

Nitrogen and phosphorus addition differentially enhance seed production of dominant species in a temperate steppe

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Abstract

Previous studies have demonstrated changes in plant growth and reproduction in response to nutrient availability, but how investigations of such responses to multiple levels of nutrient enrichment remains unclear. In this study, we manipulated nitrogen (N) and phosphorus (P) availability to examine seed production responses to three levels each of N and P addition in a factorial experiment: no N addition (0 g N m⁻² yr⁻¹), low N addition (10 g N m⁻² yr⁻¹), high N addition (40 g N m⁻² yr⁻¹), and no P addition (0 g P m⁻² yr⁻¹), low P addition (5 g P m⁻² yr⁻¹), high P addition (10 g P m⁻² yr⁻¹). Low N addition enhanced seed production by 814%, 1371%, and 1321% under ambient, low, and high P addition levels, respectively. High N addition increased seed production by 2136%, 3560%, and 3550% under ambient, low, and high P addition levels, respectively. However, P addition did not affect seed production in the absence of N addition, but it did enhance it under N addition. Furthermore, N addition enhanced seed production mainly by increasing the tiller number and inflorescence abundance per plant, whereas P addition stimulated it by decreasing the plant density yet stimulating height of plants and their seed number per inflorescence. Our results indicate seed production is limited not by P but rather by N in the temperate steppe, whereas seed production will be increased by P addition when N availability is improved. These findings enable a better understanding of plant reproduction dynamics of steppe ecosystems under intensified nutrient enrichment and can inform their improved management in the future.

1 INTRODUCTION

Anthropogenic-driven nutrient inputs, namely nitrogen (N) and phosphorus (P) enrichment of terrestrial ecosystems, have been increasing intensively since the Industrial Revolution (Harpole et al., 2011; Liu et al. 2021; Phoenix et al., 2012). Global cycles of N and P have been respectively amplified by c.100% and c. 400%, respectively, due to intensified human activities (Elser et al., 2007). Being two crucial nutrient elements for growing plant, N and P enrichment can profoundly influence plant growth, survival, and reproduction, with subsequent impacts on community structure and ecosystem functioning (Zhao et al., 2018a). Reproduction is an essential function in the life cycles of plants that determine their fitness (Willson, 1983). Seed production is an important index of reproduction that strongly influences the relative ability of species to disperse and establish as seedlings (Liu et al., 2012; Pierce et al., 2014). In addition, seed production can affect the size and extent of soil seed banks, and contribute to the maintenance of plant diversity and species composition (Luzuriaga et al., 2005). Therefore, understanding the effects of N and P enrichment upon seed production is critical for predicting plant community structure, and consequently, ecosystem functioning.

Nitrogen is a limiting nutrient for plant reproduction in terrestrial ecosystems. Numerous studies have demonstrated that N enrichment tends to augment seed production in plants (Bogdziewicz et al., 2017; Li

et al., 2016a; Ma & Herath, 2016; Shi et al., 2017). For example, in a temperate steppe, *Leymus chinensis* produced more seeds via N enrichment through enhanced spikelet and flower differentiation (Wang et al., 2010). P plays a key role in regulating plant reproductive processes because it can significantly affect the partitioning of assimilation products, flowering phenology, root growth, and seed maturation (Petraglia et al., 2014; Wang et al., 2017). Previous studies have reported that plants under P-deficient conditions allocate little to reproductive growth, which manifests as in a shortened flowering period, reduced seed yield, and weakened dispersal ability (Fujita et al., 2014; Groom & Lamont, 2009). Furthermore, the positive, negative or neutral effects of P enrichment on seed production can all occur in terrestrial ecosystems (Sims et al., 2012b; Singh et al., 2018; Wang et al., 2017). For example, although P enrichment negligibly affected the seed production of *Stipa krylovi*, it did increase that of *Artemisia frigida* in a temperate steppe (Li et al., 2017).

The availability of N and P availability, however, may jointly affect seed production since plant growth is predicted to be co-limited by multiple resources (Graciano et al., 2006; Harpole et al., 2016; Harpole & Suding, 2011; Long et al., 2016; Peñuelas et al., 2013). Simultaneously adding N and P enhances ecosystem primary productivity much more than adding either of them alone (Elser et al., 2007; Harpole et al., 2011; Solis et al., 2013). By contrast, such an interactive effect between N and P addition was not found for plant reproduction in a temperate steppe (Li et al., 2017). Further, the growth and reproduction of plants may respond differentially to the levels of nutrient enrichment (Bowman et al., 2006; Tang et al., 2017). A meta-analysis found that plant productivity in meadow steppe is positively related to N addition under low N addition level, but it decreases with increasing N addition under high N addition level (Tang et al., 2017). Nonetheless, few attempts have been made to empirically investigate how N and P addition rates and their interaction could affect plant reproduction allocation and seed production in terrestrial ecosystems, because most nutrient addition studies only include two levels of nutrient treatments (i.e., control vs. nutrient enrichment).

Grassland is one of the major terrestrial ecosystems and covers 40% of the world's land area (Adams et al., 1990). In this respect, the temperate steppe in northern China is representative of the typical vegetation of the Eurasian grassland biome (Bai et al., 2010; Su et al., 2018). A comprehensive project that used three levels of N and P addition was begun in April 2012, aiming to examine the effects of nutrient enrichment on community structure and ecosystem functioning in a typical temperate steppe of Inner Mongolia, northern China. As part of this long-term project, the present study was done to examine the interactive effects of N and P addition rates upon seed production of the dominant species *Stipa krylovii*, which is the most common perennial grass in typical temperate steppe ecosystems in China. We sought to address the following specific questions: (1) Do changes in N and P availability and in N:P ratio alter the seed production of the dominant species in temperate steppe ecosystem? (2) Which factors determine seed production of the dominant species under the different nutrient addition treatments?

2 MATERIALS AND METHODS

2.1 Site description

This experimental site is located in a fenced temperate steppe in Duolun County (42°02'N, 116°17'E, 1324 m a.s.l), Inner Mongolia, China. For the years 1960 to 2014, its mean annual temperature was 2.1°C and mean annual precipitation was 371 mm. Soil is classified as chestnut soil according to the Chinese classification, and Calcis-orthic Aridisol in the US Soil Taxonomy classification, with sand, silt, and clay contents of 62.8%, 20.3%, and 16.9%, respectively. *Stipa krylovii* is the most common herbaceous species in this temperate steppe; other common species include *Artemisia frigida*, *Agropyron cristatum*, *Cleistogenes squarrosa*, *Allium bidentatum*, and *Potentilla acaulis* (Su et al., 2018).

2.2 Experimental design

A randomized block design with complete factorials for N and P addition was used in this experiment, which was set up in early April 2012. Three levels of N addition were crossed with three levels of P addition, producing nine different nutrient addition treatments. For the N addition, the three levels were ambient

N (no N added, $N \text{ m}^{-2} \text{ yr}^{-1}$), low N addition ($N \text{ m}^{-2} \text{ yr}^{-1}$), and high N addition ($N \text{ m}^{-2} \text{ yr}^{-1}$); likewise for P addition, its three levels were ambient P (no P added, $P \text{ m}^{-2} \text{ yr}^{-1}$), low P addition ($P \text{ m}^{-2} \text{ yr}^{-1}$), and high P addition ($P \text{ m}^{-2} \text{ yr}^{-1}$). The field experiment consisted of four blocks, with each having nine plots randomly assigned to the nine nutrient addition combinations. Each plot was x in size and the distance between any two adjacent plots within each block was $.$ Nutrient addition was applied to plots annually, in July. Ammonium nitrate (NH_4NO_3) and calcium superphosphate ($\text{CaH}_4\text{P}_2\text{O}_8$) were used in the N and P addition plots, respectively.

2.3 Plant sampling

The dominant plant species, *S. krylovii*, was chosen to examine the effects of N and P addition upon seed production in this study. Being a widespread perennial tussock grass in typical temperate steppe, it is an important fodder species used in China, Mongolia, Kazakhstan, and Russia (Ronnenberg et al., 2011; Wu & Raven, 2006). The vegetative and reproductive tillers of *S. krylovii* can attain heights of 50 cm during the growing season (Li et al., 2017).

In the experiment, three plant individuals were selected in each plot to measure tiller number per individual, inflorescence number per individual, and seed number per inflorescence in late August, from 2015 to 2017. Meanwhile, maximum plant height and density were investigated in a randomly selected subplot (1 m x 1 m) within each plot. Seed production per individual was calculated as the product of seed number per inflorescence and inflorescence number per individual (Brys et al., 2005).

2.4 Data analysis

Three-way analyses of variances (ANOVAs) were employed to test the main and interactive effects of year, N addition, and P addition upon six response variables: seed production, seed number per inflorescence, inflorescence number, tiller number, density, and maximum height of *S. krylovii*. Significant difference in means for seed production, seed number per inflorescence, inflorescence number, tiller number, density, and maximum height of *S. krylovii* among the three levels of N or P addition were compared by Duncan's multiple range test. All the data were log-transformed to meet normality assumptions for ANOVAs before these analyses, which were carried out using SAS software (Proc Mixed, SAS 8.1; SAS Institute Inc., Cary, NC, USA). In addition, confirmatory analyses based on structural equation modeling (SEM) were conducted to quantify the direct and indirect impacts of N and P addition upon seed production. This SEM analysis was carried out in AMOS 21.0 (IBM, SPSS, Armonk, NY, USA).

3 RESULTS

3.1 Seed production under the N and P addition treatments

Across the three sampling years, seed production was consistently and significantly affected by N and P addition in the temperate steppe (both $P > 0.05$; Figure 1, Table 1). The low N addition enhanced seed production by 814%, 1371%, and 1321% under ambient, low, and high P addition treatments, respectively (all $P < 0.05$; Figure 1 left insert). The high N addition increased seed production by 2136%, 3560%, and 3550% under ambient, low, and high P addition treatments, respectively (all $P < 0.05$; Figure 1 left insert). However, neither a low nor high P addition altered seed production under ambient N (both $P > 0.05$; Figure 1 right insert). The low and high P addition augmented seed production by 65% and 65% under low N addition, and by 68% and 74% under high N addition treatments, respectively (all $P < 0.05$; Figure 1 right insert).

3.2 Reproductive traits changed under the N and P addition treatments

Seed number per inflorescence and inflorescence number were significantly influenced by N addition (both $P < 0.001$), whereas P addition significantly affected seed number per inflorescence ($P < 0.05$) but not inflorescence number ($P > 0.05$; Figure 2, Table 1). The low N addition stimulated seed number per inflorescence by 120%, 145%, and 175% under ambient, low, and high P addition conditions, respectively (all $P < 0.05$; Figure 2a left insert, Table 1). The high N addition increased seed number per inflorescence

by 183%, 187%, and 247% under the ambient, low, and high P addition treatments, respectively (all $P < 0.05$). Although the low P addition did not affect seed number per inflorescence under ambient, low, or high N addition conditions (all $P > 0.05$). However, high P addition enhanced seed number per inflorescence by 29% under the low N, and 27% under the high N addition treatments (both $P < 0.05$), but not under ambient N ($P > 0.05$; Figure 2a right insert).

The low N addition increased the inflorescence number by 317%, 513%, and 396% under the ambient, low, and high P addition treatments, respectively (all $P < 0.05$), but this effect was stronger under high N addition with corresponding increases of 719%, 1187%, and 934% under ambient, low, and high P addition conditions, respectively (all $P < 0.05$; Figure 2b left insert). However, either a low or high P addition did not influence inflorescence number under the ambient or low N addition treatments (all $P > 0.05$; Figure 2b right insert). Yet the low P addition was able to significantly augment inflorescence number by 49% under the high N addition treatment (Figure 2b right insert; $P < 0.05$).

3.3 Plant growth responses to the N and P addition treatments

Tiller number, plant density, and maximum plant height were significantly affected by N and P addition across the three surveyed years (all $P < 0.05$; Figure 3, Table 1). The low N addition increased the tiller number by 168%, 170%, and 177% under the ambient, low, and high P addition treatments, respectively (all $P < 0.05$; Figure 3a left insert), as did the high N addition, but almost twice as strongly, with corresponding percentages of 313%, 397%, and 446% under ambient, low, and high P addition conditions, respectively (all $P < 0.05$; Figure 3a left insert). Although the low and high P addition negligibly affected tiller number under the ambient or low N addition treatments (all $P > 0.05$; Figure 3a right insert), they did raise tiller number by 38% and 40% under the high N addition treatments, respectively (both $P < 0.05$; Figure 3a right insert).

The low N addition did not influence the density of *S. kryloii* under ambient and low P addition treatments (both $P > 0.05$), but suppressed it by 53% under high P addition ($P < 0.05$; Figure 3b left insert). By contrast, high N addition enhanced plant density by 35% and 64% under ambient and low P addition, respectively (both $P < 0.05$), but did not affect it under high P addition ($P > 0.05$; Figure 3b left insert). Across the three years, low P addition decreased the density, on average, by 49% under ambient N conditions ($P < 0.05$). While low and high P addition respectively reduced plant density by 46% and 71% under low N addition (both $P < 0.05$), the effect of high P addition was weakened by the high N addition, so that plant density decreased by 50% ($P < 0.05$; Figure 3b right insert).

The low N addition enhanced the maximum plant height by 36%, 52%, and 58% under ambient, low, and high P addition treatments, respectively (all $P < 0.05$), but the corresponding effects were stonger, at 69%, 72%, and 83% for the high N addition (Figure 3c left insert; all $P < 0.05$). Although the low and high P addition did not influence the maximum height under ambient or high N addition conditions (Figure 3c right insert; all $P > 0.05$), they did so under the low N addition treatments by 16% and 15%, respectively (Figure 3c right insert; $P < 0.05$).

Path analysis for effects of reproductive traits and plant growth on seed production

We used SEM to examine the direct and indirect factors affecting seed production. The results revealed that tiller number, plant density, and maximum plant height were indirectly responsible for 89%, 48%, and 90% of the variation in seed production, respectively, under the N and P addition treatments ($\chi^2_{15} = 24.47, P = 0.058, RMSEA = 0.134$; Figure 4). Direct contributions to changes in seed production arose from the seed number per inflorescence ($R^2 = 0.88, P < 0.001$) and inflorescence number ($R^2 = 0.12, P = 0.044$). The N addition promoted seed production of *S. kryloii* mainly via enhanced tiller number and an accompanying enhancement in the plants' inflorescence number (Figure 4). The P addition increased seed production differently, mainly by reducing the density of *S. kryloii*, thereby enabling individuals to a greater maximum height and consequently a greater seed number per inflorescence (Figure 4).

4 DISCUSSION

4.1 Impact of N addition on seed production

The general positive effects of N addition on the seed production of *Stipa krylovii* and this plant's greater seed production under high N addition relative to low N addition, suggest that seed production of this dominant species' fecundity is limited by N availability in the temperate steppe. This enhanced seed production under N addition is consistent with previous field studies done in grasslands (HilleRisLambers et al., 2009; Li et al., 2017; Shi et al., 2017). Adding N addition to soil can stimulate the plants' development of its progeny via increased reproductive traits and qualities, such as a greater inflorescence number, or seed number per inflorescence (HilleRisLambers et al., 2009; Shi et al., 2017), and by raising the nutrient concentration of structures for production (DiManno & Ostertag, 2016). However, some researchers have reported that N addition is irrelevant for seed production (DiManno & Ostertag, 2016; Ostertag, 2010). Some plant species may produce plenty of flower nectar, this being rich in amino acids and acting as N storage pool for fruits, leaves, or roots development, which would greatly weaken the promoted allocation of N to seed formation (DiManno & Ostertag, 2016). In our studied steppe grassland, *S. krylovii* cannot develop flower nectar, which permits the allocation of nutrients to vegetative or reproductive growth rather than storing them.

Nitrogen addition can promote biomass accumulation via enhanced photosynthesis (Domingues et al., 2015), roots' extension and expansion (Ruffel et al., 2011), and plant growth (Sims et al., 2012a). In our study, we found that tiller number, plant density, and plant height all increased in N addition conditions, which agrees with other studies finding that N enrichment enhances plant productivity (Tang et al., 2017; Xu et al., 2015)(Xu et al. 2015). Along with N-induced vegetative growth, plants usually allocate proportionally more resources to reproductive structures (Allison, 2002; Willis & Hulme, 2004; Xia & Wan, 2013), leading to more reproductive tillers and less aborted in abortion of flowers and fruits (Stephenson, 1981; Marcelis et al., 2004). In this study, we found that most tillers in N addition plots attained a high reproductive capacity, which greatly enhanced their inflorescence number, and thereby contributed substantially to boosting seed production per capita (Figure 4).

4.2 Impact of P addition on seed production

In our study, although the main effect of phosphorus upon seed production of *S. krylovii* was significant, neither low nor high P addition influenced seed production in the absence of N addition, suggesting that seed production is not limited by P availability in the temperate steppe. This finding is consistent with previous research (Li et al., 2017; Yang et al., 2014). Both low and high P addition increased seed production in the presence of N addition, and the increment was significantly higher under high N addition than under low N addition, indicating that a P limitation of seed production can be triggered by N addition. This phenomenon is supported by model simulation work (Menge & Field, 2007) as well several field experiments (Marklein & Houlton, 2012; Zheng et al., 2018). Plants capable of a high growth rate under N-rich conditions will require a greater allocation of P-rich rRNA to support macromolecular (protein, rRNA) synthesis (Niklas et al., 2005). The demand for P increases with N addition-induced growth (Li et al., 2016b). Accordingly, fertilization with P would allow for an increased allocation of P to reproductive structures inflorescences.

Phosphorus is not only a structural element of cell organelles (such as mitochondria and chloroplast) but also the primary constituent of phospholipids (ATP and NADPH) that are used for energy metabolism in light and dark reactions. Indeed, P is indispensable for plant photosynthesis and respiration, such that changes in the P concentration available for plant uptake would alter their vegetative and reproductive growth (George et al., 2016; Patel et al., 2017). An external P addition usually tends to enhance plants' internal P concentration, accelerating their photosynthetic efficiency, and thus promoting biomass accumulation (Graciano et al., 2006; Suriyagoda et al., 2014). P enrichment can indirectly promote plant height growth and thereby augment the seed number per inflorescence (Figure 4). Higher levels of P to plants can result in more spikelets per fertile tiller (Wang et al., 2017) and an earlier plant flowering date (Petraglia et al., 2014). Both outcomes may subsequently enhance overall fecundity and prolong the seed development period, and eventually stimulate seed production.

In addition, soil P availability is highly responsive to local available N (Marklein & Houlton, 2012). Even

a minor increase in available N addition can increase soil P availability by stimulating greater root surface phosphatase activity and facilitating P dissolution, which alleviates P limitation (Crowley et al., 2012; Johnson et al., 1999; Vitousek et al., 2010). Although N fertilizer can promote P cycling, the increased available P is insufficient to balance the greater plant demand for P (Li et al., 2016b); hence, P limitation will gradually predominate become predominant (Peng et al., 2017; Peñuelas et al., 2013).

4.3 Implication for fertilization management

Although numerous studies have found that N and P interact to control plant growth, nutrient absorption, and reproductive allocation under conditions of N and P addition (Li et al., 2017; Long et al., 2016; Zhao et al., 2018b), the effect of multi-level N/P addition on plant reproduction is still unclear, especially under various the other nutrient addition levels. In our study, the effects of N addition upon seed production did not differ significantly different under the low or high P addition. A low P addition is sufficient to balance the increased P demand of plant growth while a high P addition cannot be fully utilized by plants, leading to a similar effect arising between these two levels of P addition. The leaf N:P ratio tends to balance out at a soil available N:P supply ratio of approximately 20 (Zhan et al., 2017). Thus, our low rate of P addition ($5 \text{ g m}^{-2}\text{yr}^{-1}$) may need $100 \text{ g N m}^{-2}\text{yr}^{-1}$ to balance the N demand from plant growth in this temperate steppe.

Our study demonstrates the importance of N and P enrichment in regulating the seed production of dominant species in a temperate steppe. Seed production in response to changing available nutrients in soil can profoundly determine plant community structure and dynamics (Basto et al., 2015). Our findings of increased seed production of the dominant species under both N and P addition treatments, coupled to their additive effects, suggest that the *S. krylovii* will become more dominant under accelerating N and P enrichment regimes.

Producing more seeds confers advantage in dispersal and fecundity, resulting in higher probability of colonizing new favorable habitats. Due to the higher reproductive growth of the dominant species, the species would increase its capacity of dispersal capacity and occupy more living space. More resources, such as light and water, would be consumed by such dominant species, and this restricts the survival spaces of other co-occurring species in the community, effectively squeezing them out, over time leading to species losses in nutrient amendment conditions. Nutrient enrichment has an obvious promoting effect on the seed production of dominant species in natural ecosystems, which provides new insight into the mechanisms of biodiversity loss in the context of intensifying human activities in grasslands especially.

5 CONCLUSIONS

Seed production of the dominant species in the temperate steppe was enhanced by N addition, but a high level of N addition stimulated seed production more than a low N addition. Whereas seed production was unchanged by P addition alone, it was increased when the latter was in the presence of N addition. Seed production was enhanced mainly through an increasing of tiller and inflorescence numbers under N addition, and by decreased plant density stimulating plant height growth and enabling seed number per inflorescence under P addition. Our results indicate that N availability is the main factor limiting seed production, but seed production can become limited by P availability as N enrichment increases in the temperate steppe. These timely findings can facilitate better understanding of grassland seed banks and plant community structure responses to simultaneous multiple nutrient enrichment under future nutrient enrichment scenarios.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Guoyong Li designed the research, conducted the field measurements, and analyzed the data. Chengming You, Qun Guo, and Zhongmin Hu designed the research and secured the funding. Zhongling Yang provided ideas for writing. Lei Su and Mengzhou Liu conducted the data analysis and wrote the manuscript, it was then heavily edited by all the authors.

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DATA AVAILABILITY STATEMENT

The data of this study are available in Dryad. DOI: 10.5061/dryad.8sf7m0cms

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TABLE 1 Results (*F* ratios) of three-way ANOVAs on the effects of N and P addition on the seed production, inflorescence number, seed number per inflorescence, tiller number, density, and height in a temperate steppe of Inner Mongolia, China.

Effects	df	Seed production	Seed number per inflorescence	Inflorescence number	Tiller number	Density	Height
N	2	509.65***	204.99***	412.01***	287.38***	11.49***	24.49***
P	2	4.39*	3.67*	2.96	3.82*	23.75***	4.73**
N×P	4	1.15	1.01	1.55	1.16	2.31	1.81
Y	2	17.15***	8.38**	19.00***	34.59***	13.66**	28.11***
N×Y	4	3.81**	2.41	2.98*	4.50**	0.48	0.99
P×Y	4	1.28	0.89	3.32*	1.93	0.40	2.33
N×P×Y	8	2.23*	0.32	2.84**	1.19	0.50	1.10

Significant level: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

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