Original Article

Resolving the ‘nitrogen paradox’ of arbuscular mycorrhizas: fertilization with organic matter brings considerable benefits for plant nutrition and growth

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ABSTRACT

Arbuscular mycorrhizal fungi (AMF) can transfer nitrogen (N) to host plants, but the ecological relevance is debated, as total plant N and biomass do not generally increase. The extent to which the symbiosis is mutually beneficial is thought to rely on the stoichiometry of N, phosphorus (P) and carbon (C) availability. While inorganic N fertilization has been shown to elicit strong mutualism, characterized by improved plant and fungal growth and mineral nutrition, similar responses following organic N addition are lacking. Using a compartmented microcosm experiment, we determined the significance to a mycorrhizal plant of placing a 15N-labelled, nitrogen-rich patch of organic matter in a compartment to which only AM hyphae had access. Control microcosms denied AMF hyphal access to the patch compartment. When permitted access to the patch compartment, the fungus proliferated extensively in the patch and transferred substantial quantities of N to the plant. Moreover, our data demonstrate that allowing hyphal access to an organic matter patch enhanced total plant N and P contents, with a simultaneous and substantial increase in plant biomass. Furthermore, we demonstrate that organic matter fertilization of arbuscular mycorrhizal plants can foster a mutually beneficial symbiosis based on nitrogen transfer, a phenomenon previously thought irrelevant.

Key-words: arbuscular mycorrhiza; growth; nitrogen; nitrogen paradox; organic matter; symbiosis.

INTRODUCTION

The arbuscular mycorrhizal (AM) association is the most common type of mycorrhizal symbiosis and forms between c. two-thirds of all land plant species and soil fungi in the phylum Glomeromycota. The fungus receives photosynthetically fixed carbon (C) while, in return, the fungus confers a number of benefits to its associated host plant, the most well-established being that of increased acquisition of phosphorus (P) (Smith & Read 2008). More recently, however, there has been renewed interest in the ability of arbuscular mycorrhizal fungi (AMF) to supply nitrogen (N) to their associated host plant and the implications this may have for N cycling (reviewed by Hodge & Storer 2015).

While it has been shown that AMF can transfer N to their associated host (Ames et al. 1983; Hodge et al. 2001; Barrett et al. 2011) significant doubts remain as to the ecological relevance of such a AMF–N uptake pathway (see Read 1991; Smith & Smith 2011). In particular, regarding the exact mechanism of N transfer and, more importantly, the amounts of N transferred via the AMF compared to the N requirements of the plant (Smith & Smith 2011). Although results from root organ culture studies suggest values of up to 50% of root N may be acquired via the AMF route (Govindaraju et al. 2005), ideal as these systems are for unpicking mechanisms involved in nutrient exchange, it may be unwise to infer much about whole plant nutrient dynamics. Source–sink relationships, for example, are undoubtedly unrealistic given the growth conditions employed (Smith & Smith 2015). More realistic experiments, using whole plants and adding N as organic matter patches, have shown that AMF contribution to plant N uptake can be as high as 15–20% (Leigh et al. 2009; Barrett et al. 2014). Although this may suggest a significant nutritional contribution to the plant, the total plant N content (Hodge et al. 2000a, Hodge 2001, Leigh et al. 2009) and plant biomass (Hodge et al. 2001; Herman et al. 2012) are usually unaffected. In some cases, the plant may even suffer a reduction in biomass (Reynolds et al. 2005), implying providing N fertilization to N-limited symbioses may be deleterious.

Johnson (2010) proposed the ‘trade balance model’ to explain the apparent ‘nitrogen paradox’, where nitrogen fertilization of AM plants causes apparent mycorrhizal parasitism of partner plants. Fundamentally, the model states that the relative supply of C from the plant and availability of N and P in the soil determines the extent to which the AM-route for N uptake is mutually beneficial. The model suggests that fertilization with N is only beneficial if the plant is limited by P and will therefore benefit from providing C to the roots and mycorrhizal fungi.

Positive growth responses to N fertilization have been shown in plants receiving inorganic N inputs (Johnson et al. 2014), but corroborating evidence for an AM-mediated plant growth response after being fertilized with organic N is lacking. Addressing this knowledge gap is now pressing, given the ecological role of AMs in nitrogen cycling (Hodge, 2014;...
Hodge & Storer 2015) and the nature of soil N. Most rhizosphere N is bound in complex, organic material (Bremner 1949, Stevenson, 1994) and only a small, ephemeral pool of inorganic nitrogen exists at any given time, and inorganic N turnover in soil is rapid (Jackson et al. 1988). The integrity of the trade balance model in systems fertilized with organic N is thus far untested.

Organic N fertilization is receiving increased attention in both research and agriculture with the adoption of more sustainable agricultural practices not only in Europe but across the world (Matson et al. 1997). Inorganic N fertilization may reduce mycorrhizal inoculum potential of agricultural soil (Liu et al., 2012); increase pathogen severity (Matson et al. 1997) and boost greenhouse gas fluxes from agricultural soils (McSwiney & Robertson 2005). Combining organic N fertilization and the mycorrhizal symbioses may be useful in negating some of these problems and increasing assimilation of fertilizer N into plants which is currently limited to around 40–60% in crop plants to which inorganic N is applied (Huber & Watson 1974, Paustian et al. 1995).

Given the abundance of AMFs in temperate soils and the range of plant species they may colonize (Smith & Read 2008), it is surprising that we do not comprehensively know which forms of soil nitrogen can be utilized by the fungus. It is well established that AMF acquire inorganic N as NH₄⁺ and NO₃⁻ (Govindaraju et al. 2005; Leigh et al. 2011; Johnson et al. 2014), which represent the most abundant available inorganic sources in the hyphosphere (Tinker & Nye 2000). How commonly AMF utilize organic N directly is less well known. Experiments have shown that AMF may be capable of direct glycine uptake (Hawkins et al., 2000; but see Hodge 2001; Whiteside et al. 2012), and Cappelazzetto et al. (2008) identified an amino acid permease in Glomus mosseae, a mechanism by which an AMF may acquire organic N directly from soil substrates. Similarly, Belmondo et al. (2014) show evidence for potential uptake of organic N by a dipeptide transporter in the extraradical mycelium of Rhizopagus irregularis. However, AMF seem not to acquire organic N exclusively or indeed preferentially, as ¹³C enrichment is usually not detected in AM hyphae or plant tissue following hyphal access to ¹³C;¹⁵N dual labelled organic matter (Hodge & Fitter 2010, Nuccio et al. 2013).

By their very nature, complex organic matter patches contain a mixture of organic and inorganic sources of N. Both inorganic N and the simplest organic N components are likely to be relatively labile and more easily mobile in the soil than larger organic, nitrogenous constituents (Nemeth et al. 1987). In microcosm experiments with separate root and hyphal compartments, the potential for the N-rich, labile fraction from organic matter patches to leach from one compartment to another presents uncertainty. This is compounded as there remains in the literature a lack of patch analysis to show the relative composition of the patch (organic versus inorganic N).

In this experiment a patch of ¹⁵N labelled algal material was used in order that the amount of N acquired by the plant from the patch could be measured. Algae was used owing to its low C:N ratio of 7:1, representing a rich N source. Compartmented microcosms were employed to investigate the effect of a discrete zone or ‘patch’ of N-rich organic matter to a sand and clay growth medium of low-N and low-P availability. Mycorrhizal plants were contained in one compartment while AMF hyphae were permitted access to a second compartment containing the algal patch. Control microcosms in which the AMF could not access the patch were included in order that N movement via mass flow and diffusion could be determined.

**MATERIALS AND METHODS**

**Microcosm design**

Microcosm units were constructed by fastening together two polypropylene boxes, adapted from Hodge & Fitter (2010). The plant compartment measured 7 × 14 × 16 cm, and the patch compartment 14 × 14 × 16 cm. A window cut in the abutting sides of the boxes created an aperture (4 × 6 cm) that was covered with a double-ply mesh barrier. The ‘Arbuscular Mycorrhizal Access’ (AMA) units used a 20 μm mesh (John Stanier and Co., Whitefield, Manchester, UK) barrier, which prevented root access but allowed AMF hyphal access. The ‘No Arbuscular Mycorrhizal Access’ (NAMA) units used a 0.45 μm mesh (Anachem, Bedfordshire, UK) that prevented the access of both roots and AMF hyphae to the patch compartment. This 0.45 μm mesh barrier does not retard the diffusion of solutes from the patch compartment to the plant compartment, but the AMF mycelium cannot encounter the organic matter patch directly in NAMA microcosms. Into the bottom of each compartment, four holes were drilled and covered with 20 μm mesh to permit drainage. Both compartments of the microcosms were filled with a 1:1 (v/v) mix of silica sand and AgSorb® [a calcinated, attapulgite clay soil conditioner, Oil-Dri, Cambridgeshire, UK (formerly TerraGreen®; see Hodge et al. 2000a)]. Both sand and AgSorb® were washed 3 times in de-ionized water prior to mixing, in order to minimize mobile mineral ions in the growth medium. Within the patch compartment of the microcosm units, the organic matter was contained inside a PVC pipe of diameter 2 cm and height 7 cm, which has two windows cut into the sides, creating two apertures each with dimensions 4 cm (H) × 1 cm (W). These apertures were covered in the same 20 μm mesh as detailed above (and see Field et al. 2012). Such a setup ensures a uniform patch size across all microcosms, permits AMF hyphal access and allows easy placement of the organic matter patch (Fig. 1).

**Plantago lanceolata L.** was selected as the host plant owing to its ability to become highly colonized by AMF. Seeds of *P. lanceolata* (Emorsgate Wild seeds, Nottingham, UK) were surface sterilized in a 5% (w/v) calcium hypochlorite solution, after which they were germinated on filter paper in a sterile Petri dish. At week 0, 2-week-old seedlings were transferred into each plant compartment, 4 cm from the mesh aperture (three seedlings were planted into each microcosm, subsequently thinned to a single seedling at week 2). The plant compartment was watered daily, as required, with de-ionized water.

Except for an aperture through which the plants grew, microcosms were enveloped in aluminium foil to reduce the influx of contaminating organisms. The microcosms were planted on 30
experiment, the nutrient solution added to the plant compartment provided 112 mg N and 2.16 mg P. The bone meal provided 23 mg N and 58 mg P. The patch compartment received no further nutrient additions after the patches had been placed.

Patch material

After 16 weeks of plant growth in the microcosms, patches of organic litter were added to the patch compartment, 6 cm away from the mesh aperture between compartments. Each patch contained 0.075 g of 98 Atom% 15N-labelled algae (obtained from Sigma-Aldrich, St Louis, MO, USA) in a matrix of 0.8 g homogenized algal matter (Chlorella variabilis – PinkSun Essentials and Organics, Clayton, Yorkshire, UK). The patch contained 59 mg N (8.85 mg of which was 15N), 26 mg P, 413 mg C and the C:N ratio of the organic matter patch was 7:1.

The organic patch was mixed into 20 g of the silica sand: AgSorb® mix, which was then placed into the PVC pipe, filling the bottom 5 cm. The remaining 2 cm of PVC core was filled with the sand: Agsorb® growth medium only. The PVC pipe was placed into the patch compartment to a depth of 7 cm, such that the top of the core was flush with the level of the growth medium in which it sat.

Labile nitrogen as ammonium or nitrate in the algal patch was quantified by spectrophotometer (CECIL 100 spectrophotometer, Spectronic Analytical Instruments, Leeds, UK) and calculation from standard curve, created using standards containing 10 mg N L−1 made from NH4Cl and KNO3. Briefly, 0.2 g algal material was mixed with 10 mL de-ionized water and incubated for 60 min at 50 °C. This preparation was then centrifuged at 5000 g for 15 min, after which the supernatant was decanted. Labile nitrate was measured as detailed in Cataldo et al. (1975). Briefly, a 0.2 mL aliquot of supernatant was placed in a 50 mL Erlenmeyer flask, to which 0.8 mL 5% (w/v) salicylic acid in 96% (v/v) H2SO4 was added. After cooling to 20 °C the flask received 19 mL of 2 M NaOH to raise the pH above 12. Absorbance was measured at 410 nm, after samples had cooled to 20 °C. Labile ammonium quantification required the use of the solutions ‘A’ and ‘B’, with details of preparation given below. A 0.05 mL aliquot of supernatant was mixed with 1 mL ‘solution A’, 0.25 mL ‘solution B’ and 2.5 mL de-ionized H2O. Both solutions ‘A’ and ‘B’ were prepared using de-ionized water. Solution A contained 20 g trisodium citrate dihydrate, 17 g salicylic acid, 5 g NaOH and 0.2 g sodium nitroprusside, made up to 500 mL. Solution B, also made up to 500 mL, contained 5 g NaOH and 0.4 g dichloroisourea. Absorbance was measured by spectrophotometer at 650 nm.

Harvest

At 23 weeks after planting, the systems were destructively harvested. The P. lanceolata was separated into shoots and roots, and dried at 80 °C for 48 h. A subsample of the extracted roots was retained to assess root length colonization by the AMF. After drying, root and shoot material was ground and

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homogenized in a ball mill (Retsch MM400, Retsch GmbH, Haan, Germany), for analysis by Isotope Ratio Mass Spectrometry (PDZ 2020, Sercon Ltd, Crewe, UK).

Phosphorus content was measured using X-ray fluorescence spectrometry (XRF). Briefly, dried plant material was milled and homogenized as described above, before being pressed into a pellet and analysed with a portable X-ray fluorescence spectrometer (as Reidinger et al. 2012).

Mycorrhizal roots were stained using the method of Kormanik & McGraw (1982). Roots were cleared in 10% (w/v) KOH, acidified in 1% (v/v) HCl, stained with acid fuchsin and then stored in destain solution (lactic acid, glycerol, distilled H2O 10:1:1). All procedures were incubated at 20 °C, as per the ‘no heating’ variation of the method, detailed by Kormanik & McGraw (1982).

To quantify AMF extraradical mycelium, 5 g samples of growth medium were taken from the plant compartment, from within the PVC pipe containing the organic matter patch, and from the ‘bulk’ growth medium (i.e. within the patch compartment but outside the PVC core). Hyphal extraction was carried out by the modified membrane filter technique of Staddon et al. (1999), and hyphal length assessed using the gridline intercept method from which hyphal length densities were then calculated (Hodge 2003).

Statistical analysis

All data were analysed using SPSS 21 (IBM SPSS Inc. Armonk, NY, USA), utilizing Levene’s test for equality of variance. Data for HLD in bulk versus plant compartments were analysed using Paired-Sample T Tests, while all other data were analysed using Independent-Samples T Test. Data were transformed to satisfy Kolmogorov–Smirnov and Shapiro–Wilk tests of normality. Percentage data were square root-arcsine transformed before analysis.

RESULTS

Total plant dry weight increased substantially when AMF hyphae were allowed access to the patch compartment (3.44 ± 0.21 g with access versus 2.09 ± 0.23 g without access, \( T_{1,37} = 4.33, P < 0.001 \)). This increase in plant dry weight was driven by an increase in both the shoot and root mass, which increased by 62% and 73%, respectively, compared with those plants whose AMF partner was not permitted access to the organic patch (Fig. 2). There was, however, no significant difference in the root weight ratio (RWR: ratio of root dry weight to total plant dry weight) between any treatments, suggesting that allocation of biomass between roots and shoots did not change as a result of the plants’ AMF partner having access to the organic material substrate.

Allowing AMF hyphal access to the patch greatly increased the plant uptake of \( ^{15} \text{N} \), measured both in the shoot and the root of the partner plants (Table 1). In total, plants with AMF access to the patch contained 1.10 ± 0.25 mg \( ^{15} \text{N} \) compared with 0.34 ± 0.12 mg (\( T_{1,37} = 4.91, P < 0.001 \)) in the plants whose AMF partner was denied access to the patch. The presence of \( ^{15} \text{N} \) in the No AMF Access microcosm plants (Table 1) is ascribed to the mass flow and diffusion of \( ^{15} \text{N} \)-containing molecules through the 0.45 μm mesh from the patch to the plant compartment. Such a difference in plant \( ^{15} \text{N} \) content between AMF Access and No AMF Access microcosms highlights the important role AMF can play in nutrient acquisition from nutrient-rich areas placed at significant distances beyond the rhizosphere. Similarly, the contribution made by patch N to overall plant N was greatly increased when plants had AMF access to the patch (Fig. 3): 18 ± 3%, compared to 9 ± 1% (\( T_{1,37} = 3.57, P = 0.001 \)). \( P. \text{lanceolata} \) benefited greatly from this AMF contribution acquired from the patch as demonstrated by the 68% increase in shoot N content when AMF had access to the patch compartment, corroborated by an 80% increase in root N content (Table 1). Thus total N in the whole plant was increased 76%. Although total plant N content increased, plant N concentration was not significantly different between the two AMF access treatments. The proportion of the patch N acquired by the plant increased from 4% to 12% when AMF were permitted access to the patch compartment (\( T_{1,37} = 4.98, P < 0.001 \)), suggesting that the AMF were adept...
at exploiting a newly available patch of organic matter and transferring the N acquired to their plant partner. Allowing AMF hyphal access to the patch resulted in 4.69 mg extra N in the roots and 4.61 mg in the shoots which greatly outweighs the 0.001124 mg of ammonium-N and 0.0003976 mg nitrate-N extractable from the patch.

Root P concentration increased by 28% when AMF had access to the patch ($T_{1,34} = 3.31, P = 0.002$), but the shoot P concentration was not affected by allowing AMF access to the patch. The increase in root P concentration was not substantial enough to change the total plant P concentration ($T_{1,34} = 0.16, P = 0.88$), but combined with an increased root mass, root P content increased by 135%. Similarly, despite no increase in P concentration, shoot P content was 94% greater in AMF access plants than in no AMF access plants (Table 1). Plants with AMF access to the patch had marginally higher N:P ratios (total plant N content/total plant P content) than plants with no patch access although this was only weakly significant ($T_{1,34} = 1.98, P = 0.060$). Mean AMA plant N:P was 2.18 ± 0.15, compared with mean NAMA plant N:P of 1.84 ± 0.10.

Although low levels of fungal hyphae ($0.01 ± 0.01 \text{mg } g^{-1} \text{DW}$) were found in the organic matter patches where AMF were denied access, hyphal length densities (HLD) were significantly greater ($T_{1,37} = 18.67, P < 0.001$) in the treatments that permitted AMF hyphal access to the organic matter patch ($1.54 ± 0.19 \text{mg } g^{-1} \text{DW}$). Hyphal growth in the plant compartment was 21% greater when the AMF partner was denied access to the patch compartment than when access was permitted (Fig. 4). In AMA microcosms, hyphal proliferation in the bulk growth medium was significantly greater than in the plant compartments ($T_{1,18} = 4.94, P < 0.001$) suggesting that the C supply from the plant was limited and that the fungus was optimizing distribution of its hyphal network – into the patch compartment instead of the plant compartment. Calculating total hyphal length (by extrapolating from the HLD in compartments, assuming equal distribution of hyphae within compartments) shows that the AMA microcosms supported in excess of three times the hyphae seen in the NAMA microcosms (Fig. 4). The higher HLD in the NAMA plant compartments suggests that this was not because of reaching a maximum attainable density in this compartment, and supports the notion of limited C supply to the AMF mycelium.

$^{15}$N content in the growth medium outside the patch did not change between treatments, even in the plant compartment, suggesting that $^{15}$N lost from the patch was either lost as volatile constituents to the atmosphere, or that the AMF was very successful at acquiring N from the patch. Unfortunately it was not possible to quantify the root length colonization because of disintegration of the root material during the clearing process.

**DISCUSSION**

We show for the first time that both total N content and total dry weight of plants increased as a result of allowing AMF access to an organic matter patch. Our data show that an organic N source can elicit the ‘strong mutualism’ scenario predicted by the ‘trade balance model’ of Johnson (2010), whereby both plant and fungi benefit from the addition of a rich N source in a P-limited system. Previous work has shown mutual benefit, but only following inorganic N

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**Table 1.** The consequence of the arbuscular mycorrhizal fungus (AMF) *Glomus intraradices* hyphae being permitted access to the patch on *Plantago lanceolata* nutrient acquisition. Data presented are values per plant, for microcosms allowing AMF Access (AMA) versus No AMF Access to the patch (NAMA), measured 16 weeks after patch addition. Allowing AMF access to the organic matter patch allowed the plant greater uptake of $^{15}$N, phosphorus (P) and nitrogen (N). Data were analysed by Independent-samples T Test, and data shown are means ($n = 19$ for N measurements; $n = 17$ for P measurements) ± S.E.

|                | Shoot $^{15}$N content (mg) | Root $^{15}$N content (mg) | Shoot N content (mg) | Root N content (mg) | Shoot P content (mg) | Root P content (mg) |
|----------------|----------------------------|-----------------------------|----------------------|---------------------|---------------------|---------------------|
| AMA            | $0.57 ± 0.15$              | $0.53 ± 0.11$               | $11.43 ± 1.11$       | $10.53 ± 0.69$      | $9.92 ± 0.93$       | $3.48 ± 0.33$       |
| NAMA           | $0.20 ± 0.08$              | $0.14 ± 0.04$               | $6.82 ± 1.46$        | $5.84 ± 0.69$       | $5.12 ± 0.59$       | $1.48 ± 0.14$       |
| Test statistics| $T_{1,37} P$               | $T_{1,37} P$                | $T_{1,37} P$         | $T_{1,34} P$        | $T_{1,34} P$        | $T_{1,34} P$        |
|                | $3.99 < 0.001$             | $5.96 < 0.001$              | $3.77 < 0.001$       | $5.02 < 0.001$      | $4.98 < 0.001$      | $5.83 < 0.001$      |

**Figure 3.** The proportion of plant N that was derived from the organic patch was greater in plants with Arbuscular Mycorrhizal fungal Access (AMA) to the patch than in those plants with No Arbuscular Mycorrhizal fungal Access (NAMA) to the patch ($P = 0.001$). Data shown are means ± SE, $n = 19$. Different letters above bars indicated significantly different means ($P < 0.05$).
The increased contribution of patch N to the plants’ total N when AMF were allowed access the patch was similar to that shown by Leigh et al. (2009), suggesting that the AMF was at least as able to exploit algal patches as *Lotium perenne* patches, as used by Leigh et al. (2009). Where that study showed increased plant N concentration however, we saw increased total N content and plant mass, but no difference in N concentration between treatments. Differences in patch composition may explain different responses of the AM plant, despite using the same plant and fungal symbiont species. The low C:N ratio of our patch compared to that used by Leigh et al. (2009) makes our patch more N-rich, and should therefore allow more rapid loss of the N it contained (Hodge et al. 2000b). Rapid efflux of N from the patch is suggested by the reasonably high level of $^{15}$N detected in the plant tissue from NAMA microcosms (Table 1). Movement of labile N sources by mass flow and diffusion across the 0.45 μm membrane from patch to plant compartment is implicated, but higher N and P levels in AMA treatments confirm the importance of AMF mediated nutrient transfer. Although it remained inside the PVC tube, the algal powder settled and mixed with the sand and AgSorb® during the course of the experiment, and became inseparable from the latter by the time the microcosms were harvested. As such, the retrieval of the patch at the end of the experiment was not possible. This prevented patch analysis to determine the extent of decomposition.

The contribution of patch N to total plant N varies among different studies using similar experimental systems: from <7% (Hodge & Fitter 2010; Barrett et al. 2011; Herman et al. 2012) to >15% (Barrett et al. 2014; Leigh et al. 2009; this study). Some of these differences can be explained by variation among different AMF symbionts (e.g. Leigh et al. 2009; Barrett et al. 2014). However, the AMF may also benefit the plant from acquiring ‘extra’ N from sources other than the patch (Herman et al. 2012). Increased P uptake by AMA plants is perhaps expected, given the amount of P present in the patch and that AMs probably evolved to improve the uptake of immobile ions, such as phosphate, from soil beyond the rhizosphere (Smith & Read 2008). In this study, the N:P ratios were remarkably low, but not without precedent for forbs (Maloney & Lamberti 1995), and indicate that the plants were severely N-limited. The increase in N:P ratio in AMA plants compared with NAMA plants suggests that the AMF reduced the extreme N limitation the plants were experiencing and in so doing facilitated growth benefits for the plant. Leigh et al. (2009) showed no difference in N:P between AMA and NAMA plants, suggesting that the AMF in that case did less to lift the plant from N-limitation, and offering an explanation as to why no growth response was observed there.

Increased P content in AMA plants suggests that the patch represented a significant source of P for the fungus (see also Barrett et al. 2014). Cavagnaro et al. (2005) demonstrated that *G. intraradices* proliferated in high P patches, while reducing P uptake from low P areas. Hyphal proliferation in the high-N, high-P patch compartment and reduced AMF growth in the...
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