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Transport of ^{15}N from a soil compartment separated by a polytetrafluoroethylene membrane to plant roots via the hyphae of arbuscular mycorrhizal fungi

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SUMMARY

We studied the transport of ^{15}N from a soil compartment separated from a plant root compartment by a hydrophobic polytetrafluoroethylene (PTFE) membrane to plants in the presence and absence of arbuscular mycorrhizal fungi (AMF). We have previously shown that this type of membrane efficiently inhibits mass flow and diffusion of mobile ions in the soil solution in an abiotic system, but can be penetrated by the hyphae of mycorrhizal fungi. Mycorrhizal tomatoes (*Lycopersicon esculentum*) colonized by *Glomus mosseae* were grown at two N fertilizer concentrations in a root compartment. A PTFE membrane was placed between the root compartment and an adjoining soil compartment that was inaccessible to the roots but accessible to the AMF hyphae (hyphal compartment). Additional N was applied to the hyphal compartment using uniformly ^{15}N -labelled NH_4NO_3 . There was a flux of ^{15}N from the hyphal compartment to the plants even in the absence of mycorrhizal fungi. However, this flux was much higher in mycorrhizal plants, which had much higher N concentrations in their shoots and roots than did the non-mycorrhizal control plants. This was particularly apparent when the root compartment had a low N fertilizer concentration. Of the total N content of mycorrhizal plants, c. 42 and 24% at the low and high N fertilizer concentrations, respectively, were estimated to originate from the hyphal compartment by transport through AMF hyphae. In the presence of mycorrhizal fungi, the flux of ^{15}N was about three times higher than in their absence. The results show that AMF can access a soil compartment separated by a PTFE membrane, and can contribute substantially to N uptake by plants.

Key words: extraradical hyphae, *Glomus mosseae*, hydrophobic membranes, nitrogen uptake, ^{15}N tracer.

INTRODUCTION

Symbiosis with arbuscular mycorrhizal fungi (AMF) can significantly increase the nutritional status of plants, particularly with respect to phosphorus (Koide & Schreiner, 1992; Marschner & Dell, 1994). Using ^{15}N tracer techniques it has been shown that N uptake can also be enhanced in plants colonized by

AMF (Johansen *et al.*, 1992, 1993, 1994; Frey & Schüepp, 1993; Tobar *et al.*, 1994). Recently, nitrate transfer from extraradical AMF hyphae to roots has been shown to occur directly in mycorrhizal root organ cultures grown axenically in a Petri dish system with two compartments (Bago *et al.*, 1996).

Container systems in which the soil compartment with the mycorrhizal roots (root compartment) is separated by a fine screen (30–60 μm mesh) from a second soil compartment which is not accessible to the roots but only to fungal hyphae (hyphal com-

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partment) have frequently been used to study plant nutrient acquisition by AMF (Mäder *et al.*, 1993 and references therein). However, in these systems the extent to which extraradical AMF hyphae can contribute to nutrient acquisition of plants remains difficult to determine because of unknown transport by diffusion and mass flow in the soil solution of mobile ions such as nitrate (Johansen *et al.*, 1992, 1996; Frey & Schüepp, 1993). To reduce diffusion and mass flow between the soil compartments, Faber *et al.* (1991) used air gaps, paraffin wax or corn oil barriers, whilst Bethlenfalvay *et al.* (1991) worked with a root-free soil bridge. Johansen *et al.* (1994) applied ^{15}N -labelled ammonium in combination with a nitrification inhibitor to prevent transformation of ammonium, which exhibits little mobility in the soil in contrast to the readily mobile nitrate.

We have introduced the use of polytetrafluoroethylene (PTFE) membranes which completely prevent mass flow and diffusion of ions in an abiotic system, but can be penetrated by AMF hyphae (Mäder *et al.*, 1993). These membranes have been developed to permit exchange of water vapour through waterproof clothing and consist of a microporous, symmetrically structured PTFE layer (confering hydrophobicity), which is coated onto a polyester tissue (Allan, 1982; Schneider & van Gassel, 1984). Here we present results of ^{15}N tracer experiments showing that these membranes efficiently, although not completely, inhibit diffusion and mass flow of N in a biotic system containing roots and microorganisms. The membranes readily allow passage of N via AMF hyphae that are able to penetrate the membranes. A preliminary account of our results, including data from a separate experiment with maize plants colonized by *Glomus intraradices*, has been published (Frey *et al.*, 1998).

MATERIALS AND METHODS

Biological material and inoculum

Tomato seeds (*Lycopersicon esculentum* Mill. cv. Money maker, F_1 hybrid, kindly provided by Geissler AG, Zürich, Switzerland) were surface sterilized in 5% sodium hypochlorite for 5 min, rinsed three times with sterile water and sown in autoclaved vermiculite (Vermex M/803, size 2–4 mm, Vermica AG, Bözen, Switzerland). The plants were pre-cultured for 14 d at 25°C in the glasshouse, and during this time they were fertilized twice with Hoagland solution containing only one-third of the standard phosphorus concentration. Plants were inoculated with *Glomus mosseae* ((Nicol. & Gerd.) Gerd. & Trappe) (Banque Européenne des Glomales: BEG 12). The inoculum consisted of air-dried soil containing AMF hyphae, spores and AMF-colonized roots of *Tagetes* sp. grown in a

mixture of equal volumes of quartz sand and Lecadan clay (Wyss *et al.*, 1991).

Growth substrate

The growth substrate for the plants was an autoclaved 2/1/1 (v/v/v) mixture of quartz sand (Type K30, size 0.2–0.5 mm, Trafor AG, Basel, Switzerland), nutrient-poor subsoil Loess from Therwil, Switzerland (25% clay content, 2 mm sieved), and expanded clay (Lecadan, size 2–4 mm, Leca GmbH, Pinneberg, Germany). The mixture had a pH (0.1 N KCl) of 7.6, an organic carbon content of 0.35% (d. wt basis) and a total N content of 0.023% (d. wt), and contained 0.28 and 12.9 mg N kg⁻¹ dry soil as NH_4^+ and NO_3^- , respectively, in a water extract. The phosphorus and potassium contents of the mixture, as measured in a double lactic acid extract according to Egner *et al.* (1960), were 5.7 mg P and 41.5 mg K kg⁻¹ dry soil.

Container system

The container system used (Fig. 1) was a modification of the design by Wyss *et al.* (1991). The root compartment containing AMF-inoculated roots, and the hyphal compartment accessible to fungal hyphae but not to the roots, were separated by a PTFE membrane (Gore-Tex®, GSC No. BIB910033, pore diameter 5 µm, Gore GmbH, Putzbrunn, Germany, 22 × 12 cm) which was protected on both sides by 60 and 30 µm mesh Nylon screens (Zürcher Beuteltuchfabrik AG, Rüslikon, Switzerland). The Gore-Tex PTFE membranes were laminated on a polyester support which was positioned adjacent to the root compartment. A border of 1 cm of the PTFE membrane protruded on each side of the container system to prevent any capillary transport of nutrients in the soil solution around the membrane edges. The root and hyphal compartments were connected with screw clamps.

Experimental design and growth conditions

The root and hyphal compartments were both filled with 730 g of the growth substrate and 14-d-old tomato seedlings were planted in each of the five divisions of the root compartment. The roots were inoculated with *G. mosseae* using 6 g inoculum per plant. Non-mycorrhizal tomato plants were produced by the use of a non-mycorrhizal 'inoculum' consisting of *Tagetes* roots that were not colonized by AMF. In addition, 1 ml aliquots of a bacterial filtrate of the AMF inoculum were applied to all compartments to provide a general bacterial population. The bacterial filtrate was prepared by suspending 100 ml AMF inoculum in 250 ml distilled water, followed by three consecutive periods of

Fig. 1. A modification of the container system of Wyss *et al.* (1991) was used in the experiment. A tomato plant was grown at each of the five positions in the subdivided root compartment (RC). The adjacent hyphal compartment (HC) was separated from the root compartment by a 5 µm polytetrafluoroethylene (PTFE) Gore-Tex membrane protected by 30 µm mesh nylon screens on both sides, and additional 60 µm nylon screens mounted on the container PVC elements of the root and hyphal compartments. The dimensions of the root and hyphal compartment containers were 20 × 10 × 2 cm.

shaking for 30 min and filtering (Type LS 14.5 filters from Schleicher & Schuell AG, Switzerland). The plants in the root compartment were fertilized six times with 4 ml modified Hoagland nutrient solution per plant at 7 d intervals either with one-third (low N fertilizer) or full strength (high N fertilizer) N concentration of a standard Hoagland solution. The solution consisted of: 79.2 mg (low N) and 237.6 mg (high N) NH_4NO_3 , respectively, 431.1 mg K_2SO_4 , 73.5 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 136.0 mg KH_2PO_4 , 493.0 mg $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg NaFeEDTA , 1.5 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.2 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.08 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 1.3 mg BH_3O_3 and 0.1 mg $\text{H}_{24}\text{Mo}_7\text{N}_6\text{O}_{24} \cdot 4\text{H}_2\text{O l}^{-1}$. After 21 d of growth, the hyphal compartment was fertilized three times at 7 d

intervals with 20 ml of a 515 ppm N solution containing $1.43 \text{ g } ^{15}\text{NH}_4^{15}\text{NO}_3 \text{ l}^{-1}$ with 49 atom% ^{15}N excess (Cambridge Isotope Laboratories, Innerberg, Switzerland). In total, 14.3 mg ^{15}N was applied to each hyphal compartment. Since the soil solution in the hyphal compartment contained 9.6 mg N in mineral form, the actual ^{15}N abundance at the beginning of the experiment was calculated to be 37 atom% ^{15}N . Soil moisture in the root compartment and the hyphal compartment was maintained near field capacity by watering the compartments once or twice each day. Water was added volumetrically by pipette from above.

Plants were grown in a glasshouse at the University of Basel in Switzerland from late May until the beginning of July. Automatic control systems operated at temperatures $>25^\circ\text{C}$ and $<18^\circ\text{C}$. Supplementary lights extended the day length to at least 14 h. The position of the growth containers in the glasshouse was re-randomized at 3 d intervals.

There were eight treatments: two N fertilizer concentrations, each with or without AMF inoculation and with or without a hyphal compartment. There were three replicates: three root compartments, each containing five plants per treatment, giving a total of 24 assemblies in a randomized block. Fresh and dry weight and N concentration were determined by single plant analyses (15 plants per treatment), and means of five plants grown in one root compartment were calculated. The atom% ^{15}N of plants and AMF root colonization were performed on aliquots from five plants grown in one root compartment.

Harvest and assays

Plants were harvested 42 d after planting, 7 d after the last application of ^{15}N . The roots were washed with tap water before the plant material was dried at 70°C for 48 h and dry mass was measured. A weighed fresh subsample from each root compartment was stained with aniline blue in lactophenol according to Phillips & Hayman (1970). The percentage of root length colonized by AMF was determined microscopically using the gridline intersect method of Giovannetti & Mosse (1980).

Shoots and roots were ground to a fine powder using a ball mill and total N content was measured with a Leco CHN analyser. ^{15}N was determined by mass spectrometry using an ANCA MS system at the Analytical Service of Europa Scientific Ltd, Crewe, UK. To calculate the atom% ^{15}N excess in plant material, the atom% ^{15}N of tomato plants not supplied with ^{15}N served as a natural abundance control. The ^{15}N recovery in plants was calculated as percentage of ^{15}N mass in plant tissue (shoot and root) in relation to the ^{15}N mass applied to the hyphal compartment.

RESULTS

In the presence of a *G. mosseae* inoculum, the percentage of root length of the plants colonized by the fungus at harvest ranged between 49 and 61%. Differences did not depend on N fertilizer concentration (Table 1). There was no mycorrhiza formation in the absence of inoculum. Shoot d. wt was not affected by AMF, but was enhanced by the higher N fertilizer concentration in the treatments with and without an adjacent hyphal compartment. Root d. wt was not affected by AMF or N fertilizer concentrations.

In the absence of a hyphal compartment, N

concentrations of shoots and roots of mycorrhizal and non-mycorrhizal plants were only very slightly different. However, they were strongly enhanced in the presence of AMF in the presence of a hyphal compartment at both N fertilizer concentrations (Table 2). The presence of a hyphal compartment had no significant effect on the plant N concentrations in non-mycorrhizal treatments. This suggests that the hyphal compartment was the main source of the surplus N contained in the mycorrhizal plants compared with the non-mycorrhizal controls. The surplus of N was more pronounced when the plants were fertilized in their root compartment at a low N fertilizer concentration (Fig. 2), indicating

Table 1. Dry weights of shoots and roots and mycorrhizal colonization of tomato plants associated (M+) or not (M-) with *Glomus mosseae*

Treatment			D. wt (g per plant)		Root length colonized by AMF (%)
<i>G. mosseae</i>	N fertilizer concentration	Hyphal compartment	Shoot	Root	
M-	1/3 N	HC+	0.14 ab*	0.05 a	0†
M+	1/3 N	HC+	0.14 ab	0.05 a	61 a
M-	3/3 N	HC+	0.19 d	0.06 a	0
M+	3/3 N	HC+	0.17 cd	0.06 a	53 a
M-	1/3 N	HC-	0.12 a	0.05 a	0
M+	1/3 N	HC-	0.12 a	0.05 a	49 a
M-	3/3 N	HC-	0.15 bc	0.06 a	0
M+	3/3 N	HC-	0.15 bc	0.05 a	56 a

Plants were grown in a root compartment at two N fertilizer concentrations one-third or full-strength (3/3) N of standard Hoagland solution: see the Materials and Methods section) with (HC+) or without (HC-) an adjacent hyphal compartment (see Fig. 1). The two compartments were separated by a PTFE membrane.

*Values (means of three replicates) with different letters differ significantly ($P = 5\%$ by analysis of variance; $n = 3$).

†Zero % colonization values of the M- treatments excluded in the ANOVA.

Table 2. Effects of *Glomus mosseae* on N concentration, atom % ^{15}N excess and percentage recovery of total ^{15}N applied in tomato plants grown in a two compartment container system separated by a PTFE membrane (see Fig. 1)

Treatment			N concentration (% d. wt)		Atom % ^{15}N excess		Percentage of total ^{15}N applied
<i>G. mosseae</i>	N fertilizer concentration	Hyphal compartment	Shoot	Root	Shoot	Root	Total plant
M-	1/3 N	HC+	0.71 ab*	0.86 ab	12.0 a	9.1 a	6 a
M+	1/3 N	HC+	1.57 d	1.35 c	16.3 b	16.0 b	17 c
M-	3/3 N	HC+	0.88 ab	0.88 ab	10.2 a	9.7 a	8 ab
M+	3/3 N	HC+	1.33 c	1.35 c	10.8 a	11.6 a	12 b
M-	1/3 N	HC-	0.55 a	0.75 a	0	0	-†
M+	1/3 N	HC-	0.61 a	0.94 ab	0	0	-
M-	3/3 N	HC-	0.70 ab	0.80 ab	0	0	-
M+	3/3 N	HC-	0.74 ab	1.03 b	0	0	-

Plants were grown in the root compartment at two N fertilizer concentrations (one-third N or full-strength (3/3) N: see the Materials and Methods section) with (M+) or without (M-) *G. mosseae*. ^{15}N was supplied as $^{15}\text{NH}_4^{15}\text{NO}_3$ to an adjacent compartment accessible only to AMF hyphae (HC+). Control treatments had no adjacent hyphal compartment (HC-).

*Values with different letters in a column differ significantly ($P = 5\%$ by analysis of variance; $n = 3$).

†Not calculated because ^{15}N fertilized HC was absent in this treatment.

Fig. 2. Analysis of total N content and calculated origin of N of tomato plants (shoots and roots) which were kept without mycorrhizal fungi (M-) or colonized (M+) by *Glomus mosseae*. Plants were grown at two N fertilizer concentrations (one-third N; full-strength or 3/3 N) in the root compartment. Half of the root compartments were joined with ^{15}N fertilized hyphal compartments (HC+), the other half were not (HC-). The root compartments were separated from the hyphal compartments by polytetrafluoroethylene (PTFE) membranes (see Fig. 1). Error bars are standard errors of means of total N content ($n = 3$). Total N content values with different letters differ significantly ($P = 5\%$ by analysis of variance). Shading on bars: black, source (1; see the Results section for details of source numbering) N derived from root compartment taken up by roots; grey, (2) N derived from root compartment due to *Glomus mosseae*; white, (3) ^{15}N derived from hyphal compartment by passive transport without mycorrhizal contribution; cross-hatched, (4) ^{15}N derived from hyphal compartment via transport by mycorrhizal hyphae; diagonally hatched (5) N not accounted for (probably derived from hyphal compartment by mycorrhizal hyphae).

that these plants could profit more from the hyphal compartment via AMF-mediated transport of N across the PTFE membrane.

Since the N in the hyphal compartment was labelled with ^{15}N , it was possible to assess the flux from the hyphal compartment to the plant directly. Somewhat surprisingly, there was transfer of ^{15}N from the hyphal compartment to the plant even in the absence of AMF (Table 2). However, there was a larger increase in ^{15}N abundance of shoots and roots in the AMF treatments compared with the non-mycorrhizal controls, particularly at the low N fertilizer concentration (Table 2). Whilst at the low N fertilizer concentration the ^{15}N recovery expressed as a percentage of total ^{15}N supplied to the hyphal compartment was around three times higher in the mycorrhizal than in the non-mycorrhizal plants, this ratio was only *c.* 1.5 times higher at the high N fertilizer concentration (Table 2). However, at the high N concentration, atom% ^{15}N excess in mycorrhizal as compared with non-mycorrhizal plants was not significantly different.

The data shown in Tables 1 and 2 were used to estimate the contribution of different sources to the

N nutrition of the plant (Fig. 2). As illustrated, two N sources can be distinguished in treatments without an adjoining hyphal compartment (right-hand side of Fig. 2), as follows.

(1) N derived from the root compartment taken up by roots in the absence of AMF (black segments in Fig. 2). This N fraction is represented by the N content of plants grown without AMF.

(2) The estimated N surplus in plants from the root compartment due to AMF (grey segments in Fig. 2). This value corresponds to the difference between the total N content of plants of the treatments with and without AMF.

In the treatments with both a root compartment and a hyphal compartment, five different sources of N can be assessed (left-hand side of Fig. 2), as follows, (1) and (2) corresponding to those already mentioned.

(1) N derived from the root compartment taken up by the roots. This value is calculated by subtracting the ^{15}N content of non-mycorrhizal plants from the total N content. As expected, this value is similar to the corresponding value in the absence of a hyphal compartment.

(2) N derived from the root compartment taken up by the AMF. This cannot be directly assessed, but is assumed to be identical with the corresponding value in the absence of a hyphal compartment.

(3) ^{15}N derived from the hyphal compartment by unknown mechanisms, as calculated by the abundance of ^{15}N in plants grown without AMF and with hyphal compartment (white segments in Fig. 2).

(4) ^{15}N derived from the hyphal compartment via transport by mycorrhizal hyphae. This N fraction can be calculated as total ^{15}N content of plants of the treatments with AMF minus ^{15}N content of plants of the treatments without AMF (cross-hatched segments in Fig. 2). Calculations were done on the basis of 37 atom% ^{15}N of the soluble mineral N in the hyphal compartment, corresponding to the beginning of the experiment (see the Materials and Methods section). Mineral N contents in a water extract and in a 2 M KCl extract of soils from the hyphal compartment at harvest were too low to allow determination of ^{15}N abundance by the ANCA MS system. Nitrogen contributions (1–4) listed accounted for 84% of the total N content at the low and 87% at the high N fertilizer concentration.

(5) Residual N in mycorrhizal plants not accounted for by these calculations was calculated as the difference from the total N content of the plants (diagonally hatched segments in Fig. 2). This amount can probably also be ascribed to AMF: calculations of passive ^{15}N transport (3) and transport via mycorrhizal hyphae (4) were made on the assumption that ^{15}N abundance would remain constant during the experiment, but this was unlikely to be the case. The atom% ^{15}N in the hyphal compartment could be expected to decrease during the 42 d of the experiment due to dilution by N mineralized from the organic matter.

DISCUSSION

In the experiment described, the N concentration of mycorrhizal (as compared with non-mycorrhizal) tomato plants was enhanced only when the root compartment was combined with an N-fertilized hyphal compartment. This finding clearly suggests that N was acquired by the AMF hyphae from the hyphal compartment, and cannot be attributed merely to better exploitation of N contained in the root compartment. Of the total N content of mycorrhizal tomato plants, c. 42 and 24% at the low and high N fertilizer concentrations, respectively, were estimated to originate from hyphal uptake in the hyphal compartment. The significant ^{15}N enrichment of mycorrhizal tomato plants compared with the non-mycorrhizal plants found in this experiment confirms the capability of extraradical AMF hyphae to acquire N and supply it to host plants. Using a PTFE membrane between the root

compartment and hyphal compartment as a barrier to mobile ions, almost identical results were obtained in a simultaneously conducted experiment with maize (*Zea mays*) colonized by *Glomus intraradices* (Frey *et al.*, 1998). Similar findings have been reported previously by Ames *et al.* (1983), Johansen *et al.* (1992, 1993, 1994, 1996), Frey & Schüepp (1993) and Tobar *et al.* (1994). At a high N fertilizer concentration, the AMF-mediated transport of ^{15}N from the hyphal compartment was reduced, as also observed by Johansen *et al.* (1994). These authors hypothesized that AMF hyphal N transport might be regulated according to the N fertilizer content of the host plant: they found that a high N status in mycorrhizal cucumber plants led to a decrease in the ^{15}N recovery to about half of the recovery in plants grown at a low N status.

Using an abiotic experimental system, only negligible transport was observed of ions such as K^+ , Cl^- and Cu^{2+} across PTFE membranes (Mäder *et al.*, 1993; Frey *et al.*, 1998). In the present biotic experimental systems, however, with plant roots growing in soil, some ^{15}N transport across the PTFE membranes also occurred independently of the AMF hyphae. It is possible that hydrophilic pores were introduced into the hydrophobic PTFE membrane by soil microorganisms. Fungi might form bridges by hyphae crossing the membrane or by excretion of hydrophobins rendering the membrane hydrophilic (Wessels, 1996). The membrane might simply have been damaged mechanically by fine sand particles. Any of these factors would have lowered the minimum water entry pressure of the membrane, thereby making it permeable to solutes in the soil solution. Nevertheless, the passive ^{15}N transport from hyphal compartment to root compartment was reduced by 85% using the PTFE membrane, compared with a soil compartment system with Nylon screens alone and an additional root-free buffer zone (Frey *et al.*, 1998), an experimental setup frequently employed in ^{15}N transport studies with AMF (Frey & Schüepp, 1993; Johansen *et al.*, 1994).

The N fertilizer concentration had no effect on AMF root colonization of the tomato plants. Johansen *et al.* (1994) similarly observed no change of AMF colonization of cucumbers even when applying 100, 200 or 400 mg N per plant as NH_4^+ . By contrast, Chambers *et al.* (1980) found that AMF colonization of subterranean clover was decreased by application of 56–168 mg N as NH_4^+ per plant. In a study by Azcón *et al.* (1982), AMF colonization of maize was inhibited when the plants received 60 mg N as NO_3^- . Thus different fungus/host plant combinations might react differently to the host plant N status.

PTFE membranes have been proposed for reducing mass flow and diffusion of mobile ions in soil compartment systems used for studying AMF-

mediated nutrient acquisition by plants (Mäder *et al.*, 1993). The results presented here show that these membranes are reasonably useful tools, despite the fact that ^{15}N can pass across them by unknown (probably biotic) mechanisms, even in the absence of mycorrhizal fungi. They could help in the rapid testing of different AMF isolates with respect to their efficiency in assisting plant N acquisition. The advantage of the compartment system is evident since it permits calculations on the origin of N derived from the hyphal and root compartments. However, it should be noted that the tomato plants used in our experiments were rather small, partly because of the small soil volume in the root compartments and partly because of the N-deficient conditions imposed (plant N concentrations were low). The compartment system is somewhat artificial, as in field soils some of the inorganic N might be in the mobile nitrate form. Nevertheless, the mycorrhizal contribution to the N nutrition of plants in the field might be substantial, especially in dry soils as shown by Tobar *et al.* (1994) or in acid soils, where N occurs mainly in the less-mobile ammonium form. Investigations on AMF-mediated N transfer from an N-fixing plant species to a non-fixing plant species is another potential field of application (Bethlenfalvay *et al.*, 1991; Frey & Schüepp, 1992; Tømm *et al.*, 1994). To avoid problems in the calculations of N transfer due to N mineralized during the experiment, we suggest that only quartz sand should be used as a plant growth substrate, not a mixture that includes field soil (Frey *et al.*, 1998).

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