Plant Soil (2016) 407:279–292
DOI 10.1007/s11104-016-2994-y

REGULAR ARTICLE

Allocation trade-off between root and mycorrhizal surface defines nitrogen and phosphorus relations in 13 grassland species

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Received: 7 April 2016 / Accepted: 13 July 2016 / Published online: 8 August 2016

Abstract
Background and aims In oligotrophic ecosystems efficient nutrient uptake mechanisms, like extensive root systems or the association with belowground symbionts (e.g. arbuscular mycorrhizal fungi, AMF), are crucial. Pursuing root- or AMF-dominated foraging may result in diverging success regarding nitrogen (N) and phosphorus (P) nutrition. In this study we identify species- and functional group-specific belowground allocation strategies and disentangle the role of root vs. hyphal allocation for N and P nutrition.
Methods Allocation patterns to both root and AM hyphal surface together with plant P- and N-relations were measured in non-mycorrhizal and mycorrhizal individuals of 13 common grassland species belonging to the functional groups of forbs, grasses, legumes and non-mycotrophic *Brassicaceae*.
Results The trade-off between predominant investments into either roots or hyphae showed high species- and functional group-specificity and clearly defined plant N:P relations, with root strategists gaining larger N- and lower P-benefits than mycorrhizal strategists. Further, P-delivery by AMF was accompanied by strong fungal N-competition.
Conclusions Our results demonstrate high relevance of the allocation trade-off between root and mycorrhizal surface for N- and P-nutrition in grassland species. Low soil N:P ratios may only allow for positive AMF effects in mycorrhizal strategists, whereas root strategists may experience negative effects, likely being linked to N-limitation in the AM-state.

Keywords Arbuscular mycorrhizal fungi · Allocation · Phosphorus · Nitrogen · Plant nutrition · Grassland species

Responsible Editor: Duncan D. Cameron.

Electronic supplementary material The online version of this article (doi:10.1007/s11104-016-2994-y) contains supplementary material, which is available to authorized users.

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Introduction

Efficient exploitation of limiting resources is a prerequisite for the successful establishment, growth and survival of plant populations in oligotrophic habitats, consequently affecting competition and in turn successional progress (e.g. Tilman 1985). A textbook example for belowground resources, such as water and mineral nutrients, being the main limiting factors related to plant's success are dry sandy grasslands (Weigelt et al. 2005; Bartelheimer et al. 2006; Le Bagousse-Pinguet et al. 2013), where selective pressure for life under nutrient deficiency has promoted the dominance of plant species with efficient nutrient uptake mechanisms (Lambers et al. 2008; Richardson et al. 2009; Höpfner et al. 2014). With large carbon-investments into root biomass being one of the most successful strategies to overcome

nutrient-limitation, many species in these ecosystems develop remarkably high root-to-shoot ratios (e.g. Wilson 1988). Furthermore, root-morphological strategies like formation and maintenance of high proportions of fine roots and root hairs have the potential to substantially enlarge the soil depletion zone and thus improve plant nutrition (Ryser and Lambers 1995). This strategy is successfully employed by a wide range of plant species, many of which are grassland species, and particularly members of the Pooideae, where high fine root proportions frequently are combined with extensive root architecture (Bartelheimer et al. 2006; Höpfner et al. 2014). However, in contrast to high carbon-investments into an autonomous root system, nutrient acquisition may be optimized by allocation to belowground mutualists such as the exudation of sugars to stimulate growth of nutrient-mobilizing soil bacteria (Dakora and Phillips 2002) or C-transfer to mycorrhizal fungi. Particularly important are here arbuscular mycorrhizal fungi (AMF), which are associated with about 80 % of all land plants (Smith and Read 2008), and are the dominant type of mycorrhiza in grasslands. Similar to fine roots and root hairs, the biomass-to-surface ratio of mycorrhizal hyphae is favourable for efficient nutrient acquisition and similar to extensive fine root systems, the formation of dense mycelia create large nutrient depletion zones for efficient exploitation of oligotrophic soils. Indeed, AMF have been characterized as a functional equivalent of fine roots and root hairs (e.g. Brundrett 2002).

The degree to which a plant is nutritionally dependent on AMF is described by the term 'mycotrophy' (Janos 2007). Highly mycotrophic plants usually develop coarse and narrow root systems, with their successful establishment being dependent on carbon-allocation into AMF (e.g. Höpfner et al. 2014). However, there is a broad spectrum of mycotrophy levels ranging from obligately mycotrophic to completely non-mycotrophic species. Given these contrasting nutrient acquisition strategies, a trade-off in belowground carbon-investment between either roots or AMF is predefined, and potentially related to evolution of the AMF symbiosis, with highly mycotrophic plant taxa decreasing biomass allocation to roots in comparison to less mycotrophic taxa (Azcón and Ocampo 1981; Johnson 2010, Höpfner et al. 2014).

There is experimental evidence that the ancestors of vascular plants were invariably arbuscular mycorrhizal (Wang et al. 2010). However, evolutionary detachment from AMF dependency and a consequent stronger investment into other means of nutrient acquisition, as performed e.g. by the *Brassicaceae* (Brundrett 2009; Lambers and Teste 2013) may have brought about several advantages: First, there may be disadvantages in competitive interactions associated with mycorrhiza, as in contrast to the root system being an exclusive nutrient acquisition organ, the extraradical AMF mycelium is often not exclusive due to the development of common mycelial networks (CMNs) interlinking several host plants (Leake et al. 2004; Merrild et al. 2013). Indeed, Höpfner et al. (2015) showed that AMF-dominated foraging can be less effective than root-dominated foraging in competitive interactions, particularly under high nutrient availabilities. Second, mycorrhizal advantages decrease with increasing soil phosphorus (P) availability and the low carbon cost to P-benefit ratio can, under these circumstances, cause the generally mutualistic relationship to turn to mycorrhizal parasitism (Johnson 2010). Particularly the nitrogen: phosphorus (N:P) stoichiometry of the soil may affect this continuum between mutualism and parasitism, with negative effects of AMF becoming more likely under low than under high soil N:P ratios (Johnson 2010).

Ecology and evolution of the mycorrhizal symbiosis is a field with many open questions, in example what mechanisms evolved to maintain stability in the symbiosis, and is there reciprocal control by plants and fungi, i.e. the ability to regulate resource exchange and to exclude non-beneficial partners (van der Heijden et al. 2015). In order to shed more light on potential reasons for the development of differing mycorrhizal responsiveness in the same ecosystem type the present study links allocation patterns to root vs. mycorrhizal surface area of 13 common sandy grassland species to their mycotrophic level and their nutritional benefits regarding nitrogen and phosphorus. We used measurements of hyphal and root surface area as a proxy for belowground carbon-investment. We hypothesized a clear allocation trade-off with carbon-investment into either roots or hyphae being dependent on the species-specific mycorrhizal responsiveness: i.e. smaller root and larger hyphal surface area in a more responsive species as compared to a less responsive species. In addition, we hypothesized a positive relationship between phosphorus-uptake and relative carbon-allocation to AMF hyphae as well as between nitrogen-uptake and relative carbon-allocation to roots. The hypothesized relationship between mycorrhizal responsiveness and the C-allocation trade-off between absorptive root-surface vs. AMF-surface and the

related consequences for N vs. P nutrition has importance in explaining the occurrence of root vs. mycorrhizal strategists in habitats with diverse soil nutritional conditions.

Materials and methods

Plant material and cultivation

A controlled pot experiment was carried out with mycorrhizal (AM) and non-mycorrhizal (NM) individuals of 13 European sandy grassland species encompassing four functional groups: (i) six forbs: *Hieracium pilosella* (HP) and *Hypochaeris radicata* (HR; *Asteraceae*), *Plantago lanceolata* (PL; *Plantaginaceae*), *Dianthus carthusianorum* (DC; *Caryophyllaceae*), *Erodium cicutarium* (EC; *Geraniaceae*), *Potentilla argentea* (PA; *Rosaceae*); (ii) three grasses: *Festuca ovina* (FO), *Corynephorus canescens* (CC) and *Anthoxanthum odoratum* (AO; *Poaceae*); (iii) two legumes: *Anthyllis vulneraria* (AV) and *Trifolium arvense* (TA; *Fabaceae*) and (iv) two non-mycotroph *Brassicaceae*: *Isatis tinctoria* (IT) and *Teesdalia nudicaulis* (TN). Seeds of each species (Blauetikett-Bornträger GmbH, Offstein, Germany; Botanical Gardens of the universities of Münster and Hamburg, Germany) were sown and started in boxes with sterilized (120 °C for 1·5 h) sand. Two weeks after germination seedlings were transplanted to small pots (0·5 L) with sterilized sand. During transplantation, roots of AM treatment seedlings were inoculated using 20 g of an inoculum-sand-mixture (*Rhizophagus irregularis*, INOQ GmbH, Schnega, Germany), while the NM treatment received 20 g of sterilized sand and 5 mL of a microbial wash, which was extracted from the inoculum by sieving the supernatant of a water-inoculum-mixture through a 20 μm sieve (Koide and Li 1989). After four weeks 5 replicates of the respective AM and NM plants of each species were transplanted again in 3 L containers, resulting in a total of 130 pots. Plants were cultivated during another eleven weeks in a greenhouse at a photosynthetic photon flux density of ~400 μmol m^{-2} s^{-1}, a temperature of ~25 °C and a releative humidity of ~55 %. Plants were watered regularly to keep relative soil water content at ~6 % and twice a week, 20 mL of a modified Hoagland fertilizer solution (1·5 mmol KNO_3, 0·5 mmol $Ca(NO_3)_2$, 0·25 mmol $(NH_4)_2SO_4$, 0·25 mmol $(NH_4)_2HPO_4$, 0·5 mmol $MgSO_4$, 0·25 mmol KCl, 0·25 mmol $FeC_6H_5O_7$, 0·00,625 μmol H_3BO_3, 0·0005 μmol $MnSO_4$, 0·0005 μmol $ZnSO_4$, 0·000,125 μmol $CuSO_4$, 0·000,125 μmol MoO_3; Hoagland and Arnon 1950) was applied.

Harvest and analysis of plant and fungal material

At an age of 16 weeks all plants were harvested and separated into above- and belowground material. After cleaning roots from substrate, both root and shoot material was dried at 60 °C and weighed. Total dry weights of AM and NM plants were used for calculation of the species-specific mycorrhizal growth dependency (MGD, Eq. 1), according to Smith et al. (2003):

$$MGD = 100\frac{AM-\overline{NM}}{AM} \quad (1)$$

where AM is the dry weight of an individual AM plant and $\overline{NM}$ is the mean dry weight of the corresponding NM plants. This index is based on the equation of Plenchette et al. (1983), resulting in values ranging from -∞ to +100 %, but was further adapted according to Gange and Ayres (1999), allowing for calculation of variances.

Quantification of mycorrhizal root colonization

Representative subsamples of the extracted roots of both, AM and NM plants were analyzed for mycorrhizal colonization. The roots were bleached in 10 % KOH at 90 °C for 10 min, rinsed with deionized water and stained with an ink-acetic-acid solution (1:1:8 = ink: 10 % acetic-acid: H_2O) at 90 °C for 15 min (Phillips and Hayman 1970). The root fragments were then transferred to microscope slides and the percentage of root length colonized by AMF was estimated at × 250 magnification using a modified intersect method (McGonigle et al. 1990), scoring a minimum of 100 intersections per sample for the presence of hyphae, vesicles and arbuscules.

Determination of root and hyphal surface areas

Representative subsamples of fresh roots were selected by cutting all root material into ~1 cm fragments and, after mixing the material in 250 mL distilled water, collecting a volume of ~20 mL containing root fragments of all size classes. The subsamples were scanned at 600 dpi (Snapscan 1236s, Agfa, Mortsel, Belgium) and analyzed using WinRhizo Pro (Version 2003 b, Regent Instruments Inc., Quebec, Canada) in order to

determine root surface area and root diameter distribution. Total root surface of the subsamples analyzed averaged 12.4 ± 4.7 cm^2. The sample was then dried at 60 °C and total root surface area was determined by relating dry weight of the subsample to total root dry weight.

Extraradical hyphae were quantified in all AM plants, utilizing an aqueous extraction and membrane filter technique adapted from Jakobsen et al. (1992). Twenty g of dried substrate were suspended in a solution of 100 mL deionized water and 12 mL sodium hexametaphosphate solution (35 g L^{-1}), vigorously shaken for 30 s. After 1 h, the suspension was transferred to a 40 μm sieve. The material on the sieve was rinsed gently with deionized water to remove clay particles and transferred to a 250 mL Erlenmeyer flask which was subsequently filled with 200 mL deionized water. The flask was shaken thoroughly for 5 s to flotate the hyphae. After 60 s, an aliquot of 10 mL was taken from a defined height (5 cm) of the supernatant and drawn through a 25 mm membrane filter (0·45 μm pore size). Fungal material on the filter was specifically stained with a Trypan Blue solution (5:1 = (2:1:2 = lactic acid: glycerin: H_2O): Trypan Blue (0.4 %, Sigma-Aldrich Chemie GmbH, Germany)) for 5 min. After rinsing with deionized water, the filter was transferred to a microscope slide and hyphal density expressed as length per soil dry weight was determined according to Miller et al. (1995) at × 250 magnification. Additionally, one representative replicate reflecting the average hyphal length was chosen for each species to measure the average hyphal diameter at × 400 magnification. This value was then used for calculation of hyphal SA per soil dry weight (*A*, Eq. 2).

$$A = R \cdot 2\pi r \quad (2)$$

where *R* is the hyphal length per soil dry weight and *r* is the radius of hyphae. The average hyphal diameter (4·7 μm; *r* = 2·35 μm) was induced as a constant in all calculations, as there was no significant species effect on hyphal diameter detected.

Quantification of plant N and P

Root and shoot fractions of dried plant material were ground in a ball-mill (Retsch MM 301, Retsch, Haan, Germany) prior to further analysis. 2–4 mg of ground plant material were transferred to an elemental analyzer (EuroVector, HEKAtech, Wegberg, Germany) and analyzed for total elemental C and N. Plant P content was measured using high-temperature oxidation and colorimetrical quantification according to Watanabe and Olsen (1965). Dried plant material was ashed at 500 °C for 4 h in a muffle furnace and, after cooling, 2–4 mg of ash was digested in 10 % nitric acid. The extracts were diluted with bidestilled water and analyzed for orthophosphate concentration using flow injection analysis at 880 nm (FIA-Lab II, MLE GmbH, Dresden, Germany). Tissue P and N concentrations were calculated by relating the results to total plant dry weight.

Statistical analyses

Statistical analyses were performed using Statistica 6.0 (StatSoft Inc., Tulsa, USA). Data was tested for normal distribution (Shapiro-Wilk test) and homogeneity of variances (Brown-Forsythe test). Data that did not satisfy the assumptions of normal distribution was square root or log transformed prior to analysis. One-way ANOVA was performed on data of MGD, colonization level as well as on root and relative hyphal surface (factor: species). Two-way ANOVA was performed on total biomass, tissue N and P, and N:P ratio (factors: species / functional group and mycorrhization). When significant differences were found for main effects, Fisher's LSD post-hoc pair wise comparison was applied to determine individual differences between means. T-test against zero was used on MGD data. Correlations of MGD with colonization level and relative hyphal surface area as well as of N:P ratio with relative hyphal surface area were tested. Residual analyses were performed to detect potential outliers from the regression, with data points showing studentized residuals larger than 1.5 being flagged as outliers.

Results

Significant effects on biomass caused by mycorrhization were found in six of the 13 species (Fig. 1a). Strongest positive growth effects of AMF were found for *H. pilosella*, *T. arvense* and *H. radicata*, with 18·5-, 6·7- and 6·1-fold larger total dry weight in mycorrhizal than in non-mycorrhizal individuals, respectively. *P. argentea* and *P. lanceolata* exhibited lower dry weight increases with AMF of the 2·6- and 1·6-fold, respectively. Non-significant trends to higher biomasses in AM

individuals were detected in *A. vulneraria* and *C. canescens*. All other species showed slightly decreased biomasses when mycorrhizal, with this effect being significant in the grass *A. odoratum* (Fig. 1a).

The mycorrhizal growth dependencies of the 13 species, depicted in Fig. 1b, reflect these relative biomass differences, with the forbs *H. pilosella*, *H. radicata*, *P. argentea* and *P. lanceolata*, and the legume *T. arvense*, showing significant positive differences from 0 ($p < 0{\cdot}001$), with MGD in this group exhibiting a large range between 26 and 94 %. The grasses *F. ovina* and *A. odoratum*, as well as the forb *E. cicutarium* and, surprisingly, the non-mycotroph *I. tinctoria* showed significant negative MGDs between −28 and −31 % (Fig. 1b).

AMF hyphal colonization was highest in the forbs *H. radicata*, *P. lanceolata*, *H. pilosella* and *E. cicutarium*, and in the grass *A. odoratum*, with all of these species exhibiting relative colonization levels of above 80 % (Fig. 2). Intermediate colonization between 50 and 70 % was observed in the forb *P. argentea*, both legumes *T. arvense* and *A. vulneraria* and the grass *C. canescens*. *F. ovina* showed lower colonization levels of about 33 %, while lowest colonization was observed in *D. carthusianorum* (~7 %) and the non-mycotrophs *I. tinctoria* and *T. nudicaulis* (~0 %). Interestingly, small amounts of mycorrhizal hyphae were found in the roots of individuals of *T. nudicaulis*. As expected, we found a significant positive relationship between AMF hyphal colonization and mycorrhizal growth dependency (Fig. 3). Best results were given by a logarithmic fit. The correlation revealed significance ($p < 0{\cdot}05$) and an R^2 of 0·44. Outliers from this relationship were *A. odoratum* and *E. cicutarium* with high colonization levels together with negative MGDs and *T. arvense* with comparatively low colonization corresponding to an MGD of 85 %.

Total belowground absorptive surface provided by both hyphae and roots varied strongly across species (Fig. 4). Largest total surface areas were obtained by the forbs *P. lanceolata* and *H. radicata* with ~50 and ~37 $mm^2\ g^{-1}$ soil, respectively. The other forb species and the grasses reached intermediate total absorptive surface areas between ~12 and ~25 $mm^2\ g^{-1}$ soil. Lowest values were obtained by the legumes,

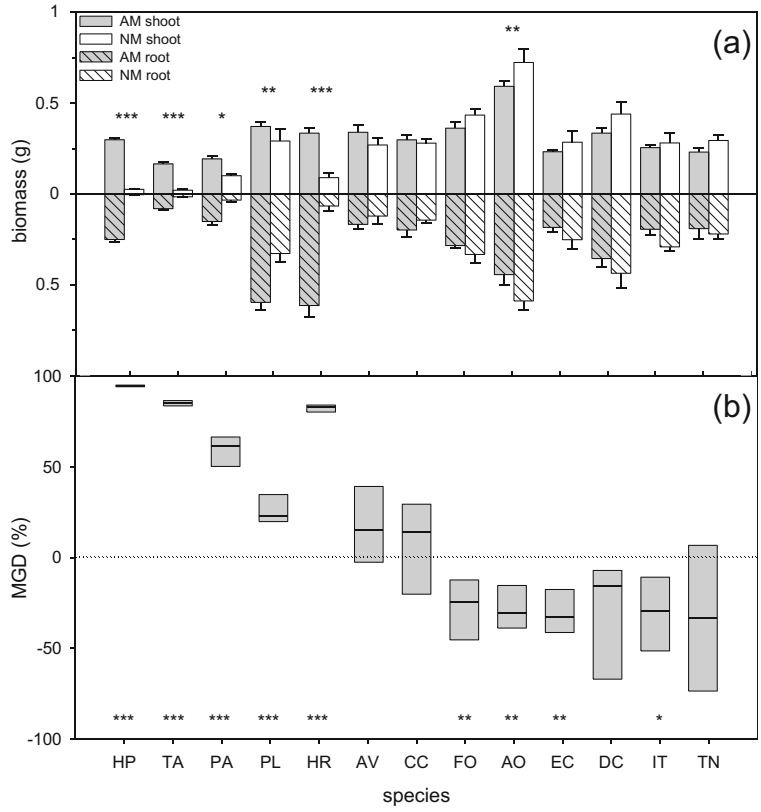

Fig. 1 **a** Aboveground (unhatched) and belowground (hatched) biomass of non-mycorrhizal (NM, white) and mycorrhizal (AM, gray) individuals. **b** Mycorrhizal growth dependency (MGD) as calculated from relative biomass differences between AM and NM individuals. Boxes visualize the 25 % and 75 % quartiles and the median values (line). 0-response is indicated by a *dotted line*. *Asterisks* mark significant differences from 0 (* $p < 0{\cdot}05$, ** $p < 0{\cdot}01$, *** $p < 0{\cdot}001$), $n = 5 \pm$ SE

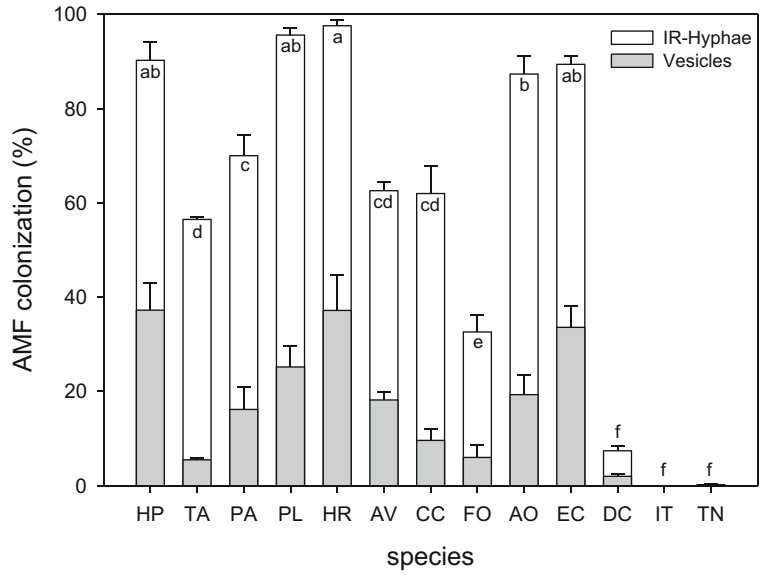

Fig. 2 Relative AMF root colonization levels with intraradical hyphae (*white*) and vesicles (*grey*). *Different letters* indicate significant differences of colonization levels at $p < 0{\cdot}05$, $n = 5 \pm SE$

the *Brassicaceae* and the forb *P. argentea* (~7 - ~9 mm^2 g^{-1} soil). Large variation between species was also observed in the relative contribution of extraradical hyphae on total belowground absorptive surface (Fig. 4). Largest relative absorptive surface areas occurred in the legume *T. arvense* (68 %), followed by the forb species *H. pilosella*, *E. cicutarium* and *P. argentea* with 50, 48 and 43 %, respectively. Grasses generally exhibited lower values between 17 and 29 %. Astonishingly, extraradical hyphae were found in low amounts in both *Brassicaceae*, accounting for 12 and 20 % of belowground absorptive surface in *I. tinctoria* and *T. nudicaulis*, respectively (Fig. 4). Particularly low counts of extraradical hyphae, with only 5 % contribution to total belowground surface area, were found in *D. carthusianorum*.

As hypothesized, there was a significant positive relationship between mycorrhizal responsiveness and relative belowground allocation to hyphae (Fig. 5; $R^2 = 0{\cdot}59$, $p < 0{\cdot}01$). *E. cicutarium* again (compare Fig. 3) deviated from this relationship, with exceptionally low MGD given the large investment into extraradical hyphae.

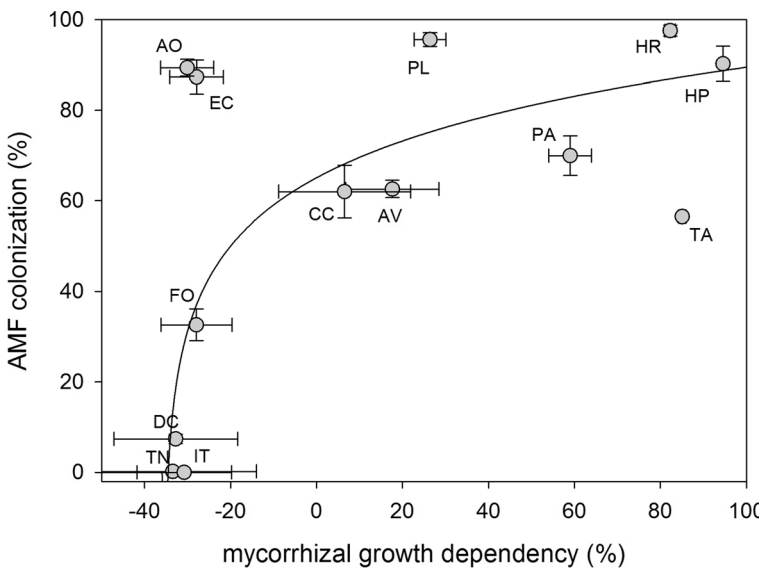

Fig. 3 Relationship between AMF root colonization levels and mycorrhizal growth dependency of all species ($n = 13$, $R^2 = 0{\cdot}44$, $p < 0{\cdot}05$)

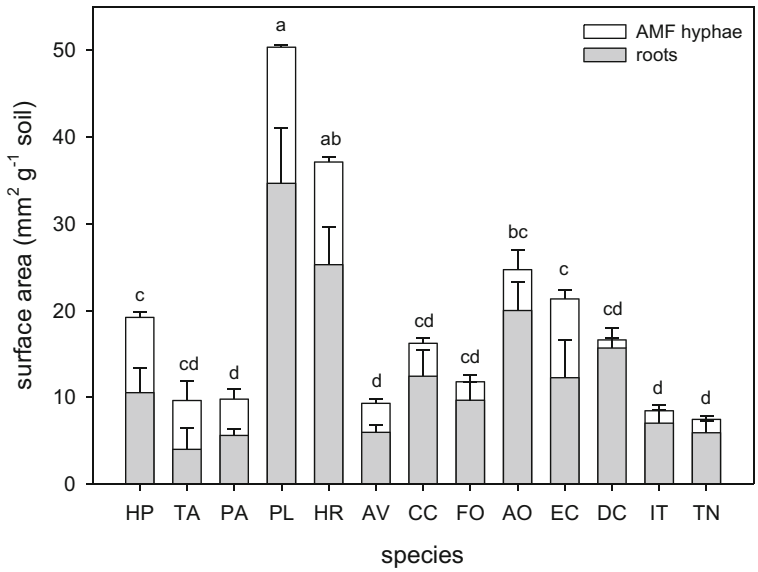

Fig. 4 Absolute belowground surface area provided by roots (*grey*) and AMF hyphae (*white*). *Different letters* indicate significant differences of total belowground surface area at $p < 0{\cdot}05$, $n = 5 \pm SE$

Both, plant N and P contents were significantly affected by the factors functional group ($p < 0{\cdot}05$) and mycorrhization ($p < 0{\cdot}05$). Additionally, a highly significant interaction between both factors was found ($p < 0{\cdot}001$). Total plant N and P contents were mainly influenced by biomass and found to be highest in mycorrhizal individuals of forbs and non-mycorrhizal individuals of grasses and *Brassicaceae*, while lowest values were detected in non-mycorrhizal legumes (Fig. 6a, b). Significant differences between mycorrhizal and non-mycorrhizal individuals for N were found only in the functional group of grasses, with on average 20 % higher N-contents in non-mycorrhizal grasses (Fig. 6a). In contrast plant P-contents were significantly higher in mycorrhizal forbs and legumes as compared to non-mycorrhizal individuals, while there was no difference detected in grasses (Fig. 6b). The differences between plant P and N contents reflected significantly on plant N:P ratio, with non-mycorrhizal plants of the highly mycotrophic functional groups forbs and legumes exhibiting N:P ratios above the range of 14 to 16, indicative for a strong P limitation, while mycorrhizal plants of these functional groups exhibited very low values of ~7, indicative for N limitation (Fig. 6c). The low or non-mycotrophic functional groups of *Brassicaceae* and grasses, on the other hand did not show significant differences in N:P ratios but exhibited generally higher N:P ratios than mycorrhizal forbs and legumes (~9–13, Fig. 6c). The differential behaviour of high and obligate mycotrophs, facultative mycotrophs and non-mycotrophs regarding N and P relations resulted in a strong negative relationship of N:P ratio with relative contributions of hyphal surface area to total belowground surface area ($R^2 = 0{\cdot}69$, $p < 0{\cdot}001$, Fig. 7).

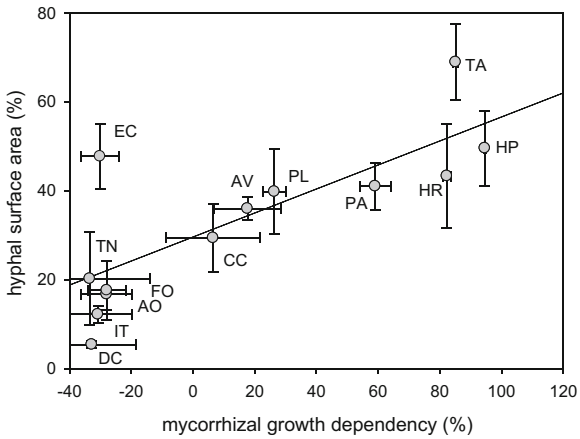

Fig. 5 Relationship between relative hyphal belowground surface area and mycorrhizal growth dependency of all species ($n = 13$, $R^2 = 0{\cdot}59$, $p < 0{\cdot}01$)

Discussion

Mycorrhizal responsiveness is species- and functional group-specific

The studied sand ecosystem species exhibited a large range of mycorrhizal responsiveness (Fig. 1b). According to Wang and Qiu (2006) a species can be either

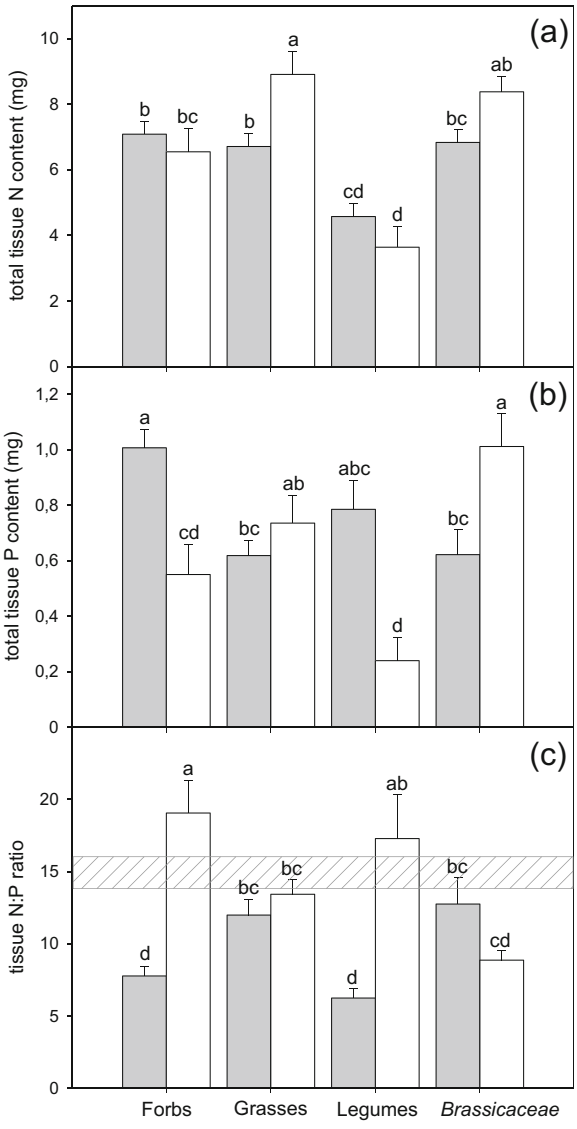

Fig. 6 **a** Mean total plant tissue N- (unhatched) and (**b**) P-contents in mg (NM white, AM gray) and (**c**) plant tissue N:P ratios of the four functional groups forbs, grasses legumes and *Brassicaceae* (NM white, AM gray). The hatched box indicates the shift in N:P ratio between N- to P-limitation. *Different letters* indicate significant differences at $p < 0{\cdot}05$, $n = 10–30 \pm$ SE

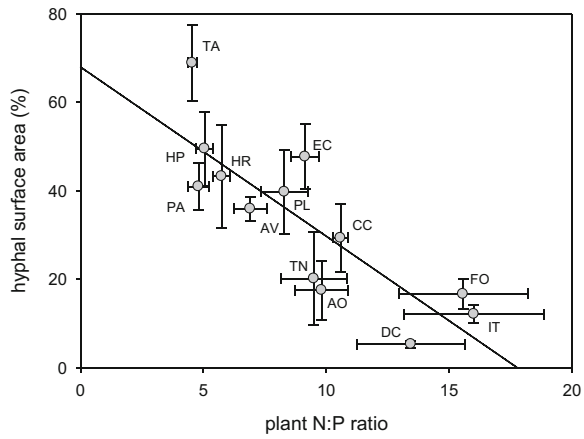

Fig. 7 Relationship between relative hyphal belowground surface area and plant N:P ratio of AM-individuals of all species ($n = 13$, $R^2 = 0{\cdot}69$, $p < 0{\cdot}001$)

obligately mycorrhizal (AM), facultatively mycorrhizal (AM + NM) or non-mycorrhizal (NM), depending on the formation of mycorrhiza occurring either under all circumstances, in one habitat but not in another or never, respectively. In a recent study Hempel et al. (2013) published a database containing literature information on mycorrhizal status of 1758 species (MycoFlor). According to this study the species used in our experiment fall into the following mycorrhizal categories: (i) AM - *H. pilosella*, *T. arvense*, *P. argentea*, *P. lanceolata* and (ii) AM + NM - *H. radicata*, *A. vulneraria*, *C. canescens*, *F. ovina*, *A. odoratum*, *E. cicutarium*, *D. carthusianorum*, *I. tinctoria*. Although *I. tinctoria* is listed as AM + NM, it was characterized as completely NM by Wang and Qiu (2006) after Harley and Harley (1987) and also by Akhmetzhanova et al. (2012). None of these data bases provide information on mycorrhizal status of *T. nudicaulis*. We found that all AM-classified species had a significant positive mycorrhizal growth dependency, while AM + NM classified species showed neutral or negative responses to AMF, with the exception of the forb *H. radicata*. The mycorrhizal status AM + NM of *H. radicata* from Wang and Qiu (2006) relates to *H. radicata* being facultatively mycotrophic (Titus and del Moral 1998), while there is no available information of *H. radicata* occurring naturally non-mycorrhizal. However, the information on mycorrhizal status given by the literature does not necessarily reflect nutritional dependency on mycorrhization, which the term "mycotrophic" relates to (Janos 2007; Höpfner et al. 2014). Therefore, *H. radicata* may also be considered exclusively AM, which is supported by the very high mycorrhizal growth dependency found for this species here (Fig. 1b) and in other studies (Höpfner et al. 2014). Thus, in general, mycorrhizal status as reported in the literature and mycorrhizal responsiveness found in this study showed good agreement.

When looking at functional group-specific MGD, forbs and legumes on average showed positive growth responses, while grasses and *Brassicaceae* showed neutral or negative growth responses to mycorrhization.

This was expected as, while there is vast literature information on high mycorrhizal advantages in many forbs (e.g. Hetrick et al. 1992; Wilson and Hartnett 1998; Höpfner et al. 2014) and legumes (e.g. Chalk et al. 2006; Sprent and James 2007; Saia et al. 2014), a common trait of the *Pooideae* is unresponsiveness to mycorrhization (Reinhart et al. 2012) and *Brassicaceae* in general do not develop mycorrhiza (e.g. Lambers and Teste 2013). However, there are exceptions, where certain *Brassicaceae* species have been reported to be colonized with endomycorrhiza (e.g. Orlowska et al. 2002; Regvar et al. 2003; Veiga et al. 2013). Indeed, very low colonization was found in *T. nudicaulis* and low amounts of extraradical mycelia were found in both *Brassicaceae* species. Growth effects of mycorrhiza in non-host plants have been described as generally negative (e.g. Allen et al. 1989; Veiga et al. 2013), with the only possible mechanism responsible in a single-host-situation being the activation of defensive mechanisms in the non-host after infection with mycorrhiza, resulting in a loss of plant fitness (Allen et al. 1989; Veiga et al. 2013). This may be an explanation for the significantly negative MGD observed in *I. tinctoria* (Fig. 1b).

AMF colonization correlated well with mycorrhizal responsiveness (Fig. 3), which has been shown by other studies (e.g. Treseder 2013) and is not unexpected, as high root colonization levels in the mycotrophic species are a prerequisite for a functional symbiosis, and AM-unresponsive species have developed other means of efficient P-uptake, such as adequate root traits (Smith and Smith 2011a), and consequently can reduce C-P trade with the fungus leading to lower colonization. However, exceptions from the positive relationship between colonization level and MGD where observed for the facultative mycotrophic species *A. odoratum* and *E. cicutarium*, with exceptionally high AMF colonization corresponding to a significantly negative MGD and the obligate mycotrophic legume *T. arvense* with comparatively low AMF colonization considering its very high MGD. Negative MGDs are generally considered to result from growth depressions caused by the fungus, resulting from a C-P trade imbalance in harm of the host plant (Smith and Smith 2011a) under for the symbiosis unfavorable soil conditions, such as high soil P-contents (Johnson 2010). However, as P-contents in this experiment were kept similarly high in all species, species differences in the fungal mutualism to parasitism continuum caused by the soil conditions alone may be excluded. Data on hyphal surface area rather suggests that under unfavorable C-P trade conditions some species can control AMF colonization better than others, i.e. high investment into mycorrhizal structures seems to be partially responsible for negative MGDs in *A. odoratum* and *E. cicutarium* but not in other species. Indeed, particularly with lacking fungal biomass, negative MGDs at low root colonization, as found for *D. carthusianorum*, *F. ovina* and the *Brassicaceae*, cannot likely be explained with large C-costs to the plant (Smith et al. 2009). There is some evidence that negative MGDs may not necessarily be linked to critical C-drain from the plant under higher soil P-levels, but rather that the AMF may suppress the root P-uptake pathway (Smith et al. 2009; Smith and Smith 2011b). This explanation is supported by strongest P-limitation found for the species with low colonization and negative growth responses, *D. carthusianorum*, *F. ovina* and *I. tinctoria* (Fig. 7).

Belowground allocation trade-off correlates with mycorrhizal responsiveness

Many studies have described root morphology to be linked to mycorrhizal responsiveness, with facultative mycotrophs and non-mycotrophs developing larger fine-root systems, higher branching or root hair densities than obligate mycotrophs to compensate for the lack or the inadequacy of AMF for nutrition (Baylis 1970; Miller et al. 1995; Schweiger et al. 1995; Brundrett 2002; Höpfner et al. 2014). Based on this, we assumed a clear belowground allocation trade-off, with allocation to either roots or hyphae being dependent on the species-specific mycorrhizal responsiveness. While total belowground allocation was unrelated with MGD or functional group (Fig. 4) but rather depended on total biomass ($R^2 = 0{\cdot}68$) and root / shoot ratio ($R^2 = 0{\cdot}71$), the trade-off was clearly visible in a significant correlation between MGD and relative belowground allocation to hyphae (Fig. 5). Here, highly mycotrophic species exhibited highest relative investments into hyphal surface, while relative investment into root surface was highest in facultative and non-mycotrophic species (Fig. 5). Again, the forb *E. cicutarium* deviated from the correlation, with large investments into hyphal surface area but negative MGD. Despite this species matching the traits of a highly mycotrophic species in all morphological aspects, it did not show any mycorrhizal benefits. This can be explained by *E. cicutarium* having on average 43 % lower P-benefits as compared

to other species with similarly high investments into AMF. Kiers et al. (2011) found carbon investment in the species *Medicago truncatula* and *Allium porrum* to depend on cooperativeness in P-delivery of different fungal symbionts, concluding that a generally reciprocal exchange of nutrients stabilizes the symbiosis. As this may be true for most of the here studied species, our results indicate that there may be exceptions for this finding, with *E. cicutarium* exhibiting unproportionally high hyphal allocation for low P-gain from *R. irregularis*. However, as highlighted by Smith and Smith (2011a), mycorrhizal responsiveness may change over a plants life span (Li et al. 2005) and the supposedly negative high allocation to AMF in *E. cicutarium* may perhaps be seen as a preliminary investment to gain larger benefits in the reproductive state.

Belowground allocation trade-off defines plant N: P relations

N- and P-stoichiometry of soils is known to affect the cost-benefit ratio of the AMF symbiosis to a plant (Johnson 2010). AMF provide nutritional benefits mainly through the delivery of immobile nutrients such as orthophosphate to the plant (Koide et al. 2000; Smith and Smith 2011b). P-nutrition via AMF is often more effective than via roots, because hyphae can scavenge immobile P far away from roots, when root uptake is faster than P-recharge of the depletion zone (Smith et al. 2011). In addition hyphae can grow faster and demand lower costs in terms of belowground C investment than roots (Fitter 1991; Jakobsen et al. 1992; Schweiger et al. 1995) and are able to exploit small-scale nutrient patches due to their smaller size (Hodge 2004). Therefore, it is not surprising that P-relations in this study were closely related to MGD and relative allocation to hyphal surface with tissue P-content showing significant positive correlations with these parameters (R^2 = 0·63 and 0·33, respectively). These results are in line with Höpfner et al. (2014), who found P-depletion from soil being dependent on hyphal surface area growth rate in five of the here studied species.

In contrast, nitrogen supply by AMF to a host plant has generally been considered to be of minor importance (Read 1991; Hodge and Storer 2015). Particularly, the advantage of hyphae to rapidly scavenge immobile phosphate from locations far away from roots does not seem to apply for the more mobile inorganic forms of NH_4^+ and NO_3^- (Smith and Smith 2011b) and the general opinion does not include saprophytic abilities of AMF to obtain organic N (Smith and Read 2008). However, there is a growing body of evidence for substantial uptake and transfer of N by AMF from both organic (e.g. Hodge et al. 2001; Leigh et al. 2009; Hodge and Fitter 2010; Whiteside et al. 2012) and inorganic sources (e.g. Govindarajulu et al. 2005; Tanaka and Yano 2005; Ashgari and Cavagnaro 2012; Fellbaum et al. 2012). Nevertheless, although AMF can acquire nitrogen, even if only for their own sustenance (Hodge and Storer 2015), the importance of AMF in N-nutrition of plants in comparison to N-nutrition via roots to date is still a matter of debate. Although a few studies found considerable mycorrhizal N-provision to the plant (George et al. 1992; Tanaka and Yano 2005; Leigh et al. 2009), the ecological significance of the mycorrhizal pathway for N-nutrition is doubtful (Smith and Smith 2011b), as the majority of studies report no clear advantage to the plant with AMF-induced N-nutrition (e.g. Ames et al. 1983; Cui and Caldwell 1996; Hawkins et al. 2000; Reynolds et al. 2005; Hodge and Fitter 2010) and there is a large variability of N-uptake and transfer observed in different studies (Hodge and Storer 2015). It is argued that enhanced N-status in mycorrhizal plants may be a side-effect of enhanced P-status leading to larger N-acquisition by the direct root uptake pathway (Reynolds et al. 2005). On the other hand, the existence of a hidden N-uptake by plants via the AMF mycelium has been argued, where analogous to P, the fungal N-uptake pathway may inhibit the direct root uptake pathway, although the experimental evidence is lacking (Smith and Smith 2011a).

Data obtained in this study suggest that, in contrast to P-nutrition, N-nutrition was not enhanced by AMF, particularly in the highly mycotrophic functional groups of forbs and legumes, while AMF had a significant negative effect on N-contents of the facultative mycotrophic group of grasses, with the same trend being detected in the non-mycotrophic *Brassicaceae*, although here not significant (Fig. 6a, b). The contrasting species- and functional group-specific effects of AMF on plant P- and N-contents reflected strongly on plant N:P ratio, with AM-plants of highly mycotrophic groups being strongly N-limited, while the NM-controls showed a significant P-limitation (Fig. 6c). In contrast, there was no difference in N:P ratio between AM- and NM-individuals of less mycotrophic and non-mycotrophic species, with both groups showing N:P ratios indicative

for a moderate N-limitation (Fig. 6c). These contrasting responses resulted in a significant negative correlation of plant N:P ratio with MGD ($R^2 = 0{\cdot}67$) as well as with relative allocation to hyphal surface area ($R^2 = 0{\cdot}69$; Fig. 7). It has been suggested earlier that N:P ratio is a decisive factor in C-allocation to AMF (Johnson et al. 2003; Treseder and Allen 2002; Johnson 2010). Johnson et al. (2003) showed that nitrogen fertilization decreased allocation to AM structures at sites with ample soil P, but it increased allocation to mycorrhiza at sites with P-deficient soils. Thus, with increasing soil N:P ratios C-allocation to AM can be either reduced or increased, depending on P being limiting or not. These findings were in accordance with the functional equilibrium model (Brouwer 1983) predicting that plant photosynthate is preferentially allocated toward structures that acquire resources that are most limiting. In this study, with substrate N:P ratios of approximately ~0·17, phosphate was not a limiting factor, whereas nitrogen was. Thus, allocation to AMF in this design is generally not expected to be highly beneficial to the plant. The trade-balance model published by Johnson (2010) predicts commensalism between fungus and plant under such soil conditions, because plants have nothing to gain from C-for-P trade, but C-demand by AMF is kept in check because fungal growth is N-limited. However, our data suggests that the outcome of the trade-balance model is highly species-specific: Positive mycorrhizal growth responses were found for highly mycotrophic species, with presumably poor P-uptake performance in the NM state. Although mycorrhizal P-uptake was beneficial for these species, large allocation to AMF resulted in strong N-limitation. Facultative and non-mycotrophic species with lower investment into AMF, on the other hand, showed neutral or negative growth responses. Here, lower N-contents in the AM-state probably also resulted in lower P-supply by limiting additional root growth. In both cases data suggests N-limitation rather than C-limitation due to increased fungal allocation as a driving factor for the outcome of the symbiosis.

N-limitation in mycorrhizal individuals can be explained by the low C:N ratios, and the consequently high N-demand of the fungal symbiont itself (e.g. Hodge and Fitter 2010). Given that the direct pathway of nutrients in root-mediated foraging gives sole access of the plant to the nutrients taken up, enhanced C-allocation to AMF and predominant nutrient-uptake by the fungal symbiont may pose a large disadvantage to the plant under low soil N:P ratios through N-competition by the symbiont. However, data suggests that plants do not always have a choice, as evolutionary attachment to P-nutrition via AMF in obligately mycotrophic species, such as many of the here studied forbs and legumes, does not allow growth in the NM-state. Particularly, the forb *E. cicutarium* with obvious disadvantages posed by the symbiosis was not able to decrease colonization and hyphal surface area allocation, suggesting some dominance of the fungal partner in the interaction. Thus, the functional equilibrium model does not seem to apply in the AMF-symbiosis under all circumstances and rather than a trade-off, allocation to hyphae vs. roots may be a species- and functional group-specific predisposition. Further, evolutionary detachment of plants from AMF in many species may be explained in part by disadvantages posed by fungal competition.

In line with our results, and under similar experimental conditions, Höpfner et al. (2015) showed that AMF-mediated foraging may even pose a disadvantage in competitive interactions with species favouring a more root-dominated nutrition, which may be partially caused by N-limitation through fungal N-acquisition. This suggests that the occurrence of root vs. mycorrhizal strategists in habitats with diverse soil nutritional conditions, namely N:P ratios, may partly be driven by mycorrhizal benefits balancing competitive interactions and thus, play an important role in successional progress of nutrient-poor sand dune ecosystems.

Conclusions

Our study showed strong relationships of mycorrhizal growth dependency with allocation to hyphal vs. root surface area in 13 European sand dune species. The trade-off between predominant investments into either roots or hyphae was clearly related to the plant N:P relations, with root strategists gaining larger N- and lower P-benefits than mycorrhizal strategists. Data showed that P-delivery by the AMF *R. irregularis* was accompanied by strong N-competition from the fungus. The low soil N:P ratio, typical for sand dune ecosystems, thus only allowed for positive mycorrhizal growth dependencies in mycorrhizal strategists, where strong evolutionary attachment to P-nutrition via AMF does not allow growth in the NM-state. Therefore, rather than C-limited parasitism through AMF, negative growth

effects can likely be explained by N-limitation in presence of AMF under N-limiting soil conditions. This may have ample significance for the occurrence of root vs. mycorrhizal strategists in habitats with diverse soil nutritional conditions, where mycorrhizal benefits may drive competitive interactions and in turn successional progress.

Acknowledgments The authors wish to thank Elke Furlkröger, Christine Schlüter and Barbara Teichner for technical support with plant cultivation and laboratory work.

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