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Comparison of two test systems for measuring plant phosphorus uptake via arbuscular mycorrhizal fungi

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Abstract Plant phosphorus uptake via external hyphae of arbuscular mycorrhizal fungi has been measured using compartmented systems where a hyphal compartment is separated from a rooting compartment by a fine mesh. By labelling the soil within the hyphal compartment with a radioactive phosphorus (P) isotope, hyphal uptake of P into the plant can be traced. The objective of this growth chamber study was to test two hyphal compartments of different design with respect to their suitabilities for measurement of hyphal P uptake. One hyphal compartment was simply a nylon mesh bag filled with ^{32}P-labelled soil. The labelled soil in the other hyphal compartment was completely surrounded by an 8–10 mm layer of unlabelled soil that served as a buffer zone. Mycorrhizal and non-mycorrhizal subterranean clover plants were grown in pots with a centrally positioned hyphal compartment. Uptake of radioactive P by non-mycorrhizal control plants was 25% of that by mycorrhizal plants with the mesh bag but only 3% when including the buffer zone. Based on this good control of non-mycorrhizal P uptake from within the hyphal compartment and its greater ease of handling once produced, we judged the hyphal compartment including a buffer zone to be superior to the mesh bag.

Key words Arbuscular mycorrhizal fungi · Hyphal phosphorus uptake

Introduction

Knowledge about the widespread occurrence of arbuscular mycorrhizal (AM) fungi in natural and agricultural ecosystems, combined with the well-documented ability of these fungi to increase plant growth and phosphorus (P) uptake under controlled conditions, has spurred interest in measuring and quantifying their functional significance for plant growth in the field (Hall 1988; Sieverding 1991; Jeffries and Barea 1994). Of the field studies conducted so far, the majority concluded that root colonization with AM fungi increases plant growth and P uptake of field-grown plants (Trent et al. 1989; Thompson 1990; Weber et al. 1993).

Soil fumigation was used in many of these studies to eliminate the naturally occurring population of AM fungi. The growth and P uptake of plants grown in the fumigated plots was then compared to plant growth and P uptake either (i) in plots reinoculated with native (Weber et al. 1993) or exotic (Sylvia et al. 1993) AM fungi or (ii) in non-fumigated plots (Trent et al. 1989). Other methods have aimed at disrupting or hampering hyphal P uptake and transport to the host plant by the use of fungicides (Merryweather and Fitter 1996) or varying the proportion of root length colonized in the treated plants by agronomic practices (Miller et al. 1995) or inoculation (Dodd et al. 1990). Differences in growth between plant mutants unable to form the mycorrhizal symbiosis but otherwise assumed not to differ from the control plants have also been used to quantify the contribution of AM fungi to plant growth (Srivastava and Mukerji 1995).

Some of the above methods do not, however, exclusively affect plant P uptake via AM fungi and, therefore, show undesirable side-effects (see Jakobsen 1994). None of them give a direct measure of the contribution to P nutrition of field-grown crops by the native field populations of AM fungi. Direct measurements of hyphal P transport using compartmented systems in combination with the use of a radioactive P isotope were, therefore, advocated by Jakobsen (1994, 1995). Compartmented systems consist of a hyphal compartment separated from a rooting compartment by a fine mesh that prevents roots from entering, but allows free passage of hyphae. These systems have been successfully used to measure hyphal P uptake separately from root

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P uptake in studies performed under controlled conditions (see Li et al. 1991; Jakobsen et al. 1994).

Employing a compartmented system, Jakobsen (1992, 1995) was able to measure directly hyphal P uptake into field-grown peas, associated either with the native field population of AM fungi or with an inoculant AM fungus. However, a small amount of the radioactive isotope used to label the soil inside the hyphal compartment was also detected in the plants growing in the plots from which the AM fungi had been eliminated by fumigation. Uptake of P from within the hyphal compartment by root hairs penetrating the fine mesh or diffusion of radiolabel out of the hyphal compartment followed by plant uptake were given as possible reasons for this observation.

A prerequisite for measurements of hyphal P uptake from root-free hyphal compartments is the spread of hyphae from the rooting compartment into the hyphal compartment. Jakobsen (1995) observed spread of external hyphae of an inoculant AM fungus into hyphal compartments under field conditions, while in the same study fungi of the native AM fungal community failed to do so (Jakobsen, unpublished data). The length of external hyphae spreading into unexplored volumes of soil such as hyphal compartments will depend on which plant and fungus species form the association, hyphal production and possibly nutrient status of that soil. Hyphal length density in a specific plant-fungus association is positively correlated with colonized root length density (Abbott and Robson 1985). External hyphae may also proliferate in unexplored volumes of soil enriched in nutrients, as was suggested by Cui and Caldwell (1996). High rooting densities in the rooting compartment would deplete that volume of soil of nutrients, which could lead to proliferation of external hyphae in the nutrient rich, unexplored soil of the hyphal compartment.

The aim of this present study was to compare the suitability of two compartmented systems under controlled conditions for measurement of hyphal P uptake by a native AM fungal community later to be tested in the field. The two systems tested were (i) the mesh bag system as described by Jakobsen (1992) and (ii) a novel system further referred to as buffer system, designed to prevent both root hairs from accessing ^{32}P and diffusion of ^{32}P out of the hyphal compartment. The effect of varying mycorrhizal root length densitiy in the rooting compartment on ingrowth of external hyphae into the hyphal compartment and their P uptake was also tested. Additionally, the hyphal P uptake capabilities of the native AM fungal community and a well-known AM fungal isolate were compared.

Materials and methods

Design of the test systems

Two compartmented systems were tested in this study. The mesh bag system was simply a bag made out of 25-μm nylon mesh designed to hold 100 g of soil. The mesh size prevents roots but not AM fungal hyphae from entering the interior of the bag. The bag was filled with 80 g soil labelled with ^{32}P at a specific activity of 3 kBq g^{-1} soil covered by 20 g of unlabelled soil. The buffer system was a soil cylinder completely enclosed by nylon mesh with a central volume of 270 g soil labelled with ^{32}P at a specific activity of 3 kBq g^{-1} surrounded by an 8- to 10-mm-wide layer of unlabelled soil on all sides. The nylon mesh was glued onto the side and bottom of a piece of PVC tubing (75 mm outer diameter, 80 cm high) into the sides of which two wide windows had been cut. The resulting nylon cup was filled with unlabelled soil. A stainless-steel pipe (63 mm outer diameter) was pushed into the whole length of the soil cylinder, and the soil from inside the pipe was removed. Unlabelled soil was filled inside the steel pipe to give a 1-cm-thick bottom layer on top of which the labelled soil was added. The steel pipe was then pulled out of the soil, taking care not to disturb the side layers of unlabelled soil. After filling the cup to the rim with unlabelled soil, a nylon mesh was affixed to the top of the cup. In total, the buffer system contained approximately 500 g soil. The width of the buffering layer of unlabelled soil was chosen to ensure that all of the added ^{32}P lay outside the maximal expected P-depletion zone around a root actively absorbing nutrients and growing in immediate contact with the nylon mesh.

Experimental setup

The soil (Calcic Luvisol, pH 6.8, 50% sand, 32% silt, 16% clay, 1.4% C, 24 ppm $NaHCO_3$-extractable P (Olsen et al. 1954)) used in this study was taken from an organic cropping system at Snubbekorsgård, Taastrup, Denmark (Magid and Kølster 1995) and sieved to <5 mm. The soil was partially sterilized by irradiation (10 kGy, 10 MeV electron beam) when used in the non-mycorrhizal control treatments (treatments 3 and 5; see Table 1) and the

Table 1 Treatment parameters

Treatment	Type of hyphal compartment	Fungus	P added (mg kg^{-1} soil)	Plants per pot	Pot volume (l)	Rooting compartment (g soil per pot)
1	Buffer system	Native	0	3	1.5	1273
2	Buffer system	*Glomus claroideum* (BEG 14)	0	3	1.5	1273
3	Buffer system	None	20	3	1.5	1273
4	Meshbag	Native	0	3	1.5	1665
5	Meshbag	None	20	3	1.5	1665
6	Meshbag	Native	0	1	3	3526
7	Meshbag	Native	0	5	3	3526
8	Meshbag	Native	0	12	3	3526

treatment in which the plants were inoculated with a known mycorrhizal fungal isolate (treatment 2). Non-irradiated soil was used in the remaining treatments 1, 4, 6, 7 and 8. Soil used in the hyphal compartments had been sieved to <2 mm and mixed with quartz sand at 9:1 (w:w). This soil/sand mixture was also irradiated before being used in the hyphal compartments. A set of basal nutrients without P was mixed into the soil and soil/sand mixture at the following concentrations (mg kg^{-1}): 23 mg N as NH_4NO_3; 31 mg K as K_2SO_4; 25 mg Ca as $CaCl_2$; 0.56 mg Cu as $CuSO_4.5H_2O$; 1.14 mg Zn as $ZnSO_4.7H_2O$; 3.25 mg Mn as $MnSO_4.H_2O$; 0.07 mg Co as $CoSO_4.7H_2O$; 0.09 mg Mo as $NaMoO_4.2H_2O$; 2 mg Mg as $MgSO_4.7H_2O$. The soil used for the rooting compartment in the non-mycorrhizal control treatments 3 and 5 was amended with P as KH_2PO_4 at a concentration of 20 mg P kg^{-1} soil. Fifty grams of soil inoculum from a pot culture of *Glomus claroideum* Schenck & Smith (BEG 14) was mixed through the soil of each pot used in treatment 2. A mesh bag or buffer system was placed in the centre of a 1.5-l (treatments 1 to 5) or 3-l (treatments 6 to 8) pot and soil was packed around it to ensure good contact between the mesh and the surrounding soil. Each treatment was replicated three times.

The pots were watered to 60% of water holding capacity by weight and kept for 6 days at 20 °C until planting. Depending on the number of seedlings wanted after thinning, twice that number of pregerminated clover seeds (*Trifolium subterraneum* L. cv. Mount Barker) were sown into the rooting compartments. The pots were transferred to a growth chamber at 20/16 °C with a 16/8 h light/dark cycle, with Osram daylight lamps providing a 500–550 μmol m^{-2} s^{-1} PAR photon flux density. The pots were watered with deionized water to 60% of water holding capacity by weight every second day. The pots were randomly distributed in the growth chamber and were rearranged every day.

Harvest

Five weeks after sowing, the shoots were cut at the soil surface and dried at 80 °C. The hyphal compartments were taken out of the soil, cleared of adhering roots and kept at 7 °C until the next day. Roots were washed free of soil and weighed. Subsamples of the total root mass were cleared for 25 min in 10% KOH at 90 °C and stained with 0.05% trypan blue in lactoglycerol (Kormanik and McGraw 1982). The remaining root mass was dried at 80 °C.

Samples of ground, dried shoots and roots were digested in nitric/perchloric acid (4:1, v/v). The ^{32}P activity in these digests was measured in a Packard TR1900 liquid scintillation counter. The P concentration in the digests was measured by the molybdate blue method (Murphy and Riley 1962). Total root length was assessed on the cleared, stained root sample employing the method of Newman (1966) at $\times 20$ magnification and the presence or absence of AM root colonization noted (Giovannetti and Mosse 1980).

For the measurement of external hyphal lengths, the soil from inside a hyphal compartment was emptied into a paper bag, dried at 80 °C and stored until the radioactivity in that sample had decreased to a level suitable for further processing. Two replicate 5–9 soil samples taken from the soil of each hyphal compartment were shaken in a solution containing 3.8 g sodium metaphosphate l^{-1} for 30 min and washed onto a 38-μm sieve. Membrane filters were prepared from the material retained on the sieve using the method described by Abbott et al. (1984). The length of hyphae on the membrane filters was assessed using the grid-line intercept method (Tennant 1975) at $\times 200$ magnification.

Data analysis

The complete set of data for plant growth and root colonization was examined for significant treatment effects by one-way analysis of variance. Data were log-transformed prior to analysis except for the data for relative amount of root length colonized by AM fungi, which were arcsin-transformed. Significant differences between treatments were tested by the Scheffé test. Data for hyphal length in hyphal compartments and plant ^{32}P uptake obtained in treatments 1–5 were examined for significant treatment effects using two-factor (hyphal compartment, mycorrhizal treatment) analysis of variance. Significant differences between treatments were tested by *t*-test or one-way analysis of variance. Data on length-specific hyphal ^{32}P uptake were examined by *t*-test and one-way analysis of variance. All analyses were performed in StatGraphics. Linear relationships were sought for the data on hyphal length density with mycorrhizal root length density and the data on plant ^{32}P content with hyphal length density obtained in treatments 4, 6, 7 and 8.

Results

The growth of mycorrhizal plants was unaffected by their fungal symbionts and the type of test system used (treatments 1, 2 and 4) (Table 2). Adding P to the irradiated soil more than compensated for the absence of AM fungi in terms of plant growth (treatments 3 and 5). Increasing the planting density to 12 plants growing in a 3-l pot (treatment 8) resulted in significantly reduced growth of the individual plants (Table 2) but the highest biomass production per pot. The roots of the plants grown in the untreated soil were heavily colonized by the native AM fungi. Inoculation with *G. claroideum* (treatment 2) resulted in an only slightly higher percentage of root length colonized. Irradiation of the soil effectively eliminated sources of viable mycorrhizal inoculum.

Treatments differed significantly in total plant content of ^{32}P (Table 3). There were, however, large differences in variance between treatments even after log-transformation of the data (Bartlett's test: $P=0.057$). Plants colonized by the native AM fungal community grown with either of the two compartmented systems took up a large amount of ^{32}P (treatments 1 and 4). The ^{32}P content of plants grown with the buffer system (treatment 1) was significantly higher than in plants grown with the mesh bag in treatment 4 (*t*-test; $P<0.05$), which is most likely due to the three times greater amount of labelled soil in treatment 1 than in treatment 4. Uptake of ^{32}P was significantly reduced in the respective non-mycorrhizal control treatments 3 and 5. There was no significant difference in plant ^{32}P content between these two treatments ($P=0.08$), which may in part be due to the more than 40 times greater variance in treatment 5 than in treatment 3. Plant ^{32}P uptake in the non-mycorrhizal treatments as a proportion of that in the respective native mycorrhizal treatments was 25% with the mesh bag system and 3% with the buffer system. The buffer system was significantly ($P<0.01$) superior to the mesh bag system in this control of non-hyphal P uptake.

Hyphal lengths in the non-mycorrhizal treatments were significantly lower than in the respective mycorrhizal treatments (Table 3). Hyphal lengths inside the buffer systems appeared greater than inside the mesh bags when comparing equivalent treatments (Table 3; compare treatment 1 with 4) but the difference was not

Table 2 Plant growth data and root colonization. Values are mean (±SD). Values in columns followed by the same letter are not significantly different (Scheffé test; $P>0.05$)

Treatment	Shoot dry wt. (g plant^{-1})		Root dry wt. (g plant^{-1})		Root length (m pot^{-1})		Root colonization (%)	
1	0.60 (0.07)	B	0.25 (0.03)	B	85.9 (12.4)	B	65 (2)	BC
2	0.64 (0.07)	B	0.26 (0.05)	B	110.2 (48.4)	B	79 (5)	C
3	0.99 (0.01)	C	0.49 (0.01)	C	223.4 (27.7)	CD	1 (1)	A
4	0.65 (0.07)	B	0.26 (0.04)	B	76.2 (4.1)	B	55 (8)	B
5	1.11 (0.07)	C	0.55 (0.04)	C	251.7 (50.1)	CD	0 (0)	A
6	0.63 (0.03)	B	0.28 (0.03)	B	29.4 (3.4)	A	56 (4)	B
7	0.58 (0.01)	B	0.29 (0.04)	B	133.7 (14.6)	BC	68 (6)	BC
8	0.38 (0.01)	A	0.19 (0.01)	A	267.2 (27.4)	D	60 (1)	B

Table 3 Hyphal length in hyphal compartment, plant ^{32}P content and hyphal ^{32}P uptake. Values are mean (±SD). Values in columns followed by the same letter are not significantly different (Scheffé test; $P>0.05$)

Treatment	Hyphal compartment	Fungus	Hyphal length (m g^{-1} soil)		Plant ^{32}P content (kBq)		Hyphal ^{32}P uptake (Bq m^{-1} hyphae)	
1	Buffer system	Native	8.62 (1.83)	B	45.08 (6.71)	B	30.18 (7.47)	A
2	Buffer system	*G. claroideum* (BEG 14)	5.47 (1.3)	B	74.91 (11.65)	B	124.08 (37.66)	B
3	Buffer system	None	3.01 (0.47)	A	1.37 (0.19)	A		
4	Meshbag	Native	5.80 (0.32)	B	21.41 (6.33)	B	63.30 (23.57)	AB
5	Meshbag	None	2.57 (0.53)	A	5.09 (3.35)	A		

significant. Estimates for length-specific hyphal ^{32}P uptake (Bq m^{-1} hyphae) were calculated after subtracting plant ^{32}P content and hyphal length in the non-mycorrhizal control treatments from the values obtained in the respective native mycorrhizal treatments. Length-specific hyphal ^{32}P uptake thus estimated over the whole experimental period was approximately double when using mesh bags compared with buffer systems (Table 3; compare treatment 4 with 1).

Inoculation with *G. claroideum* (treatment 2) resulted in a significantly increased root P concentration (data not shown) but otherwise did not differ in its effect on plant growth parameters from the native AM fungal population (treatment 1; Table 2). *G. claroideum* colonized a greater fraction of total root length than the native AM fungal population but showed a lower hyphal production (Table 3; *t*-test; $P<0.05$). The ^{32}P uptake by *G. claroideum* was 66% greater than that of the native AM fungal population. Thus, the estimate of length-specific hyphal P uptake in this treatment was four times higher than that obtained for treatment 1 (Table 3).

Increasing planting densities significantly increased total plant biomass production per pot, which was paralleled by an increase in root length (Table 2). Colonized root length also increased, as the percentage of root length colonized was unaffected by planting density. There was a positive correlation between hyphal length in the hyphal compartments (Table 4) and colonized root length in the rooting compartments (Fig. 1). Plant ^{32}P uptake increased with an increase in hyphal length in the hyphal compartment (Fig. 2). Estimates for length-specific hyphal P uptake in treatments 6–8 were quite variable but not significantly affected by increases in rooting density in the soil surrounding the hyphal compartment (Table 4). These estimates appeared lower than the estimate for length-specific hyphal P uptake in treatment 4 but the difference was not significant.

Discussion

The main objective of this study was to compare two compartmented systems for measurements of AM hyphal P uptake and transport to the plant. The buffer system with a ^{32}P-free soil layer prevented non-mycorrhizal plants from accessing ^{32}P from inside the hyphal compartment. Similarly, layers of unlabelled soil in hyphal compartments were previously shown to help con-

Table 4 Hyphal length in hyphal compartment, plant ^{32}P content and hyphal ^{32}P uptake as affected by available soil volume per plant. Values are meal (±SD)

Treatment	Soil volume (ml plant^{-1})	Hyphal length (m g^{-1} soil)	Plant ^{32}P content (kBq)	Hyphal ^{32}P uptake (Bq m^{-1} hyphae)
6	3000	4.06 (0.68)	8.68 (4.07)	19.83 (34.08)
7	600	9.25 (2.94)	24.01 (4.82)	38.24 (14.04)
8	250	11.16 (0.38)	28.74 (2.29)	34.48 (4.43)

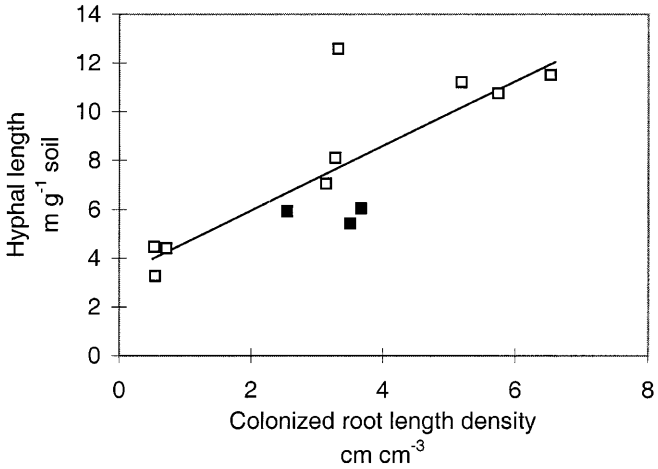

Fig. 1 Relationship between hyphal length in the hyphal compartment and density of colonized root length in the rooting compartment. *Open symbols* 3-l pot volume; *closed symbols* 1.5-l pot volume. *Fitted line*: $y = 1.32*x + 3.31$; $R^2 = 0.66$

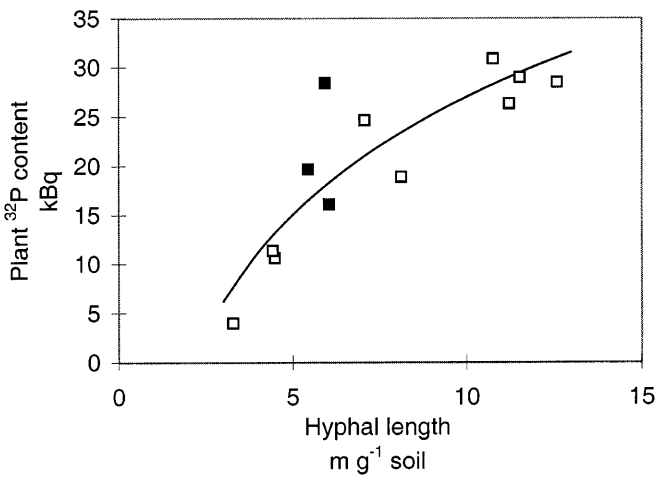

Fig. 2 Relationship between plant ^{32}P content and hyphal length inside the hyphal compartment. Only the soil inside the hyphal compartment was labelled with ^{32}P. *Open symbols* 3-l pot volume; *closed symbols* 1.5-l pot volume. *Fitted line:* $y = 17.26* \ln(x) - 12.75$; $R^2 = 0.76$

trol non-hyphal uptake of ^{15}N-labelled ammonium (Frey and Schüepp 1993). Uptake of P from within the labelled soil volume by non-mycorrhizal plants was reduced to 3% of that taken up by mycorrhizal plants. The buffer system was in this respect superior to the mesh bag system, since uptake of P from within the labelled soil volume by non-mycorrhizal plants grown with the mesh bag system was 25% of the amount taken up by mycorrhizal plants. This high non-mycorrhizal P uptake from inside the hyphal compartment necessitates a control treatment when using the mesh bag system. Addition of the fungicide carbendazim to the soil inside the hyphal compartment has been used previously as a control treatment (Thingstrup personal communication). Carbendazim was shown to effectively inhibit hyphal P uptake when applied directly to external mycorrhizal hyphae (Kling and Jakobsen 1997), but its effectiveness when mixed through the soil was inconsistent (Thingstrup personal communication). Carbendazim, which does not specifically inhibit AM fungi and has negative effects on a wide range of fungi (Edgington et al. 1971), thus has potentially undesirable side-effects.

The two systems also differ to some extent in the characteristics of the soil volume from which hyphal P uptake is measured. While hyphal P uptake in the mesh bag system is measured from both rhizosphere and bulk soil, hyphal P uptake in the buffer system is mainly or exclusively measured from bulk soil. The AM fungal contribution to plant P uptake has most frequently been explained by external hyphae absorbing P from the bulk soil outside the P-depletion zone around actively-absorbing roots (Bolan 1991). However, there are some indications that the P contained in rhizosphere soil with its considerably different chemical, physical and biological properties (Bowen and Rovira 1991) may also be an important source of P taken up by external hyphae (George et al. 1995). The large difference in length-specific hyphal P uptake between the mesh bag and the buffer system was most likely due to differences in design between the two systems, such as the 8- to 10-mm-wide buffer zone of unlabelled soil which external hyphae growing into the buffer system need to bridge before taking up any ^{32}P. The difference, however, may also reflect the importance of rhizosphere processes for hyphal P uptake.

The mesh bag system was easy to set up, whereas preparation of the buffer system was more time-consuming and required two people. However, the necessity to include a control treatment when using the mesh bag system means that double the number of hyphal compartments must be assembled and double the number of samples analyzed compared with the buffer system. Additionally, from a radiation safety viewpoint,

the buffer system was less of a hazard since most of the radiation emitted by the labelled soil was absorbed by the surrounding layer of unlabelled soil.

External hyphae of the native AM fungal community grew into the hyphal compartments and reached greater lengths than external hyphae of a well-known isolate of *G. claroideum*. Length-specific hyphal P uptake by *G. claroideum* was, however, four times higher than that by the native AM fungi. The *Glomus* isolate used is known to have a very high hyphal P uptake capability (Kling and Jakobsen 1997), ranging from approximately 2- to 20-fold higher than other AM fungal isolates. Thus, the hyphal P uptake by the native AM fungal community in the present study lies within the range of values recorded for cultured AM fungal isolates, at least under these growth conditions.

Increasing the planting density and, thereby, the root length density in the rooting compartments led to a concomitant linear increase in hyphal lengths inside the hyphal compartments. This linear increase does not support the suggestion that external hyphae proliferate in unexplored volumes of soil relatively enriched in nutrients (Cui and Caldwell 1996), but is in good agreement with results showing no effect of soil P concentration in the hyphal compartment on hyphal length (Li et al. 1991). In the present study, the soil P concentration in the hyphal and rooting compartments was not measured after the experimental period. However, we expect that differences in soil P concentration between the hyphal and rooting compartments would have increased with increases in rooting density.

Increases in hyphal length in the hyphal compartments were associated with increases in plant P uptake from inside the hyphal compartments. These data were used to estimate length-specific hyphal P uptake. These estimates were not affected by variations in root length density in the rooting compartment. Variations in root length density around a hyphal compartment are, however, expected to affect estimates for length-specific hyphal P uptake and estimates for the contribution of AM to total plant P uptake in two ways. Very low planting densities may result in very variable and, therefore, unreliable estimates for hyphal P uptake, as observed in this study. Very high root length densities, on the other hand, may lead to an overestimation of the hyphal contribution to total plant P uptake. The soil in the hyphal compartment in contrast to the soil in the rooting compartment, would not be depleted of P and would, therefore, allow for concentration-unlimited P uptake by the hyphae (George et al. 1995). Estimates for length-specific hyphal P uptake can, therefore, not be converted to a value for the hyphal contribution to total plant P uptake.

In conclusion, the buffer system provided better control of non-hyphal ^{32}P uptake than the mesh bag system. Based on this and its greater ease of handling once produced and the lack of need for a fungicide-treated control, we selected the buffer system for further testing and use under field conditions.

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