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REGULAR ARTICLE

Plant carbon limitation does not reduce nitrogen transfer from arbuscular mycorrhizal fungi to *Plantago lanceolata*

Haiyang Zhang · Waldemar Ziegler · Xingguo Han · Susan Trumbore · Henrik Hartmann

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Abstract
Aims The stress-gradient-hypothesis predicts that interactions among organisms shift from competition to facilitation as environmental stress increases. Whether the strength of mutualism will increase among symbiotically associated organisms when partners are forced into resource limitation remains unknown. Plants exchange photosynthetic carbohydrates (plant C) for nutrients in mycorrhizal symbiosis but how this exchange varies with plant C limitation is not fully understood.
Methods We investigated the influence of plant C availability and of arbuscular mycorrhizal fungi (AMF) on plant nitrogen (N) uptake and resource allocation using ^{13}C and ^{15}N labeling. We grew *Plantago lanceolata* with and without AMF *Rhizophagus irregularis* under ambient (400 ppm, AC) and low (100 ppm, LC) atmospheric [CO_2] and physically restricted plant root but not mycorrhizal access to soil N.
Results We found that plants grown under LC used AMF to obtain the same amount of N as those grown under AC, but the amount of newly fixed C correlated with the acquisition of N only under LC. The LC plants allocated more of their C to aboveground tissues.
Conclusions Overall our results suggest a more beneficial role of symbiosis under C limitation. The tight reciprocal control on N transfer and C allocation under C limited conditions supports the stress-gradient hypothesis of mutualistic symbiotic functioning.

Keywords Symbiosis · Resource allocation · Plant carbon limitation · Stable isotopes ^{13}C and ^{15}N · Stress-gradient hypothesis

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Introduction

Arbuscular mycorrhizal fungi (AMF) can form parternerships with over two-thirds of terrestrial plants and provide numerous resources (e.g., water, nutrients) in exchange for photosynthetic carbohydrates (plant C) (Smith and Smith 2011). AMF are generally thought to be responsible for taking up immobile nutrients (such as phosphorus (P), copper or zinc) but recent work also highlights their role in nitrogen (N) uptake (Leigh et al. 2009). N and P, heterogenously distributed in soils, limit plant growth in most ecosystems (Vitousek and Howarth 1991). Via their extraradical mycelium, AMF increase plant root arsorbing surface area with positive consequences for plant survival and growth (Smith and Read 2008). The fact that AMF transfer N to their hosts

has been shown in both laboratory (Tanaka and Yano 2005) and field experiments (Blanke et al. 2011; Cavagnaro et al. 2012). However, the underlying mechanisms regulating AMF-mediated N transfer remain unclear (Fitter et al. 2011; Govindarajulu et al. 2005; Jin et al. 2005).

Growth and survivial of AMF rely on the provision of photosynthates from their host plants (Heinemeyer et al. 2006; Johnson et al. 2002) and recent studies using root organ cultures showed that plant C availability influenced nutrient transfer in plant-mycorrhizal symbiosis (Fellbaum et al. 2012; Hammer et al. 2011; Kiers et al. 2011). The concentration and types of plant carbohydrates determined nutrient uptake and transfer via AMF (*Glomus intraradices*) in axenic cultures of transformed carrot (*Daucus carota*) roots (Bücking and Shachar-Hill 2005). However, these root culture studies supplied plant tissues with extraneous carbohydrates and hence do not allow conclusions on whole-plant C-N exchange mechanisms. AMF nutrient transfer to host plants (*Plantago lanceolata*), using whole-plant system, did not change when plants were grown at 440 ppm as opposed to 1500 ppm [CO_2]. In contrast, AMF permitted more plant nutrition gain per unit of C invested into fungi for plants grown under low CO_2 (Field et al. 2012). In another study using whole-plant system, differences in sucrose investment did not affect nutrient (N, P, Zn) uptake by plants via arbuscular mycorrhizal fungi (Gabriel-Neumann et al. 2011). Thus, plant C supply may not be the only factor controlling AMF nutrient transfer.

According to the stress-gradient hypothesis (Bertness and Callaway 1994; Brooker et al. 2008), interactions among symbiotically associated organisms shift from competition to facilitation as environmental stress increases for one or both symbiotic partners. However, whether the functional complementarity between plant and AMF, in terms of C supply and N transfer, are reinforced under extreme environmental conditions has not been fully investigated. Cost and benefit in symbiotic exchanges depend on the relative resource availability and their balance between the symbiotic partners (Grman 2012; Grman and Robinson 2013) but how this mutualistic relationship changes when both symbiotic partners are forced into resource limitation remains unknown.

To address these issues, we devised an experiment where plant-AMF systems were forced into C limitation by strongly reducing ambient [CO_2]. Previous studies that relied on shading to reduce C supply showed that plants allocated more C to above- than below-ground organs and regulated C transfer to AMF by adjusting C allocation (Bethlenfalvay and Pacovsky 1983; Son and Smith 1988; Fellbaum et al. 2014). However, shading can cause shifts in C allocation to enhance shoot elongation for light capture independent of AMF (Pierik et al. 2009). In contrast, direct manipulation of atmospheric [CO_2] can force plants into C limitation without inducing such artifacts (Hartmann et al. 2013). Under such conditions, changes in C allocation among plant organs and/or between plant and AMF could extend results from shading experiments and provide deeper insights into the mechanisms that controling plant-AMF interactions.

Within the plant-mycorrhiza-soil system, we investigated N transfer dynamics under ambient and under induced reductions in plant C availability. We used C and N stable isotope labelling to quantify net C gain of plants and N fluxes from mycorrhiza to plants under experimental conditions. The main goals were to investigate: (1) whether sudden changes in plant (*P. lanceolata*) C supply influenced AMF (*Rhizophagus irregularis*) mediated N transfer and the cost-benefit ratio of the symbiotic exchange; and (2) whether internal plant C and N allocation changed under C limited conditions. Because plant carbohydrate supply to AMF determined nutrient uptake and transfer in vitro root cultures (Bücking and Shachar-Hill 2005; Kiers et al. 2011), we hypothesized that: (1) less N would be transferred from AMF to host plants under plant C limitation; (2) plants would receive more N per unit C invested under C limitation thereby decreasing the cost-benefit ratio, (3) internal plant resource allocation would shift to above-ground tissues in order to compensate for C limitation, and (4) AMF-mediated N transfer plays a more important role in plant growth and survival when plants are forced into C limitation.

Materials and methods

Microcosm design

Microcosms were made of PVC material (14 cm height × 17 cm length × 12 cm width) and separated into two compartments (Fig. 1, modified from (Hodge et al. 2001): HOST (11 cm length) and LABEL (6 cm length) separated by a 3-mm thick plate also made of PVC). The

plates were perforated evenly with 144 holes (6 mm radius) with the lowest row 3 cm higher than the base to prevent transfer of water and dissolved nutrients between chambers. To allow hyphae but prevent roots to penetrate, we glued a 20-μm nylon mesh on the HOST side and a 65-μm nylon mesh on the LABEL side of the plates (Walder et al. 2012). An air gap formed between the mesh membranes on either side of the 3-mm thick PVC plates thereby preventing both mass flow and nutrient diffusion across plates (tested in a pre-experiment). A visual water level indicator (float, Technoplant GmbH, Germany) was fixed in the corner of each compartment and ~2 cm of gravel was added to the bottom of each compartment to allow soil drainage. HOST compartments were filled with 1000 g, LABEL compartments with 500 g of sterile growth substrate (treated at 170 °C for 2 h) consisting of a carbon-free 1:1 vermiculate: sand mixture (Hartmann et al. 2013).

Biological material and growth conditions

Seeds of *P. lanceolata L.* were surface sterilized with a 2.5 % HCl solution for 10 min, then rinsed with distilled H_2O and germinated on moist filter paper in a Petri dish (Olsson and Johnson 2005). Three 1-week-old seedlings were planted in each HOST compartment and thinned 60 days after planting, leaving two seedlings per microcosm. Thinning minimized biomass variation across microcosms. Each microcosm was treated with 2 g of inoculum containing expanded clay and *R. irregularis* spores (110 infective units per gram) or with 2 g autoclaved inoculum in the nonmycorrhizal controls (Walder et al. 2012). In addition, soil microbial organisms, excluding AMF, were added to HOST compartments to homogenize microbial communities (Koide and Li 1989). To do so, we first washed 100 g field soil (collected from native *P. lanceolata* habitat) with deionized water and sieved the supernatant liquid through a 32-μm sieve to obtain 1 L of microbial wash. We added 10 mL of this microbial wash to each pot. The microcosms were then randomly arranged and evenly spaced on a glasshouse table (90 days after planting). Plants were left to grow under controlled environmental conditions on a 16–8 h day–night cycle. Day and night temperatures were set at 24 and 20 °C, respectively. Photosynthetically active radiation (PAR) measured daily at the leaf surface ranged from 450 to 550 μmol photons m^{-2} s^{-1}. Microcosms were irrigated twice a week with 50 mL of a nutrient solution (1/10 of N and P content as in Thornton and Bausenwein 2000, i.e. 0.15 mmol/L NH_4NO_3, 2.1 mmol/L $CaCl_2$, 0.75 mmol/L $MgSO_4$, 0.5 mmol/L K_2SO_4, 0.0307 mmol/L NaH_2PO_4, 0.0026 mmol/L Na_2HPO_4, 50 μmol/L H_3BO_3, 10 μmol/L $FeC_6H_5O_7$, 8.6 μmol/L $MnSO_4$, 2 μmol/L $ZnSO_4$ and 1 μmol/L $CuSO_4$).

Experimental design

Eight glass growth chambers (80 cm height×75 cm length×45 cm width, c. 250 L volume) were placed on a glasshouse table to provide controllable headspace for manipulation of atmospheric [CO_2]. The table was covered with closed-cell rubber foam mats of ethylene propylene diene monomer creating an airtight seal (details see Hartmann et al. 2013). The chambers were equipped with an automated watering system (drippers) that could be activated without opening the chambers. On August 15 (i.e. 90 days after planting), we transferred four microcosms into each chamber (two treated with active AMF inoculum and two with autoclaved inoculum, Fig. 1) and randomly arranged them on a plastic tray within the growth chambers. Plants were allowed to acclimate for 1 week. We applied a ^{15}N-labeled solution (2 g NH_4NO_3 with $\delta^{15}N$= 171.3‰, in 10 ml distilled water) to the LABEL compartment 97 days after planting. During the experimental period each HOST compartment was watered twice a week until water level indicator readings indicated soil saturation. As the volume of LABEL compartment was only 1/4 of the size of the HOST compartment, each LABEL compartment received 1/4 of the amount of water applied to the HOST compartment. Microcosms were also irrigated every second week with 50 mL of the nutrient solution only at HOST side.

Chambers were flushed with mixtures of CO_2-free air (Gamnitzer et al. 2009; Hartmann et al. 2013) and fixed amounts of ^{13}C-depleted ($\delta^{13}C$=−36.41±0.005‰) from a gas bottle with pure CO_2. Air inlet and outlet pipes went through the greenhouse table inside the chambers (Fig. 1), and the airflow was fixed at 10 L min^{-1} independent of CO_2 concentration. We started by flushing all eight chambers with ambient [CO_2] (i.e. 400 ppm, hereafter AC) for 1 week after plants were installed (90 days after planting). Subsequently, we progressively decreased [CO_2] in half of the chambers down to 100 ppm. This concentration was identified as the whole-plant C compensation point where no measurable difference between the daily sum of assimilated C and

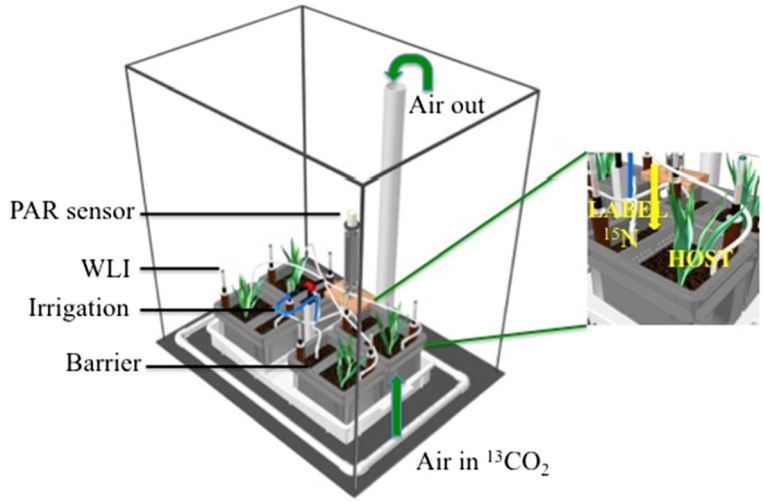

Fig. 1 Schematic view of four microcosms within one chamber showing the ventilation and irrigation systems. Two pots for mycorrhizal plants were treated as one replicate for each chamber, while the other two pots for non-mycorrhzal plants was treated as control. PAR, photosynthetically active radiation; WLI, water level indicator; Barriers were attached with nylon mesh screens (25 μm for HOST and 65 μm for LABEL compartment side, respectively)

the daily sum of respired C could be observed over a 2-week period. Thereafter, we continued to use 100 ppm [CO_2] as the level of the C limitation treatment (hereafter LC treatment) and maintained this concentration until the end of the experiment. The ambient C treatment (AC) maintained a level of 400 ppm CO_2 throughout the experiment.

Sample collecting and analysis

Three mycorrhizal (M) and three non-mycorrhizal (NM) microcosms were destructively harvested 90 days after planting – i.e. before the start of the experiment and as a measure for pre-treatment condition. Two M and two NM microcosms within each of the four chambers per treatment (representing one replicate) were also harvested 146 days after planting (i.e. after a 7 days acclimation period to chamber conditions and an additional 49 days of treatment). Plants were harvested quickly (within 3 min) from experimental chambers to reduce the influence of outside atmospheric CO_2 on the remaining microcosms.

Shoots were clipped at the soil surface and roots were carefully rinsed with water to remove adhering sand particles, and the total fresh weight of each recorded. Root subsamples (~2 g fresh roots) were randomly picked and cut into 2 cm lengths for the assessment of mycorrhizal colonization. The remaining shoots and roots were weighed and then dried at 70 °C for 48 h and weighed again to determine the fresh to dry biomass ratios.

Root subsamples were cleaned with 10 % KOH, acidified with 1 % HCl and stained with lactoglycerol mixture (1:1:1 lactic acid, glycerol and water), as described in Phillips and Hayman (1970). The proportion of root length colonized by arbuscular, vesicles, and hyphae was estimated using the magnified intersections method (Brundrett et al. 1996). At least 100 root intersections for each sample were inspected under a compound microscope at ×200 magnification. To quantify hyphal length density in the LABEL compartment, we used the modified membrane filter method (Jakobsen et al. 1992) and at least 50 views were counted at ×125 magnification using a compound microscope (Carl Zeiss, Inc., Axiolab, Jena, Germany). Hyphal length (in m g^{-1} soil) was estimated following the modified Newman formula (Tennant 1975).

Dried plant material was milled to a fine powder. For both shoots and roots, C and N concentrations and isotopic ratios were determined with an isotope-ratio mass spectrometer (IRMS; Deltaplus XP and Delta C prototype Finnigan MAT, respectively, Finnigan MAT, Bremen, Germany; 0.1‰ precision).

Mycorrhizal responses were calculated as MR= 100×(M−NM)/NM, where M are values for mycorrhizal plants, and NM are paired values for nonmycorrhizal plants grown within the same chamber. Mycorrhizal growth response was calculated with M and NM based on whole plant dry weight while mycorrhizal N response was determined with M and NM derived from whole plant N content.

Calculation of newly fixed-C and transferred-N

The CO_2 supplied to the chamber system had a very distinct isotopic signature ($\delta\ ^{13}C_{chamber}$=−36.41±0.005 ‰, compared to $\delta\ ^{13}C_{atmosphere}$~−8.5 ‰). Before we transplanted the pots into the chambers, we measured $\delta^{13}C$ and $\delta^{15}N$ on sacrificed plant tissues, and these are $\delta^{13}C_{t0\_plant}$ and $\delta^{15}N_{t0\_plant}$. The $\delta^{15}N$ of the nutrient solution added to the LABEL compartment ($\delta^{15}N_{LABEL}$) at the beginning of the experiment was 171.3‰.

We assumed that fractionation from CO_2 fixation was constant during the experiment and calculated the proportion of newly fixed-C in the plant C pool as:

$$\text{Newly fixed}-\text{C} = \frac{\left(\delta^{13}C_{t1_{plant}} - \delta^{13}C_{t0_{plant}}\right)}{\left[\left(\delta^{13}C_{chamber} - \delta^{13}C_{fractionation}\right) - \delta^{13}C_{t0_{plant}}\right]} \quad (1)$$

where $\delta\ ^{13}C_{chamber}$ is the isotopic signature of air leaving the chamber, $\delta^{13}C_{t0\_plant}$ is the isotopic signature of the plant tissue before we moved them into the chambers, $\delta\ ^{13}C_{t1\_plant}$ is the isotopic signature of plant tissues at each sampling time, and $\delta\ ^{13}C_{fractionation}$ was calculated as $\delta\ ^{13}C_{atmosphere}$ - $\delta^{13}C_{t0\_plant}$.

A similar mixing model equation was used for calculating the proportion of transferred-N:

$$\text{Transferred}-\text{N} = \frac{\left(\delta^{15}N_{t1_{plant}} - \delta^{15}N_{t0_{plant}}\right)}{\left(\delta^{15}N_{LABEL} - \delta^{15}N_{t0_{plant}}\right)} \quad (2)$$

where $\delta^{15}N_{LABEL}$ was 171.3‰, $\delta^{15}N_{t0\_plant}$ is the isotopic ^{15}N signature for the plants before being moved into the chambers, and $\delta\ ^{15}N_{t1\_plant}$ is the isotopic signature for the plants at each sampling time.

Statistical analysis

We analyzed our data with linear mixed models (LMMs) as implemented in package lme4 (Bates et al. 2013), using CO_2 treatment, AMF, and their interaction as fixed factors, and individual chamber as random factor. We set each chamber as our biological replicate units and thus had four replicates per treatment. To meet assumptions of normality of errors (Shapiro-wilk test) and homogeneity of variances (Levene's test), we transformed data using arcsine [square-root] (mycorrhizal root colonization, arbusculars, vesicles) or log10 (newly-fixed C, plant N, transferred N) when necessary. We used linear regression on the mycorrhizal N response (MNR) with the mycorrhizal growth response (MGR) and also on the newly fixed-C with transferred-N to test for correlations. All statistical analyses were conducted in R (v2.15.3, The R Foundation for Statistical Computing).

Results

Plants with AMF showed larger increases in below- but not above-ground biomass under AC (Fig. 2). In contrast, the presence of AMF had no significant effect on shoot and root biomass of plants under LC (Fig. 2, Table 2). The mycorrhizal colonization rate was 44.23 (± 42.18)% and 33.28 (± 11.30)% of root length for M plants grown under AC and LC, respectively, with no significant differences. Mycorrhiza-infected plants grown under AC and LC had no significant differences in hyphal length density (HLD), root length colonization, vesicles or arbuscules (Table 1).

AMF, but not [CO_2], had a significant effect on total plant N and also on the total amount of N transferred by AMF (Fig. 3, Table 2). Patterns seen in total biomass were also seen in total C (Fig. 4a, b, Table 2); i.e. AFM-infected plants grwon under AC ended the experiment with more C in below- but not above-ground tissues, while M and NM plants under LC did not differ in the final mass of shoot or root C. M plants grown under LC had significantly less total root C than those grown under AC, though shoots contained the same total amount of C (Fig. 4a, b, Table 2).

Under AC, plants with AMF fixed more total C in shoots and roots during the experiment (as determined by the amount of ^{13}C label incorporated) (Fig. 4). Under LC, however, plants with AMF contained more C only in shoots not in roots (Fig. 4c, Table 2).

AMF significantly increased newly-fixed C allocation ratios (shoot/root) under LC, but had no effect under AC (Fig. 5a). For NM plants under LC and all plants under AC, newly-fixed C allocation ratios were not significant different from one (Fig. 5a). Transferred N allocation ratios (shoot/root) were significantly greater under LC than that under AC for M plants (Fig. 5b).

LC significantly reduced plant nitrogen use efficiency (the amount of biomass (g) produced per unit of total N (mg), Fig. 6). Mycorrhizal growth responses had no relationship with mycorrhizal nitrogen response under AC, but showed a positive correlation under LC (although not significant, R^2=0.64, P=0.13; Fig. 7a).

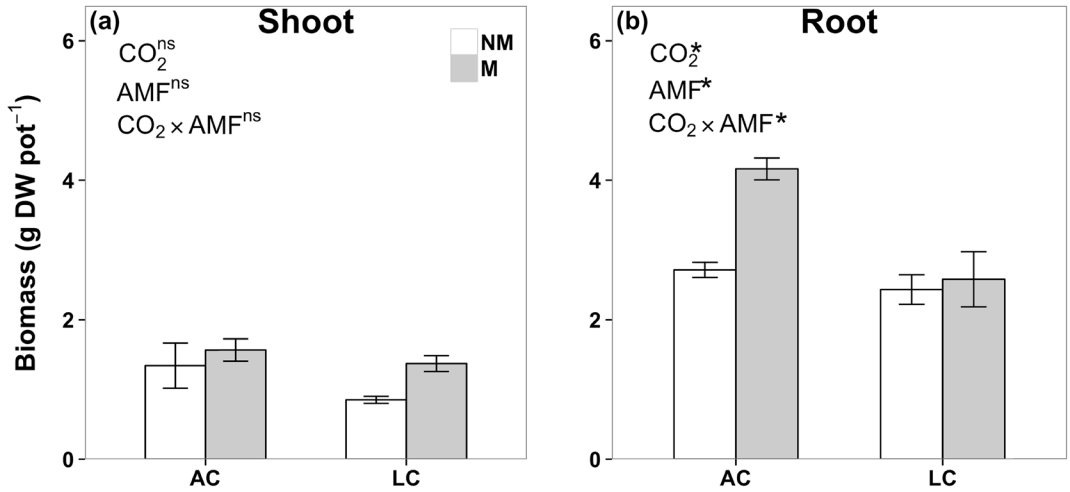

Fig. 2 Final shoot (**a**) and root (**b**) biomass (DW pot^{-1}) for non-mycorrhizal (NM, *white boxes*) and mycorrhizal (M, *grey boxes*) *P. lanceolata* grown for 49 days under ambient (AC, 400 ppm) and low CO_2 (LC, 100 ppm), respectively. Values are means (n=4)±1 SE. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant). DW, dry weight

Furthermore, transferred-N was positively correlated with newly fixed-C content (R^2=0.92, P<0.05) for M plants grown under LC but not AC (Fig. 7b).

Discussion

Our results showed that plant C limitation did not reduce net N transfer from fungi to plants. However, C-limited host plants with AMF symbionts optimized the use of resources by increasing both C and N allocation to shoots to alliviate C limitation. The positive relationship between transferred-N and newly fixed-C under LC suggests that AMF-mediated N transfer plays an essential role in plant net C gain under C limitation.

N and C transfer under plant C limitation

Contrary to our initial hypothesis, the amount of new-N transferred to plants by AMF was independent of plant C availability (Fig. 3). This is in accord with a study by Hodge and Fitter (2010), in which shading had no effect on AMF-mediated N transfer to *P. lanceolata*. In another study, shaded *Medicago truncatula* maintained high AMF colonization rates and did not reduce gene expression of a putative ammonium transporter in the plant host (Fellbaum et al. 2014), also indicating that AMF maintained the rate of N transfer to C limited plants. In all of these studies, including ours, N and P concentrations of added fertilizer (except the additional labled-N) were in much lower concentrations than under normal field conditions and forced plants to rely on the AMF

Table 1 Arbuscular mycorrhizal fungal colonization parameters: extraradical hyphal length density (HLD, m g^{-1}) in the LABEL compartment; root length colonization (RLC, %), arbuscular (%) and vesicle (%) in the HOST compartment under ambient (AC, 400 ppm) and low (LC, 100 ppm) CO_2 for mycorrhizal (M) and non-mycorrhizal (NM) plants. Values are means±SD (n=4)

	AC		LC	
	NM	M	NM	M
HLD (m g^{-1})	0.00 (± 0.00)^a	6.85 (± 5.63)^b	0.00 (± 0.00)^a	2.20 (± 2.27)^b
RLC (%)	0.00 (± 0.00)^a	44.23 (± 42.18)^b	0.10 (± 0.79)^a	33.28 (± 11.30)^b
Arbuscular (%)	0.00 (± 0.00)^a	6.93 (± 5.80)^b	0.00 (± 0.00)^a	4.78 (± 1.61)^b
Vesical (%)	0.00 (± 0.00)^a	12.32 (± 9.75)^b	0.14 (± 0.28)^a	6.92 (± 7.32)^b

Different superscripts indicate significant differences between treatment levels

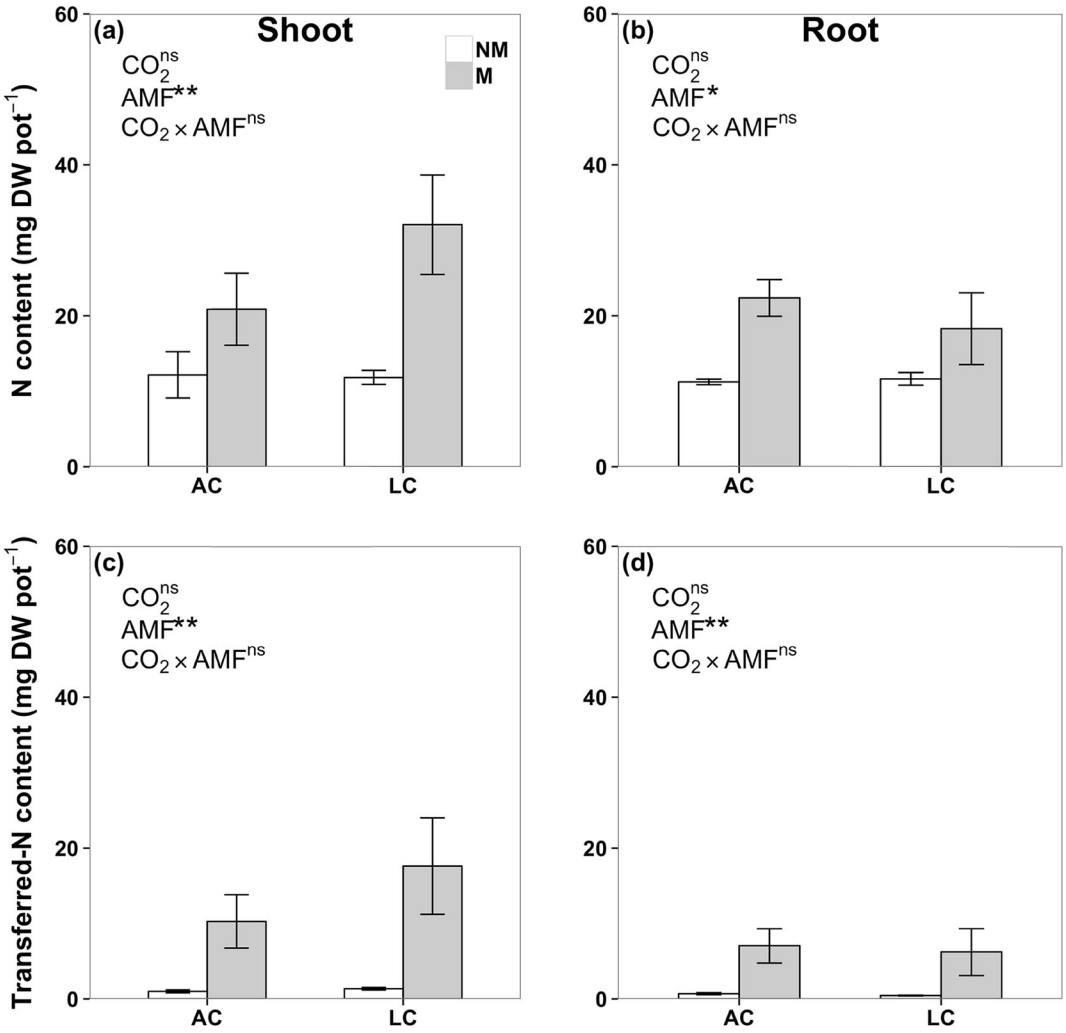

Fig. 3 Total nitrogen and transferred-N (i.e. ^{15}N-labeled, mg DW pot^{-1}) for shoots (**a**, **c**) and roots (**b**, **d**) of non-mycorrhizal (NM) and mycorrhizal (M) *P. lanceolata* under ambient (AC, 400 ppm) and low CO_2 (LC, 100 ppm), respectively. Values are means (n= 4)±1 SE. Asterisks indicate significant differences between NM and M plants. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant). DW, dry weight

Table 2 ANOVA F ratios for the fixed effects of CO_2 concentration, AMF association and their interaction on plant biomass (dry weight), carbon (C) content, newly fixed-C (NewC), nitrogen (N) content, and transferred-N (NewN) for *Plantago lanceolata*

Source of variation		Biomass $F_{1,6}$	C content $F_{1,6}$	NewC $F_{1,6}$	N content $F_{1,6}$	NewN $F_{1,6}$
Shoot	CO_2	3.22	3.88^	30.39**	1.50	1.22
	AMF	3.79^	3.01	12.34*	14.43**	63.81***
	Interaction	0.60	0.56	0.09	0.95	0.05
Root	CO_2	9.66*	9.73*	95.86***	0.90	1.29
	AMF	21.38**	23.32**	0.29	13.04*	48.95***
	Interaction	14.20**	16.17**	6.58*	1.36	0.01

^P< 0.1; *P< 0.05; **P< 0.01; ***P< 0.001

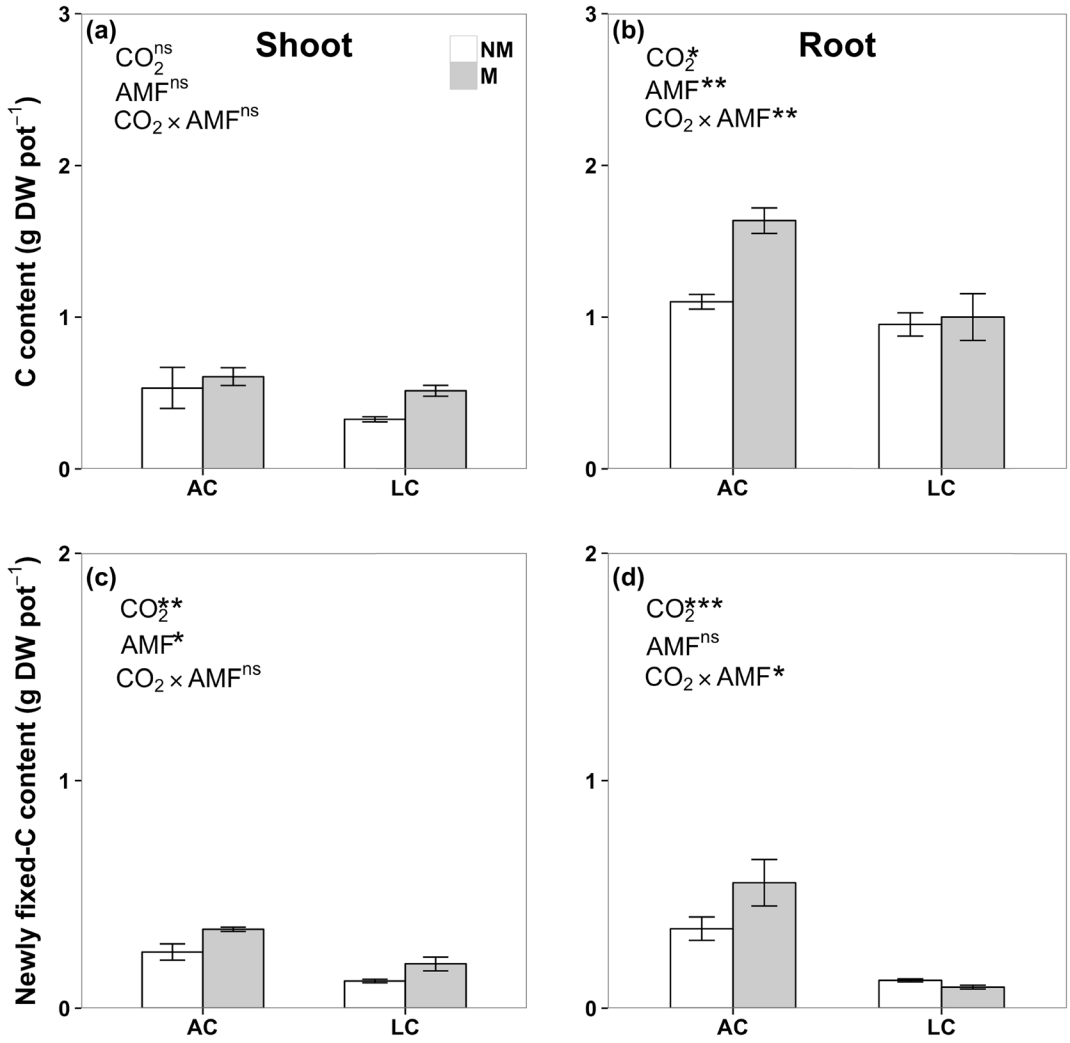

Fig. 4 Total plant C (**a**, **b**) and newly fixed-C (**c**, **d**, g pot^{-1}) for non-mycorrhizal (NM, *white boxes*) and mycorrhizal (M, *grey boxes*) *P. lanceolata* under ambient (AC, 400 ppm) and low CO_2 (LC, 100 ppm), respectively. Values are means (n=4)±1 SE. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant). DW, dry weight

symbiosis for nutrient supply. Therefore, N transfer was maintained when plants had the chance to interact with AMF, even when C supply decreased.

Low [CO_2] did not reduce mycorrhizal root colonization in our study. Growth of mycelium under low carbohydrate levels and low soil nutrient availability was also observed by Olsson et al. (2014) and in plants where C availability was reduced by shading (Knegt et al. 2014). AMF are obligate biotrophs and colonizing and transferring N, even to low-quality hosts, secures a sustained C suply to fungi. Hence, under both C and N limitation, maintaining plant-AMF exchange may be a high priority for both plant hosts and symbionts because it provides a means for C-limited plants to get nutrients for survival and growth and represents a "strategic investment to retain future bargaining power" for AMF (Fellbaum et al. 2014).

In contrast, results from in vitro root organ cultures suggested that C availability controlled nutrient transfer in AM symbioses (Bücking and Shachar-Hill 2005; Fellbaum et al. 2012). It is important to consider, however, that these studies did not consider shoot-mediated effects on nutrient sink strength and uptake (Smith and Smith 2011). Shoots also play a key role in determining C transfer to AMF because plant shoots are the actual C source for both the plant and AMF (Gabriel-Neumann

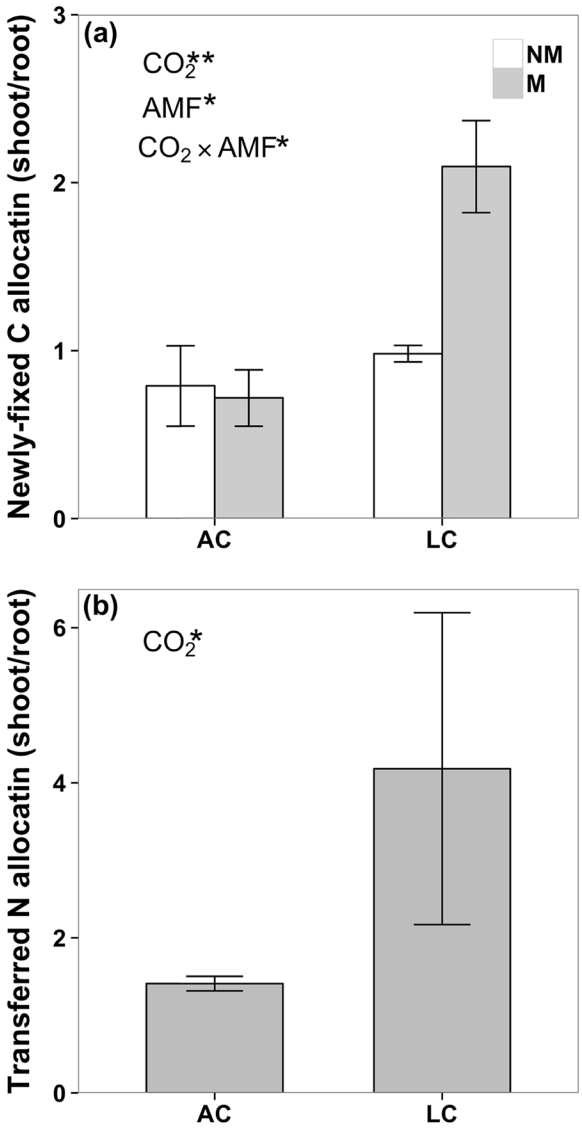

Fig. 5 Newly fixed-C (**a**) and transferred-N (**b**) allocation for shoots and roots for non-mycorrhizal (NM, *white boxes*) and mycorrhizal (M, *grey boxes*) *P. lanceolata* under ambient (AC, 400 ppm) and low (LC, 100 ppm) CO_2, respectively. Values are means (n=4)±1 SE. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant)

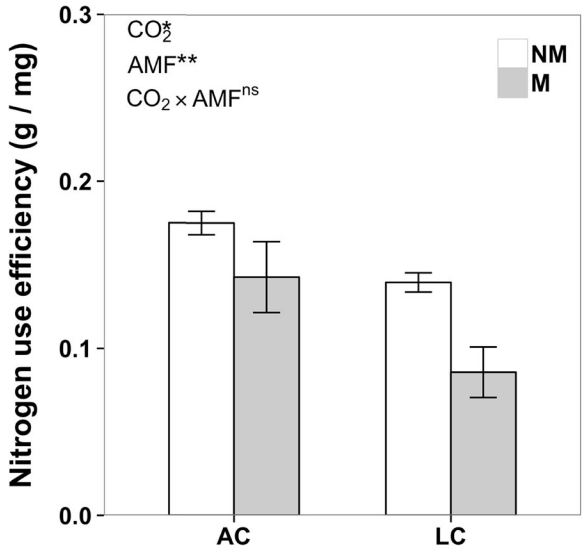

Fig. 6 Whole-plant nitrogen use efficiency (g / mg, productivity per unit N) for non-mycorrhizal (NM, *white boxes*) and mycorrhizal (M, *grey boxes*) *P. lanceolata* under ambient (AC) and low (LC) CO_2, respectively. Values are means (n=4)±1 SE. Statistically significant effects of single factors (CO_2 and AMF) and interactions (CO_2 × AMF) are shown with treatment letters (*** if P<0.0001, ** if P<0.001, * if P<0.05, ns if not significant)

et al. 2011). The contrasting results from shading (Hodge and Fitter 2010) and axenic culture experiments (Fellbaum et al. 2012) may be due to the fact that C availibility was extremly low in the latter study (80–90 % of original root C had been exhausted before adding sucrose). Once the amount of added sucrose to the root compartment went beyond a critical level (i.e., 5 mM), N transfer by the extraradical mycelium did not increase anymore and gene expression only showed moderate changes (Fellbaum et al. 2012). This suggests that C availibility may not control N transfer unless the plant and mycorrhizal symbiosis are extremely C limited. The fact that both low CO_2 in our experiment and shading in Hodge and Fitter (2010) did not reduce plant nutrient uptake in a whole-plant system highlights the influence of shoots as strong nutrient sinks (Stonor et al. 2014). In addition, increasing carbohydate availibility under elevated CO_2 reduced N acquisition in *Pinus halepensis* via its ectomycorrhizal fungi, *paxillus involutus* (Kytöviita et al. 2001), but increased AMF-mediated plant ^{15}N uptake for three arbuscular mycorrhizal fungi, *Gigaspora margarita*, *Glomus clarum* and *Acaulospora morrowiae* (Cheng et al. 2012). These results suggest that C availibity does not always control AMF-mediated N transfer and that the overall effect may depend on mycorrhizal types.

Plant resource allocation strategy

Plants preferentially allocate C to tissues responsible for acquiring the most limiting resources (Johnson et al. 2013). Consequently, plants grown under C limitation

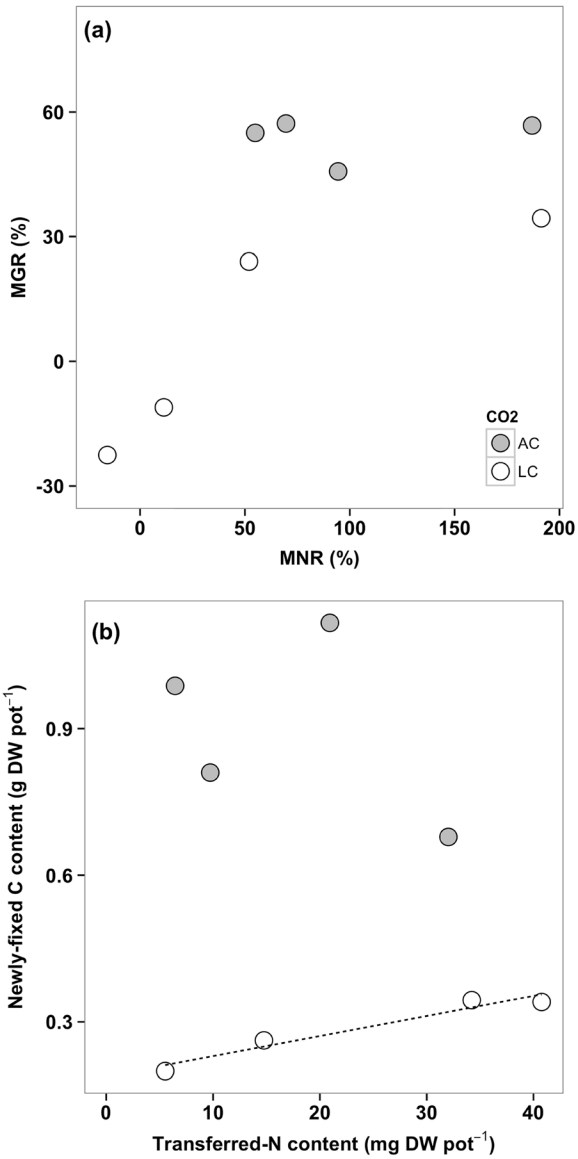

Fig. 7 Mycorrhizal nitrogen response and mycorrhizal growth response for (**a**), newly fixed plant-C content (g pot^{-1}) and (**b**) AMF-transferred-N (mg pot^{-1}) for *P. lanceolata* grown under ambient (AC, *grey*) and low CO_2 (LC, *white*) conditions. Only under LC, newly fixed-C showed positive relationship with AMF-transferred-N (*slashed line*, R^2=0.92, P<0.05)

are expected to allocate more biomass to aboveground tissues to enhance C assimilation (Sage and Coleman 2001). As shading itself can shift C allocation to enhance shoot elongation (Pierik et al. 2009), inferring regulatory mechanisms of plant C transfer to AMF from C allocation data in shading experiments may be problematic (Bethlenfalvay and Pacovsky 1983; Son and Smith 1988). We found that under LC only M plants but not NM plants allocated more resources to shoots (Fig. 5). In contrast, we found no preferential allocation of newly fixed-C between shoots and roots for NM plants under either CO_2 level. Thus, the observed shift in C allocation towards shoots in M plants under LC apparently resulted from the interaction with AMF rather than from resource allocation optimization alone.

Mechanisms that control the transfer of C between plant and AMF are not well understood. The total amount of C allocated to shoots was the same under both CO_2 levels, while allocation to roots was less under LC than that under AC, even though levels of AMF root colonization were not different. If shoots control C supply to AMF, we suggest as a possible mechanism that plants shifted C allocation to shoots in order to maintain C supply to AMF under plant C and N limitation. However, a proof for such a mechanism would require investigating metabolomics (i.e. genes encoding for carbohydrate transporters) and how they respond to AMF colonization under varying C availability.

Plant-AMF interaction under nutrient limitation

Our results raise the intriguing question why the bilateral exchange of plant-supplied C for microbially-acquired N was sustained even under low C availability. We found overall lower N use efficiency (new C fixed per unit transferred N) in plants under LC than under AC (Fig. 6). The plant growth response mediated by AMF (mycorrhizal growth response, MGR) can be positive or negative and depends on the mycorrhizal nutritional response (MNR). We found that the mycorrhizal growth response showed a positive trend with mycorrhizal nutritional response (although not significant, R^2=0.64, P=0.13) only under LC. In contrast, plants under AC did not grow more biomass even when they received more N via AMF (likely due to limitations from otherelements, such as P, Fig. 7a) and the amount of transferred-N mediated by AMF was positively correlated with newly-fixed C at LC but not AC (Fig. 7b). These results suggest a more synergistic relationship between plants and mycorrizae when environmental constraints intensify.

Consistent with the stress gradient hypothesis (Bertness and Callaway 1994; Brooker et al. 2008), our results indicated that the strength of mutualism between symbiotically associated organisms increased when habitat stress increased. Indeed, plants invested more C in AMF and received more benefits in return

under stressful (C limited) than optimal (high CO_2) conditions (Aghili et al. 2014). Shading had a significant effect on AMF-mediated N transfer in roots and shoots of plants associated with *Glomus aggregatum* but not with *R. irregularis* (Fellbaum et al. 2014), indicating that decreasing C availibility does not reduce benefits for plants associated with *R. irregularis* as in our study. When not given the choice of their plant host, even *G. aggregatum*, transferred more nutrients per unit C to C-limited (shaded) hosts, causing a shift in the cost-to-benefit ratio in favor of the shaded plant (Fellbaum et al. 2014). Similarly, *P. lanceolata* grown at a gradient representing the Palaeozonic CO_2 decline (from 1500 to 440 ppm) received proportionally more nutrients from AMF under lower [CO_2], i.e. the symbiotic relationship became more beneficial under declining C availability (Field et al. 2012). These results suggest that the mycorrhizal symbiosis may function as an insurance strategy that allows the plant to maintain productivity even when C becomes limiting. In turn, the N investment by AMF could also be an importrant stragegy to maintain or increase plant C assimilation and consequently to enhance the probability of a future investment return.

Conclusion

Terrestrial plants with mycorrhizal symbionts have co-evolved for 400 million years. However, details of how and why these interactions change with atmospheric CO_2 levels remains an important question. Here we showed that forcing plants to their photosynthetic compensation point did not reduce AMF-mediated N transfer. Plants under C limitation with AMF increased their C allocation to shoots, presumably to increase future C supply. Whether plants can control C supply to AMF by changing C allocation among different organs requires further investigation. However, the mutualistic relationship between symbiotically associated organisms became stronger, rather than weaker, under C stress, suggesting mycorrhizal symbiosis must have a high priority for stressed plants.

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