassland on the Tibetan plateau for three years, we observed that N and P, both alone and in combination, drastically changed the performance of legumes. Our results were consistent with some, but not all, of our hypotheses.

Negative effects of N addition on legumes

In support of our first hypothesis, N addition significantly suppressed legume biomass. This result is consistent with the observations in Park Grass Experiments (Bobbink 1991), a temperate grassland (Yang et al. 2011b) and an alpine grassland in the same region (Song et al. 2012; Sun et al. 2016; Xu et al. 2014). Several factors may have caused this effect. First, as in previous studies, N increased ANPP (Fig. 1a) and community height (Fig. 1b), but decreased light transmission (Fig. 3) and thereby shifted the competitive advantage for light (Bobbink 1991; Hautier et al. 2009; Silvertown et al. 2006; Vitousek and Field 1999) among different functional groups, especially grasses and legumes (Suding et al. 2005; Xu et al. 2014), and thus decreased legume biomass. Moreover, the consistent responses of relative coverage and relative density to N addition (Supplementary Fig. S1b and c), could also partially explain such ‘top–down’ control of legume performance by light.

Second, we speculated that N addition aggravated P limitation of legumes’ growth and N-fixation, because grasses would need more P for N-induced faster growth. However, when supplied with sufficient N, grasses have the upper hand against legumes in competition for P (Bobbink 1991; Hacker et al. 2015; Silvertown et al. 2006).

Third, previous studies indicated that N-induced soil acidification (Lu et al. 2011) also suppressed legume performance. However, after four years nutrients addition, we did not observe significant soil acidification in this study (Supplementary Table S1 and Fig. S2), suggesting that soil acidification was unlikely to have influenced legume performance, at least during the study period.

Positive effects of P addition on legumes

In support of our second hypothesis, we found that P addition significantly increased legume biomass and other performance metrics. It is clear that the enhancement of soil P availability is the most likely factor driving these changes (see foliar [P] of two main legume species in Fig. 5c and d). These results are consistent with previous reports (Bobbink 1991; Grünzweig and Korner 2003; Novotny et al. 2007), and provide strong evidence that legumes growing in alpine grasslands are severely P limited. In addition, we speculated that the inherent N limitation of other non-leguminous plants, which must rely solely on soil nitrogen, presented a favourable condition for legumes to fix nitrogen and get a competitive edge in light exposure to increase productivity (Suding et al. 2005; Temperton et al. 2007). Moreover, N addition increased the foliar [N] in T. himalaica (Fig. 5a) in 2012, suggesting that legumes in alpine grassland may co-limited by soil P and N content, but P’s essential role in N-fixation probably makes it the ultimate limiting nutrient for legume production. This speculation was consistent with our results that P addition only increased legume biomass (Fig. 2a), but did not increase ANPP (Fig. 1a). These results are inconsistent with results from previous studies that legumes promoted ANPP and N availability (Marquard et al. 2009; Mulder et al. 2002; Oelmann et al. 2011). Our results on legume biomass and ANPP have three implications. First, at severe P limitation, legumes growing in N-poor alpine grasslands rely strongly, but not completely, on N-fixation.
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(Liu et al. 2013; Xu et al. 2014). Second, despite facilitation of legume growth by P addition, the low soil temperatures in alpine grasslands, which are far below the optimum of 20–35°C for nitrogenase activity (Dart and Day 1971), may reduce the magnitude of facilitation (Yang et al. 2011a). Third, in alpine grassland, the facilitation of legumes to other plants in the community may take some time as the decomposition of those leguminous plant litter may take longer than the study timeframe. This could be another explanation for the observed positive effect of P addition on legume biomass, but negligible effect on ANPP, in our study.

**P did not alleviate the negative effect of N on legume performance**

Compared to the individual effects of N and P addition, the combined effect of N and P on legumes performance has received less attention in grassland ecosystems. Although there are several studies addressing how combined N and P addition influences legumes, their results remain controversial (Avolio et al. 2014; Grünzweig and Korner 2003; Sun et al. 2016).

Contrary to our third hypothesis, NP addition suppressed legume performance more than N addition alone, indicating that P did not alleviate the negative effects of N on legume performance (Fig. 2). This result is consistent with a recent study conducted in an alpine grassland (Sun et al. 2016), which found that NP addition decreased legume biomass, irrespective of amount of which the P added with N. Although the addition of N and P together precluded nutrient competition between legumes and grasses, the more rapid increase in community height resulting from grasses growth (Fig. 1b) increased light competition to a higher degree than what was seen following N addition (Fig. 3). Moreover, the negative correlation between legume biomass and litter stock found in our

**Figure 5:** Effects of N and P additions on foliar N (a, b), P concentrations (c, d) of two legumes, Tibetia himalaica (left) and Medicago archiducis-nicolaii (right) (mean ± SE) in 2012. ANOVA P-values are reported (ns: no significant, *P < 0.05). Letters denote significant differences at *P < 0.05. Abbreviation: ANOVA, analysis of variance; SE, standard error.
study (Fig. 4) suggests that the litter accumulated after nutrient enrichment can aggravate light competition. Together, these data suggest that light competition is the major mechanism driving P-induced changes in legume performance, with legumes and dominant grasses having the advantage in low-N (P-addition) and high-N (N- and NP-addition) scenarios, respectively. These findings highlight the importance of height-mediated light competition in determining legume performance under N addition.

Neutral effect of N and P on legumes richness

In general, increased N availability enhances ANPP and reduces species richness (Song et al. 2011; Suding et al. 2005; Sun et al. 2016). Moreover, legumes have a higher probability of being lost from communities with N additions than non-leguminous species (Isbell et al. 2013; Suding et al. 2005). In contrast, P additions may favour legumes by releasing them from P limitation without releasing other plant groups from N limitation. However, contrary to our hypothesis that nutrient additions would affect legume species richness, we found no evidence that N enrichment decreased, or P addition increased, legume species richness significantly. Therefore, the alterations in biomass in our study were not from species loss or immigration, but from large reductions or increases in the abundance of legumes. It is not clear why nutrient addition did not affect legume species richness, but perhaps the fertilization effect was not completely apparent within just three years because the legumes in our study are perennials. Therefore, a longer experiment with more detailed analysis is required to determine whether these nutrient additions could affect the species richness of legumes.

CONCLUSIONS

In summary, this study provides further evidence of the importance of P as a co-limiting nutrient in alpine grasslands, in contrast to the traditional view that N limitation predominates in such regions. These results improve our understanding of the potential importance of legumes in alpine meadows (in which their contribution to community biomass depends on nutrient availability). Our findings suggest that the effects of N and P on legumes are mediated by competition with grasses for light, and that legumes are likely to respond to N and P co-limitation or enrichment differently than other functional groups because of their ability to fix N. The finding that legume abundance depends on nutrient availability may partially explain why legumes contribute little biomass to both alpine and temperate grasslands. It also provides important insights into potential changes in the performance of legumes in nutrient-limited grasslands following N and P enrichment with anthropogenic changes in nutrient inputs. Moreover, the contrasting and interactive effects of N and P addition on legume performance indicate that P alone does not alleviate the negative effect of N on legume performance, even though N and P both limit community biomass in alpine grasslands. The rapid change in functional group performance under nutrient amendment might lower community stability and thus, future ecosystem function. Therefore, special cautions should be taken when adding nutrients to improve community productivity in alpine grasslands on the Tibetan plateau.

SUPPLEMENTARY MATERIAL

Supplementary material is available at Journal of Plant Ecology online.

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