RESEARCH ARTICLE

The synergistic response of primary production in grasslands to combined nitrogen and phosphorus addition is caused by increased nutrient uptake and retention

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Abstract

Background and aims A synergistic response of aboveground plant biomass production to combined nitrogen (N) and phosphorus (P) addition has been observed in many ecosystems, but the underlying mechanisms and their relative importance are not well known. We aimed at evaluating several mechanisms that could potentially cause the synergistic growth response, such as changes in plant biomass allocation, increased N and P uptake by plants, and enhanced ecosystem nutrient retention.

Methods We studied five grasslands located in Europe and the USA that are subjected to an element addition experiment composed of four treatments: control (no element addition), N addition, P addition, combined NP addition.

Results Combined NP addition increased the total plant N stocks by 1.47 times compared to the N treatment, while total plant P stocks were 1.62 times higher in NP than in single P addition. Further, higher N uptake by plants in response to combined NP addition was associated with reduced N losses from the soil (evaluated based on soil $\delta^{15}N$) compared to N addition alone, indicating a higher ecosystem N retention. In contrast, the synergistic growth response was not associated with significant changes in plant resource allocation.

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Conclusions Our results demonstrate that the commonly observed synergistic effect of NP addition on aboveground biomass production in grasslands is caused by enhanced N uptake compared to single N addition, and increased P uptake compared to single P addition, which is associated with a higher N and P retention in the ecosystem.

Keywords Synergistic growth effect · Nitrogen addition · Phosphorus addition · Grasslands · NutNet · Nitrogen losses · ¹⁵N natural abundance

Introduction

Aboveground plant biomass production in terrestrial ecosystems is commonly co-limited by nitrogen (N) and phosphorus (P) (Elser et al. 2007; Harpole et al. 2011; Fay et al. 2015). In many grasslands, the increase in aboveground plant biomass caused by a combined NP addition is higher than the sum of the increases caused by single N and P additions, which is called synergistic growth response or synergistic co-limitation (Elser et al. 2007; Craine and Jackson 2010; Harpole et al. 2011; Fay et al. 2015). The mechanisms driving this synergistic response to NP addition are still not well understood (Davidson and Howarth 2007; Harpole et al. 2011; Schleuss et al. 2020). Several interactive mechanisms from plant to ecosystem level may contribute to the synergistic response to NP additions in co-limited grasslands (Bloom et al. 1985; Davidson and Howarth 2007; Craine and Jackson 2010; Schleuss et al. 2020).

These potential mechanisms include: (1) greater aboveground biomass allocation in response to NP addition compared to single nutrient-addition, (2) increased plant N uptake in response to NP addition compared to the single N addition leading to a higher plant biomass production as well as (3) elevated plant P uptake in response to NP addition compared to P addition, and (4) reduced N and P losses from the

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ecosystem compared to single N or P addition, promoting the N and P retention and recycling, which increases their availability for further plant biomass production.

More specifically, the observed synergistic response of aboveground biomass production to NP addition might be caused by changes in plant biomass allocation patterns, according to the theory of optimal biomass allocation of plants (Bloom et al. 1985; Poorter et al. 2012; Cleland et al. 2019). This means that NP addition makes plants overcome their growth limitation by both nutrients, and thus enhances growth limitation by aboveground resources such as light. Investment in tissues for light acquisition increases the relative amount of biomass allocated aboveground compared to belowground. This reduction of the root mass fraction (the fraction of total biomass allocated belowground) could potentially explain the observed synergistic response of aboveground biomass production to NP addition (Yuan and Chen 2012; Cleland et al. 2019).

Both N and P are closely connected by the biological stoichiometry of cells (Elser et al. 2007), thus the lack of N can limit the uptake of P and vice versa (de Groot et al. 2003; Elser et al. 2007). Phosphorus is essential for energy storage (ATP) and cell growth (ribosomal RNA) (de Groot et al. 2003; Elser et al. 2007). Thus, low P availability can likely limit plant N uptake due to a low ATP concentration and P limitation of RNA production (Schjørring 1986; de Groot et al. 2003). Similarly, N is beneficial for building N-rich proteins such as P transporters, which is important for P uptake (Zeng et al. 2012; Perini and Bracken 2014). Thus, low N availability can limit the P uptake (Zeng et al. 2012; Perini and Bracken 2014). Therefore, the combined NP addition can increase simultaneously the N and P uptake and plant biomass production if plants are NP co-limited (Craine and Jackson 2010; Bracken et al. 2015; Schleuss et al. 2020). Otherwise, the N uptake is limited by the lack of P in single N addition and vice versa, P uptake can be limited by low N availability (de Groot et al. 2003; Bracken et al. 2015).

The higher N and P uptake by plants in response to NP addition can reduce ecosystem nutrient loss rates compared to a single N or P addition which can enhance the aboveground biomass production (Craine and Jackson 2010). Increased N uptake in response to NP addition compared to single N addition, might reduce N loss from the ecosystem since it can reduce

inorganic N leaching (NO_3^- and NH_4^+) and gaseous losses (N_2O, N_2, etc) (Craine and Jackson 2010; Baral et al. 2014; Mehnaz et al. 2019). Likewise, increased plant P uptake in response to NP addition compared to single P addition, might reduce P loss from the ecosystem due to leaching and runoff (Perring et al. 2008; Craine and Jackson 2010). The reduced nutrient losses due to higher plant N and P uptake can lead to an accumulation of organic N and P in the soil due to the higher nutrient inputs via plant litter which could become available for plants upon mineralization (Craine and Jackson 2010; Schleuss et al. 2020). In turn, the mineralization of the accumulated N and P facilitates an efficient nutrient recycling, and thus can promote the primary production and might foster a synergistic growth in response to combined NP addition (Craine and Jackson 2010; Schleuss et al. 2020).

Increased N retention should be reflected in the $\delta^{15}N$ of soil, which provides an integrated measurement of the N transformations in the ecosystem, in particular reflecting the past N losses (Watzka et al. 2006; Kriszan et al. 2014). Many N transformation processes in soils like nitrification, denitrification or organic N mineralization discriminate against the heavier ^{15}N isotope, leading to ^{15}N enrichment of the substrate (Högberg 1997; Denk et al. 2017). Thus, if the process is associated with a loss of the resulting ^{15}N-depleted N species from soil, for instance due to leaching or gaseous losses, it causes ^{15}N enrichment in the soil (Högberg 1997; Denk et al. 2017). Therefore, if the combined NP addition reduces the N losses compared to N added alone, the $\delta^{15}N$ of soil should be lower under NP than N addition.

While each of these mechanisms has been invoked to explain the synergistic responses to NP addition of aboveground biomass production in grasslands, the relative importance of these mechanisms remains unclear. Here we examine evidence for multiple mechanisms that could potentially cause a synergistic plant growth response to combined N and P additions in grassland ecosystems. For this purpose, we selected five grassland sites from a globally replicated nutrient addition experiment with evidence of synergistic NP co-limitation. At each of the five sites (three in the United States and two in Europe), the same experimental design is replicated including four treatments: N addition, P addition, N and P addition, and a control without element addition. We hypothesized that one or several of the following responses can be observed at the grassland sites that could potentially explain the synergistic growth effect of NP addition on plant aboveground biomass.

1) NP addition decreases the root mass fraction compared to single N and P addition.
2) NP addition increases the N uptake by plants compared to single N addition.
3) NP addition increases the plant P uptake compared to single P addition.
4) NP addition reduces N losses and hence the soil $\delta^{15}N$ compared to single N addition.

Material and methods

Study sites

The five study sites included in the present study (Table 1) are part of the Nutrient Network Global Research Cooperative (NutNet, https://nutnet.org) (Borer et al. 2014, 2017) and were chosen because they demonstrated evidence of synergistic NP colimitation of biomass across the last five years of sampling (Figs. S1-S2) and the nutrients had been added for at least eight seasons at the time of sampling. Further, this subset of sites was selected, because the sites represent a wide range of abiotic characteristics, allowing us to test the generality of mechanisms across environments. Sites are located between 200 and 2320 m above sea level (Table 1). Sites span a mean annual precipitation (MAP) range between 470 and 1166 mm and a mean annual temperature (MAT) range between 0.3 and 16.6 °C (Table 1). All study sites are natural and semi-natural grasslands, with two sites in Europe (Switzerland and Portugal) and three sites in the United States of America.

At each site, four treatments were studied: control without element addition (Ctrl), N addition of 100 kg N ha^{-1} yr^{-1} as slow-release urea (N), P addition of 100 kg P ha^{-1} yr^{-1} as triple super phosphate (P), and combined addition of 100 kg ha^{-1} yr^{-1} of both N and P (NP), following the same protocol and experimental design (Borer et al. 2014). All treatments were replicated three times ($n = 3$) in plots (5 m × 5 m = 25 m^2 $plot^{-1}$) organized in a randomized block design. At the time of sampling, the element addition treatments had been repeated for eight to 13 seasons (see Table 1). The aboveground

Table 1 Continent, country, region/state, site name, latitude and longitude, elevation, mean annual precipitation (MAP), mean annual temperature (MAT), estimated atmospheric nitrogen deposition (Ndep) and the seasons of element addition (i.e., years from establishment to the sample collection) of the five sites included in the study

Continent	Country	Region/State	Site	Latitude (°)	Longitude (°)	Elevation (m)	MAP (mm)	MAT (°C)	Ndep (kg N ha^{-1})	Seasons of element addition
North America	USA	Kansas	Konza.us	39.1 N	96.6 W	440	835	11.9	9.8	13
North America	USA	Kentucky	Spin.us	38.1 N	84.5 W	271	1166	12.5	10.7	13
North America	USA	Nebraska	Cdpt.us	41.2 N	101.6 W	965	470	9.5	3.1	13
Europe	Portugal	Ribatejo	Comp.pt	38.0 N	8.0 W	200	641	16.6	3.0	8
Europe	Switzerland	Graubünden	Valm.ch	46.6 N	10.4 E	2320	950	0.3	21.7	12

plant biomass was clipped and removed from the plots annually at the time of peak of biomass since the establishment of the experiment using a common protocol (Borer et al. 2014). The main soil properties of each site were analyzed before the first nutrient addition with methods described in Seabloom et al. (2021) (Table S2). The climate data are derived from Hijmans et al. (2005) and the N deposition was estimated using the model of Ackerman et al. (2019) for the last year of estimation (2016).

Plant and soil sampling and processing

Plant and soil samples were collected in 2020 during the peak of standing biomass using a common protocol. The aboveground plant biomass (called aboveground biomass hereafter) was clipped at the soil surface in two strips of 10×100 cm and sorted into live and dead biomass. The live biomass was dried at 60 °C until constant mass and weighed to the nearest 0.01 g. A representative aliquot of each aboveground biomass sample was sent to the University of Bayreuth (Germany) for further analyses. The cover estimates of grasses, forbs and legumes, and the four most abundant plants species of the control plots for 2020 at each site are summarized in Table 2. The cover for each plant species was estimated visually to the nearest 1% for each species rooted within a designated 1×1 m subplot at the time of peak of biomass. Total cover estimate may exceed 100% because species cover is estimated independently for each species. The aboveground plant biomass during the period 2015–2019 used to select the sites for the main sampling was collected following the same protocol.

Soil samples in 2020 were collected with a 3 cm diameter auger from the upper 0–15 cm soil depth within 24 hours after plant collection. The samples were collected from three different spots allocated within the plot avoiding the outer 50 cm of the plots and were combined to one composite sample per plot. The soil samples were collected in the space between plants trying to avoid the root crowns. The soil samples were kept moist in plastic bags and sent to the University of Bayreuth by express mail (one to two weeks of shipping time).

At the University of Bayreuth, the moist soil samples were sieved (<2 mm) and roots were picked out with tweezers, washed with deionized water, dried at

Table 2 Mean cover estimates of grasses, forbs and legumes and the four most abundant plants species in the three control plots of each site for the year 2020. Bold numbers in brackets are mean values of cover estimates (%) of each species in the control plots

Country	Site	Cover (%)			Most abundant plant species and their cover (%)
		Grasses	Forbs	Legumes	
USA	Konza.us*	117	30	4	*Schizachyrium scoparium* (MICHX.) NASH **(57)**
					Andropogon gerardii VITMAN **(23)**
					Symphyotrichum ericoides (L.) G.L. Nesom **(17)**
					Bouteloua curtipendula (MICHX.) TORR **(13)**
USA	Spin.us	62	39	1	*Poa pratensis* L. **(28)**
					Festuca arundinacea SCHREB. **(26)**
					Viola sp. L. **(15)**
					Dactylis glomerata L. **(8)**
USA	CdPt.us	50	17	3	*Carex filifolia* NUTT. **(16)**
					Hesperostipa comata (Trin. & Rupr.) Barkworth **(12)**
					Chondrosum gracile KUNTH **(10)**
					Artemisia frigida WILLD. **(8)**
Portugal	Comp.pt	48	65	12	*Tuberaria guttata* (L.) FOURR. **(17)**
					Avena barbata POTT *EX* LINK **(17)**
					Bromus diandrus ROTH **(13)**
					Plantago bellardi All. **(7)**
Switzerland	Valm.ch	29	65	8	*Hieracium pilosella* L. **(11)**
					Helianthemum nummularium (CAV.) LOSA & RIVAS GODAY **(10)**
					Vaccinium vitis-idaea L. **(7)**
					Festuca rubra L. **(7)**

*For the site Konza.us, the data shown is from 2021 season because plant cover data from 2020 was not available

60 °C until constant mass and weighed to the nearest 0.01 g. The root biomass was expressed in $g\ m^{-2}$ considering the soil bulk density measured using intact soil cores (Table S2). One soil aliquot (50 g) was dried at 50 °C, another aliquot (10 g) was used for the determination of the gravimetric water content after drying at 105 °C, and the remaining soil (150 g) was stored at 4 °C for further analyses. The water holding capacity (WHC) of soil samples was determined according to Öhlinger (1996).

Plant and soil chemical analyses

Total organic carbon (TOC) and total nitrogen (TN) concentrations of dried and milled samples of soil, above- and belowground plant biomass were determined using a CN element analyzer (Flash EA 1112, Thermo Fisher Scientific, Waltham, MA, USA). The total P (TP) concentration of dried and milled samples of above- and belowground plant biomass was determined by ICP-OES (Vista-Pro radial, Varian, Aschaffenburg, Germany) after nitric acid digestion (1 mL HNO_3 + 9 mL H_2O for 8 h at 170 °C). The soil

available P (Bray-P) was extracted using the Bray and Kurtz (1945) method and determined by spectrophotometry (UV-1800, Shimadzu Corporation, Kyoto, Japan) according Murphy and Riley (1962). Soil pH was determined in H_2O (1:5; weight:volume). Total organic P (TOP) was determined in dried and milled soil samples following the method described by Saunders and Williams (1955).

The $\delta^{15}N$ value of soil was measured in dried and milled soil samples using a continuous-flow isotope ratio mass spectrometer (NA 1108 elemental Analyzer, CE Instruments, Milano, Italy) coupled via a ConFlo III open-split interface (Finnigan MAT, Bremen, Germany) to a delta S isotope ratio mass spectrometer (Finnigan MAT, Bremen, Germany) at the Laboratory of Isotope Biogeochemistry of the University of Bayreuth. The isotopic N composition was expressed in δ notation and calculated as ^{15}N atom%. Soil net N mineralization and nitrification rates were determined after 28-days incubation of soil samples at 60% WHC. Further details about the soil N mineralization and nitrification rates can be found in the supplementary material.

Calculations and statistical analyses

The root biomass content was calculated in g kg^{-1} soil considering the root dry weight and the total soil dry weight of each sample. Furthermore, it was expressed in g of root biomass m^{-2} based on the soil bulk density and the rock fragments content. The root biomass expressed as g m^{-2} will be referred to as belowground plant biomass hereafter. The total plant biomass is the sum of above- and belowground plant biomass. The root mass fraction was calculated as the belowground biomass divided by the total biomass. The N and P stocks of aboveground and belowground biomass were calculated as the product of the respective element concentration (g kg^{-1}) and the corresponding plant biomass (g m^{-2}). The total plant N and P stocks were calculated as the sum of above- and belowground N and P stocks, respectively.

We calculated the normalized response (NR) of the parameters determined to facilitate the comparison of nutrient addition effects among sites as follows:

$$NR = \left(\frac{Y\ treatment}{Y\ control} \right) - 1$$

where $Y_{treatment}$ is the value measured for each parameter in the N, P or NP addition treatment and $Y_{control}$ is the value in the control treatment in the same block. Hence, normalized response values >0 represent a positive response to nutrient addition and normalized response values <0 represent a negative response.

Differences in normalized response (NR) were analyzed using a linear mixed model (LMM) with the software SPSS 27 (IBM SPSS, Inc., Chicago, USA). Since NR data were not normally distributed (Shapiro-Wilk-test, p > 0.05) all variables were log transformed ($\log_{10}(NR + 1)$) prior to run the LMM analysis. The different element addition treatments (Ctrl, N, P, NP) were used as fixed factor, while site and block (nested within site) were considered as random factors. The NR of the control treatment was equal to 0. When a significant treatment effect ($p < 0.05$) was found, LSD post hoc test ($p < 0.05$) was used for comparison of means of the element addition treatments.

Results

Plant biomass production and allocation

In the period 2015–2020, NP addition across all five sites increased the aboveground biomass by 273 g m^{-2} compared to control, while the sum of the increases due to single N and P addition was 99 g m^{-2} (Graphical abstract, Table 3 and Figs. S1-S2). Thus, the sites showed a clear synergistic NP co-limitation.

Table 3 Mean aboveground biomass in the control and the element addition treatments, together with the summed aboveground biomass response of the single nitrogen and phosphorus additions (N + P) and the combined nitrogen and phosphorus addition (NP) on aboveground biomass at each site ($n = 18$) and the means across the five sites ($n = 90$) for the period 2015–2020. N + P effect was calculated as the sum of aboveground biomass response to single N and to single P addition, and NP was calculated as the aboveground biomass response to combined NP addition. The response to each element addition treatment was calculated by substracting the aboveground biomass value of the control from the aboveground biomass value of each element addition treatment in the same block. Mean ± standard deviation

Site	Aboveground biomass 2015–2020				Aboveground biomass response 2015–2020	
	g m^{-2}				g m^{-2}	
	Ctrl	N	P	NP	N + P	NP
Konza.us	612 ± 258	653 ± 209	670 ± 338	889 ± 344	98 ± 348	277 ± 230
Spin.us	539 ± 204	640 ± 163	555 ± 193	826 ± 242	117 ± 425	287 ± 298
Cdpt.us	181 ± 85	288 ± 119	232 ± 101	354 ± 157	159 ± 179	174 ± 161
Comp.pt	312 ± 166	389 ± 186	316 ± 177	796 ± 331	82 ± 199	484 ± 244
Valm.ch	215 ± 99	219 ± 119	238 ± 108	329 ± 179	26 ± 11	114 ± 147
Mean	377 ± 233	447 ± 214	404 ± 241	648 ± 313	99 ± 281	273 ± 274

Ctrl, control; N, Nitrogen addition; P, Phosphorus addition; NP, combined N and P addition

In 2020, when the main samples for this study were taken, the aboveground biomass in the five sites ranged from 79 g m^{-2} in Valm.ch (Switzerland) to 762 g m^{-2} in Spin.us (USA) in the control treatment (Table S1). The NP addition increased aboveground biomass by 44% compared to control. Across all sites, the mean aboveground biomass in the control was 353 g m^{-2}, and in the NP treatment it was 509 g m^{-2} (Table S1). Thus, in 2020, NP addition increased aboveground biomass on average by 156 g m^{-2} compared to control, while the sum of the changes in aboveground biomass due to single N and single P addition was slightly negative (-38 g m^{-2}), which together indicates a synergistic NP co-limitation (Table S1, S3). Combined NP addition increased plant biomass by 47% compared to the N treatment and by 62% compared to single P addition (Fig. 1A, Table S4). Thus, across all the sites, we observed a clear synergistic NP co-limitation. The addition of N or P alone did not significantly affect the aboveground biomass compared to the control across all sites (Fig. 1A, Tables S1, S4).

When the aboveground biomass production is evaluated site by site, two of the sites (Cdpt.us and Spin. us) did not show a synergistic response to NP addition in 2020 (Table S1) despite the clear synergistic effect observed during the previous years (Table 3). Furthermore, according to the cover estimate, the plant species composition of the control treatment for the three sites located in USA was dominated by grasses, while Valm. ch was dominated by forbs and Comp.pt. showed a similar proportion of grasses and forbs (Table 2). Legumes had a cover of 12% in Comp.pt., 8% in Valm.ch and below 4% in the three sites in USA (Table 2).

Across all the sites, belowground biomass in the NP treatment did not significantly differ from the control treatment, while the single addition of N or P significantly decreased belowground biomass compared to the control by 13 and 11%, respectively (Fig. 1B, Tables S1, S4). Belowground biomass did not differ significantly among the three nutrient addition treatments (N, P, NP) (Fig. 1B, Tables S1, S4). None of the three nutrient addition treatments affected the total biomass (sum of above- and belowground
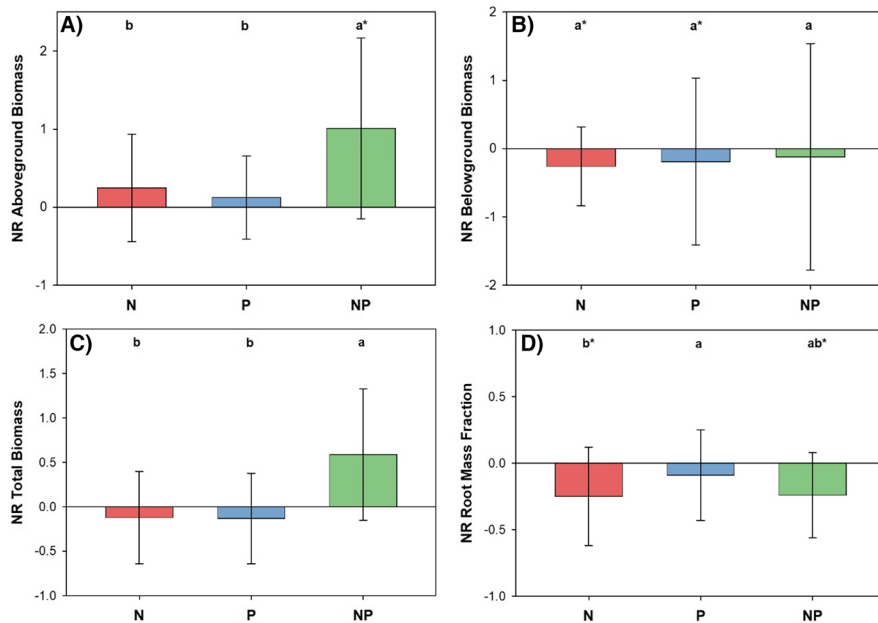

Fig. 1 Normalized response (NR) of aboveground biomass (**A**), belowground biomass (**B**), total biomass (the sum of above- and belowground biomass; **C**, and the root mass fraction (i.e., the proportion of the total biomass allocated belowground; **D** to nitrogen (N), phosphorus (P) and their combined addition (NP) across all five sites ($n = 15$). The normalized response was calculated dividing values of the treatments (N, P or NP) by the value of the control and subtracting 1. Columns show means, and error bars indicate standard deviations. Significant differences ($p < 0.05$) among element addition treatments (N, P and NP) are indicated by different letters, and among element addition treatments and control (Ctrl = 0) by an asterisk (*). Prior to analysis, all variables were log transformed

biomass) significantly compared to control (Fig. 1C, Tables S1, S4). However, total biomass in the NP treatment was significantly higher than in the N and P treatments (Fig. 1C, Tables S1, S4).

The mean root mass fraction across the five sites was 0.63 and ranged from 0.98 in Valm.ch (Switzerland) to 0.24 in Spin.us (USA) in the control treatment (Table S1). The root mass fraction (i.e., the portion of the total biomass allocated belowground) was significantly reduced by the N and NP treatments compared to control (Fig. 1D, Tables S1, S4). In addition, N addition reduced the root mass fraction compared to P addition, while no significant difference between P and NP was observed (Fig. 1D, Tables S1, S4).

Nitrogen and phosphorus contents in plant biomass

Across all sites, N and P concentrations (in mg g^{-1}) of above- and belowground biomass were significantly increased compared to control in the N and P treatment, and the strongest effects were observed in Konza. us and Valm.ch (Tables S4-S6). More specifically, the N concentration of aboveground biomass was significantly increased by both N and NP addition compared to the control (by 50% and 42%, respectively) and compared to the P treatment (by 49% and 41%, respectively) (Tables S4-S6). Similarly, the P concentration of aboveground biomass was significantly increased by P and NP addition compared to the control (by 71% and 80%, respectively) and compared to the N treatment (by 79% and 88%, respectively) (Tables S4-S6). The N concentration of belowground biomass was increased by N and NP addition compared to the control and P treatment. The P concentration of belowground biomass was higher in the P and NP treatments compared to the control and N treatment (Tables S4-S6).

The biomass stocks of N and P (in g m^{-2}) were significantly increased by NP addition compared to the control across all the sites (Fig. 2, Table S4) and the largest differences were observed in Konza.us, Comp. pt. and Valm.ch (Table S6). Particularly, the NP addition significantly increased the total plant N stock compared to the control by 65% and compared to the N and P treatments by 47 and 90%, respectively (Fig. 2E, Tables S4, S6). The N added alone significantly decreased the total P stock compared to the other treatments while the combined NP addition induced the significantly highest total plant P stock (Fig. 2F, Tables S4, S6).

Soil chemical properties

The TOC concentration in the uppermost 15 cm of the soils in the control plots ranged from 11.5 in Comp.pt. to 71.9 g kg^{-1} in Valm.ch (Table S8). The combined NP addition increased the TOC and TN concentrations in the 0–15 cm soil layer significantly compared to control and P treatment across the five grasslands (Fig. 3A, Tables S4). TOC and TN concentrations did not differ significantly between N and NP treatments (Fig. 3, Table S4). The soil pH decreased due to nutrient addition compared to control, particularly when N was added which decreased the soil pH by approximately 0.5 pH units across all sites (Tables S4, S7-S8). The addition of P, alone or combined with N, tripled the concentration of soil available P compared to the control and N treatment (Tables S4, S7-S8). However, no significant difference between treatments was observed in the TOP (Tables S4, S7-S8).

The δ^{15}N of soil (0–15 cm) in the control treatment of the five study sites ranged between 1.26 and 4.53‰ in Valm.ch and Konza.us (Table S8). The addition of N alone increased δ^{15}N (3.55‰) significantly compared to the control (2.86‰) while P and NP addition did not significantly affect the δ^{15}N compared to control (Fig. 3C, Table S4). The δ^{15}N of soil was significantly lower when N was added in combination with P (NP treatment, 3.14‰) than when N was added alone across all the sites (Fig. 3C, Table S4).

In the control treatment, the net N mineralization rate and the net nitrification rate ranged from 0.09 mg N kg^{-1} day^{-1} (Konza.us) to 1.85 mg N kg^{-1} day^{-1} (Valm.ch) and from 0.07 mg N kg^{-1} day^{-1} (Konza.us) to 1.90 mg N kg^{-1} day^{-1} (Valm.ch), respectively (Table S9). The addition of N, either alone or combined with P, significantly increased both net N mineralization and net nitrification rates in comparison to the control and the P treatment (Fig. 4, Tables S4, S9).

Discussion

Our results revealed that the synergistic response of biomass production in the NP treatment across the five studied grasslands is mainly driven by enhanced plant N and P uptake. Specifically, in the NP treatment, plant N uptake was significantly higher than in the N addition

Fig. 2 Normalized response (NR) of nitrogen and phosphorus stocks of aboveground (**A**, **B**), belowground (**C**, **D**) and total biomass (the sum of above- and belowground stocks) (**E**, **F**) to nitrogen (N), phosphorus (P) and their combined addition (NP) across all five sites ($n = 15$). The normalized response was calculated dividing values of the treatments (N, P or NP) by the value of the control and subtracting 1. Columns show means, and error bars indicate standard deviations. Significant differences ($p < 0.05$) among element addition treatments (N, P and NP) are indicated by different letters, and among element addition treatments and control (Ctrl=0) by an asterisk (*). Prior to analysis, all variables were log transformed
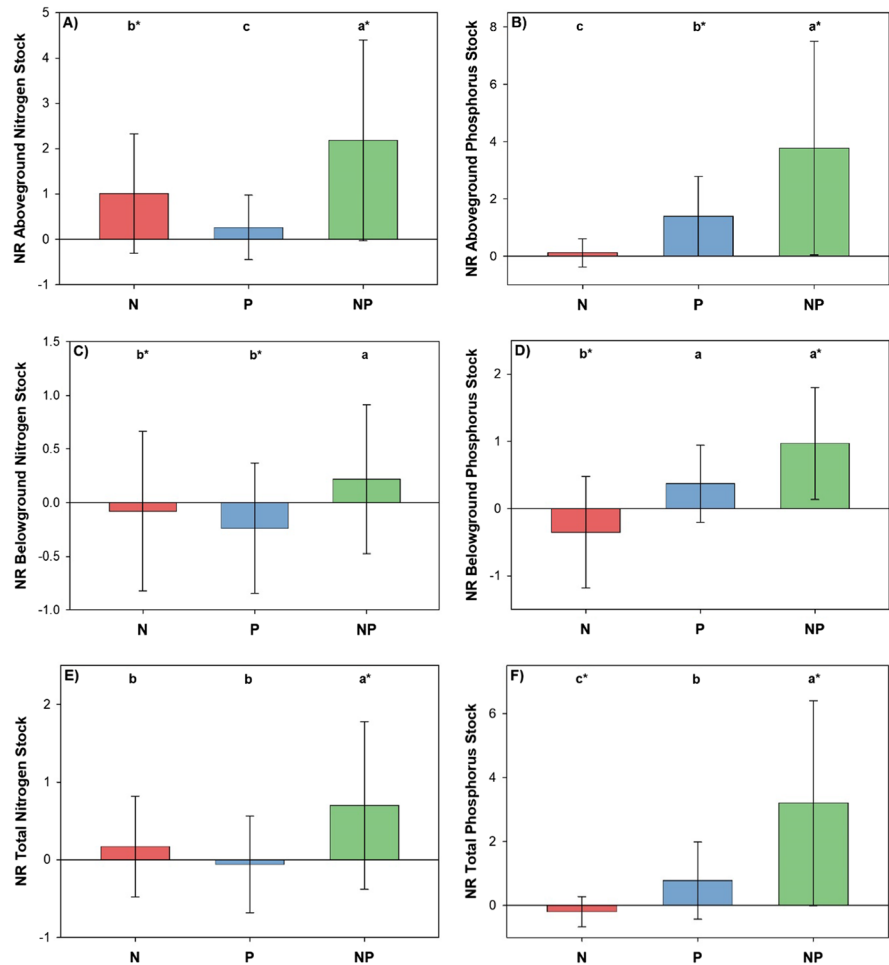
treatment, while plant P uptake in the NP treatment was significantly higher than in the P addition treatment. The enhanced N uptake by plants in the NP treatment compared to the N treatment is associated with a higher N retention in the grassland ecosystem. In contrast, the synergistic growth response was not associated with changes in plant resource allocation, i.e., changes in the ratio of aboveground-to-belowground biomass. By evaluating several interactive mechanisms from plant to ecosystem level, we reveal the relative importance of some mechanisms over others to explain the synergistic response to NP addition of biomass production.

Nutrient addition effects on plant biomass production and allocation

We observed a synergistic response of aboveground biomass to NP addition across the five sites (i.e.,

NP > N+P) at the main year of sampling (2020) (Fig. 1A) and during previous years (2015–2019) (Graphical Abstract, Table 3, Figs. S1-S3). The synergistic response of aboveground biomass to NP addition is consistent with previous studies showing a synergistic response to NP addition of aboveground biomass in grasslands (Elser et al. 2007; Harpole et al. 2011; Fay et al. 2015; Schleuss et al. 2020). Individually, all the sites also showed a synergistic response to NP addition for the 2015–2020 period (Table 3). However, two of the sites (Cdpt.us and Spin.us) did not show a synergistic response to NP addition in the year 2020 (Table S1). Addition of nutrients increases the plant biomass productivity but weakens the temporal stability of productivity (Hautier et al. 2014; Wang et al. 2017), which might contribute to the lack of synergistic effect in two of the sites in the year 2020 despite the clear synergistic effect observed during the previous years (Table 3).
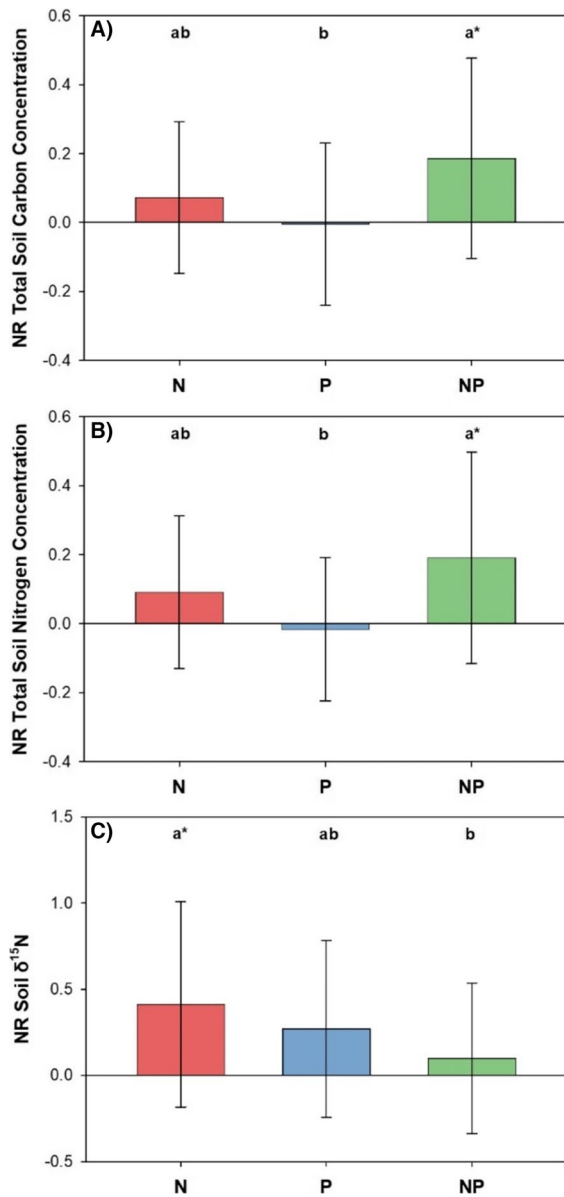
Fig. 3 Normalized response (NR) of total soil organic carbon (**A**) and total nitrogen (**B**) concentrations and $\delta^{15}N$ of soil samples (**C**) (0–15 cm) to nitrogen (N), phosphorus (P) and their combined addition (NP) across all five sites (*n* = 15). The normalized response was calculated dividing values of the treatments (N, P or NP) by the value of the control and subtracting 1. Columns show means, and error bars indicate standard deviations. Significant differences (*p* < 0.05) among element addition treatments (N, P and NP) are indicated by different letters, and among element addition treatments and control (Ctrl = 0) by an asterisk (*). Prior to analysis, all variables were log transformed

In addition, we relate the lack of synergistic effect at the site Cdpt.us in 2020 to the dry conditions in this year (40% lower precipitation in 2020 than in the period 2015–2019) that could have hampered the effect of nutrient addition on plant biomass production. A previous study performed in Cdpt.us observed that the effect of nutrient addition in plant biomass production is only observable in wet years when the water availability is not a limiting factor (Wang et al. 2017). In the following, and considering the robust synergistic response to NP addition observed in the five sites for the 2015–2020 period, we evaluate the potential underlying mechanisms that might have caused the synergistic growth response.

Our first hypothesis of reduced root mass fraction by NP addition compared to single N and P addition does not provide a strong explanation for the synergistic response of aboveground biomass production to NP addition in the five studied grasslands. We observed that the addition of N and NP decreased the root mass fraction compared to control (Fig. 1D), confirming changes in biomass allocation upon nutrient additions as described by the theory of optimal biomass allocation for plants (Bloom et al. 1985; Poorter et al. 2012; Cleland et al. 2019). However, across the five sites the NP addition did not change the root allocation response compared to both single N and P addition, indicating that a change in the biomass allocation is not the main mechanism driving the synergistic response to NP addition of aboveground biomass in the five studied grasslands.

The lower root mass fraction in the single N treatment compared to the control was caused by a decrease in the belowground biomass rather than an increase in the aboveground biomass (Fig. 1A-B). The reduction of root biomass in response to N addition is concordant with previous findings from element addition experiments in grasslands (Li et al. 2011). Combined NP addition did not significantly affect belowground plant biomass compared to control (Fig. 1A-B), which may indicate that another belowground resource (e.g., nutrient or water) may constrain aboveground plant growth in the NP treatment. Thereby, the lower root mass fraction in NP compared to the control was caused by increased aboveground plant biomass rather than by decreased belowground biomass as observed in the single N addition treatment. However, we only evaluated
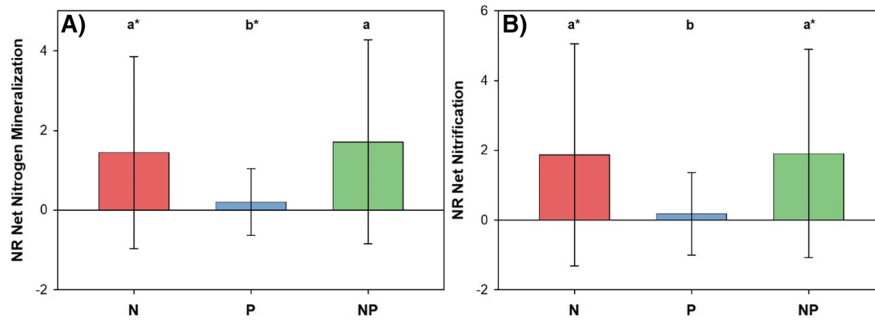
Fig. 4 Normalized response (NR) of net nitrogen mineralization (**A**) and net nitrification (**C**) of soil samples (0–15 cm) to nitrogen (N), phosphorus (P) and their combined addition (NP) across all five sites ($n = 15$). The normalized response was calculated dividing values of the treatments (N, P or NP) by the value of the control and subtracting 1. Columns show means, and error bars indicate standard deviations. Significant differences ($p < 0.05$) among element addition treatments (N, P and NP) are indicated by different letters, and among element addition treatments and control (Ctrl = 0) by an asterisk (*). Prior to analysis, all variables were log transformed

the root biomass in the first 15 cm of soil profile. While this is typically the soil increment with the highest root density, the sampling in the uppermost 15 cm may have underestimated the root biomass.

The response of the root biomass fraction to nutrient addition differed among the five sites (Table S1). We observed a lower effect of nutrient addition at the sites with higher root biomass fraction (Valm.ch and Cdpt.us) than at the sites with lower biomass fraction (Konza.us, Comp.pt. and Spin.us) (Table S1) which we relate to elevation of the sites (Table 1). Elevation can shape the plant biomass allocation at higher altitudes in order to increase the biomass allocated in storage organs as an adaptation to harsh environmental conditions (Körner and Renhardt 1987). Indeed, we observed an altitudinal pattern in our five sites with a high root biomass fraction (0.98 and 0.85 in the control treatment) in Valm.ch (2320 m) and Cdpt.us (965 m) respectively, intermediate root biomass fraction (0.62) in Konza (440 m), and low root biomass (0.48 and 0.24) in Comp.pt. (200 m) and Spin.us (271 m), respectively (Table 1 and Table S1). Thus, the response of root mass fraction to nutrient addition may have ben also affected by elevation. This assumption could be supported by Keller et al. (2023) who observed that the response of root biomass fraction to nutrient addition was weaker at the sites located at higher elevations compared to lower elevations in other nine NutNet sites.

Increased N uptake by plants upon NP addition

The total plant N stock was significantly higher in NP addition than in single N addition across the five sites (Fig. 2E) which is consistent with our second hypothesis. A similar result was described by Craine et al. (2008) in five South African grasslands. The reason for the higher total plant N stock in the NP treatment than in the N treatment is likely the alleviation of any potential P limitation of the uptake of the (added) N, which is beneficial for plant biomass production (Craine and Jackson 2010). In contrast low P availability in the N addition treatment might limit plant N uptake, thus hampering plant growth (Schjørring 1986; de Groot et al. 2003; Luo et al. 2022). Despite the general trend observed across the five sites, the increased N uptake by plants upon NP addition was not observed in Cdpt.us and Spin.us due to the lack of synergistic response of biomass production in the NP treatment in 2020 (Table S6). Because we did not observe differences in the N concentration of aboveground and belowground plant biomass between N and NP addition (Table S5), we assume that the increased N uptake by plants upon NP addition is only observable when a synergistic response of aboveground to NP addition occurs.

The N concentration of aboveground and belowground plant biomass was significantly increased by both N and NP addition compared to control (Table S5) as described by Firn et al. (2019) about the nutrient concentration in aboveground biomass of 27 grasslands. The increase in N concentration in the N treatment without subsequent plant growth can arise from luxury consumption of nutrients (Chapin 1980). In such situation, plants build up internal plant reserves to be used during period of nutrient scarcity

(Chapin 1980). No significant effect of single P addition on the N concentration was observed, confirming the NP co-limitation of the studied grasslands.

Combined NP addition increases plant P uptake

The total plant P stock was increased by NP addition compared to single P addition (Fig. 2F) confirming our third hypothesis. The highest total (above- and belowground) plant P stock was observed in NP compared to the other three treatments, indicating an enhanced P uptake by plants when P is added together with N. The higher P uptake when N and P were added together is concordant with previous studies in grasslands showing an increase of plant P uptake when N and P were combined (Long et al. 2016; Schleuss et al. 2020).

The positive effect of combined NP addition on P stocks may arise from the stimulation of the P transporter synthesis (which are proteins, hence rich in N) by the co-application of N and P, which in turn increases the P uptake by plants (Zeng et al. 2012; Perini and Bracken 2014). In addition, continuous N addition decreased soil pH (Table S7), which might enhance the dissolution of calcium-phosphates increasing the P availability for plants (Wang et al. 2022). Finally, N added alone reduced the total plant P stocks compared to control due to the lower belowground P stocks (Fig. 2). This indicates that the previously described positive effect of N addition on plant P uptake is only observed when N is added together with P, whereas single N addition can have a negative effect on plant P stocks. Finally, the P concentration of above- and belowground biomass was increased by P and NP and unaffected by N addition, compared to control (Table S5). This result confirms the NP co-limitation of the studied sites as discussed in section 4.2 for N concentration in plant biomass.

The observed higher P uptake by plants in the NP than in the P treatment might reduce the ecosystem P losses via leaching and runoff by storing the added inorganic P in plant biomass and soil organic P (Perring et al. 2008; Schleuss et al. 2020). These P losses via runoff could be particularly important at sites with high MAP or torrential precipitation events. However, based on our data we cannot confirm whether P retention is greater in NP compared to P added alone. No significant difference between treatments was observed in the soil TOP pool (Table S7), contrasting with results obtained by Schleuss et al. (2020) who found a substantial increase in soil TOP after 66 years of combined NP addition compared to only P addition. The difference between the present study and Schleuss et al. (2020) might be the different duration of the P addition treatment.

Our results have important implications for nutrient and fertilizer management in grasslands because they show that plants take up more P when P is added together with N compared to P added alone. This is important for a more sustainable use of the finite resource of phosphate rock used for P fertilizer production in managed grasslands.

Combined NP addition reduces N losses compared to single N addition

The combined addition of N and P significantly reduced the $\delta^{15}N$ of the soil compared to single N addition (Fig. 3C), confirming our fourth hypothesis. The reduced $\delta^{15}N$ of the soil suggests lower N losses in the NP treatment compared to the N treatment despite the same amount of N being added. This result was also consistent with six additional sites subjected to the same experiment that also had lower $\delta^{15}N$ in the NP (4.00‰) than in the N treatment (4.37‰) (Fig. S3, Table S10, for information about the sites see Schleuss et al. 2021). The reason for this might be that the NP addition alleviates any potential P limitation for plants or microorganisms caused by N addition alone, stimulating their N uptake and storage, and reducing the risk of N losses (Davidson and Howarth 2007; Baral et al. 2014; Mehnaz et al. 2019). The significantly higher plant total N uptake in NP than in the N treatment likely reduces the amount of N accumulated in the soil prone to gaseous and leaching losses. Other processes affecting the $\delta^{15}N$ of the soil like N_2 fixation by legumes do not seem to explain the observed differences between N and NP due to the low presence of legumes at the study sites (Table 3). This is also supported by a recent study showing low N_2 fixation rates in N and NP treatments at three of the study sites (Comp.pt., Valm.ch and Spin.us) in a recent study (Vázquez et al. 2022).

The higher N retention in the NP treatment increased the soil N concentration in NP compared to control and P addition. The increased litter inputs caused by the elevated plant biomass can contribute

to the build-up of a soil organic N stock, which becomes available for plants upon N mineralization. This higher N mineralization may be particularly important to supply inorganic N to plants in periods of reduced N inputs and hence, contributing to the synergistic response to NP addition of plant growth via N recycling. This is supported by the higher soil TN concentration (Fig. 3B), net N mineralization and nitrification in NP compared to control and P treatment, although no differences were observed between the N and NP treatment (Fig. 4), similarly as described by Schleuss et al. (2021). Our results contrast with previous studies (He and Dijkstra 2015; Mehnaz and Dijkstra 2016) which showed a stimulation of N mineralization and nitrification when N and P are added together compared to N added alone which the authors linked to higher N losses from the ecosystem. The reason for the contrasting results could be that plants at our sites were synergistically co-limited by NP instead of P limited as in the mentioned studies, which increased the N uptake and retention by plants in response to NP addition in our study. Otherwise, the stimulation of N mineralization and nitrification by NP addition could lead to higher N losses if the N mineralized and nitrified is not taken up by plants.

Finally, we observed a higher soil TOC concentration in the NP treatments than in control and P treatment (Fig. 3A) indicating an accumulation of organic matter in soils as a consequence of the higher biomass production. This finding contrasts with two previous studies evaluating the effect of N, P and NP addition on soil TOC storage in the short-term (2–4 years) and medium-term (a decade of nutrient addition) (Crowther et al. 2019; Keller et al. 2022). In both studies, no effect on soil TOC storage by N, P and NP addition was observed compared to control. Is it likely that the increase in the soil TOC concentration by NP addition observed in our study is because we selected sites with a synergistic response of aboveground biomass growth to NP addition.

Conclusions

We evaluated the different mechanisms potentially involved in the synergistic response of aboveground biomass production to NP addition in five grasslands that are part of a coordinated global experiment and

were selected because of their synergistic growth response. Our results support three of our hypotheses: NP addition increased the N uptake by plants compared to N added alone (second hypothesis), the combined NP addition increased the plant P uptake compared to P added alone (third hypothesis) and the higher N uptake in NP than in N reduced the N losses (fourth hypothesis). In contrast, changes in relative plant biomass allocation (between above and belowground) did not cause the synergistic growth effect in aboveground biomass in the studied grasslands, providing no support for our first hypothesis. While we found patterns across the five studied sites, there were differences in the responses to element addition across sites. Sites located at lower elevations showed stronger effects of nutrient addition on plant biomass allocation. Furthermore, an increased N and P uptake in NP was only observable at the sites that showed a synergistic response to NP addition of aboveground biomass production in the main year of study.

In summary, we show that the commonly observed synergistic effect caused by combined NP addition on aboveground grassland biomass production is caused by enhanced N uptake compared to N addition alone, and increased P uptake compared to P addition alone. The interaction of the element cycles that fosters plant nutrient uptake also increased C, N and P storage in the ecosystem, which has a positive feedback on plant nutrition and element storage. Our results highlight the importance of holistic studies using comparable management protocols in contrast to meta-analysis studies where the relative importance of the mechanisms could be obscured by methodological differences in the studies.

Declarations

Conflict of interest The authors declare no conflict of interest.

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