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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 fertilisation has been shown to elicit strong mutualism, characterised 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 ^{15}N-labelled, nitrogen-rich patch of organic matter in a compartment to which only AMF 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. Moreover, 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.

Keywords: symbiosis, growth, arbuscular mycorrhiza, nitrogen, organic matter, nitrogen paradox.

43 Introduction

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

52 While it has been shown that AMF can transfer N to their associated host (Ames *et al*. 1983;
53 Hodge *et al.* 2001; Barrett *et al*. 2011) significant doubts remain as to the ecological
54 relevance of such a AMF-N uptake pathway (see Read 1991; Smith & Smith 2011). In
55 particular, regarding the exact mechanism of N transfer and, more importantly, the amounts
56 of N transferred via the AMF compared to the N requirements of the plant (Smith & Smith
57 2011). Although results from root organ culture studies suggest values of up to 50 % of root
58 N may be acquired via the AMF route (Govindarajulu *et al*. 2005), ideal as these systems are
59 for unpicking mechanisms involved in nutrient exchange, it may be unwise to infer much
60 about whole plant nutrient dynamics. Source-sink relationships, for example, are undoubtedly
61 unrealistic given the growth conditions employed (Smith & Smith 2015). More realistic
62 experiments, using whole plants and adding N as organic matter patches, have shown that
63 AMF contribution to plant N uptake can be as high as 15-20% (Barrett *et al*. 2014; Leigh *et*
64 *al*. 2009). Although this may suggest a significant nutritional contribution to the plant, the
65 total plant N content (Hodge 2001, Hodge *et al.* 2000a, Leigh *et al*. 2009) and plant biomass
66 (Herman *et al*. 2012; Hodge *et al*. 2001) is 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 fertilisation 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 a mycorrhiza-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 fertilisation 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 (Huber, 1981 cited in Matson *et al*. 1997), and boost greenhouse gas fluxes from agricultural soils (McSwiney & Robertson 2005). Combining organic N fertilisation and the mycorrhizal symbioses may be

91 useful in negating some of these problems and increasing assimilation of fertilizer N into
92 plants which is currently limited to around 40-60% in crop plants to which inorganic N is
93 applied (Huber & Watson 1974, Paustian *et al*. 1995).

94 Given the abundance of AMFs in temperate soils and the range of plant species they may
95 colonise (Smith & Read 2008), it is surprising that we do not comprehensively know which
96 forms of soil nitrogen can be utilised by the fungus. It is well established that AMF acquire
97 inorganic N as NH_4^+ and NO_3^- (Govindarajulu *et al*. 2005; Leigh *et al*. 2011; Johnson *et al*.
98 2014), which represent the most abundant available inorganic sources in the hyphosphere
99 (Tinker & Nye 2000). How commonly AMF utilise organic N directly is less well known.
100 Experiments have shown that AMF may be capable of direct glycine uptake (Hawkins *et al*.
101 2000; Whiteside *et al*. 2012; but see Hodge 2001), and Cappallazzo *et al* (2008) identified an
102 amino acid permease in *Glomus mosseae*, a mechanism by which an AMF may acquire
103 organic N directly from soil substrates. Similarly, Belmondo *et al*. (2014) show evidence for
104 potential uptake of organic N by a dipeptide transporter in the extraradical mycelium of
105 *Rhiozphagus irregularis*. However, AMF seem not to acquire organic N exclusively or
106 indeed preferentially, as ^{13}C enrichment is usually not detected in AM hyphae or plant tissue
107 following hyphal access to ^{13}C:^{15}N dual labelled organic matter (Hodge & Fitter 2010,
108 Nuccio *et al*. 2013).

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

115 remains in the literature a lack of patch analysis to show the relative composition of the patch
116 (organic vs. inorganic N).

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

125

126 Materials and Methods

127 Microcosm Design

128

129 Microcosm units were constructed by fastening together two polypropylene boxes, adapted
130 from Hodge & Fitter (2010). The plant compartment measured 7 x 14 x 16 cm, and the patch
131 compartment 14 x 14 x 16 cm. A window cut in the abutting sides of the boxes created an
132 aperture (4 x 6 cm) that was covered with a double-ply mesh barrier. The 'AMA' (Arbuscular
133 Mycorrhizal Access) units used a 20 μm mesh barrier, which prevented root access but
134 allowed AMF hyphal access (John Stanier and Co., Whitefield, Manchester, UK). The
135 'NAMA' (No Arbuscular Mycorrhizal Access) units used a 0.45 μm mesh (Anachem,
136 Bedfordshire, UK) that prevented the access of both roots and AMF hyphae to the patch
137 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) x 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 colonised by AMF. Seeds of *P. lanceolata* (Emorsgate Wild seeds, Nottingham, UK) were surface sterilised 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 (3 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 August 30th, 2013, and maintained in a heated, lit glasshouse and re-randomised weekly to avoid environmental artefacts. From planting to harvesting, the experiment ran for 23 weeks.

163

164 AMF Inoculum

165 Into the plant compartment, 50 g of *Glomus intraradices* inoculum comprising macerated *P.*
166 *lanceolata* and *Trifolium repens* L roots inoculated with *G. intraradices* (isolate BB-E;
167 Biorhize, Dijon, France) and growth medium (sand and AgSorb® mix described above) was
168 added. The mycorrhizal inoculum was 9 months old when added to microcosms. While we
169 acknowledge that changes in the nomenclature of AMF species have been recommended (see
170 Redecker *et al*. 2013), here, we retain the previous name '*G. intraradices*', given that the
171 exact phylogenetic position of this particular isolate is uncertain.

172

173 Nutrient Addition

174 Each week, the plant compartment of each microcosm received 50 ml of a low-N and low-P
175 nutrient solution (as Leigh *et al*. 2009) containing 2.5 mmol l^{-1} N as NH_4NO_3 and 0.034
176 mmol l^{-1} P as NaH_2PO_4. The pH of this nutrient solution was adjusted to 7.0 with KOH. The
177 plant compartment also received 0.25 g l^{-1} bone meal (Vitax, Leicestershire, UK), a complex
178 N and P source which encourages AM establishment (Hodge & Fitter 2010). Bone meal was
179 added only once, at the start of the experiment. Over the course of the experiment, the
180 nutrient solution added to the plant compartment provided 112 mg N and 2.16 mg P. The
181 bone meal provided 23 mg N and 58 mg P. The patch compartment received no further
182 nutrient additions after the patches had been placed.

183

184 Patch Material

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

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

196 Labile nitrogen as ammonium or nitrate in the algal patch was quantified by
197 spectrophotometer (CECIL 100 spectrophotometer, Spectronic Analytical Instruments,
198 Leeds, UK) and calculation from standard curve, created using standards containing 10 mg N
199 l^{-1} made from NH_4Cl and KNO_3. Briefly, 0.2 g algal material was mixed with 10 ml de-
200 ionised water and incubated for 60 minutes at 50 °C. This preparation was then centrifuged at
201 5,000 *g* for 15 minutes, after which the supernatant was decanted. Labile nitrate was
202 measured as detailed in Cataldo *et al*. (1975). Briefly, a 0.2 ml aliquot of supernatant was
203 placed in a 50 ml Erlenmeyer flask, to which 0.8 ml 5% (w/v) salicylic acid in > 96 % (v/v)
204 H_2SO_4 was added. After cooling to 20 °C the flask received 19 ml of 2 M NaOH to raise the
205 pH above 12. Absorbance was measured at 410 nm, after samples had cooled to 20 °C. Labile
206 ammonium quantification required the use of the solutions 'A' and 'B', with details of
207 preparation given below. A 0.05 ml aliquot of supernatant was mixed with 1 ml 'solution A',
208 0.25 ml 'solution B' and 2.5 ml de-ionised H_2O. Both solutions 'A' and 'B' were prepared
209 using de-ionised water. Solution A contained 20 g trisodium citrate dihydrate, 17 g salicylic

210 acid, 5 g NaOH, and 0.2 g sodium nitroprusside, made up to 500 ml. Solution B, also made
211 up to 500 ml, contained 5 g NaOH and 0.4 g dichlorosyonurate. Absorbance was measured
212 by spectrophotometer at 650 nm.

213

214 Harvest

215 At 23 weeks after planting, the systems were destructively harvested. The *P. lanceolata* was
216 separated into shoots and roots, and dried at 80°C for 48 hours. A subsample of the extracted
217 roots was retained to assess root length colonisation by the AMF. After drying, root and shoot
218 material was ground and homogenized in a ball mill (Retsch MM400, Retsch GmbH, Haan,
219 Germany), for analysis by Isotope Ratio Mass Spectrometry (PDZ 2020, Sercon Ltd, Crewe,
220 UK).

221

222 Phosphorus content was measured using X-Ray fluorescence spectrometry (XRF). Briefly,
223 dried plant material was milled and homogenised as described above, before being pressed
224 into a pellet and analysed with a portable X-ray fluorescence spectrometer (as Reidinger *et al*.
225 2012).

226 Mycorrhizal roots were stained using the method of Kormanik & McGraw (1982). Roots
227 were cleared in 10% (w/v) KOH, acidified in 1% (v/v) HCl, stained with acid fuchsin and
228 then stored in destain solution (lactic acid, glycerol, distilled H_2O 10:1:1). All procedures
229 were incubated at 20°C, as per the 'no heating' variation of the method, detailed by
230 Kormanik & McGraw (1982).

231 To quantify AMF extraradical mycelium, 5 g samples of growth medium were taken from the
232 plant compartment, from within the PVC pipe containing the organic matter patch, and from

233 the ‘bulk’ growth medium (i.e. within the patch compartment but outside the PVC core).
234 Hyphal extraction was carried out by the modified membrane filter technique of Staddon *et al*
235 (1999), and hyphal length assessed using the gridline intercept method from which hyphal
236 length densities were then calculated (Hodge 2003).

237

238 Statistical Analysis

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

244

245 Results

246 Total plant dry weight increased substantially when AMF hyphae were allowed access to the
247 patch compartment (3.44 ± 0.21 g with access versus 2.09 ± 0.23 g without access, $T_{1,37}$ =
248 4.33, $P < 0.001$). This increase in plant dry weight was driven by an increase in both the
249 shoot and root mass, which increased by 62% and 73% respectively compared with those
250 plants whose AMF partner was not permitted access to the organic patch (Fig. 2). There was
251 however, no significant difference in the root weight ratio (RWR; ratio of root dry weight to
252 total plant dry weight) between any treatments, suggesting that allocation of biomass between
253 roots and shoots did not change as a result of the plants’ AMF partner having access to the
254 organic material substrate.

255 Allowing AMF hyphal access to the patch greatly increased the plant uptake of ^{15}N,
256 measured both in the shoot and the root of the partner plants (Table 1). In total, plants with
257 AMF access to the patch contained 1.10 ± 0.25 mg ^{15}N compared with 0.34 ± 0.12 mg ($T_{1,37}$
258 = 4.91, $P < 0.001$) in the plants whose AMF partner was denied access to the patch. The
259 presence of ^{15}N in the No AMF Access microcosm plants (Table 1) is ascribed to the mass
260 flow and diffusion of ^{15}N-containing molecules through the 0.45 μm mesh from the patch to
261 the plant compartment. Such a difference in plant ^{15}N content between AMF Access and No
262 AMF Access microcosms highlights the important role AMF can play in nutrient acquisition
263 from nutrient-rich areas placed at significant distances beyond the rhizosphere. Similarly, the
264 contribution made by patch N to overall plant N was greatly increased when plants had AMF
265 access to the patch (Fig. 3): 18 ± 3%, compared to 9 ± 1% ($T_{1,37} = 3.57$, $P = 0.001$). *P.*
266 *lanceolata* benefitted greatly from this AMF contribution acquired from the patch as
267 demonstrated by the 68% increase in shoot N content when AMF had access to the patch
268 compartment, corroborated by an 80% increase in root N content (Table 1). Thus total N in
269 the whole plant was increased 76%. Although total plant N content increased, plant N
270 concentration was not significantly different between the two AMF access treatments.

271 (Table 1 here.)

272 The proportion of the patch N acquired by the plant increased from 4% to 12% when AMF
273 were permitted access to the patch compartment ($T_{1,37} = 4.98$, $P < 0.001$), suggesting that the
274 AMF were adept at exploiting a newly available patch of organic matter and transferring the
275 N acquired to their plant partner. Allowing AMF hyphal access to the patch resulted in 4.69
276 mg extra N in the roots and 4.61 mg in the shoots which greatly outweighs the 0.001124 mg
277 of ammonium-N and 0.0003976 mg nitrate-N extractable from the patch.

278 Root P concentration increased by 28% when AMF had access to the patch ($T_{1,34} = 3.31$, $P =$
279 0.002), but the shoot P concentration was not affected by allowing AMF access to the patch.
280 The increase in root P concentration was not substantial enough to change the total plant P
281 concentration ($T_{1,34} = 0.16$, $P = 0.88$), but combined with an increased root mass, root P
282 content increased by 135%. Similarly, despite no increase in P concentration, shoot P content
283 was 94% greater in AMF access plants than in no AMF access plants (Table 1). Plants with
284 AMF access to the patch had marginally higher N:P ratio (total plant N content / total plant P
285 content) than plants with no patch access although this was only weakly significant ($T_{1,34} =$
286 1.98, P = 0.060). Mean AMA plant N:P was 2.18 ± 0.15, compared with mean NAMA plant
287 N:P of 1.84 ± 0.10.

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

303 ^{15}N content in the growth medium outside the patch did not change between treatments, even
304 in the plant compartment, suggesting that ^{15}N lost from the patch was either lost as volatile
305 constituents to the atmosphere, or that the AMF was very successful at acquiring N from the
306 patch. Unfortunately it was not possible to quantify the root length colonisation due to
307 disintegration of the root material during the clearing process.

308

309 Discussion

310 We show for the first time that both total N content and total dry weight of plants increased as
311 a result of allowing AMF access to an organic matter patch. Our data show that an organic N
312 source can elicit the 'strong mutualism' scenario predicted by the 'trade balance model' of
313 Johnson (2010), whereby both plant and fungi benefit from the addition of a rich N source in
314 a P-limited system. Previous work has shown mutual benefit, but only following inorganic N
315 addition (Johnson et al 2014). Here, *G. intraradices* was adept at acquiring N from patches of
316 algal material and transferring a significant fraction of patch N to the partner plant. The
317 quantity of nitrogen transferred here, and the increased contribution made by the ERM in
318 AMA microcosms support the argument that AMF can be a significant conduit for plant N
319 uptake, a position not universally supported in the literature (e.g. Reynolds *et al.* 2005).
320 Although AM uptake of N from organic matter patches has been reported, (Barrett *et al*.
321 2014, Leigh *et al.* 2009), previous studies generally have not displayed increased total plant
322 N, as we show here, and a concurrent increase in plant biomass as found in this study is
323 unprecedented. The increase of ^{15}N in the plant shoots (Table 1) is noteworthy as it indicates
324 genuine transfer of patch N to the plant via the AMF, whereas it cannot be determined what
325 proportion of ^{15}N in roots remains in the intraradical mycelium of the AMF. We assumed no
326 fractionation of ^{15}N and ^{14}N during uptake by the fungus or transfer to the plant.

327 Ectomycorrhizal fungi may transfer ^{14}N preferentially to a partner plant, so the mycelium
328 becomes relatively ^{15}N enriched (Hobbie & Colpaert 2003). Were such a phenomenon to
329 have taken place here, our calculations for N transfer would be underestimating the
330 contribution made by the AMF to plant N.

331 The increased contribution of patch N to the plants' total N when AMF were allowed access
332 the patch was similar to that shown by Leigh *et al.* (2009), suggesting that the AMF was at
333 least as able to exploit algal patches as *Lolium perenne* patches, as used by Leigh *et al.*
334 (2009). Where that study showed increased plant N concentration however, we saw increased
335 total N content and plant mass, but no difference in N concentration between treatments.
336 Differences in patch composition may explain different responses of the AM plant, despite
337 using the same plant and fungal symbiont species. The low C:N ratio of our patch compared
338 to that used by Leigh *et al.* (2009) makes our patch more N-rich, and should therefore allow
339 more rapid loss of the N it contained (Hodge *et al.* 2000b). Rapid efflux of N from the patch
340 is suggested by the reasonably high level of ^{15}N detected in the plant tissue from NAMA
341 microcosms (Table 1). Movement of labile N sources by mass flow and diffusion across the
342 0.45 μm membrane from patch to plant compartment is implicated, but higher N and P levels
343 in AMA treatments confirm the importance of AMF mediated nutrient transfer. Although it
344 remained inside the PVC tube, the algal powder settled and mixed with the sand and
345 AgSorb® during the course of the experiment, and became inseparable from the latter by the
346 time the microcosms were harvested. As such, the retrieval of the patch at the end of the
347 experiment was not possible. This prevented patch analysis to determine the extent of
348 decomposition.

349 The contribution of patch N to total plant N varies among different studies using similar
350 experimental systems: from < 7 % (Barrett *et al*. 2011; Herman *et al*. 2012; Hodge & Fitter
351 2010) to > 15 % (Barrett *et al*. 2014; Leigh *et al*. 2009; this study). Some of these differences

352 can be explained by variation among different AMF symbionts (e.g. Barrett *et al*. 2014;
353 Leigh *et al*. 2009). However, the AMF may also benefit the plant from acquiring 'extra' N
354 from sources other than the patch (Herman *et al*. 2012). Increased P uptake by AMA plants
355 is perhaps expected, given the amount of P present in the patch and that AMs probably
356 evolved to improve the uptake of immobile ions, such as phosphate, from soil beyond the
357 rhizosphere (Smith & Read 2008). In this study, the N:P ratios were remarkably low, but not
358 without precedent for forbs (Maloney & Lamberti 1995), and indicate that the plants were
359 severely N-limited. The increase in N:P ratio in AMA plants compared with NAMA plants
360 suggests that the AMF reduced the extreme N limitation the plants were experiencing and in
361 so doing facilitated growth benefits for the plant. Leigh *et al.* (2009) showed no difference in
362 N:P between AMA and NAMA plants, suggesting that the AMF in that case did less to lift
363 the plant from N-limitation, and offering an explanation as to why no growth response was
364 observed there.

365 Increased P content in AMA plants suggests that the patch represented a significant source of
366 P for the fungus (see also Barrett *et al*. 2014). Cavagnaro *et al*. (2005) demonstrated that *G.*
367 *intraradices* proliferated in high P patches, while reducing P uptake from low P areas. Hyphal
368 proliferation in the high-N, high-P patch compartment and reduced AMF growth in the plant
369 compartment in AMA microcosms (Fig. 4) suggests the fungus behaved similarly here.

370 Reduced hyphal length in the plant compartment of AMA microcosms may suggest C
371 limitation, as previous studies with similar experimental design (Hodge *et al*. 2001) showed
372 increased growth of AMF in plant and patch compartments. Here, the fungus may have been
373 unable to obtain enough C from the plant to maintain the hyphal biomass in the plant
374 compartment when it was also supporting a mycelium in the patch compartment, and thus co-
375 ordinated its hyphal growth for the greatest benefit - the mineral nutrition from the patch, a
376 phenomenon that is well documented in root allocation (Drew 1975).

Previously, experimental evidence for strong mutual benefit of AMs was obtained only by inorganic N addition to mycorrhizal plants. Our findings demonstrate that AMF can provide considerable benefit to plant N and P nutrition following the addition of organic matter, followed by substantial increases in biomass, both for plant and fungus.

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Conflict of Interest

The Authors declare no conflict of interest.

References

Ames R.N., Reid C. P. P., Porter L. K. & Cambardella C. (1983) Hyphal uptake and transport of nitrogen from 2 ^{15}N-labeled sources by *Glomus mosseae*, a vesicular arbuscular mycorrhizal fungus. *New Phytologist* **95,** 381–396.

397 Barrett G., Campbell C.D., Fitter A.H. & Hodge A. (2011) The arbuscular mycorrhizal
398 fungus *Glomus hoi* can capture and transfer nitrogen from organic patches to its associated
399 host plant at low temperature. *Applied Soil Ecology* **48**, 102-105.

400 Barrett G., Campbell C.D. & Hodge A. (2014) The direct response of the external mycelium
401 of arbuscular mycorrhizal fungi to temperature and the implications for nutrient transfer. *Soil*
402 *Biology & Biochemistry* **78,** 109-117.

403 Belmondo S., Fiorilli V., Pérez-Tienda J., Ferrol N., Mermeisse R. & Lanfranco L. (2014) A
404 dipeptide transporter from the arbuscular mycorrhizal fungus *Rhizophagus irregularis* is
405 upregulated in the intraradical phase. *Frontiers in Plant Science* **5**, 436.

406 Bremner J.M. (1949) Studies on soil organic matter. 1. The chemical nature of soil organic
407 nitrogen. *Journal of Agricultural Science* **39,** 183-193.

408 Cappellazzo G., Lanfranco L., Fitz M., Wipf D. & Bonfante P. (2008) Characterisation of an
409 amino acid permease from the endomycorrhizal fungus *Glomus mosseae*. *Plant Physiology*
410 **147,** 429-437.

411 Cataldo D.A., Haroon M., Schrader L.E. & Youngs V.L. (1975) Rapid colorimetric
412 determination of nitrate in plant tissue by nitration of salicylic acid. *Communications in Soil*
413 *Science and Plant Analysis* **6,** 71-80.

414 Cavanagro T.T., Smith F.A., Smith S.E. & Jakobsen I. (2005) Functional diversity in
415 arbuscular mycorrhizas: exploitation of soil patches with different phosphate enrichment
416 differs among fungal species. *Plant, Cell and Environment* **28,** 642-650.

417 Drew M.C. (1975) Comparison of the effects of a localised supply of phosphate, nitrate,
418 ammonium and potassium on the growth of the seminal root system, and the shoot, in barley.
419 *New Phytologist* **75**, 479-490.

Field K.J., Cameron D.D., Leake J.R., Tille S., Bidartondo M.I. & Beerling D.J. (2012) Contrasting arbuscular mycorrhizal responses of vascular and non-vascular plants to a simulated Palaeozoic CO_2 decline. *Nature Communications* **3,** 835.

Govindarajulu M., Pfeffer P.E., Jin H., Abubaker J., Douds D.D., Allen, J.W., Bucking, H., Lammers, P.J. & Shachar-Hill, Y. (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. *Nature* **435***,* 819-823.

Hawkins H.J., Johansen A. & George E. (2000) Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. *Plant and Soil* **226,** 275-285.

Herman D.J., Firestone M.K., Nuccio E. & Hodge A. (2012) Interactions between an arbuscular mycorrhizal fungus and a soil microbial community mediating litter decomposition. *FEMS Microbiology Ecology* **80,** 236-247.

Hobbie E.A. & Colpaert J.V. (2003) Nitrogen availability and colonization by mycorrhizal fungi correlate with nitrogen isotope patterns in plants *New Phytologist* **157,** 115-126.

Hodge A. (2001) Arbuscular mycorrhizal fungi influence decomposition of, but not plant nutrient capture from, glycine patches in soil. *New Phytologist* **151,** 725-734.

Hodge A. (2003) Plant nitrogen capture from organic matter as affected by spatial dispersion, interspecific competition and mycorrhizal colonization. *New Phytologist* **157,** 303-314.

Hodge A. (2014) Interactions between arbuscular mycorrhizal fungi and organic material substrates. *Advances in Applied Microbiology* **89,** 47-99.

Hodge A. & Fitter A. H. (2010) Substantial nitrogen acquisition by arbuscular mycorrhizal fungi from organic material has implications for N cycling. *Proceedings of the National Academy of Sciences of the United States of America* **31,** 13754-13759.

442 Hodge A. & Storer K. (2015) Arbuscular mycorrhiza and nitrogen: implications
443 for individual plants through to ecosystems. *Plant and Soil* **386,** 1-19.

444 Hodge A., Robinson D. & Fitter A.H. (2000a) An arbuscular mycorrhizal inoculum enhances
445 root proliferation in, but not nitrogen capture from, nutrient-rich patches in soil. *New*
446 *Phytologist* **145,** 575-584.

447 Hodge A., Stewart J., Robinson D., Griffiths B. S. & Fitter A. H. (2000b) Competition
448 between roots and soil micro-organisms for nutrients from nitrogen-rich patches of varying
449 complexity. *Journal of Ecology* **88**,150-164.

450 Hodge A., Campbell C. D. & Fitter A. H. (2001) An arbuscular mycorrhizal fungus
451 accelerates decomposition and acquires nitrogen directly from organic material. *Nature* **413,**
452 297-299.

453 Huber D.M. & Watson R.D. (1974) Nitrogen form and plant disease. *Annual Review of*
454 *Phytopathology* **12,** 139-165.
455 Jackson L.E., Schimel J.P. & Firestone M.K. (1988) Short term partitioning of ammonium
456 and nitrate between plants and microbes in an annual grassland. *Soil Biology & Biochemistry*
457 **21,** 409-415.

458 Johnson N.C. (2010) Resource stoichiometry elucidates the structure and function of
459 arbuscular mycorrhizas across scales. *New Phytologist* **185,** 631-647.

460 Johnson N.C., Wilson G.W.T., Wilson J.A., Miller R.M., & Bowker M.A. (2014)
461 Mycorrhizal phenotypes and the Law of the Minimum. *New Phytologist* **205,** 1473-1484.

462 Kormanik P.P. & McGraw A.C. (1982) Quantification of vesicular-arbuscular mycorrhizae in
463 plant roots. In: *Methods and Principals of Mycorrhizal Research* (ed. N.C. Schenk), pp. 37–
464 46. American Phytopathological Society, St Paul, MN, USA.

Leigh J., Hodge A. & Fitter A.H. (2009) Arbuscular mycorrhizal fungi can transfer substantial amounts of nitrogen to their host plant from organic material. *New Phytologist* **181,** 199-207.

Leigh J., Fitter A.H. & Hodge A. (2011) Growth and symbiotic effectiveness of an arbuscular mycorrhizal fungus in organic matter in competition with soil bacteria. *FEMS Microbiology Ecology* **76,** 428-438.

Liu Y.J., Shi G.X., Mao L., Cheng G., Jiang S.J., Ma X.J., An L.Z., Du G.Z., Johnson N.C. & Feng H.Y. (2012) Direct and indirect influences of 8 yr of nitrogen and phosphorus fertilization on Glomeromycota in an alpine meadow ecosystem. *New Phytologist* **194**, 523-535.

Maloney D. C. & Lamberti G. A. (1995) Rapid decomposition of summer-input leaves in a Northern Michigan stream. *American Midland Naturalist* **1,** 184-195.

Matson P.A., Parton W.J., Power A.G. & Swift M.J. (1997) Agricultural intensification and ecosystem properties. *Science* **277,** 504-509.

McSwiney C.P. & Robertson G.P. (2005) Nonlinear response of N_2O flux to incremental fertilizer addition in a continuous maize (*Zea mays* L.) cropping system. *Global Change Biology* **11,** 1712-1719.

Nemeth K., Bartels H., Heuer C. & Maier J. (1987) Determination by means of EUF of the inorganic and organic soil-nitrogen available to plants. *Zuckerindustrie* **112,** 223-226.

Nuccio E.E., Hodge A., Pett-Ridge J., Herman D.J., Weber P.K. & Firestone M.K. (2013) An arbuscular mycorrhizal fungus significantly modifies the soil bacterial community and nitrogen cycling during litter decomposition. *Environmental Microbiology* **15,** 1870-1881.

487 Paustian K., Robertson G.P. & Elliott E.T. (1995) Management impacts on carbon storage
488 and gas fluxes (CO_2, CH_4) in mid-latitude cropland ecosystems. In: *Soil Management and*
489 *Greenhouse Effect* (eds R. Lal, J. Kimble, E. Levine & B.A. Stewart), pp. 69-84. CRC Press,
490 Florida, USA.

491 Read D.J. (1991) Mycorrhizas in ecosystems. *Experientia* **47,** 376-391.

492 Redecker D., Schuβler A., Stockinger H., Stürmer S., L., Morton J.B. & Walker C. (2013) An
493 evidence-based consensus for the classification of arbuscular mycorrhizal fungi
494 (Glomeromycota) *Mycorrhiza* **23,** 515-531.

495 Reidinger S., Ramsey M.H. & Hartley S.E. (2012) Rapid and accurate analyses of silicon and
496 phosphorus in plants using a portable X-ray fluorescence spectrometer. *New Phytologist* **195,**
497 699-706.

498 Reynolds H.L., Hartley A.E., Vogelsang K.M., Bever J.D. & Schultz P.A. (2005) Arbuscular
499 mycorrhizal fungi do not enhance nitrogen acquisition and growth of old-field perennials
500 under low nitrogen supply in glasshouse culture. *New Phytologist* **167,** 869-880.

501 Smith S. E. & Read D.J. (2008) *Mycorrhizal Symbiosis* 3rd edn. Academic Press, London,
502 UK.

503 Smith F.A. & Smith S.E. (2011) Mycorrhizas in plant nutrition and growth: new paradigms
504 from cellular to ecosystem scales. *Annual Review of Plant Biology* **62**, 227-250.

505 Smith F.A. & Smith S.E. (2015) How harmonious are arbuscular mycorrhizal symbioses?
506 Inconsistent concepts reflect different mindsets as well as results. *New Phytologist* **205,** 1381-
507 1384.

508 Staddon P.L., Fitter A.H. & Graves J.D. (1999) Effect of elevated atmospheric CO_2 on
509 mycorrhizal colonization, external mycorrhizal hyphal production and phosphorus inflow in
510 *Plantago lanceolata* and *Trifolium repens* in association with the arbuscular mycorrhizal
511 fungus *Glomus mosseae. Global Change Biology* **5,** 347-358.

512 Stevenson F.J. (1994) *Humus Chemistry. Genesis, Composition, Reactions*. 2nd edition. John
513 Wiley & Sons, New Jersey, USA.

514 Tinker P.B. & Nye P.H. (2000) Solute movement in the rhizosphere. Oxford University
515 Press, New York, USA.

516 Whiteside M.D., Garcia M.O. & Treseder K.K. (2012) Amino acid uptake in arbuscular
517 mycorrhizal plants. *PLoS One* **7,** e47643.
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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	$T_{1,37}$ 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 1. Diagram of the microcosm design. An individual *Plantago lanceolata* plant, colonised by *Glomus intraradices* was contained within one compartment of the microcosm, and a ^{15}N-labelled organic matter patch was placed into an adjoining patch compartment. Patches were contained within a PVC core, and retained within mesh sides which allow arbuscular mycorrhizal fungal (AMF) hyphal entry. Half of the microcosm units contained a 0.45 μm mesh rather than a 20 μm mesh, to prevent the roots and the AMF hyphae crossing from the plant compartment to the patch compartment. This allowed for any mass flow and diffusion of ^{15}N from the patch across the barrier to be accounted for, rather than genuine transfer via the AMF hyphae.

Figure 2. Dry weight (DW) (g) of *Plantago lanceolata* colonised by *Glomus intraradices* in Arbuscular Mycorrhizal fungal Access (AMA) and No Arbuscular Mycorrhizal fungal Access (NAMA) microcosms. **A)** Allowing arbuscular mycorrhizal fungal hyphal access to the patch compartment resulted in a significant increase in shoot DW ($P = 0.001$). **B)** Plant root DW was significantly greater for AMA plants than for NAMA ($P < 0.001$). Data shown are means ± S.E., $n = 19$. Different letters above bars indicated significantly different means ($P < 0.05$).

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 ± S.E., $n = 19$. Different letters above bars indicated significantly different means ($P < 0.05$).

Figure 4. Total hyphal length, extrapolated from hyphal length density (HLD) measurements. HLD was calculated from growth medium in the PVC core, from the plant compartment and the bulk growth medium that surrounded the core. Plants with Arbuscular Mycorrhizal fungal

569 Access (AMA) supported substantially more total hyphal length than those plants with No
570 Arbuscular Mycorrhizal Access (NAMA), despite plant compartment hyphal length being
571 higher in NAMA microcosms than AMA. Total hyphal length in the bulk growth medium
572 surrounding the core was higher than in the plant compartment when AMF hyphae were
573 permitted access to the compartment containing the patch ($T_{1, 18} = 4.94$, $P < 0.001$). (Data
574 shown are means ± S.E., $n = 19$).

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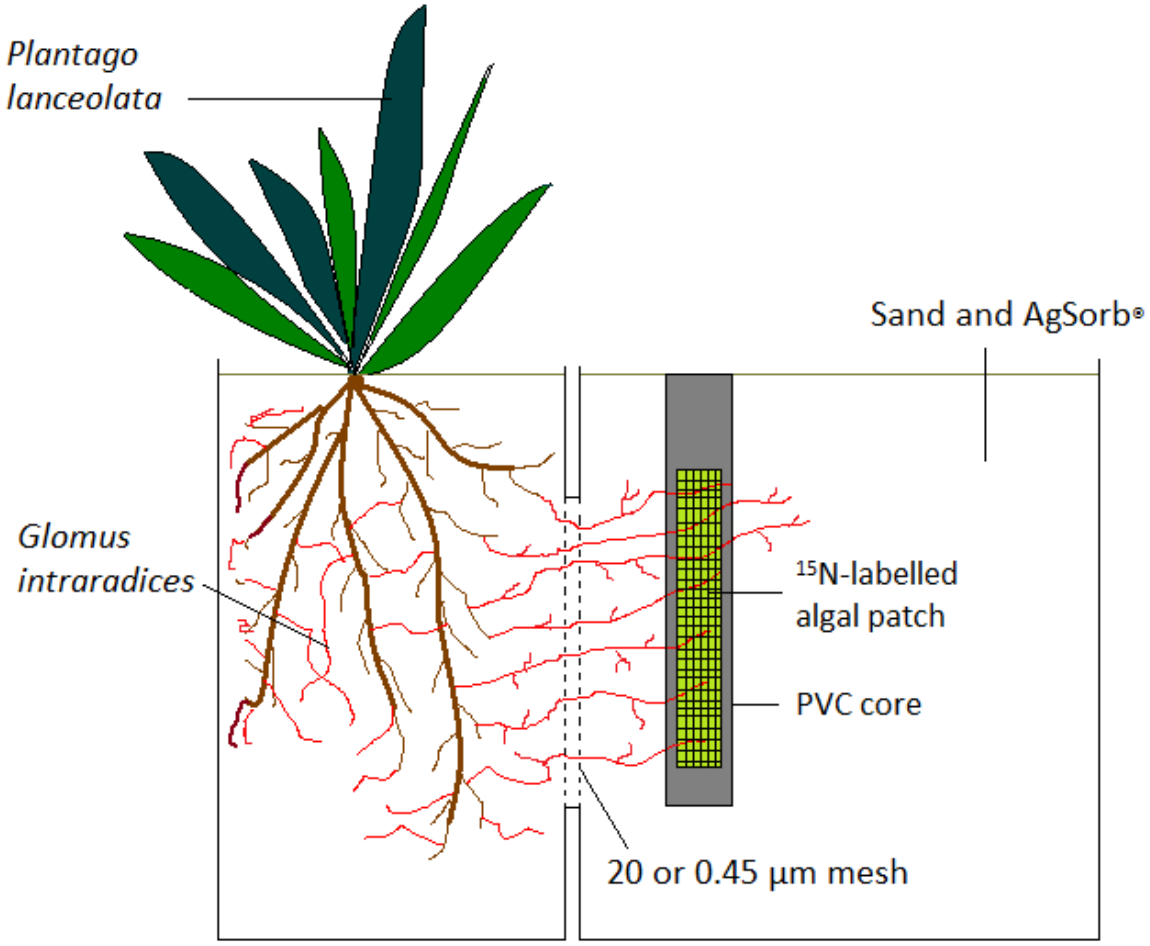

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577 **Figure 1**

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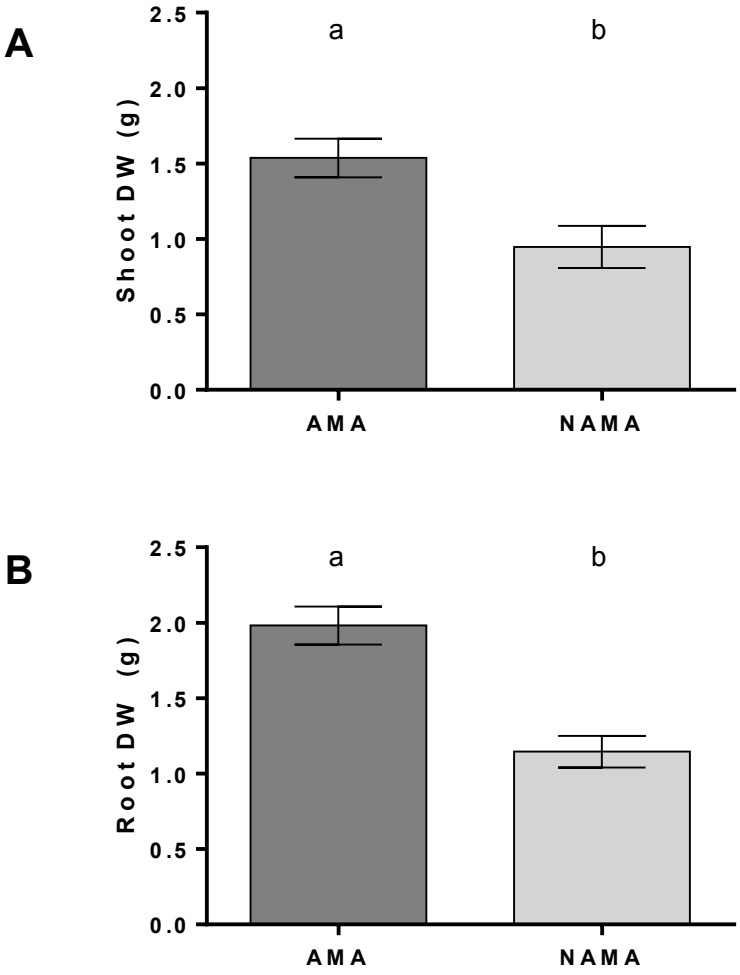

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580 **Figure 2**

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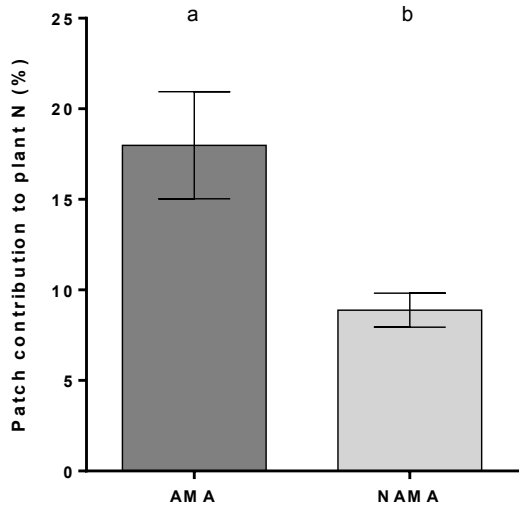

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583 **Figure 3**

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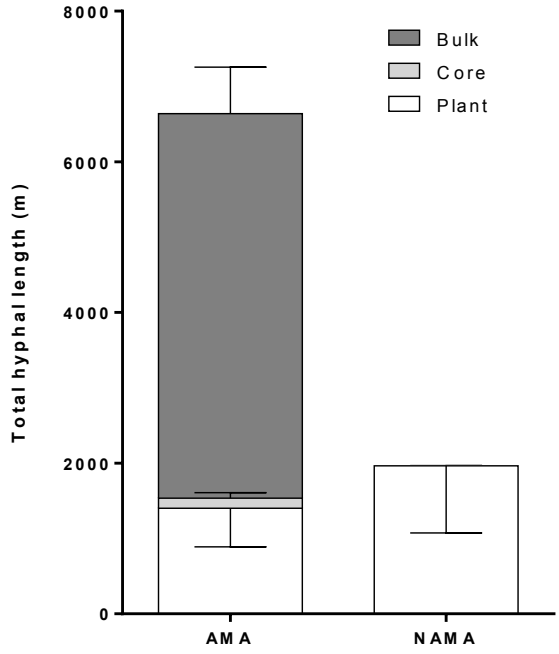

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586 **Figure 4**