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ORIGINAL PAPER

Influence of different mineral nitrogen sources (NO_3^--N vs. NH_4^+-N) on arbuscular mycorrhiza development and N transfer in a *Glomus intraradices*–cowpea symbiosis

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Abstract Labeled nitrogen (^{15}N) was applied to a soil-based substrate in order to study the uptake of N by *Glomus intraradices* extraradical mycelium (ERM) from different mineral N (NO_3^- vs. NH_4^+) sources and the subsequent transfer to cowpea plants. Fungal compartments (FCs) were placed within the plant growth substrate to simulate soil patches containing root-inaccessible, but mycorrhiza-accessible, N. The fungus was able to take up both N-forms, NO_3^- and NH_4^+. However, the amount of N transferred from the FC to the plant was higher when NO_3^- was applied to the FC. In contrast, analysis of ERM harvested from the FC showed a higher ^{15}N enrichment when the FC was supplied with $^{15}NH_4^+$ compared with $^{15}NO_3^-$. The ^{15}N shoot/root ratio of plants supplied with $^{15}NO_3^-$ was much higher than that of plants supplied with $^{15}NH_4^+$, indicative of a faster transfer of $^{15}NO_3^-$ from the root to the shoot and a higher accumulation of $^{15}NH_4^+$ in the root and/or intraradical mycelium. It is concluded that hyphae of the arbuscular mycorrhizal fungus may absorb NH_4^+ preferentially over NO_3^- but that export of N from the hyphae to the root and shoot may be greater following NO_3^- uptake. The need for NH_4^+ to be assimilated into organically bound N prior to transport into the plant is discussed.

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Introduction

Many studies have reported that the extraradical mycelium (ERM) of arbuscular mycorrhizal (AM) fungi can take up and transfer considerable amounts of N from both organic (Hawkins et al. 2000; Hodge et al. 2001; Leigh et al. 2009; Hodge and Fitter 2010) and inorganic (Johansen et al. 1996; Tanaka and Yano 2005; Subramanian and Charest 1999; Govindarajulu et al. 2005; Jin et al. 2005) sources to the host plant. The most common forms of N taken up by AM are nitrate and ammonium. However, under some conditions ammonium (NH_4^+) supply, compared with nitrate (NO_3^-) supply, has been shown to decrease mycorrhizal activity (Valentine and Kleinert 2006; Ngwene et al. 2010).

The importance of AM fungi for plant N nutrition is a topic of controversy. The existing experimental evidence has recently been summarized by Smith and Smith (2011). Some authors have argued that the potential N uptake and transport rates of roots are much higher than those of hyphae so that in soils with high mineral N supply, the contribution of AM fungi to shoot N content is likely to be small compared with uptake by the roots (Hawkins et al. 2000; Hodge 2003). However, the case may be different when AM fungal hyphae have access to N sources that are not accessible to roots (George et al. 1992; Frey and Schuepp 1993; Hodge 2003; Leigh et al. 2009). An understanding of N dynamics between AM fungi and host plants may help not only to quantify N uptake processes in individual plants but also to describe plant and AM fungal contribution to global N cycles.

Although studies on N transfer by AM fungi to host plants have shown that N can be transported through AM fungal hyphae, it is still not clear how different forms of N are transferred by the fungi (Smith and Smith 2011).

Govindarajulu et al. (2005) proposed that after uptake of mineral N (NO_3^- or NH_4^+), both forms are assimilated mainly into the amino acid arginine, transported through the hyphae, and then transferred in the form of NH_4^+ to the plant. In accordance with this pathway, some studies have indicated a preferred hyphal AM uptake of NH_4^+ as opposed to NO_3^- (e.g., Johansen et al. 1996; Govindarajulu et al. 2005; Jin et al. 2005). Despite these insights into plant–fungal N dynamics, inconsistencies exist between published results which may have arisen due to differences in host plant N status, root or hyphal proliferation, localization of N supply in the substrate, and substrate pH or moisture content. Additional inconsistencies could have arisen from a failure to consider that N transfer rates for NO_3^- or NH_4^+ from soil to the plant shoot via AM hyphae are controlled by several independent steps: (1) soil adsorption of NO_3^- or NH_4^+, resulting in available and fixed pools in soil; (2) absorption and assimilation by the ERM; (3) transport within the ERM to the fungal tissues within the root; (4) transfer from fungal tissue to root tissue; and (5) root-to-shoot transfer. For example, experiments using excised hyphae investigate only step 2, and those on artificial substrates or in nutrient solutions often do not consider step 1.

Since an approach to better understand AM fungal N dynamics under field conditions requires more studies with soil-grown plants (Smith and Smith 2011), the present study was undertaken using a soil-based substrate for both plant and fungal compartments to follow the transport of different N sources by the ERM of *Glomus intraradices* to cowpea plants. Small fungal compartments were placed within the plant growth substrate, with an air gap to eliminate mass flow and diffusion, to simulate soil patches containing root-inaccessible, but mycorrhiza-accessible N. The influence of different mineral N sources (NO_3^- vs. NH_4^+) on AM fungal N transfer to the host plant was investigated using ^{15}N-nitrogen. The effect of these different N sources on AM fungal development was also studied. Based on previous results from our laboratory (Neumann and George 2010), we hypothesized that the fungus may accumulate more N when supplied with NH_4^+ as compared with NO_3^- but that NO_3^- supply would lead to better mycorrhiza root colonization, ERM development, and fungal N contribution to host plant shoot N content.

Materials and methods

Precultivation of plants and AM fungal inoculation

Seeds of the African cowpea cultivar "IT 18", supplied by the International Institute for Tropical Agriculture, were germinated on a filter paper soaked in saturated $CaSO_4$ solution. Sixty seedlings with fully established primordial leaves were individually transplanted for precultivation into black, round 220-ml plastic planting pots (Teku-Terra TO 9; Pöppelmann, Germany) containing 260 g of heat pasteurized (85 °C for 48 h) dry soil substrate at a bulk density of 1.2 $g cm^{-3}$. The soil was a nutrient poor loess subsoil of a luvisol, classified as loamy sand with a pH of 7.2, sieved through a 4-mm sieve before pasteurizing. After pasteurizing, the soil was fertilized with 100 mg N kg^{-1} dry soil (in the form of NH_4NO_3), 50 mg P (KH_2PO_4), 200 mg K (K_2SO_4), 100 mg Mg ($MgSO_4$), 10.4 mg Fe (Fe-EDTA), 10 mg Zn ($ZnSO_4$), and 10 mg Cu ($CuSO_4$). Half of the plants (30) were inoculated by mixing 10 g of AM fungal inoculum with the respective growth substrate. Axenic cultures of transformed carrot roots colonized by *G. intraradices* (Schenck and Smith) DAOM 181602 (syn. *Rhizophagus irregularis*, Krüger et al. 2011) were used as an inoculum. The cultures were grown at the Institute of Vegetable and Ornamental Crops, Großbeeren, on solid M medium (Bécard and Fortin 1988) for approximately 8 weeks before experimentation. The material used for plant inoculation consisted of the AM fungal-colonized roots with the surrounding growth medium, which contained numerous spores and hyphae. Prior to its use, the inoculum was placed in a drying oven at 35 °C for 48 h and then cut into small pieces. The remaining 30 plants (non-AM) received the same amount of autoclaved (121 °C for 20 min) inoculum.

After planting, daily water loss from the pots was estimated gravimetrically and water content in the substrate was maintained at 17 % (*w*/*w*) with deionized water. Plants were kept in a greenhouse in Grossbeeren, Germany (long. 13°19′ 60″ E, lat. 51°22′0″ N); inoculation was repeated 3 weeks after planting because no colonization was observed in root samples of AM plants at that time. The inoculum was not dried before use to inoculate plants for the second time and was inserted at three points around each AM plant (20 g in total). Again, non-AM plants received the same amount of autoclaved inoculum. Three weeks after the second inoculation, plants showed AM fungal colonization in roots.

Preparation of fungal compartments and fungal compartment substrate

Fungal compartments (FCs) were constructed from 55-ml plastic net pots with a latticed wall (Teku; Pöppelmann, Germany). The outer surface of the net pots was covered with a nylon membrane with a 30-μm mesh width (Sefar AG; Heiden, Switzerland) that allowed hyphae, but not roots, to grow into the FCs. The nylon membrane was fixed to the net pots using silicon (Sista Silicon Küche; Henkel KGaA, Düsseldorf, Germany). To avoid ion diffusion between the substrate in the FCs and the surrounding pot substrate, an air gap was created by lining the inner wall of the FCs with two layers of nylon net having a 1-mm mesh width (Sefar AG; Heiden, Switzerland). Root + fungal

compartments (RFC) construction was similar to that of the FCs but without the 30-μm nylon membrane, thus allowing both roots and hyphae to grow into them.

The compartment substrate (in both FCs and RFCs) was a 1:1 mixture of wet sieved loess soil (particle size <40 μm) and glass beads (Ø 1.7–2.1 mm; Carl Roth GmbH, Karlsruhe, Germany), with 20 % *w*/*w* water (Neumann and George 2005). This mixture allows for the extraction of intact fungal ERM after harvest. The wet sieved loess soil (compartment substrate) was the same as the cultivation substrate. All nutritional elements were fertilized at the same rate and using the same salts in both the compartment and cultivation substrates, apart from N which was supplied either in the form of NO_3^- ($Ca(NO_3)_2$) or NH_4^+ ($(NH_4)_2SO_4$). To minimize the conversion of NH_4^+ to NO_3^-, a nitrification inhibitor nitrapyrin (N-Serve; Dow AgroSciences LLC; Indianapolis, USA) was applied (initially 7 mg kg^{-1} dry soil) to the fertilized compartment substrate.

Experimental setup and growth conditions

Fifty-six precultivated plants (28 AM and 28 non-AM) were individually transplanted into black, round 2-l plastic pots (Teku Container BC 17; Pöppelmann, Germany) containing 1,870 g of dry soil at a bulk density of 1.3 g cm^{-3}. The pot substrate (PS) was prepared in the same way as the substrate used for precultivation, except that N was fertilized either as NO_3^- ($Ca(NO_3)_2$) to 16 AM and 16 non-AM plants (PS_{NO3}) or as NH_4^+ ($(NH_4)_2SO_4$) to 12 AM and 12 non-AM plants (PS_{NH4}). Nitrapyrin was applied to the pot substrate in all treatments at an initial rate of 7 mg kg^{-1} dry soil. Two FCs and two RFCs were alternately inserted into each pot substrate around the plant (Fig. 1). The compartments were filled (5 mm below the top) with 110 g of fresh compartment substrate (2.0 g cm^{-3}). The four compartments in each pot were either all filled with NO_3^-- ($C_{NO3/NO3}$) or NH_4^+- ($C_{NH4/NH4}$) fertilized substrate or two (one FC and one RFC) compartments were filled with NO_3^- and the other two with NH_4^+-fertilized substrate ($C_{NO3/NH4}$). Eight replicates were prepared for the treatment $PS_{NO3}C_{NO3/NH4}$. This permitted later compartment application of ^{15}N as $^{15}NO_3^-$ to four of the eight replicates and as $^{15}NH_4^+$ to the remaining four. All other treatments also had four replicates and the experiment was set up in a completely randomized design.

As in precultivation, daily water loss from the pots was estimated gravimetrically, water content in the substrate was maintained at 17 % (*w*/*w*) with deionized water, and plants were grown in a greenhouse (July–September, day/night 24/19 °C, rh 68 %). Compartments were watered as required to keep the substrate moist. Two weeks after transplanting, all plants received an additional 50 mg N kg^{-1} dry soil applied to the pot substrate either in the form of NO_3^- ($Ca(NO_3)_2$) or NH_4^+ ($(NH_4)_2SO_4$). Nitrapyrin (4 mg kg^{-1} dry soil) was again applied to all pot and compartment substrates. This procedure was repeated after another 2 weeks when compartments received an additional 100 mg N kg^{-1} dry soil of their respective N-form. Selected FCs were given nitrogen enriched with the ^{15}N isotope at a rate of 880 μg ^{15}N per FC (amounting to 20 % of N applied to the FC). The ^{15}N-nitrogen was supplied as $Ca(NO_3)_2$ or NH_4Cl to the respective compartments. When all compartments in a pot were fertilized with the same N-form ($C_{NO3/NO3}$ or $C_{NH4/NH4}$ treatments), ^{15}N was applied to both FCs (2× 880 μg per plant). When the N-form was not the same ($C_{NO3/NH4}$ treatments), ^{15}N was applied only to one FC, either the NO_3^-- or the NH_4^+-fertilized one (1×880 μg per plant).

Harvest

Plants were harvested 2 weeks after application of ^{15}N to the FCs (6 weeks after transplanting). Shoots were separated into stem, leaf, and pod components, and fresh weights were recorded. Roots were separated from the substrate by washing, and a representative sample (about 1 g) of fresh roots

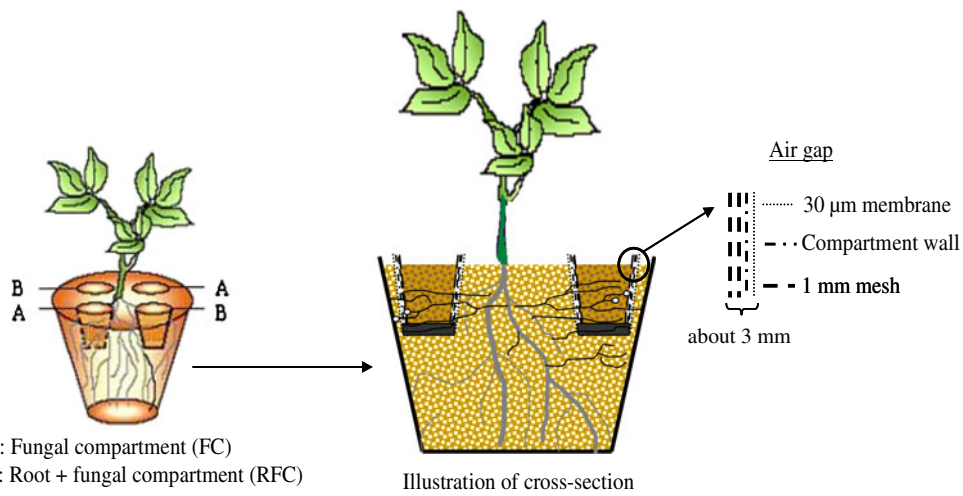

Fig. 1 Compartment placement within planting pots. To avoid ion diffusion between the substrate in the FCs and the surrounding pot substrate, an air gap was created by lining the inner wall of the FCs with two layers of a nylon net having 1-mm mesh width. Two fungal compartments (FC) and two root + fungal compartment (RFC) were inserted into the substrate of each pot around a cowpea plant. All were either NO_3^- compartments ($C_{NO3/NO3}$) or NH_4^+ compartments ($C_{NH4/NH4}$), or one of each pair NO_3^- and the other NH_4^+ ($C_{NO3/NH4}$)

was collected and stored in 15 % ethanol for staining with 0.05 % trypan blue in lactic acid after cutting into 1–2-cm length (Koske and Gemma 1989). The AM fungal-colonized root length was evaluated by a modified intersection method (Tennant 1975; Kormanik and Mc Graw 1982) using 200 or more intersections per plant.

All plant parts were oven dried at 65 °C for 3 days, and dry weights were recorded. Roots plus ERM and ERM alone were obtained from the RFCs and FCs, respectively, by washing the contents of the compartment over a 40-μm sieve (Retsch Test Sieve 3310-1; Retsch, Germany) and separating glass beads from roots and hyphae (Neumann and George 2005). Samples were then freeze-dried at −30 °C for 4 days in a freeze drier (CHRIST ALPHA; Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and dry weights were recorded.

Hyphal length and number of spores were estimated using a modified membrane filtration technique. A subsample of the freeze-dried ERM (about 0.5 mg) was stained with a few drops of 0.05 % trypan blue in lactic acid and homogenized with 300 ml of deionized water in a laboratory blender (Waring Commercial; CT, USA) for 60 s. An aliquot of the hyphal suspension was filtered on a 0.45-μm mesh width membrane filter (MicronSep; GE Water and Process Technologies, USA) using a bottle neck filtration unit (NALGENE Reusable Bottle Top Filter Unit; Nalge Company, NY, USA). The membrane filter was mounted onto a microscope slide, and hyphal length was estimated by a modified gridline intersection method observed at×200 magnification (Newman 1966; Tennant 1975). Spore numbers were counted on the membrane.

Mineral nutrient analysis

For P analysis, 500 mg samples of pulverized plant material were dry-ashed at 500 °C, oxidized with 5 ml 21.7 % HNO_3 on a hot plate, and taken up in 25 ml 2.1 % HCl. After filtration (filter circles MN 615, Macherey-Nagel, Germany), P concentration in the filtrate was analyzed colormetrically with a spectrophotometer (EPOS 5060, Eppendorf, Hamburg, Germany) set at a wavelength of 436 nm, after staining with ammonium molybdate-vanadate solution (Gericke and Kurmies 1952). For N analyses, samples of 5 to 15 mg pulverized plant material or freeze-dried fungal material were analyzed after dry oxidation (Elementar vario EL; Hanau, Germany). After total N measurement, the N fraction and helium (carrier gas) were automatically introduced to a coupled emission spectrometer (NOI 7; Fischer Analysen Instrumente, Leipzig, Germany) where ^{15}N atom percentage labeling was determined. The amount of ^{15}N (^{15}N content in microgram) in the plant and fungal material was calculated by multiplying the ^{15}N atom percentage excess with the total N (in millimole per sample) and then converting to microgram ^{15}N per sample.

Statistical analyses

All data were analyzed for normal distribution and equality of variance before being subjected to analyses of variance (ANOVA). The shoot/root ratio, ERM dry weight, and spore numbers as well as data in the form of percentages were first arcsine square root transformed before being analyzed. Mean values were compared by a one-way ANOVA/Tukey's multiple comparison, or a *t* test when appropriate. Two- and three-way ANOVAs were used to estimate whether pot substrate N fertilization, compartment N fertilization, or AM fungal inoculation, alone or in interaction, had a significant influence on the mean values. Differences were considered significant when *p* values were below 0.05. Statistics were performed using the SigmaStat 3.5 program (STATCON, Germany).

Results

Extent of root length colonized by *G. intraradices* in the pot substrate

No root colonization was observed in non-AM plants. All AM plants showed a high degree of root colonization (Fig. 2). Two-way ANOVA revealed a significant effect of the N-form fertilized to the pot substrate on the extent of AM fungal colonization of roots in the pot substrate (*p* values <0.001), with a slight reduction in root colonization levels in plants supplied with NH_4^+ (PS_{NH4}) as opposed to NO_3^- (PS_{NO3}). Colonization levels of roots in the pot substrate were not significantly affected by the N-form applied in the compartment substrate.

Plant growth

Four weeks after transplanting, leaves of the NH_4^+-fed plants (PS_{NH4}) appeared pale. At harvest, the shoot and root dry weights (DWs) of the cowpea plants were significantly influenced by AM fungal inoculation and pot substrate N fertilization (Tables 1 and 2). Inoculation with *G. intraradices* increased shoot and root DWs above those of the corresponding non-AM plants. No significant differences in shoot or root DWs were observed between non-AM plants supplied with either NO_3^- or NH_4^+. In contrast, NO_3^- fertilization increased both shoot and root DWs in AM plants, compared to NH_4^+ fertilization. This increase was smaller in the presence of NO_3^- in two or all compartments in the NH_4^+-fertilized treatments. When the pot substrate and all compartments were fertilized with NO_3^- ($PS_{NO3}C_{NO3/NO3}$), the shoot/root ratio was increased by *G. intraradices* inoculation. No significant differences in the shoot/root ratio were observed among other treatments. No nodules were observed on roots at harvest.

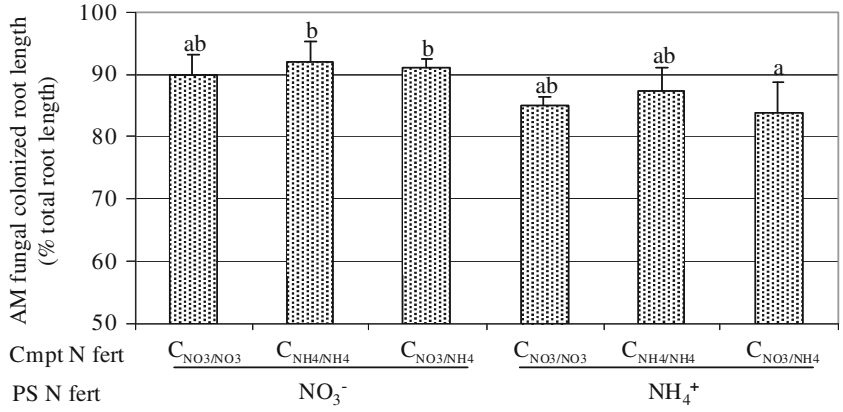

Fig. 2 Percentage of total cowpea root length colonized by *G. intraradices* in the main pot substrate (outside of the compartments) at harvest. The plants were fertilized either with NO_3^--N (NO_3^-) or with NH_4^+-N (NH_4^+) in the pot substrate (PS). The compartments (Cmpt) in each pot (two FCs and two RFCs) were either all fertilized with NO_3^--N ($C_{NO3/NO3}$) or NH_4^+-N ($C_{NH4/NH4}$), or one of each type NO_3^--N and the other NH_4^+-N ($C_{NO3/NH4}$). Mean values ± standard deviation are shown. Mean values labeled by the same letter are not significantly different

Plant P and N status

Inoculation with *G. intraradices* resulted in increased plant P concentrations (Tables 2 and 3). In AM plants, NH_4^+ fertilization resulted in an increased shoot P concentration but a decreased total plant P content compared with NO_3^- fertilization. No effect of compartment N fertilization on plant P status was observed. Non-AM plants had higher shoot N concentrations but lower total plant N contents (Table 3) when compared with AM plants. Nitrate fertilization led to an increase in the shoot N concentration of non-AM plants and an increased plant total N content in AM plants. When AM plants were supplied with NH_4^+, total plant N content was higher when the compartment substrate was fertilized with NO_3^- instead of NH_4^+. There was no significant difference in root N concentration between the different treatments. However, the three-way ANOVA results showed a positive influence of AM fungal inoculation and compartment NO_3^- fertilization on root N concentration (Table 2).

Extent of root length colonized by AM fungi and development of ERM in compartments

No ERM was found in the FCs of the non-AM plants. For AM plants, NH_4^+ fertilization led to relatively lower root and ERM DWs (data not shown) in the FCs and RFCs when compared with NO_3^- fertilization. The amount of material harvested from the compartments in the NH_4^+-fertilized treatments was too small (<15 μg cm^{-3} substrate) for any further analysis. Roots harvested from the RFCs from pots supplied with NO_3^- showed a high degree of AM fungal root colonization, irrespective of the type of compartment N fertilization (Table 4). These were,

Table 1 Total plant dry weight (DW), shoot DW, root DW, and shoot/root ratio at harvest of cowpea plants

PS N fertilization		PS_{NO3}			PS_{NH4}		
Compartments in pot		$C_{NO3/NO3}$	$C_{NH4/NH4}$	$C_{NO3/NH4}$	$C_{NO3/NO3}$	$C_{NH4/NH4}$	$C_{NO3/NH4}$
Total plant DW (g per plant)	−AM	1.41±0.18 a	1.53±0.16 a	1.43±0.30 a	1.26±0.20 a	1.30±0.26 a	1.44±0.20 a
	+AM	**11.42**±0.56 c	**11.51**±1.80 c	**10.78**±1.28 c	**6.31**±0.70 b	**4.97**±0.58 b	**6.27**±0.79 b
Shoot DW (g per plant)	−AM	1.01±0.12 a	1.16±0.13 a	1.02±0.23 a	0.95±0.19 a	0.96±0.26 a	1.02±0.19 a
	+AM	**9.81**±0.33 c	**9.71**±1.55 c	**9.05**±1.03 c	**5.25**±0.78 b	**4.01**±0.47 b	**5.11**±0.55 b
Root DW (g per plant)	−AM	0.40±0.05 a	0.37±0.05 a	0.40±0.08 a	0.31±0.05 a	0.34±0.03 a	0.42±0.04 a
	+AM	**1.61**±0.28 c, d	**1.80**±0.29 d	**1.73**±0.27 d	**1.06**±0.12 b	**0.96**±0.18 b	**1.17**±0.26 b, c
Shoot/Root ratio	−AM	2.57±0.08 a	3.16±0.29 a, b	2.53±0.15 a	3.09±0.81 a, b	2.84±0.85 a, b	2.46±0.46 a
	+AM	**6.22**±0.97 b	5.41±0.60 a, b	5.28±0.37 a, b	5.06±1.28 a, b	4.26±0.66 a, b	4.49±0.72 a, b

Mycorrhizal (+AM) and non-mycorrhizal (−AM) plants were fertilized either with NO_3^--N (PS_{NO3}) or with NH_4^+-N (PS_{NH4}) in the pot substrate. Two fungal compartments (FC) and two root + fungal compartments (RFC) were inserted into each pot. All were either NO_3^- ($C_{NO3/NO3}$) or NH_4^+ ($C_{NH4/NH4}$) fertilized, or one of each compartment type was NO_3^- while the other one was NH_4^+ ($C_{NO3/NH4}$) fertilized. Mean values ± standard deviation are shown. Mean values for AM plants (+AM) in bold are significantly different ($p<0.05$) from the corresponding non-AM plants (−AM). Mean values in rows labeled by the same letter are not significantly different

Table 2 Result of the three-way ANOVA analysis showing effects of pot substrate N fertilization (PS_N), compartment substrate N fertilization (C_N), AM fungal inoculation (M), and their interactions on different plant parameters

Parameter	Results of the three-way ANOVA						
	PS_N	C_N	M	PS_N*C_N	PS_N*M	C_N*M	PS_N*C_N*M
Total plant DW	**<0.001**	0.532	**<0.001**	**0.046**	**<0.001**	0.263	0.307
Shoot DW	**<0.001**	0.464	**<0.001**	**0.040**	**<0.001**	0.150	0.345
Root DW	**<0.001**	0.174	**<0.001**	0.306	**<0.001**	0.982	0.326
Shoot/root	**0.015**	0.074	**<0.001**	0.518	**0.012**	0.146	0.486
Shoot P conc.	**<0.001**	0.229	**<0.001**	0.370	**<0.001**	0.883	0.074
Root P conc.	0.226	0.243	**<0.001**	0.312	0.457	0.525	0.198
Plant P content	**<0.001**	0.494	**<0.001**	0.056	**<0.001**	0.501	0.127
Shoot N conc.	**<0.001**	0.108	**<0.001**	0.454	**0.009**	0.062	0.449
Root N conc.	0.596	**0.016**	**<0.001**	0.076	0.378	0.785	0.409
Plant N content	**<0.001**	0.153	**<0.001**	0.092	**<0.001**	**0.028**	**0.041**

Effects were considered significant when p values were below 0.05 (printed in bold)

however, on average colonized 13 % less than roots outside the compartment. The ERM DW and spore density in FCs obtained from the pots fertilized with NO_3^- were also not significantly affected by compartment N fertilization. However, hyphal length increased in this treatment when the compartment was supplied with NH_4^+, irrespective of whether the other compartment was also supplied with NH_4^+ or with NO_3^- (Table 4).

Nitrogen (^{15}N) uptake and transfer to the host plant by fungal ERM

Both forms of ^{15}N supplied (NH_4^+ and NO_3^-) were taken up by the ERM, and N from these forms was transferred to the plant. At harvest, ^{15}N enrichment of ERM harvested from the FCs of the NO_3^--fertilized plants was clearly higher when the FCs were fertilized with $^{15}NH_4^+$, as opposed to $^{15}NO_3^-$ (Table 4). Non-AM plants contained only traces of excess ^{15}N in plant tissues (Fig. 3). In AM plants, ^{15}N transfer from FCs to the shoot + root (including internal mycorrhizal structures) was consistently higher when FCs were fertilized with $^{15}NO_3^-$, as opposed to $^{15}NH_4^+$, irrespective of the pot N fertilization. When plants were supplied with NO_3^- and had both NO_3^-- and NH_4^+-fertilized compartments, total ^{15}N transfer to the plant was higher when the labeled N derived from the NO_3^-, compared with the NH_4^+-fertilized compartment (Fig. 3). The ^{15}N shoot/root ratio (shoot ^{15}N content/root ^{15}N content) was lower when NH_4^+ as opposed to NO_3^- was supplied to the FCs, irrespective of whether the other FC was also supplied with NH_4^+ or with NO_3^- (Fig. 4).

Table 3 P and N status of cowpea plants at harvest

PS N fertilization		PS_{NO3}		PS_{NH4}	
Compartments in pot		$C_{NO3/NO3}$	$C_{NH4/NH4}$	$C_{NO3/NO3}$	$C_{NH4/NH4}$
Shoot P conc. (mg per g DW)	−AM	0.88±0.06 a	0.86±0.05 a	0.84±0.05 a	0.89±0.03 a
	+AM	**1.96**±0.09 b	**2.23**±0.09 b	**2.70**±0.36 c	**2.58**±0.24 c
Root P conc. (mg per g DW)	−AM	1.28±0.17 a	1.43±0.06 a	1.58±0.17 a	1.25±0.35 a
	+AM	**2.73**±0.15 b	**2.69**±0.23 b	**2.43**±0.45 b	**2.40**±0.14 b
Plant P content (mg per g DW)	−AM	1.38±0.14 a	1.47±0.18 a	1.29±0.17 a	1.27±0.15 a
	+AM	**23.65**±2.01[c]	**26.45**±4.43 c	**17.13**±2.38 b	**12.65**±2.11 b
Shoot N conc. (mg per g DW)	−AM	38.7±1.4 c	35.3±1.6 b, c	32.4±3.5 b	31.0±1.0 b
	+AM	**23.6**±0.5 a	**23.8**±1.6 a	**22.3**±0.9 a	**22.4**±1.1 a
Root N conc. (mg per g DW)	−AM	20.9±1.0 a, b	20.8±1.4 a, b	22.7±1.3 a, b	19.4±1.2 a
	+AM	24.2±1.1 b	23.4±2.0 a, b	24.0±3.5 b	21.9±1.3 a, b
Plant N content (mg per plant)	−AM	47.4±5.3 a	48.6±4.1 a	37.4±3.1 a	40.4±0.9 a
	+AM	**270.3**±15.3 d	**270.9**±24.2 d	**141.7**±15.6 c	**110.5**±10.9 b

Mycorrhizal (+AM) and non-mycorrhizal (−AM) plants were fertilized either with NO_3^--N (PS_{NO3}) or with NH_4^+-N (PS_{NH4}) in the pot substrate. Two fungal compartments (FC) and two root + fungal compartments (RFC) were inserted into each pot. All were either NO_3^- ($C_{NO3/NO3}$) or NH_4^+ ($C_{NH4/NH4}$) fertilized. Mean values ± standard deviation are shown. Mean values for AM plants (+AM) in bold are significantly different ($p<0.05$) from the corresponding non-AM plants (−AM). Mean values in rows labeled by the same letter are not significantly different

Table 4 Percentage of cowpea root length colonized by *G. intraradices* in the RFC, hyphal DW, hyphal length, and spore density in the FCs removed from the NO_3^--N-fertilized pots (PS_{NO3}) at harvest

Compartments in pot	$C_{NO3/NO3}$	$C_{NH4/NH4}$	$C_{NO3/NH4}$	
Compartment analyzed	NO_3^-	NH_4^+	NO_3^-	NH_4^+
AM colonization rate (roots in RFC)	81.1±3.7 a	79.8±5.4 a	79.2±3.8 a	84.1±2.2 a
FC hyphae DW (μg cm^{-3} substrate)	81.9±64.2 a	93.8±25.3 a	113.0±170.7 a	220.3±208.7 a
FC hyphae length (m cm^{-3} substrate)	6.8±1.37 a, b	11.2±3.37 c	5.6±0.72 a	10.1±1.61 b, c
FC spore density (per cm^{-3} substrate)	582.1±973 a	408.9±302 a	145.6±213 a	1,133.2±1581 a
FC ERM ^{15}N conc. (% of N total)	0.44±0.40 a	3.94±0.79 b	Not analyzed	

Two fungal compartments (FC) and two root + fungal compartments (RFC) were inserted into each pot. All were either NO_3^- ($C_{NO3/NO3}$) or NH_4^+ ($C_{NH4/NH4}$) fertilized, or one of each compartment type was NO_3^- while the other one was NH_4^+ ($C_{NO3/NH4}$) fertilized. Mean values ± standard deviation are shown. Mean values in rows labeled by the same letter are not significantly different

Discussion

The FC substrate used in the present experiment (50 % soil material, 50 % inert glass beads) was intended to represent the chemical (not physical) conditions of a soil, in order to allow a more realistic study of hyphal nutrient uptake by an AM fungus, compared to situations in artificial growth media or on nutrient solutions. Being closer to soil chemical conditions in the field, this substrate may have affected the availability of the different N-forms applied. Both forms of ^{15}N supplied (NH_4^+ and NO_3^-) were taken up from the substrate by the ERM of *G. intraradices*, and N from these forms which was transferred to the cowpea plants reached up to 40 % of the applied ^{15}N (fraction of applied ^{15}N detected in plant material). Transport of considerable amounts of N by AM fungi to a host plant has been demonstrated in previous experiments using different soilless media (Johansen et al. 1992, 1996; Hawkins and George 1999; Subramanian and Charest 1999; Hawkins et al. 2000; Govindarajulu et al. 2005; Tian et al. 2010).

The transfer of ^{15}N by the ERM of *G. intraradices* from the FC to the cowpea plants was consistently higher with $^{15}NO_3^-$ than with $^{15}NH_4^+$, regardless of whether both FCs in the pot contained the same or different N-forms (NO_3^--N or NH_4^+-N) and irrespective of the N-form fertilized in the main pot substrate. Bago et al. (2001) proposed that absorbed N (NO_3^- or NH_4^+) is assimilated into arginine via the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle in the ERM, then compartmented in the vacuoles, and translocated to the intraradical mycelium (IRM) for storage and cytosolic pool replenishment. In the cytosol, arginine is broken down by arginase and urease to release NH_4^+, which is then transferred to the plant at the AM symbiotic interface, the fungus retaining the fixed carbon component of the arginine. This proposed pathway, which is metabolically efficient for both partners, has

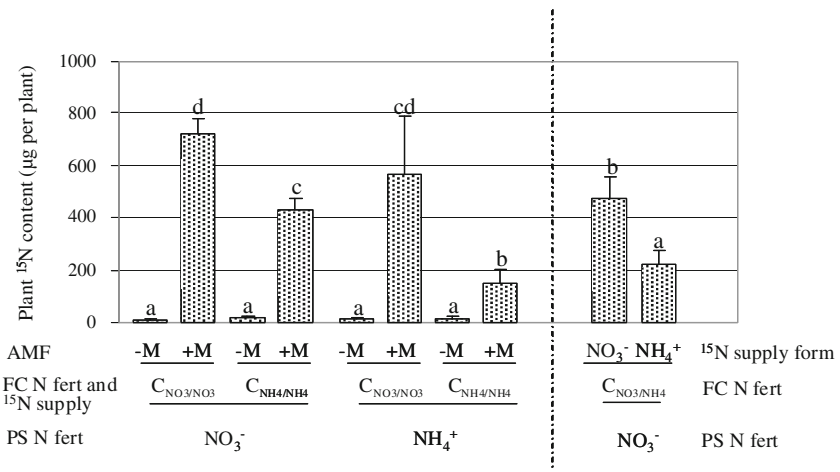

Fig. 3 The total ^{15}N content in shoot + root (including internal mycorrhizal structures) in microgram per plant at harvest. Two weeks before harvest, labeled N (^{15}N) was supplied to the ERM in the fungal compartment (FC) in the respective form (NO_3^- or NH_4^+). When both FC in a pot were fertilized with the same N-form ($C_{NO3/NO3}$ or $C_{NH4/NH4}$), ^{15}N was applied to both (*left*). When the N-form was not the same ($C_{NO3/NH4}$), ^{15}N was applied to only one FC (NO_3^- or NH_4^+) or the other (*right*). Mean values ± standard deviation are shown. Mean values labeled by the same letter are not significantly different (Tukey's multiple comparison, $p<0.05$ (*left*) and *t* test, $p<0.05$ (*right*))

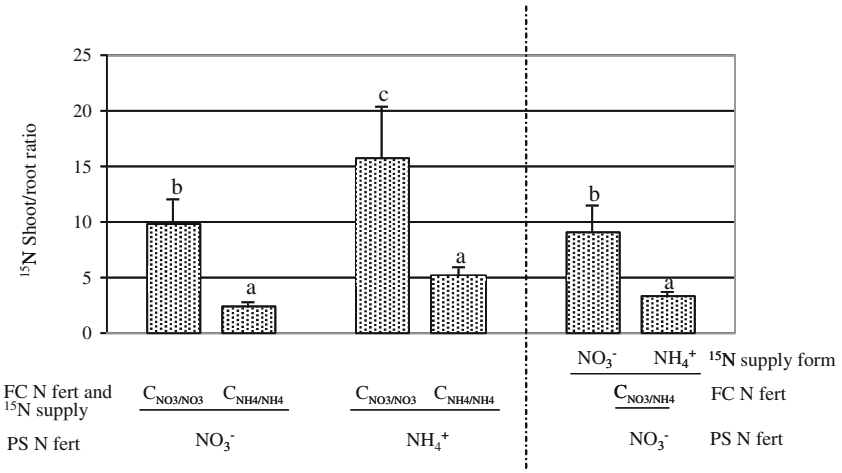

Fig. 4 The ^{15}N shoot/root ratio in AM cowpea plants at harvest. Two weeks before harvest, labeled N (^{15}N) was supplied to the ERM in the fungal compartment (FC) in the respective form (NO_3^- or NH_4^+). When both FCs in a pot were fertilized with the same N-form ($C_{NO3/NO3}$ or $C_{NH4/NH4}$), ^{15}N was applied to both (*left*). When the N-form was not the same ($C_{NO3/NH4}$), ^{15}N was applied to only one FC (NO_3^- or NH_4^+) or the other (*right*). Mean values ± standard deviation are shown. Mean values followed by the same letter are not significantly different (Tukey's multiple comparison, $p<0.05$ (*left*) and *t* test, $p<0.05$ (*right*))

received further support from other studies. Govindarajulu et al. (2005) and Jin et al. (2005) reported that after feeding ERM with ^{15}N-nitrogen, high levels of ^{15}N-labeled arginine were detected in the ERM which were then translocated to the IRM. They also observed that when ^{13}C-substrates were applied to the ERM, ^{13}C was detected in free fungal amino acids, but not in mycorrhizal roots. This indicates that translocated amino acid is broken down in the IRM and only the N-fraction (NH_4^+) is transferred to the plant while the carbon is recycled into the fungal C pool. Other studies on spatial and temporal expression of N metabolic enzymes (Cruz et al. 2007) and genes (Tian et al. 2010) have provided molecular evidence consistent with the proposed pathway.

Govindarajulu et al. (2005) also postulated that no direct transfer of NO_3^- would occur from an AM fungus to the plant and proposed that all N (from NO_3^- and NH_4^+) is transferred as described above. Although the arginine pathway has been shown to clearly function under specific conditions (Ri T-DNA-trasformed carrot roots colonized by *G. intraradices*), it is still an open question whether this is the predominant pathway in agricultural (whole plant) systems (see Smith and Smith 2011). If this is the case, then it is unlikely that NO_3^--N would be transferred faster than NH_4^+-N. However, a larger transfer of ^{15}N was observed when it was applied in the form of $^{15}NO_3^-$ compared with $^{15}NH_4^+$ in the present study, similar to that previously reported by Hawkins and George (2001). In addition to this, if arginine is the universal transport form of N in AM fungal hyphae (irrespective of NO_3^- or NH_4^+ supply to ERM), similar ^{15}N shoot/root ratios would be expected in plants where ERM was supplied with $^{15}NO_3^-$ and $^{15}NH_4^+$, but this was also not the case in the present study where higher ^{15}N shoot/root ratios (indicating higher root-to-shoot translocation) were found in the cowpea plants with $^{15}NO_3^-$ supply to ERM treatments.

Many previous studies have shown that AM fungal ERM can absorb both $^{15}NO_3^-$ and $^{15}NH_4^+$. Because of the extra energy cost involved in reducing NO_3^- prior to assimilation into amino acids, AM systems may more readily assimilate NH_4^+ (e.g., Johansen et al. 1996; Hawkins et al. 2000). The results obtained here with intact hyphae connected to a living plant support this view, in that ERM had higher ^{15}N enrichment when the FC was supplied with $^{15}NH_4^+$ than when supplied with $^{15}NO_3^-$. However, this enrichment (accumulation) of ^{15}N in the hyphae of *G. intraradices* exposed to a local ^{15}N supply is the result of a balance between ^{15}N absorption and export to other parts of the mycelium or the plant so that high ^{15}N accumulation in hyphae (after $^{15}NH_4^+$ supply) may indicate either high absorption or low export. Thus, larger absorption (assimilation) of $^{15}NH_4^+$ over $^{15}NO_3^-$ does not necessarily imply greater transfer to other parts of the ERM, to the IRM or to the plant. Considering the potentially high N demand of the AM fungus (Hodge and Fitter 2010; Hodge et al. 2010), rapid assimilation of inorganic N by AM fungi may in part be simply to satisfy the internal fungal demand, for example, during spore formation. Jin et al. (2005) found high levels of ^{15}N-labeled arginine in mature spores after feeding the ERM with ^{15}N-nitrogen. It may also be a strategy to prevent the accumulation of toxic amounts of NH_4^+ in the ERM (Chalot et al. 2006) and may not always be directly linked to N transfer to the plant. In the

current study, the higher transfer of $^{15}NO_3^-$ may suggest the existence of an additional, alternative pathway for NO_3^- transfer.

As mentioned earlier, the ^{15}N shoot/root ratio of cowpea plants supplied with $^{15}NO_3^-$ was much higher than of plants supplied with $^{15}NH_4^+$, indicative of a faster transfer of $^{15}NO_3^-$ (or its metabolic products) from the root to the shoot or higher accumulation of the metabolic products of $^{15}NH_4^+$ in the root and/or intraradical mycelium. These differences in the ^{15}N shoot/root ratio suggest that the mode of transport and transfer may not always be the same for NO_3^- and NH_4^+ taken up by hyphae and also points to a possible additional, alternative transfer pathway for NO_3^- (or its metabolic products). The present results suggest that NO_3^- may either have been assimilated by hyphae in larger amounts than NH_4^+ (perhaps due to NH_4^+ adsorption in the FC soil substrate) or, more likely, that the metabolic products from NO_3^- are transported faster in the ERM than the metabolic products from NH_4^+. A possible explanation of the present results is also that NO_3^- is transferred directly to the plant after uptake by the ERM. Hildebrandt et al. (2002) found that the transcript levels of a plant-specific NO_3^- transporter increased in AM roots, which may imply an increased NO_3^- acquisition in these roots. From their findings, these authors speculated that AM fungi transfer excess NO_3^- directly to the host plant.

Another factor that may have contributed to the difference in the amount of N transferred from NO_3^- vs. NH_4^+ was the availability of the applied N, as mentioned above. In many soilless media, the availability and mobility of applied N-forms (NO_3^- and NH_4^+) are not distinctly different, whereas NH_4^+ is less mobile compared with NO_3^- in most soils. This is particularly true for clay-rich soils, such as the one used in this study; for example, Jensen et al. (1989) observed that clay minerals can adsorb considerable amounts of NH_4^+. This phenomenon could have led to differences in the proportions of applied N available for uptake in the soil-based substrate used in the present study and thus have contributed to the observed differences in the amount of ^{15}N transferred by the fungus to the plant. It should, however, have no major influence on the ^{15}N shoot/root ratio.

The non-AM plants in the present experiment had only minimal levels of ^{15}N in their tissues (concentration and total content), and these were not influenced by the form of ^{15}N (NO_3^--N vs. NH_4^+-N) applied to the FC. This suggests that there was no significant leakage of N into the soil outside of the compartment and that the transferred ^{15}N from the compartments to the AM plants was mainly through the ERM. Although N transfer in surface water on hyphae cannot be completely excluded (as suggested for small amounts of, for example, thiophenes by Barto et al. 2011), the high level of ^{15}N detected in ERM samples (after washing the ERM from the substrate) is an indication that the main transfer route was within the ERM.

It can thus be concluded from the present data, together with those from earlier studies, that (a) at equal concentrations in the supply solution, AM fungal hyphae may take up more N from NH_4^+ than from NO_3^- ("preference" for NH_4^+), (b) at equal application rates to soil of both mineral N-forms, more NO_3^- than NH_4^+ is available for take up by AM hyphae due to soil absorption of NH_4^+, and (c) after uptake of NO_3^-, some of the NO_3^- (or a specific metabolic product of NO_3^-) may be directly translocated via the ERM to the root and then to the shoot. More experimental evidence, including experiments with other AM fungal isolates under realistic supply conditions close to those in soil, is required to test the general validity of these conclusions.

It is clear that AM fungal hyphae can transport nutrients such as P and N, but in an ecological perspective, it is also important to consider that P or N supply affect mycorrhizal colonization and the formation of the ERM. It is assumed that plants regulate the extent of AM fungal root colonization depending on their P-nutritional status (Smith and Read 2008). However, there appear to be also distinct effects of NH_4^+ and NO_3^- in this respect. Although the poor P status in the present study (seen from the deficient P-nutritional status of non-AM cowpea plants) resulted in a high degree of AM fungal root colonization in all AM plants, the two-way ANOVA results indicate an apparent negative effect of NH_4^+ supply in the pot substrate on root colonization rate. Such negative effects of increasing levels of NH_4^+ on AM fungal root colonization, as compared with a NO_3^- supply, have been previously reported by Azcon et al. (1992), Valentine and Kleinert (2006), and Ngwene et al. (2010). Proposed explanations for this effect include a reduced rhizosphere pH following plant NH_4^+ assimilation (Habte 1999; Rohyadi et al. 2004), reduced carbohydrate allocation to fungal development due to competition between root development, NH_4^+ assimilation in the root, and fungal growth (Raven and Smith 1976), or improved plant P-status that may result from NH_4^+ nutrition (Johnson et al. 1984). In the present study, a higher shoot P concentration was observed in NH_4^+-fed cowpea plants, indicating a better P-status compared with NO_3^--fed plants. It could therefore be that the better P-status of NH_4^+-fed plants contributed to this slight reduction in root colonization rate. The contribution of pH changes to this effect cannot be confirmed since AM colonization levels in root samples from compartments (RHC) in the pots supplied with NO_3^- did not show any significant difference between NO_3^--fertilized and NH_4^+-fertilized compartments. If substantiated in further studies, this would mean that decreased AM colonization of NH_4^+-fed roots is not principally linked to local changes in rhizosphere pH but to overall changes in root physiology.

A considerable amount of ERM was harvested from the FCs of nitrate-fed plants. Hyphal length (5–11 $m\,cm^{-3}$ substrate) was similar to values observed by Neumann et al.

(2009) in a comparable substrate (3.5–8.5 mcm^{-3} substrate) and was also within the range obtained by Drew et al. (2006) in a sand substrate. Spore density was up to five times higher than values reported for pot cultures using a 1:1 sand/vermiculite substrate (up to 200 spores cm^{-3} substrate) (Silva et al. 2005) and ten times the values reported from field samples (up to 100 spores cm^{-3} substrate) (Oehl et al. 2005), but within the range reported by Neumann et al. (2009). In the present study, ERM growth in the FC was clearly decreased by the supply of NH_4^+ to the pot substrate. While this could also be a result from the improved P-status in the cowpea plants supplied with NH_4^+, as described above, below-ground carbon allocation may also be a contributing factor. Since NH_4^+ is generally assimilated in the roots (Marschner 1995), there is a higher demand for carbon in NH_4^+-fed roots leading to reduced carbon allocation for fungal growth. However, this effect probably did not play a major role in the present study because there was no significant difference in the shoot/root ratio between cowpea plants supplied with NH_4^+ and plants supplied with NO_3^-.

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