

Negative effects of nitrogen override positive effects of phosphorus on grassland legumes worldwide

Pedro M. Tognetti^{1†}, Suzanne M. Prober^{2†}, Selene Báez³, Enrique J. Chaneton¹, Jennifer Firn⁴, Anita C. Risch⁵, Martin Schuetz⁵, Anna K. Simonsen^{6,7}, Laura Yahdjian¹, Elizabeth T. Borer⁸, Eric W. Seabloom⁸, Carlos Alberto Arnillas⁹, Jonathan D. Bakker¹⁰, Cynthia S. Brown¹¹, Marc W. Cadotte⁹, Maria C. Caldeira¹², Pedro Daleo¹³, John M. Dwyer¹⁴, Philip A. Fay¹⁵, Laureano A. Gherardi¹⁶, Nicole Hagenah¹⁷, Yann Hautier¹⁸, Kimberly J Komatsu¹⁹, Rebecca L. McCulley²⁰, Jodi N. Price²¹, Rachel J. Standish²², Carly J. Stevens²³, Peter D. Wragg²⁴, Mahesh Sankaran^{25,26}

¹IFEVA-CONICET, Facultad de Agronomía, Universidad de Buenos Aires, C1417DSE, CABA, Argentina

²CSIRO Land and Water, Private Bag 5, Wembley, Western Australia 6913, Australia

³ Department of Biology, Escuela Politécnica Nacional del Ecuador, Quito, Ecuador

⁴ Queensland University of Technology (QUT), Brisbane, Queensland 4001, Australia

⁵ Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zuercherstrasse 111, 8903 Birmensdorf, Switzerland

⁶ Australian National University, Canberra, Australian Capital Territory, 2601, Australia

⁷ Department of Biological Sciences, Florida International University, Miami, Florida, 33199, USA

⁸ Department of Ecology, Evolution & Behavior, University of Minnesota, St. Paul, MN 55108, USA

⁹ Department of Biological Sciences, University of Toronto Scarborough, 1265 Military Trail, Toronto, ON, M1C 2M2, Canada.

¹⁰ School of Environmental and Forest Sciences, University of Washington, Seattle, WA 98195, USA

¹¹ Department of Agricultural Biology, Graduate Degree Program in Ecology, 1177 Campus Delivery, Colorado State University, Fort Collins, Colorado 80523, USA

¹² Centro de Estudos Florestais, Instituto Superior de Agronomia, Universidade de Lisboa, 1349-017 Lisbon, Portugal

¹³ IIMyC, UNMDP-CONICET, Casilla de Correo 1260, Mar del Plata, Argentina

¹⁴ School of Biological Sciences, The University of Queensland, St Lucia QLD 4072 – CSIRO, Ecosciences Precinct, Dutton Park QLD 4102, Australia

¹⁵ USDA-ARS Grassland, Soil, and Water Research Lab, Temple, Texas 76502, USA.

¹⁶ Arizona State University - School of Life Sciences, Mail code 4601, LSA 259, Arizona, USA

¹⁷ Mammal Research Institute, Department of Zoology & Entomology, University of Pretoria, Pretoria, South Africa

¹⁸ Ecology and Biodiversity Group, Department of Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

¹⁹ Smithsonian Environmental Research Center, Edgewater, Maryland 21037, USA

²⁰ Department of Plant & Soil Sciences, University of Kentucky, Lexington, Kentucky, 40546-0312, USA

²¹ Institute of Land, Water and Society, Charles Sturt University, Albury, NSW, 2640, Australia.

²² Environmental and Conservation Sciences, Murdoch University, 90 South Street Murdoch WA 6150, Australia

²³ Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK

²⁴ University of Minnesota, Department of Forest Resources, 1530 Cleveland Ave N, St. Paul, MN 55108, USA

²⁵ National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru 560065, Karnataka, India

²⁶ School of Biology, University of Leeds, Leeds LS2 9JT, UK

†Joint first authors

* Corresponding Authors: Pedro M. Tognetti & Suzanne M. Prober

Email: tognetti@agro.uba.ar ; Suzanne.Prober@csiro.au

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55

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61 N.H., Y.H., K.J.K., R.L.M., J.P., R.J.S., C.S., and P.D.W. E.T.B. and E.W.S. are Nutrient Network
62 coordinators. Author contribution details are provided in this [LINK](#).

63 **This file includes:**

64 Main Text

65 Figures 1 to 3

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68 Supplementary figures Fig. S1 to Fig. S7

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ABSTRACT

Anthropogenic nutrient enrichment is driving global biodiversity decline and modifying ecosystem functions. Theory suggests that plant functional types that fix atmospheric nitrogen have a competitive advantage in nitrogen-poor soils, but lose this advantage with increasing nitrogen supply. By contrast, addition of phosphorus, potassium and other nutrients may benefit such species in low nutrient environments by enhancing their nitrogen-fixing capacity. We present the first global-scale experiment confirming these predictions for nitrogen-fixing legumes (Fabaceae) across 45 grasslands on six continents. Nitrogen addition reduced legume cover, richness, and biomass particularly in nitrogen-poor soils while cover of non-nitrogen-fixing plants increased. Addition of phosphorous, potassium, and other nutrients enhanced legume abundance but did not mitigate the negative effects of nitrogen addition. Increasing nitrogen-supply thus has potential to decrease the diversity and abundance of grassland legumes worldwide regardless of the availability of other nutrients, with consequences for biodiversity, food webs, ecosystem resilience and genetic improvement of protein-rich agricultural plant species.

SIGNIFICANCE STATEMENT

Predicting effects of anthropogenic nutrient enrichment on plant communities is critical for managing implications for biodiversity and ecosystem services. Plant functional types that fix atmospheric nitrogen (e.g. legumes) may be at particular risk of nutrient-driven global decline, yet global-scale evidence is lacking. Using an experiment in 45 grasslands across six continents, we showed that legume cover, richness, and biomass declined substantially with nitrogen additions. Although legumes benefited from phosphorus, potassium and other nutrients, these nutrients did not ameliorate nitrogen-induced legume decline. Given global trends in anthropogenic nutrient enrichment, our results indicate potential for global decline in grassland legumes, with likely consequences for biodiversity, food webs, soil health and genetic improvement of protein-rich plant species for food production.

100 **Introduction**

101 Anthropogenic enrichment of nitrogen (N), phosphorus (P) and other nutrients from fertilizers
102 and fossil fuel combustion is transforming natural ecosystems worldwide (1–5), leading to
103 increased terrestrial plant productivity (6, 7) and loss of biodiversity (8, 9). Resource
104 competition theory proposes that the capacity of species to persist at low levels of a limiting
105 resource is a key mechanism underpinning competitive success. Consequently, plant
106 functional types with specialized nutrient-acquisition strategies are expected to have a
107 competitive advantage in nutrient-limited environments but also to be especially vulnerable
108 to nutrient enrichment (10–13).

109 Legumes (Fabaceae) are one of the largest families of flowering plants, contributing over 650
110 genera and 19,000 taxa to global plant diversity (14). This diversity is important for
111 biodiversity conservation and for genetic improvement of protein-rich crops and forage
112 species for sustainable livestock production (15–17). Further, the ability to fix atmospheric N₂
113 is one of the most important plant functional traits for influencing ecosystem processes,
114 conferring N-fixing legumes with a disproportionately important role in ecosystem functioning
115 (18, 19). For example, litter produced by legumes is nitrogen-rich and more easily
116 decomposed by soil micro-organisms, leading to flow on effects to higher trophic levels,
117 including increased complexity of food webs and resistance of soil biophysical and chemical
118 properties to ecosystem disturbance (20). As the success of legumes often arises from this
119 capacity for symbiotic fixation of atmospheric N₂ in N-limited environments (21, 22),
120 atmospheric N-deposition and other pathways of anthropogenic N supply are expected to
121 drastically reduce their competitive advantage in plant communities (1, 5, 11, 23). This is
122 especially the case for obligate-N-fixers that cannot down-regulate N-fixation (24, 25) and
123 hence at higher soil N are disadvantaged by the high energetic cost of N-fixation (26).

124 While concerns about global nutrient enrichment are focused on impacts of N on
125 biodiversity and ecosystem productivity (1, 2, 27), changes in P and potassium (K) cycles (3, 4)
126 or altered concentrations of other nutrients, can also influence the abundance and diversity of
127 legumes in accordance with resource competition theory (10–13). Owing to the physiological
128 demands of N-fixation, N-fixing legumes often have higher requirements for P, K, and other
129 nutrients (e.g. Molybdenum (Mo), Iron (Fe), and Calcium (Ca)) than non-N fixing plants (28–
130 31), and increases in these nutrients can favor N-fixing over non-N-fixing species, particularly
131 in nutrient poor soils (21, 22). However, added nutrients may have synergistic effects (6, 32),
132 leading to uncertainties in the expected net effect of P addition on the abundance of N-fixing
133 legumes (26). For example, the phosphatases required for P-acquisition from soils are rich in
134 N; N addition may increase phosphatase investment, conferring legumes a superior
135 phosphorus acquisition capacity in P- and N-limited environments (25, 29). Conversely,
136 multiple nutrient addition is expected to allow non-legumes to compete more effectively with
137 legume species. Resulting light limitation may suppress legume growth and reduce the
138 survival and establishment of new legume individuals (8, 9), especially of those legumes that
139 are unable to reduce the costs of N-fixation through down-regulation (10, 11, 15, 33–35).

Despite these theoretical predictions, empirical evidence for the individual and interactive effects of changes in nutrient availability on legumes in natural ecosystems is limited (29, 36–39). Some experimental studies have shown decreased legume abundance with N addition and increased with P addition, but these studies are typically conducted at a single site and show both positive and negative interactive effects among nutrients (e.g. (37, 40, 41). Further, minimal evidence is available regarding the influence of K or micronutrient enrichment on legume responses (29), and the underlying mechanisms of legume responses to nutrient addition, such as soil and climatic conditions, have not been investigated at global scales (but see (26) for forest ecosystems).

Using data from the Nutrient Network global collaborative experiment (www.nutnet.org (42)), we measured the cover, richness, and biomass responses of N-fixing legumes (hereafter legumes) to standardized experimental nutrient additions in 45 grasslands across six continents (SI Appendix Fig. S1 and Table S1). Grasslands are a globally significant biome, covering more than one third of the Earth's ice-free land surface, accounting for a third of terrestrial net primary production (43), and supporting the livelihoods of more than 1.3 billion people. They are subject to chronic atmospheric nitrogen deposition due to fossil fuel combustion and are likely candidates for direct nitrogen fertilisation (44). Whilst N emissions in many regions of Europe has declined leading to plateaus or reductions in deposition (45) deposition in other world grasslands, such as the Mongolian Steppe, have increased in recent decades (e.g. (46)). Experimental sites included temperate and anthropic grasslands that spanned a broad range of geographical locations and ecological conditions, although were mostly from temperate latitudes (39; SI Appendix Table S1; Fig. S1; see Methods for details).

Three nutrients (N, P, K+) were applied in factorial combinations, resulting in eight treatments enabling evaluation of the interactive effects of N, P, and K addition (6, 8) on legumes. Over 3–6 years, 10 g m⁻² N, P, and K were added annually to their respective treatment plots at the beginning of each sites' growing season; other nutrients in the K+ treatment (sulphur (S), magnesium (Mg) and micronutrients) were applied only in the first year to avoid toxicity (42). These nutrient levels were selected to ensure they were high enough to reduce nutrient limitation at a wide diversity of sites. They are at the higher end of the range for agricultural fertilizer application rates globally (5), and higher than atmospheric nutrient deposition rates (1, 3, 41, 43). In particular, our N-addition rate was about three times maximum current N-deposition rates in European grasslands and more generally across the globe (1, 48, 49).

We used a standardized protocol (6, 42) to annually measure cover, richness and biomass of legumes, forbs and grasses in 1 m² permanent plots (see Methods), starting in the year prior to the first nutrient application (Y_{initial}). Across all years and sites, we recorded 170 species of N-fixing grassland legumes, comprising 50 genera (SI Appendix Table S2). The most species-rich genera were *Trifolium* (25 spp.), *Astragalus* (12 spp.), *Vicia* (11 spp.) and *Lupinus* (11 spp.). *Vicia sativa*, *Trifolium repens* and *Vicia hirsuta* were the most frequent species across our sites (9.1%, 5.1% and 4.9% of total occurrences, respectively). Each site contained 1–8 legume species (see Methods; SI Appendix Table S1). Most legume species were

perennials (~60%), including ten woody or shrub species (~6% of species). On average, approximately 3% and 4% of total live cover comprised annual and perennial legumes, respectively.

We present results of nutrient addition for the third and the last available sampling year (years 3 to 6) after starting nutrient application in each site (noting sites started applying experimental treatments in different calendar years and ran for different lengths of time (SI Appendix Table S1)). To measure the relative impact of N, P, and K+ addition on legumes, we calculated the log ratio (LR) of legume abundance and richness in the third or last year in each plot versus the initial (pre-treatment) value [$LR = \ln(Y_{\text{final}} / Y_{\text{initial}})$]. We used the pre-treatment legume abundance in the LR instead of control plots (50) to control for initial legume abundance and spatial variability among plots (8, 51). We also calculated measures of legume colonization and extinction in each plot, and evaluated the effect of initial soil nutrient concentrations, community structure and climatic conditions as contingencies for nutrient addition effects (See Methods for details). We analyzed the data using linear mixed-effects models (52–54), with nutrient treatments (i.e., N, P, K+ and their interactions) as fixed effects, and blocks nested within sites as random effects. Confidence intervals for model parameters were bootstrapped as a conservative method for hypothesis testing (52, 53) (See Methods for details).

Results and Discussion

Effects of nutrient addition on legumes

We expected N addition to reduce the competitive advantage of legumes, resulting in a decline in legume relative abundance and richness (11–13, 23, 30, 33, 55). This prediction was strongly supported (Fig. 1, SI Appendix Fig. S2-3 and Tables S3-5), with an average 32% decline in legume cover ($LR_N = -0.397$) after only three years of chronic N addition (SI Appendix Table S3-4). Nitrogen addition did not significantly reduce non-leguminous forb cover ($LR_N = -0.086$, SI Appendix Table S6a), and increased grass cover ($LR_N = 0.129$; SI Appendix Table S6b), showing that the negative effect of N addition was specific to legumes. Nitrogen also significantly reduced legume species richness by 12% and biomass by 43% ($LR_N = -0.129$ and $LR_N = -0.569$, respectively, Figure 1, SI Appendix Fig. S3-4 and Table S3-5).

The clear declines in legume cover, biomass, and richness with N addition occurred despite the potential for some N-fixing legumes to down-regulate N-fixation (24), further supporting our predictions. Down-regulation is proposed to underpin the ‘tropical paradox’, enabling high species richness and abundance of legumes in late successional tropical rainforests with high soil N (25). Our result indicates that in temperate grasslands around the world, (i) the high cost of fixation in obligate N-fixing legumes, and/or (ii) inferior competitive growth strategies in facultative N-fixing legumes compared with other herbaceous species, outweighed any potential amelioration of these costs through down-regulation of N-fixation. Nevertheless, it is possible that down-regulation reduced the magnitude of the negative N-addition effect. This contrasting result in grasslands compared with tropical forests highlights a need for further evaluation of the importance of down-regulation in grasslands, and

potential drivers of differences from tropical forests. For example, vertebrate herbivores, nutrient leaching, or grass-legume dynamics, could maintain ongoing or fluctuating N deficits in grasslands, that reduce evolutionary pressures for legumes to down-regulate ((56, 57); see (58) for evolutionary persistence of fixation). Further investigation could be achieved by directly assessing nodulation, N-fixation and rhizobial biomass in experimental plots (35), and more broadly by better global documentation of which legume species are facultative and which are obligate N-fixers.

We predicted that P and K⁺ addition would increase legume abundance, particularly in the absence of N addition (26, 28, 30, 31, 37, 39, 59), despite the potential for concurrent increases in non-legume competitors. Accordingly, we found that P addition (without N) significantly increased the cover of legumes by an average of 34% by the last experimental year (the average 20% increase after 3 years was not significant; Fig. 1). By contrast, K⁺ addition alone did not significantly increase legume cover (Fig. 1, SI Appendix Table S3-4 and Fig. S2-4).

Together, P and K⁺ addition increased the cover of legumes by an average of 37% after three years (32% by the last experimental year; Fig. 1; SI Appendix Table S3-5), even though P also enhanced grass cover (but not non-N fixing forbs, $LR_{P \text{ grass}} = 0.13$; SI Appendix Table S6). The increase in legume cover with P and K⁺ was additive (P x K interaction and $LR_{(K + P) \text{ vs } KP}$ contrast included zero; SI Appendix Fig. S2 and Table S3-5), suggesting that in the absence of N addition, K and/or other added nutrients became limiting to N-fixers when P requirements were met (29, 60).

A similar pattern was evident for legume biomass, with P and K⁺ addition on average across nutrient treatments leading to significant increases in legume biomass after three years (25%, $LR_P = 0.22$ and 29%, $LR_K = 0.25$ respectively, SI Appendix Table S5), although the effect of P was not significant for the final year analysis (43% for K⁺ addition; $LR_K = 0.36$, Fig. 1, SI Appendix Table S5). We did not detect significant changes in legume richness due to P or K⁺ addition (Fig. 1, SI Appendix Table S3-5).

Given the opposing effects of N versus P or K⁺ addition on legumes, effects of adding N in combination with P or K⁺ are difficult to predict, and empirical evidence is conflicting (10, 21, 22, 26, 36, 37). We hypothesised that legumes would decrease with high levels of N addition even with concurrent addition of P and K⁺, because the P and K⁺ required for N-fixation confers less advantage to legumes when N is not limiting (26, 28, 37). Consistent with this hypothesis, we found that despite the benefits of P addition, its combination with N resulted in a net reduction of legume cover and biomass that was nearly as large as the effect of N addition alone ($LR_{N \times P} = -0.24$, Fig. 1, SI Appendix Fig. S2-3 and Table S3-5). This indicates that the benefits of P were largely overridden by the suppressive effects of N (although this effect was significant only for legume cover). Similarly, statistical contrasts indicated that N addition obscured the positive effects of combined P and K⁺ addition on legumes (Contrast 6-7 in SI Appendix Fig. S4 and Table S5), again as would be expected where N-fixing capacity is no longer advantageous (29) (see also (6)). While N, P, and K⁺ have previously been

recognized as influencing the abundance of N-fixing legumes (10, 11, 37, 61), our study is the first to describe N, P and K⁺ interactions in grassland ecosystems at a global scale.

Mechanisms underpinning legume responses

We expected N addition to suppress legumes by reducing their competitive advantage over co-occurring, non-N-fixing forbs and grasses (10–13, 33, 37). Supporting this prediction, plot-scale mixed-effects models showed that cover of non-legume forbs plus grasses significantly increased with N addition (see above; SI Appendix Table S6c), and this increase was significantly associated with the reduction in legume cover (Fig. 2a, SI Appendix Table S7). Further, through increases in cover and biomass (6, 8, 9), N addition reduced photosynthetically active radiation (PAR) at the soil surface (SI Appendix Fig. S5 and Table S8), consistent with previous evidence that light reduction impairs legume performance (35, 56) (see also (9)). Taken together, these results are consistent with previous evidence (10, 11, 37, 61) suggesting nutrient-related increases in non-legume forbs and grasses may affect legumes through increased competition, particularly for light.

We also asked whether the strong negative effect of N on legumes arose through a reduction in legume establishment (e.g. due to diminished seed accumulation and/or microsite availability), or reduction in growth and survival of mature individuals (62, 63). We found evidence for both mechanisms: by the third year, N addition had significantly reduced legume species colonization rates from 34 to 15% and increased local legume species extinction from 25 to 46% (Fig. 3, SI Appendix Table S9). The proportion of annual richness was only slightly higher for legumes (mean = 0.46 ± 0.39), than forbs (0.39 ± 0.26) or grasses (0.36 ± 0.25), suggesting that a higher proportion of species with annual life history seems unlikely to solely explain observed negative N-addition impact on legumes. Indeed, the trends were consistent across both perennial and annual species of legume: extinction of perennial legume species increased from 21 to 35%, and of annual legume species from 45 to 72%. Similarly, colonization of perennial legume species decreased from 5.5 to 2.7% and from 1% to less than 1% for annuals (SI Appendix Table S9).

Potential contingencies influencing nutrient responses

We expected legume responses to nutrient addition to vary among sites because of differing background rates of edaphic resource availability (10, 12, 13). With respect to N addition, we predicted greater legume decline in soils that were initially more favourable for legumes, including soils with lower initial N (11, 13, 56) or with higher P, K or micronutrient concentrations (26, 38, 39, 56, 59). We predicted this because (i) potential for loss is limited if initial legume abundance is low, (ii) non-fixing competitors would be more responsive to N addition at low N, resulting in greater rate of increase in competition for light (6), and (iii) sites with initially higher N (or lower K or micronutrients) may already support a higher proportion of more competitive, facultative legumes that are less likely to decline with N addition (24, 25).

Analysis of site-scale data indeed showed that N addition led to greater legume decline at sites with lower initial soil N (SI Appendix Fig. S6 and Tables S10a), lower N:P_{ratio} (Fig. 2b, SI Appendix Table S7), or higher initial soil Fe (an element that is particularly critical for the legume symbiosis (39, 59, 60, 64) (SI Appendix Fig. S6, SI Appendix Table S10a), supporting our hypothesis that N addition has the greatest impact on legumes in soils more favourable for legumes (65). We did not detect a significant relationship between initial soil N or Fe and initial legume abundance, inconsistent with our first hypothesized mechanism (i) for this response, i.e. that it resulted from initially low legume abundance in high N or low Fe sites. Our second hypothesized mechanism (ii) was supported, with significantly greater increases in non-fixing competitors with N addition at lower initial soil N [N addition: 0.09 (0.05;0.13); Soil N x N addition: -0.15 (-0.25; -0.03)]. We could not test our third hypothesised mechanism (iii), that greater legume decline at sites with lower initial soil N was explained by a lower proportion of more competitive, facultative legumes in sites with initially low soil N or Fe, because we lack data on nodulation and fixation rates (35, 36). Further investigation is thus needed to test whether facultative legumes increase in or colonise N-enriched sites that were initially low in N (24, 25).

Unexpectedly, N addition was more detrimental to legume cover in sites with lower initial soil K (i.e. in soils we expected to be already limiting for N-fixation, SI Appendix Table S10a). The reason for this is unclear but could involve correlations of soil K with other soil variables that impact legume cover (e.g. soil pH, $r=0.64$, SI Appendix Fig. S7). We detected no significant effects of initial soil P on legume response to N addition.

With respect to addition of P or K⁺, we expected a greater benefit to legumes when initial soil concentrations of P or K⁺ were lower (suggesting potentially greater P or K⁺ limitation), and when initial N was lower, suggesting greater activation of N-fixation by legumes (21). We did not detect these effects directly (SI Appendix Tables S11-S12) but we did detect an increasing benefit of K⁺ addition with increasing initial soil P and decreasing initial soil K⁺ (SI Appendix Table S12), i.e. in conditions that are otherwise more favourable for N-fixation. This trend is consistent with our experimental result that legume response to addition of K⁺ was evident when combined with P addition.

Other potential contingencies influencing legume responses to nutrient addition include climate and initial community composition factors. We expected legume responses to nutrient addition to be stronger in mesic environments where moisture limitation does not constrain the benefits of N-rich leaves for photosynthesis (21) (but see also (66–68)), and in warmer environments with lower energetic constraints on N-fixation (26, 67). We did not detect any significant effects of mean annual precipitation but found that temperature significantly predicted N (although not P or K⁺) effects on legumes (SI Appendix Tables S10-S12). In particular, N addition was more detrimental to legumes at sites with higher mean annual temperatures (SI Appendix Table S10), providing further support for the hypothesis that N addition is more detrimental in environments more favourable for N-fixation (26, 56).

With respect to initial community composition, we predicted that legume response to nutrient addition may be constrained by (i) the ambient site-level cover and richness of legumes, and (ii) availability of N-responsive, non-legume competitors such as many non-native grassland species (69, 70). Consistent with our first prediction (i), plot and site-scale regressions indicated that N addition was more detrimental to legumes at sites with higher initial legume cover and richness (Fig. 2c, SI Appendix Table S9b), likely due to density dependent limits to decline at low initial cover values (11). For unknown reasons, control plots also declined in proportional legume cover over the experimental period, especially in sites with initially higher legume cover (Fig. 2c), but the decrease was 30% greater with N addition (SI Appendix Table S7). Consistent with our second prediction (ii), N addition was more detrimental to legumes in sites with higher initial non-native plant species cover, potentially indicating greater scope for suppression by N-responsive competitors (69, 70). Our assessment of effects of N, P and K⁺ addition across 45 grasslands thus emphasizes the importance of soil nutrients and plant community composition in combination with macro-climatic conditions as predictors of legume responses.

Conclusions

Our data from 45 standardized experiments in grasslands from six continents provide broad-scale evidence that the individual and interactive effects of nutrient addition on legumes correspond with predictions from resource competition theory (10, 26, 55). First, grassland legumes declined substantially with N addition at 10 g N m⁻² yr⁻¹ over at least 3 years. This finding demonstrates the generality of this response in temperate grasslands, which until now has only been documented in a range of isolated experiments and model predictions (11, 37, 40, 71). The reduction likely resulted from reduced competitive advantage of legumes over non-N-fixing plants, potentially mediated by light availability. Second, grassland legumes benefited from P and K⁺ addition in the absence of N addition, generalizing emerging localized evidence to global scales (29, 37, 41). Third, addition of P and/or K⁺ at rates exceeding current global deposition rates did little to ameliorate N-induced legume decline, providing global support for overriding impacts of high levels of N enrichment (11, 37). These results complement and support broader conclusions regarding impacts of N and P enrichment on grasslands, including increased grassland productivity (6, 72), reduced plant richness (8, 9), and changes in the spectrum of plant functional traits (73).

Anthropogenic activities are increasing N supplies through atmospheric deposition and direct fertilization, thereby transforming ecosystems across the globe (1–5). While further work is needed to investigate the role of elemental supply rates on changes in obligate and facultative legume relative abundance (24, 25, 35), our results highlight the potential for substantial impact of anthropogenic N-enrichment on the abundance and diversity of plants with specialized N-acquisition strategies. In the context of atmospheric N-deposition, we acknowledge our experiment applied N at ~ 3x estimated annual maximum global N-deposition rates (1, 48), so likely overestimates the effects of N-deposition over our experimental timeframe. On the other hand, global N deposition is expected to occur over decades to centuries, while our results were observed after only 3-6 years of nutrient

addition. Earlier work (74) provides evidence for ongoing loss of legumes with long-term N addition across a wide a range of rates (0 to 20 g m⁻² yr⁻¹), so it is conceivable that our N-addition treatments could elicit broadly comparable responses to the cumulative effects of global N-deposition. Nevertheless, further work is needed to understand implications of interactions between rates and duration of nutrient applications to better predict impacts of atmospheric N-deposition.

Finally, our results suggest that legume decline is likely in the face of significant N enrichment regardless of changes in global P or K cycles. Owing to their disproportionate effects on ecosystem functions, widespread decline in grassland legumes is likely not only to contribute directly to global loss of plant diversity, but to affect nutritional quality for other trophic levels (19, 75), and to reduce food web complexity and ecosystem resilience (18–20). Further, loss of legume genetic resources will reduce the potential for new or genetically-improved protein-rich crop and pasture species important for food production (15–17). Reduced N-fixation through loss of legumes could result in quantifiable impacts on the global N cycle that could potentially mitigate anthropogenic N-deposition. However, even if N₂ fixation ceased in all grasslands globally, this would represent only 5–10% of anthropogenic N-deposition (1, 76), falling well-short of the estimated 45–75% reduction needed to restore the global N cycle below planetary boundaries (7, 27).

METHODS

Site selection. Our experiment was replicated at 45 sites participating in the global Nutrient Network experiment (42). Sites included natural and anthropogenic grasslands from six continents, most in temperate climate zones (SI Appendix Fig. S1 and Table S1). We included all Nutrient Network sites with N-fixing legumes in their regional composition (i.e. the entire spatial and temporal span for each site) and that had applied nutrient treatments for at least 3 years (see below). In sites with longer time spans (>7 yr), we used data from years 0 to 6 only (as only 12 sites, all from the USA, had >7 years of data). N-fixer status of legume species was reported by each site principal investigator, based on local literature. Sites ranged in latitude from 54° N to 37° S (SI Appendix Fig. S1), differing in initial degree of native versus non-native species dominance (70) and spanning a broad spectrum of climates and soils. Climatic conditions ranged from 252 to 1898 mm mean annual precipitation and 0.3 to 22.1°C mean annual temperature, and pre-treatment soils varied from 0.018–1.182 % N and 9.25–227.62 mg kg⁻¹ P (SI Appendix Table S1).

Experimental treatments. Each site established eight 5 x 5 m plots per block to accommodate a single replicate of eight treatments per block, with sites mostly containing 3 blocks (SI Appendix Table S1). All sites had the same treatments, involving factorial combinations of nitrogen, phosphorus and potassium addition and an untreated control. Nutrients were added annually as follows: 10 g N m⁻² yr⁻¹ as timed-release urea ((NH₂)₂CO), 10 g P m⁻² yr⁻¹ as triple-super phosphate (Ca(H₂PO₄)₂), 10 g K m⁻² yr⁻¹ as potassium sulphate (K₂SO₄). Plots receiving potassium also received a once-off addition of other macro- and micronutrients in the first year: 100 g m⁻² of a mix containing Fe (15%), S (14%), Mg (1.5%), Mn (2.5%), Cu (1%), Zn (1%), B (0.2%) and Mo (0.05%). Nitrogen addition was approximately three times the maximum Year 2000 estimated annual global N-deposition rate (1, 48), whereas P and K were added at substantially higher rates than currently occur (3, 47). We note though that our experimental timeframes of 3–6 years were short compared with decades to centuries of expected nutrient deposition. Further, our N addition rate was also less than those used in other studies to evaluate N limitation of N fixation (e.g. 20 or 40 g m⁻² yr⁻¹; (35)).

Response variables. Plant cover and biomass were measured annually, beginning one year before treatments were applied. Within each treatment plot, the cover of each vascular plant species was estimated to the nearest 1% in a permanent 1 x 1 m subplot during the season of peak biomass at each location. Sites with two biomass peaks were measured at each peak, and data averaged between sampling dates in each year. For each plot in each year, we calculated the cumulative cover for species belonging to four key functional groups: N-fixing legumes, other forbs, graminoids (grasses + sedges). Adjacent to the 1 x 1 m cover subplot, all aboveground biomass was clipped in two 1 x 0.1 m strips (0.2 m²), dried at 60°C and weighed to the nearest 0.01 g. Biomass was sorted to the functional group level in 30 of the 45 sites.

Legume abundance and diversity were expressed as cover, richness and biomass. For each response in each plot, we calculated In-ratio (LR) of the current over the initial values in each plot ($LR = \ln [(Y_{\text{final}} + 1) / (Y_{\text{initial}} + 1)]$), where Y_{initial} and Y_{final} are the initial and final values, and final values are either the third or last year of the study. We added 1 to the numerator and denominator because zeros make LR incalculable, although they can indicate an ecologically meaningful outcome (i.e. initial absence or total loss of legumes in the plot). We used current year / initial legume values as LR ratios (50) because this allowed us to study legume changes after accounting for variation in initial legume abundance and local conditions (8, 51), and to evaluate the effects of (standardized) initial conditions. We also checked for successional trends by evaluating changes in control plots in the LR analysis. In these plots, cover, richness, and biomass did not differ from initial values, suggesting that there were no perceptible successional changes. We also analysed absolute cover richness and biomass, with initial values as covariables, with similar results as described under Statistical analyses.

Site- and plot-level covariates. We used community, soil and climatic covariates to investigate controls on N-fixing legume responses to nutrient additions at the site and plot level. In some cases, we calculated the In-ratio of the covariate, using initial and final values as above (e.g. cover of grasses: LR_G , see below).

Plant community covariates. A range of plant community-level variables were calculated from the plant cover data for the third or last year of the experiment in each plot (plot-level predictors) or across all plots (site-level predictors). The latter were measured either at the beginning of the experiment or averaged across all plots in a site. At the plot level, we considered initial legume cover (%) and legume richness (number of species), initial total non-native cover (%), initial live biomass (g m⁻²) and third or last year abundance of grasses and forbs (%). At the site level, we calculated initial mean legume cover (%), legume richness (number of species), total live plant biomass (g m⁻²), mean native and non-native plant cover (%).

Photosynthetically active radiation (PAR, $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) was measured annually in the 1 m² cover-sampling subplots, in cloud-free conditions between 10am and 2pm during peak biomass (8, 42). One above-canopy and two perpendicular ground level measurements were made in each plot using a 1-m light ceptometer. Proportion of PAR was calculated as the ratio of the average ground level and the incident above-canopy PAR readings.

Soil covariates. We measured soil chemical properties at the plot level at the beginning of the experiment in 37 of the 45 sites (SI Appendix Table S1). In each plot, two 2.5 cm diameter x 10 cm depth soil cores, free of litter and vegetation, were collected, combined, homogenized, air-dried and shipped to a single laboratory (A&L Laboratories, Memphis, Tennessee, USA) for analysis using standard methods. In particular, we measured soil pH, N:P ratio, total N (%), extractable P (ppm), extractable K (ppm), Calcium (Ca, ppm), Magnesium (Mg, ppm), Sulfur (S, ppm), Sodium (Na, ppm), Manganese (Mn, ppm), Iron (Fe, ppm), Copper (Cu, ppm), and Boron (B, ppm) (see http://www.nutnet.org/exp_protocol; see (42) for further details).

Climatic covariates. Mean annual precipitation (MAP, mm), mean annual temperature (MAT, °C), and aridity index (AI) were derived from WorldClim (77) based on site location (42).

Statistical analyses. All analyses were conducted using R version R-3.4.0 (R Core Team, 2017). We used mixed effect-models (52, 54) to evaluate the effect of nutrient treatments and community or soil covariates on N-fixing legume LR-cover, LR-richness and LR-biomass. Models included nutrient addition

and covariates as fixed effects and block within sites as nested random effects (see below). In all cases, we analyzed data from the third and last measured year (3, 4, 5 or 6) separately. We included experiment duration (i.e. years under treatment) as a co-variate but it was never significant, so we do not report it. Initial cover of N-fixing legumes was significantly positively correlated with legume biomass and richness (Spearman correlation: $r=0.88$ and $r=0.60$, respectively), as were their respective LR_s ($r=0.58$, $r=0.28$). Given that we included sites based on legume presence at the site level, not all plots within a site had initial legume cover.

To evaluate possible bias originating from these differences, we tested the same models considering only those plots that initially contained legumes. Results did not differ qualitatively across these subsets of the data, and we present results of models fitted with the larger data set. Similarly, to avoid excessive zeros and error originating from spatial patchiness (e.g. because cover and biomass were necessarily sampled in different parts of each experimental plot), N-fixing legume biomass was analysed only in the 26 sites that reported biomass data for at least two years. Moreover, to indicate whether N-fixing legume responses differed from other elements of the vegetation, we tested the response of non-N-fixing forbs, graminoids and forbs + graminoids (LR_F, LR_G and LR_{F+G}) using an identical approach.

We also analyzed results using the absolute (non-ln-transformed) cover, richness or biomass values. As with LR, we analyzed these data using the factorial design or as eight independent treatments. We used initial cover, richness or biomass as covariates. Covariates were always conserved and concurred with LR in accounting for initial values in each plot. Untransformed and LR analysis led to similar conclusions. We opted for the LR approach in the main text because LR presents the relative change independently of site values, accounts for potential successional trends and is easily comparable with other studies. In addition, LR has better statistical properties and is superior for our experimental structure (50). Untransformed data is presented in the Supplementary section (SI Appendix Table S4).

To evaluate legume responses to different combinations of nutrient additions, we performed planned contrasts based on *a priori* questions (78). Contrasts are described in Fig. S4. Estimable functions were determined on the basis of the planned contrast for the fixed effects while conserving the structure of the model with respect to random effects (78, 79). In addition, to estimate the effect of nutrient addition on legume colonization (plots where legumes were initially absent but later present) or local extinction (plots where legumes were initially present but became extinct by the end of the experiment), we used generalized linear mixed effect models (54), with a binomial error distribution and a logit link function, including blocks within sites as the random structure. Colonization was measured as the number of new legume species divided by the final number of legumes in the plot, whereas extinction was measured as the number of legume species lost in the period divided by the initial legume richness (modified from (80)). We replicated this for all legumes, and for perennial and annual/biennial legume species separately.

To evaluate plot-level drivers of N-fixing legume response to treatments, we tested the effect of changes in community variables with treatments (i.e. LR_{PAR}, LR_{biomass} and LR_{F+G}), initial N-fixing legume abundance at the site level and soil N:P ratio for each plot. In particular, we evaluated whether competition and relative community change in each plot (i.e. relative change in the cover of forbs and grasses or change in biomass as proxies for competition intensity) affected legumes, including also quadratic terms (i.e. LR_{F+G} + LR_{F+G}²). Model selection was performed through stepwise elimination (e.g. function *stepAIC* in 'mass' package, (81)), with further eliminations using AIC criteria (54). In all these cases, to test the significance of parameter estimates, we generated confidence intervals using semi-parametric bootstrapping with 9,999 randomizations (53). These results were consistent with approximate P-values, but more conservative; as a reference, we also present approximate degrees of freedom based on Satterthwaite approximation (82).

To evaluate contingencies of nutrient effects on legume cover at the site level, we first calculated the main effects of N, P and K⁺ in each site as the N addition treatment x Site, P addition x Site and K addition x Site interactions in the full model, and then used initial soil, climatic or community variables at the site level as predictors of these changes. We utilized multiple regression

using step-wise elimination (e.g. function *stepAIC* in the ‘mass’ package (81)). We checked for collinearity through variance inflation factor (v.i.f) criteria; v.i.f. were lower than 2.8. Residuals were approximately homoscedastic and normally distributed. We repeated this analysis for the third year and last year data from the experiment.

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Data Availability

Source data are provided with this paper. The plant, PAR, climate and soil nitrogen data presented in the current study are also available in the Environmental Data Initiative (EDI) repository with the identifier <https://doi.org/10.6073/pasta/b569123d4b10d0861842e5eed9ce8118>

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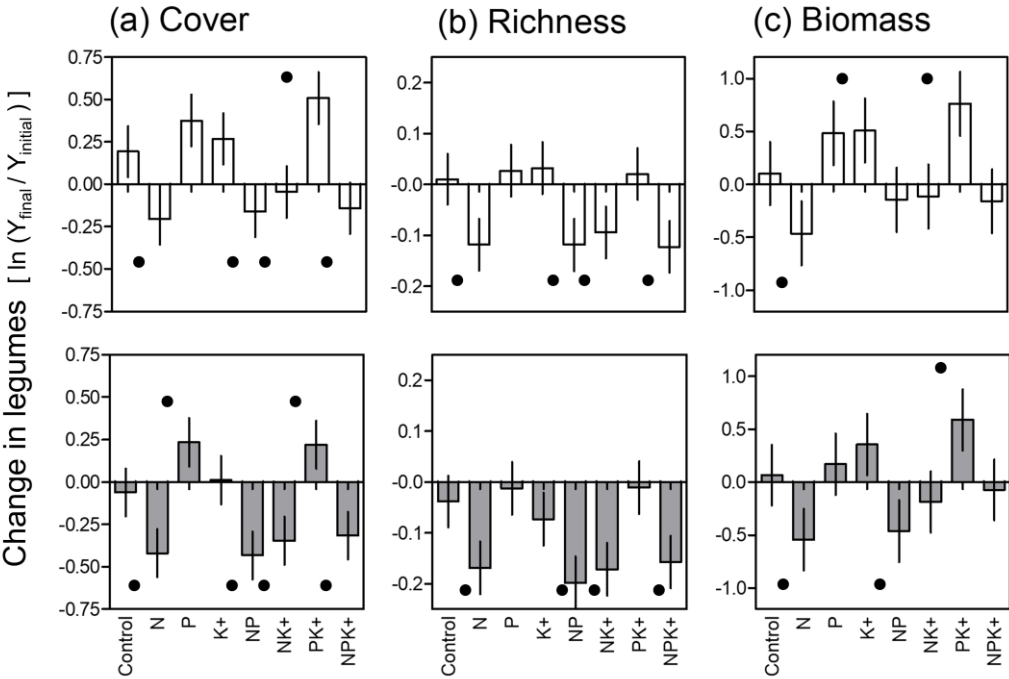
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Figure legends

Fig. 1. Change in legume cover (a), richness (b) and biomass (c) for the third year (top row) and last (third to sixth) year after initiation of the experiment (bottom row). Change were expressed as response ratios, the natural logarithm of the relative change from initial values (see Methods); positive and negative values indicate increases and decreases, respectively. Bars represent means \pm standard error of the means and dots (•) indicate treatment means that were statistically different from the controls. No response ratio in control plots were statistically different from zero, indicating that controls remained the same on average over time. Note the different Y-axis ranges. Cover and richness data were available for 45 sites and biomass data for 26 sites.

Fig. 2. Community drivers of the change in legume cover after three years of N addition. The relative change in legume cover (natural log of response ratio) was explained by nitrogen addition (red lines, versus blue lines for plots without N) and by the relative change in cover of non-leguminous forbs + grasses (LR_{F+G}) (a), the initial soil N:P ratio (b) and the initial cover of legumes of the site (c). Nitrogen (a and b) and N addition x legume cover (c) persisted as significant drivers ($P < 0.05$) after stepwise model reduction. Red symbols indicate plots without N addition, and red symbols indicate plots with N addition. Results for the last year at all sites were qualitatively similar (SI Appendix Table S7, S10-12).

Fig. 3. Colonization and extinction probabilities for legume species in plots without (blue symbols) and with (red symbols) N addition for the third and last experimental year. Symbols are estimates \pm bootstrapped 95% confidence intervals. Asterisks indicate that the logit of the confidence interval for the N effect did not include zero in the reduced model (SI Appendix Table S9). Colonization was assessed in plots where legumes were initially absent but later present, and extinction in plots where legumes were initially present but disappeared by the third or last year of the experiment. Estimates are based on 468 to 726 plots in 32 to 42 sites. SI Appendix Table S9 for further details and for colonization and extinction of annual and perennial legume species.

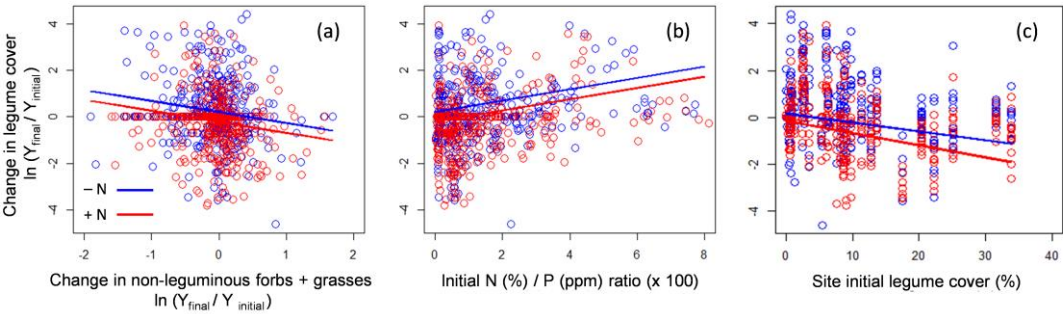


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Figure 2



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