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ORIGINAL PAPER

Nitrogen addition has a stronger effect on stoichiometries of non-structural carbohydrates, nitrogen and phosphorus in *Bothriochloa ischaemum* than elevated CO_2

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Abstract The nutrient concentrations and carbon:nitrogen:phosphorus stoichiometries of plants have been greatly altered by elevated CO_2 concentrations and nitrogen (N) deposition. Studies of these changes, however, are mostly limited to tropical and subtropical forests. In this study, a C_4 herbaceous grass (*Bothriochloa ischaemum*) was grown in a pot experiment at two CO_2 concentrations (400 and 800 μmol mol^{-1}) and three levels of N fertilization (0, 2.5 and 5 g N m^{-2} y^{-1}) in a full factorial design. Plant biomass and the concentrations of non-structural carbohydrates (NSCs), N and phosphorus (P) in the shoots and roots were determined. The elevated CO_2 concentration and N addition significantly increased total biomass, starch and NSC concentrations and root:shoot, starch:N, NSC:N, starch:P and NSC:P ratios in whole plants and roots. N addition alone decreased the soluble sugar (SS) concentration in whole plants and increased N concentration and decreased P concentration in whole plants and roots and thus decreased the SS:N ratio and increased the SS:P and N:P ratios. Our results suggested that SS, N and P concentrations and their stoichiometries responded more strongly to N addition than elevated CO_2 concentration. Plant growth in this region suffers more from P than N limitation, and N addition would exacerbate the P limitation on plant growth.

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Introduction

Atmospheric CO_2 concentrations have been increasing dramatically due to anthropogenic activities, from approximately 280 μmol mol^{-1} to the current level of 400 μmol mol^{-1}, and are projected to exceed 800 μmol mol^{-1} by 2100 (NOAA 2013). Atmospheric nitrogen (N) deposition has also increased, by three- to five-fold within the last century, and is expected to continue to rise (Galloway et al. 2008). Elevated CO_2 levels and N deposition have affected global biogeochemical cycles, particularly the carbon (C), N and phosphorus (P) cycles in terrestrial ecosystems (Heimann and Reichstein 2008; Gruber and Galloway 2008; Marklein and Houlton 2012). The altered ecosystemic nutrient cycles may lead to shifts in C:N:P stoichiometry in plant-soil systems (Yang et al. 2011; Huang et al. 2015a). The stoichiometric flexibility of elemental ratios, in return, may influence biogeochemical cycles by their effects on the material cycle in natural ecological systems (Huang et al. 2015b; Liu et al. 2015). Obtaining sufficient concentrations of nutrient elements and maintaining relatively stable stoichiometries in plant tissues is vital for health (Han et al. 2011). Our knowledge of the relationship between the global environmental changes and ecosystem stoichiometric responses is very important for understanding future biogeochemical cycles in terrestrial biosphere (Sardans et al. 2012; Huang et al. 2012; Liu et al. 2013a).

Increased atmospheric CO_2 concentration stimulates photosynthesis and plant biomass, which are affected by most environmental stresses, such as nutrient limitation and water deficits (Newingham et al. 2013; Dong et al. 2016; Xiao et al. 2016). The sustainability of ecosystemic responses to CO_2 maybe constrained by the progressive N limitation induced by the stimulation of growth from increased CO_2 levels (Luo et al. 2004; Reich et al. 2006). The interaction of elevated CO_2 levels and N addition would therefore synergistically increase plant biomass (Lee et al. 2010; Zhang et al. 2011; Novriyanti et al. 2012; Dong et al. 2016). A meta-analysis also showed that both grassland above- and belowground biomasses increased with a combination of CO_2 elevation and N fertilization (Sillen and Dieleman 2012). Plant C:N and C:P ratios usually increase due to the dilution of N and P concentrations by the accumulation of carbohydrates (Novotny et al. 2007; Stiling and Cornelissen 2007). Elevated CO_2 levels and N deposition can also alter the structures of soil microbial communities (Lee et al. 2015) and significantly increase soil respiration and nutrient (e.g. N and P) loss from decomposing litter (Deng et al. 2013; Liu et al. 2015). The availability of soil N and P can thus be increased under elevated CO_2 levels in combination with N addition (Huang et al. 2014), thereby increasing plant N and P concentrations (Huang et al. 2015a). Plant N:P ratios, however, maybe not affected (Huang et al. 2015a), may increase (Huang et al. 2012) or may decrease (Liu et al. 2013a), depending on species (Liu et al. 2013a) and soil nutrient conditions (Huang et al. 2012, 2015a). Most studies until recently have focused on elemental-nutrient stoichiometries on the leaf-level rather than the level of whole plants (Liu et al. 2012; Huang et al. 2012). Mineral elements can respond to elevated CO_2 levels and N addition more strongly in roots than leaves (Liu et al. 2013a; Huang et al. 2015c), and leaf-level responses may not be representative of whole-plant responses. The responses of different plant tissues to elevated CO_2 levels and N addition, however, remain largely unexplored.

Carbohydrates are the main products of plant photosynthesis and are be partitioned mostly into structural carbohydrates (SCs) and non-structural carbohydrates (NSCs), based on their roles (Song et al. 2016). SCs, including lignin, cellulose, hemicelluloses and pectin, are mainly used for the structural growth of plants; NSCs, mainly soluble sugar (SS) and starch, provide substrates for plant growth and metabolism (Quentin et al. 2015; Hartmann and Trumbore 2016). SS is the main form for translating carbohydrates, and starch is the pivotal non-soluble longer-term storage compound. The concentrations of NSCs strongly influence plant growth and are sensitive to environmental changes (Ibrahim et al. 2011; de Souza et al. 2013). The proportional relationships between N, P and NSCs, to a large extent, reflect the amount of available C for plant growth and its use efficiency (Yin et al. 2009; Guo et al. 2015). Previous studies have mainly focused on the foliar stoichiometries of C, N and P, but the responses of NSC, N and P stoichiometries to elevated CO_2 levels and N addition, and the ecological significance of these responses, are unclear, strongly hindering our understanding of the availability of C for plant growth and its relationships with elemental N and P.

Bothriochloa ischaemum (L.) Keng is a dominant species in natural grassland communities, which is widespread in the hilly-gully regions on the Loess Plateau. The current rate of N deposition in this area is 2.2 g N m^{-2} y^{-1} (Wei et al. 2010; Han et al. 2013) and is expected to increase in the future (Galloway et al. 2004). Elevated global levels of CO_2 and N deposition would synergistically lead to changes in the nutrients cycles in this temperate area. The levels of both soil total N and P are low on the Loess Plateau (Liu et al. 2013b) and differ greatly from those in temperate and tropical forests, which are usually regarded as N- and P-poor, respectively (Huang et al. 2012). The response of perennial grass species to elevated CO_2 levels in combination with N deposition has rarely been studied. The aim of the present research was to evaluate the concentrations and stoichiometries of NSCs, N, and P in whole plants, shoots and roots of *B. ischaemum* under elevated CO_2 levels combined with N additions. We further hypothesized that (1) roots would respond more strongly than shoots to elevated CO_2 concentration and N addition and (2) N addition would have a stronger effect than elevated CO_2 concentration on the concentrations and stoichiometries of NSCs, N and P in this nutrient-poor area.

Materials and methods

Plant material and soil

Seeds of *B. ischaemum*, a C_4 perennial herbaceous grass species, were collected in the autumn of 2013 from the experimental fields at the Ansai Research Station (ARS) (36°51′30″N, 109°19′23″E), Chinese Academy of Sciences, on the Loess Plateau of China. The station has a mean annual temperature of 8.8 °C, and receives a mean annual precipitation of 510 mm, most of which occurs from July to September. The rates of seed germination were >90% when germinated on moist filter paper in Petri dishes at 25 °C prior to the experiment.

The soil was collected from the upper 20 cm layer of a farmland at ARS. Soil gravimetric moisture content as a percentage of field capacity (FC) and the wilting point were 20 and 4%, respectively. The soil organic carbon, total nitrogen, and total phosphorus content were 1.50, 0.21, and 0.57 g kg^{-1}, respectively. The soil was sieved through a 5 mm mesh before the experiment.

Experimental design

The cylindrical plastic pots (20 cm in depth, 15 cm in diameter) with sealed bottom were filled with 3.8 kg soil. Multiple seeds were sown in the pots on 1 June 2014. All pots were well watered in order to ensure seedling establishment. After emergence, seedlings were thinned to three plant per pot. The pots were transferred to two closed climate-controlled chambers (AGC-D001P, Qiushi Corp., Hangzhou, China) on 1 August 2014. The environmental conditions of the two chambers have been described detail elsewhere (Xiao et al. 2016). Briefly, one chamber received ambient CO_2 concentration (400 µmol mol^{-1}), the other received elevated CO_2 concentration (800 µmol mol^{-1}). The pots in each chamber received N fertilizer by spraying the seedlings twice a month at rates of total NH_4NO_3–N of 0, 2.5 and 5 g N m^{-2} y^{-1}, respectively. All pots were regularly weighed, and water was added through plastic pipes to above 80% of FC until the end of the experiments. There were a total of six treatments were included: ambient CO_2 concentration and no N fertilization (AN_0), ambient CO_2 concentration and low-N fertilization (AN_1); ambient CO_2 concentration and high-N fertilization (AN_2); elevated CO_2 concentration and no N fertilization (EN_0), elevated CO_2 concentration and low-N fertilization (EN_1); elevated CO_2 concentration and high-N fertilization (EN_2). Each treatment had five replicates.

Sample collection and measurement

At the end of growing season, the three plants in each of pot were harvested. The shoots were severed from the roots at the crown. The roots were collected from the soil and carefully washed. Shoots and roots were dried at 80 °C for 48 h in oven to determine the dry weights (DW), respectively. Shoots and roots were ground using a mortar and pestle to fine powder prior to NSC, N, and P analyses.

NSCs were defined as the sum of SS and starch concentrations, which were measured using the anthrone method (Yemm and Willis 1954). The details of the extractions and measurements are described by Zhang et al. (2015). SS and starch concentrations were expressed as mg g^{-1} DW.

N and P concentrations were measured by Kjeldahl method and molybdenum blue method, respectively (Bao 2000). The N and P concentrations were presented in units of mg g^{-1} DW.

Statistical analysis

The SPSS 16.0 (SPSS Inc., Chicago, USA) was used for data analysis. Two-way analysis of variance (ANOVA) was conducted to evaluate the effects of CO_2 and N treatments and their interactions on the concentrations and stoichiometries of NSCs, N and P based on whole plants, shoots and roots. Differences between each treatment were compared by Duncan's multiple range tests at a probability level of 0.05.

Results

Plant Biomass

The elevated CO_2 concentration and N addition had significantly positive effects on total, shoot and root biomasses and the root:shoot ratio (P <0.01 or <0.001) (Table 1). The elevated CO_2 concentration increased total biomass by 47.14, 71.05 and 51.04% in the EN_0,

Table 1 Effects of elevated CO_2 concentration and nitrogen addition on plant biomass and its allocation

Treatment	Total biomass (mg)	Shoot biomass (mg)	Root biomass (mg)	Root to shoot ratio
AN_0	466.67 ± 51.32a	280.87 ± 23.58a	185.8 ± 27.83a	0.66 ± 0.05a
AN_1	1013.33 ± 247.86b	581.22 ± 153.55b	432.11 ± 97.7a	0.75 ± 0.08ab
AN_2	2716.67 ± 358.38d	1473.87 ± 110d	1242.79 ± 249.93c	0.84 ± 0.11bc
EN_0	686.67 ± 75.06ab	406.55 ± 68.04ab	280.12 ± 18.14a	0.70 ± 0.10ab
EN_1	1733.33 ± 94.52c	879.59 ± 54.56c	853.74 ± 41.76b	0.97 ± 0.03c
EN_2	4103.33 ± 290.23e	1966.89 ± 285.25e	2136.44 ± 178.17d	1.10 ± 0.19d
F values of two-way ANOVAS				
CO_2	55.751***	20.151***	55.916***	12.028**
N	263.861***	144.952***	190.546***	11.336**
CO_2 × N	10.585**	2.428	13.633**	1.840

Different letters with a column indicate significant differences (P < 0.05) based on Duncan's multiple range test

*P < 0.05

**P < 0.01

***P < 0.001

EN_1 and EN_2 treatments, respectively. N addition at the ambient CO_2 concentration increased total biomass by 117.17 and 482.14% in the AN_1 and AN_2 treatments, respectively, and N addition at the elevated CO_2 concentration increased total biomass by 152.43 and 497.57% in the EN_1 and EN_2 treatments, respectively. The interaction of the elevated CO_2 concentration and N addition significantly affected total and root biomass ($P < 0.01$).

NSC, N and P concentrations

The elevated CO_2 concentration significantly increased the starch and NSC concentrations in whole plants, shoots and roots ($P < 0.01$ or < 0.001) (Table 2; Fig. 1). N addition significantly decreased the SS concentration in whole plants and shoots and significantly increased the starch and NSC concentrations in whole plants, shoots and roots ($P < 0.01$ or < 0.001). The interaction of the elevated CO_2 concentration and N addition significantly affected only the SS concentration in the shoots, with the highest and lowest values in the AN_0 and EN_2 treatments, respectively ($P < 0.05$) (Table 2; Fig. 1b).

The elevated CO_2 concentration had a significantly negative effect on the whole-plant P concentration ($P < 0.05$) (Table 2; Fig. 1) but not the P concentrations in the shoots and roots. N addition significantly increased the N concentrations in whole plants and roots and significantly decreased the P concentrations in whole plants, shoots and roots ($P < 0.01$ or < 0.001). The interaction of the elevated CO_2 concentration and N addition significantly affected the P concentration in roots ($P < 0.05$) (Table 2).

Stoichiometries of NSCs, N and P

The elevated CO_2 concentration significantly increased the starch:N and NSC:N ratios in whole plants, shoots and roots (Table 3; Fig. 2). N addition significantly decreased the SS:N ratio in whole plants, shoots and roots and significantly increased the starch:N and NSC:N ratios in whole plants and roots. The elevated CO_2 concentration and N addition did not have a significant interactive effect on the SS:N, starch:N or NSC:N ratios.

The elevated CO_2 concentration significantly increased the SS:P ratio in roots and the starch:P and NSC:P ratios in whole plants, shoots and roots (Table 3; Fig. 3). N addition significantly increased the SS:P ratio in whole plants and shoots and the starch:P and NSC:P ratios in whole plants, shoots and roots. The elevated CO_2 concentration and N addition had interactive effects on the starch:P and NSC:P ratios in roots.

The elevated CO_2 concentration significantly increased the N:P ratio in whole plants (Table 3; Fig. 3), and N addition significantly increased the N:P ratio in whole plants, shoots and roots. The elevated CO_2 concentration and N addition did not have significant interactive effects on plant N:P ratios.

Discussion

Plant biomass

Soil nutrient availability modifies the responses of plant species to elevated CO_2 levels. Soil total N content is low on the Loess Plateau (Liu et al. 2013b), so N addition would be better for plant growth. The growth and biomass

Table 2 Significance (F-values) of the effects of elevated CO_2 concentration, N addition and their interactions on NSC, N and P concentrations (The values were presented in Fig. 1)

Source	SS	Starch	NSC	N	P
Whole plants					
CO_2	2.933	76.805***	49.846***	0.002	7.531*
N	38.267***	153.022***	80.842***	6.304**	53.706***
$CO_2 \times N$	2.282	1.398	1.992	0.175	2.433
Shoots					
CO_2	0.813	90.954***	57.183***	0.498	2.795
N	25.242***	21.227***	7.204**	2.168	38.444***
$CO_2 \times N$	3.916*	0.117	0.256	0.089	1.837
Roots					
CO_2	2.190	9.444**	14.989**	0.093	2.766
N	1.032	138.023***	160.855***	23.870***	6.489**
$CO_2 \times N$	0.088	2.004	2.726	0.336	4.029*

*$P < 0.05$

**$P < 0.01$

***$P < 0.001$

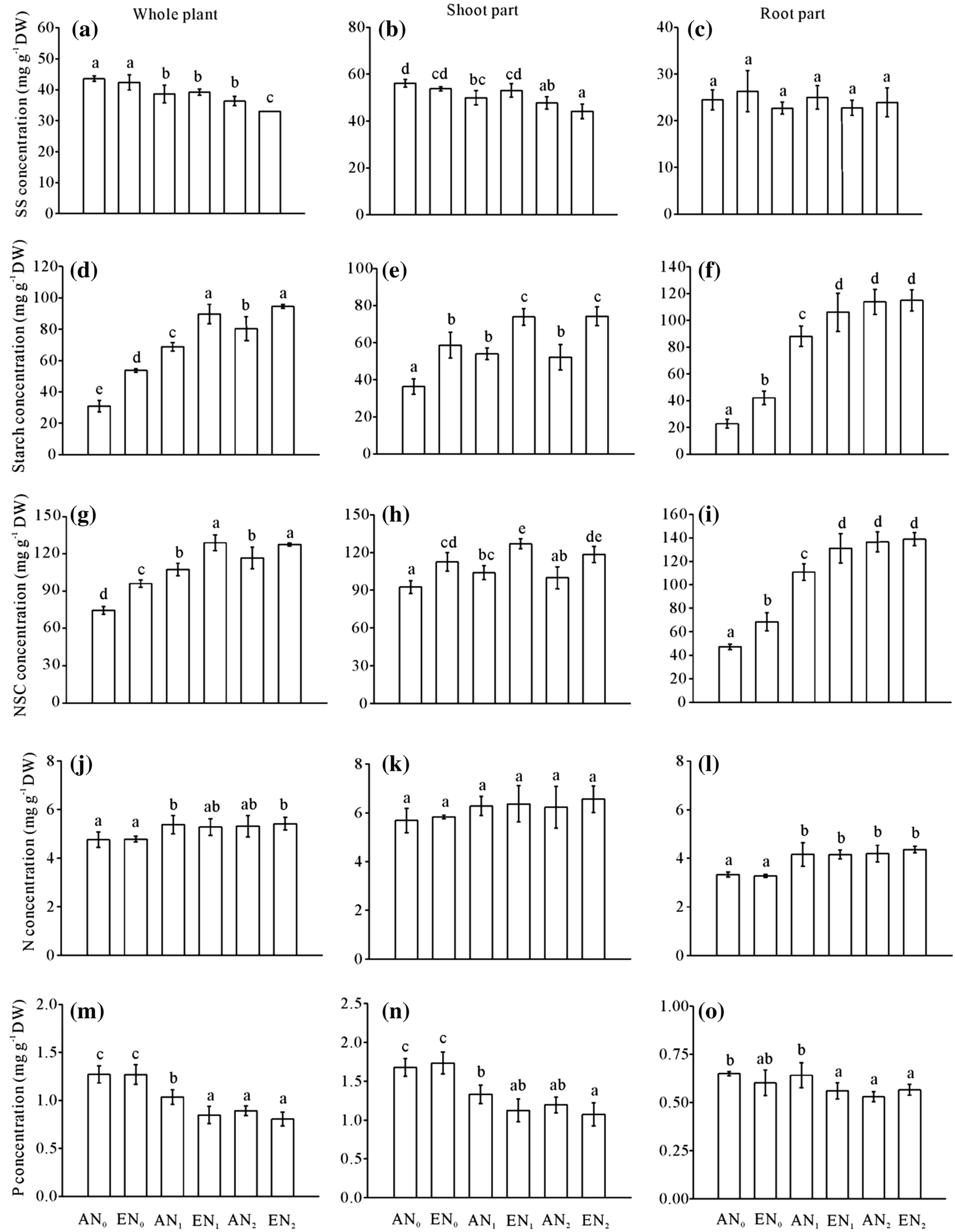

Fig. 1 SS (**a–c**), starch (**d–f**), NSC (**g–i**), N (**j–l**) and P (**m–o**) concentrations in whole plants, shoots and roots of *B. ischaemum*. Different letters indicate significant differences ($P < 0.05$) based on Duncan's multiple range test

Table 3 Significance (*F* values) of the effects of elevated CO_2 concentration, N addition and their interactions on the stoichiometries of NSCs, N and P

Source	SS:N	Starch:N	NSC:N	SS:P	Starch:P	NSC:P	N:P
Whole plants							
CO_2	0.558	34.804***	18.175***	3.600	49.309***	33.572***	5.827*
N	31.644***	44.198***	11.909***	9.305**	103.662***	71.441***	34.105***
$CO_2 \times N$	0.467	0.437	0.364	4.521*	2.742	3.383	1.265
Shoots							
CO_2	1.093	33.448***	9.676**	2.163	46.080***	24.564***	3.133
N	10.666***	3.010	0.663	8.707**	27.714***	20.716***	14.726***
$CO_2 \times N$	1.436	0.466	0.446	3.069	1.472	1.911	0.722
Roots							
CO_2	1.000	6.122*	6.548*	8.878*	9.029**	14.145**	1.891
N	11.502**	68.508***	43.085***	0.689	99.258***	113.316***	29.288***
$CO_2 \times N$	0.229	2.968	2.948	2.664	4.008*	5.940*	1.747

The values were presented in Figs. 2, 3

*$P < 0.05$

**$P < 0.01$

***$P < 0.001$

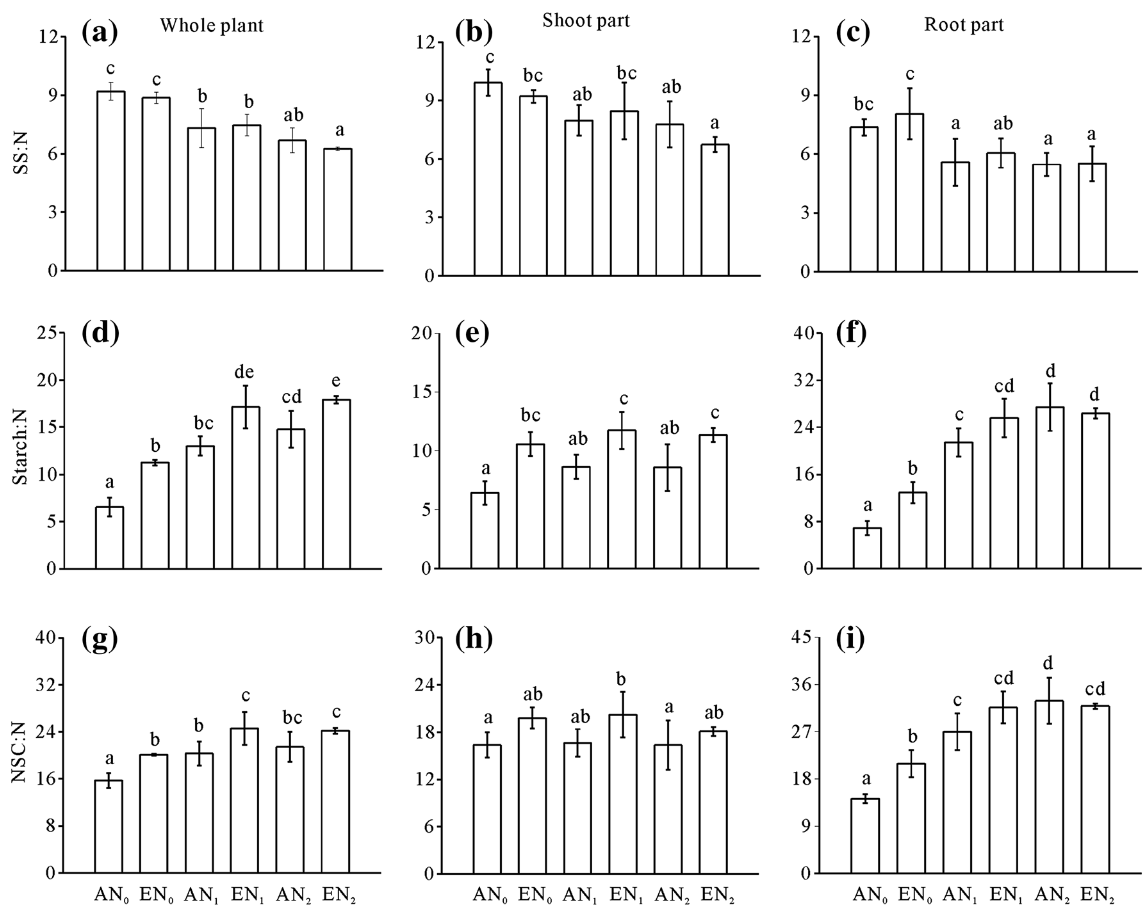

Fig. 2 SS:N (**a–c**), starch:N (**d–f**) and NSC:N (**g–i**) ratios in whole plants, shoots and roots of *B. ischaemum*. *Different letters* indicate significant differences ($P < 0.05$) based on Duncan's multiple range test

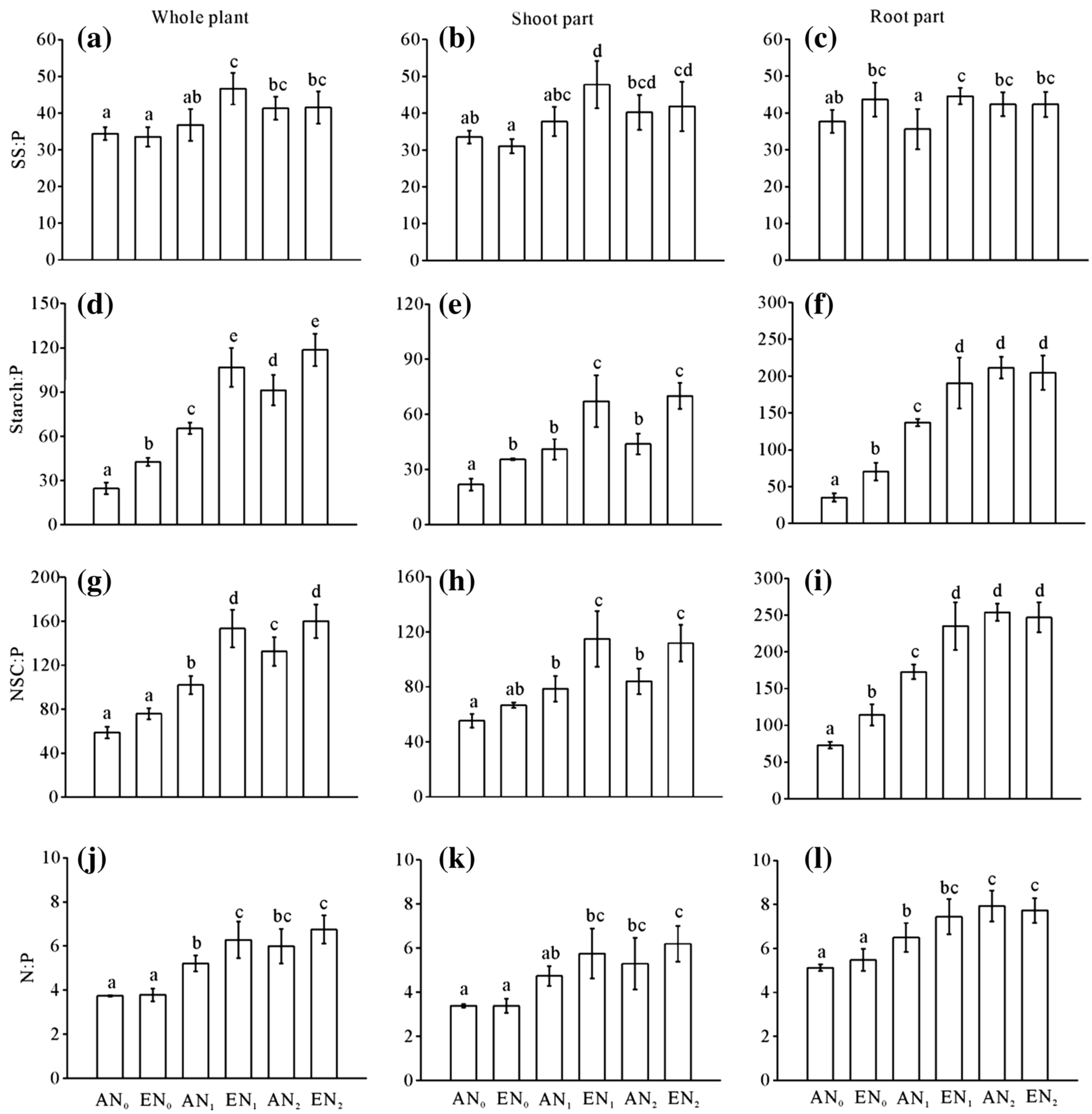

Fig. 3 SS:P (**a–c**), starch:P (**d–f**), NSC:P (**g–i**) and N:P (**j–l**) ratios in whole plants, shoots and roots of *B. ischaemum*. *Different letters* indicate significant differences ($P < 0.05$) based on Duncan's multiple range test

allocation of plants in response to elevated CO_2 levels and N addition are species-specific (Zhang et al. 2010, 2011). The significant positive effect in our study of the elevated CO_2 concentration and N addition on the root:shoot ratio implies that root growth in *B. ischaemum* is more sensitive than shoot growth to elevated CO_2 levels and N addition, which supports our first hypothesis.

NSC, N and P concentrations

NSCs are products of photosynthesis and provide energy for plant growth and metabolism. NSCs play a central role in the response of plants to environmental changes (Quentin et al. 2015). Elevated CO_2 levels and N addition can synergistically enhance plant photosynthesis (Zhang et al. 2011; Chen et al. 2016) and thus increase NSC accumulation (Zhang et al. 2008; Zhu et al. 2016). Other studies have reported that elevated CO_2 levels and/or N addition had no influence on, or even decreased, NSC concentrations due to higher carbohydrate use and the constraints of soil environmental stresses (Kakani et al. 2011; Pelletier et al. 2009; Alderman et al. 2011; Farrer et al. 2013; Sullivan et al. 2015; Liu et al. 2016). In the present study, the elevated CO_2 concentration and N addition synergistically increased NSC accumulation, mainly due to the increase in starch. The SS content did not change significantly and even decreased under the elevated CO_2 concentration and/or N addition. The elevated CO_2 concentration and N addition significantly increased root biomass, so less energy was used for assimilating soil water and nutrients, and more SS, the main direct energy source for metabolism, was converted to starch (Latt et al. 2001), which was stored in the plant tissues.

N and P are important nutrients for plant growth (Huang et al. 2012). They are commonly considered as limiting factor for primary production and other ecosystemic processes. N and/or P concentrations generally decrease in plants with elevated CO_2 levels because of nutrient dilution by the accumulation of NSCs (Loladze 2002; Deng et al. 2015). Elevated CO_2 levels, though, can stimulate soil microbial processes and increase nutrient mineralization, which contribute to the uptake of more available N and P by the plants (Huang et al. 2012; Sardans et al. 2012). Our results showed that plant N concentration was little affected by the elevated CO_2 concentration but significantly decreased the whole-plant P concentration, suggesting that *B. ischaemum* on the Loess Plateau is more limited by P than N under elevated CO_2 levels. N deposition directly increases soil N availability and can increase the capacity of plants to take up P by stimulating the activity of phosphatase in the rhizosphere (Phoenix et al. 2004; Fujita et al. 2010). Increased N deposition thus usually increases N and/or P concentrations in plant tissues. In the present study, N addition significantly increased N concentrations in whole plants and roots, supporting our first hypothesis that roots would respond more strongly than shoots to N deposition. P concentrations, however, decreased significantly with N addition in whole plants, shoots and roots, indicating that the limiting effect of P was enhanced by N addition.

Stoichiometries of NSCs, N and P

NSCs are the main energy sources for plant growth, reproduction and survival under stress (Hartmann and Trumbore 2016; Song et al. 2016). The ratio of NSCs to N or P, to a large extent, reflects the relationship between the input of N or P and the output of NSCs and their use efficiencies (Li et al. 2008a, b; Guo et al. 2015). In accordance with our second hypothesis, the elevated CO_2 concentration did not significantly influence the SS:N or SS:P ratios, and N addition significantly decreased the SS:N ratio and increased the SS:P ratio, implying that each unit of N produces less SS, and each unit of P produces more SS. The elevated CO_2 concentration and N addition both significantly increased the starch:N, NSC:N, starch:P and NSC:P ratios, indicating that each unit of N and P produced more starch and NSCs, and the use efficiencies of N and P both increased.

N:P ratios can be used as diagnostic tool for evaluate nutrient limitation in terrestrial ecosystems under future environmental scenarios (Liu et al. 2013a; Huang et al. 2015a). The elevated CO_2 concentration increased the whole-plant N:P ratio, and N addition significantly increased the whole-plant, shoot and root N:P ratios, supporting our second hypothesis. The higher N:P ratios under the elevated CO_2 concentration and N addition were mainly due to the lower P concentrations. This result is consistent with those by Huang et al. (2012), who reported that elevated CO_2 levels and N addition increased foliar N:P ratio in a subtropical model forest. Elevated CO_2 levels and N addition can decrease N:P ratios but are mainly related to the increases P concentrations (Liu et al. 2013a), consistent with the results of a previous study reporting strong control of elevated CO_2 levels and N addition on soil nutrient availability, especially P availability (Huang et al. 2014). The available-N:available-P ratio is much higher than the N:P ratio on the Loess Plateau (Jiao et al. 2013), and P mineralization cannot provide enough available P for plant growth under elevated CO_2 levels and N addition. Plant growth in this region would thus suffer more from P than N limitation (Jiao et al. 2013; Xu et al. 2016), and N addition would exacerbate the P limitation on plant growth (An et al. 2011).

Conclusions

The elevated CO_2 concentration and N addition significantly increased total biomass, starch and NSC concentrations and the root:shoot, starch:N, NSC:N, starch:P and NSC:P ratios in whole plants and roots of *B. ischaemum*. N addition alone decreased SS concentration in whole plants, increased N concentration and decreased P concentration in whole plants and roots and thus decreased the SS:N ratio and increased the SS:P and N:P ratios. Our results suggest that plant growth on the Loess Plateau suffers more from P than N limitation and that N addition would exacerbate the P limitation on plant growth.

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