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Determination of the nitrogen source for arbuscular mycorrhizal fungi by ^{15}N application to soil and plants

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Abstract The source of nitrogen in the spores of arbuscular mycorrhizal (AM) fungi was quantified by a ^{15}N-labeling technique. N was applied as coated urea to the soil and in solution to plant shoots. Soil-applied fertilizer had a significant effect on spore % ^{15}N ($P<0.01$), with a 24–75% contribution to spore N. Fertilizer applied to either alfalfa shoots or bahia grass shoots had little effect on spore % ^{15}N, accounting for 0–14% or 1–9% of spore N, respectively. These results indicate that AM fungi obtain spore N mostly from the soil. The small amount of spore N originating from shoot-applied N may have been obtained via root exudation.

Keywords Arbuscular mycorrhizal fungi · Nitrogen source · ^{15}N · Soil mineral N

Introduction

Arbuscular mycorrhizal (AM) fungi develop hyphal networks which can transfer materials over relatively large distances in the soil (Read 1992). ^{15}N-labeling techniques have great potential for the study of N cycling in the soil, where multiple N transformations occur (Hart and Hyrold 1996). ^{15}N labeling has also demonstrated N transfer from soil to plant (Ames et al. 1983) and between plants (van Kessel et al. 1985) via AM fungi.

Mineral N is the form of N thought to be available to AM fungi, and enzyme activities involved in mineral N assimilation have been detected in these fungi (Ho and Trappe 1975; Smith et al. 1985). There is no evidence that AM fungi are involved in mineralization of organic N (Smith and Read 1997).

While many studies have focused on the effects of nutrient transfer from AM fungi on host plant growth (Sanders et al. 1995; Smith and Read 1997), the effects of the soil or plants on AM fungal nutrition have received less attention. Johansen et al. (1992) found that *Glomus intraradices* hyphae depleted mineral N from the soil and varied in ^{15}N enrichment with distance from the labeling compartment. Hamel et al. (1991), however, found that *Glomus versiforme* hyphae showed little ^{15}N enrichment compared with the roots of ^{15}N-labeled soybean plants.

Spore formation by AM fungi is important for survival and dispersal. To determine the origin of the N within AM fungal spores, we conducted experiments with ^{14}N- and ^{15}N-labeled urea applied to soil in a coated form and to plant shoots in solution form. Plants were inoculated with *Gigaspora margarita* and newly formed spores were analyzed for ^{15}N.

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Materials and methods

Pot preparation and culture

Each rectangular PVC pot (188×162×150 mm) was divided into 13 compartments by screens of stainless-steel mesh (26 μm): a C_3 plant compartment (PC_3), a C_4 plant compartment (PC_4), and 11 central AM hyphal compartments (HC1–11) (Fig. 1). The fungal hyphae could pass freely through the screens (Nakano et al. 1999), but the plant roots were restricted to their respective plant compartments. The pots were filled with 3.7 l of a mixture of equal volumes of subsoil and sand. The subsoil (ca. 5 m depth) was collected from the Nagoya University Farm (Red-Yellow soil, Typic Hapludult, Soil Survey Staff 1998) and had a pH of 4.9, total C of 0.67%, total N of 0.05%, and <1 mg kg $^{-1}$ of available P (Truog 1930). The sand was washed river sand. Both subsoil and sand were air-dried and sieved through a 2-mm screen before mix-

Table 1 ^{14}N and ^{15}N treatments of plants and soil (*HC6* central hyphal compartment, *PC_3* C_3 plant compartment, *PC_4* C_4 plant compartment)

Treatment	Application site			Number of pots
	Alfalfa shoot (PC_3)	Soil (HC6)	Bahia grass shoot (PC_4)	
1	^{14}N	^{14}N	^{14}N	2
2	^{14}N	^{15}N	^{14}N	3
3	^{14}N		^{14}N	2
4	^{14}N		^{15}N	3
5	^{15}N		^{15}N	2

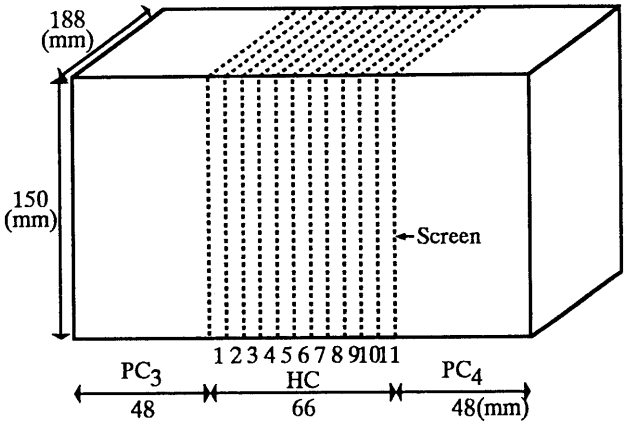

Fig. 1 PVC rectangular pot divided into 13 compartments by 26-µm mesh screens (*HC* hyphal compartment, *PC_3* C_3 plant compartment, *PC_4* C_4 plant compartment)

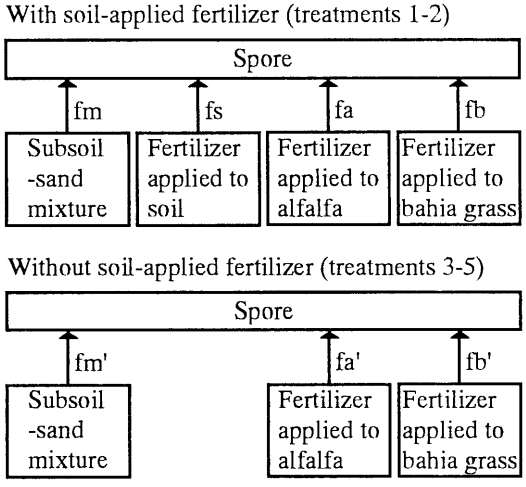

Fig. 2 Contribution of fertilizer to spore N of *Gigaspora margarita* in treatments with (1,2) and without (3–5) soil-applied fertilizer. For abbreviations, see Materials and methods

ing. The mixture was autoclaved for 1 h at 120°C and amended with 1.2 l of soil suspension (soil:water 1:100, sieved through a 38-µm screen) and 4.87 g of lime 10 days before pot preparation. The lime application increased the pH to 6.0–6.5. Fertilizer (0.38 g of 0:1:3 N:P:K) was applied to the respective plant compartments. Five N treatments were prepared (Table 1). A total of 95 mg N was applied to the respective compartments. N was applied to shoots by brushing on a 0.45% urea solution daily starting 10 days after establishing the pots. N was applied to the central hyphal compartment (HC6) as 237 mg of coated urea (40% N) (Meister-10, Chisso Corp., Japan) when the pots were established. The coated urea slowly released N into the soil over 100 days. Atom % ^{15}N ($AP^{15}N$) values of the soil (subsoil-sand mixture), ^{14}N-urea, and ^{15}N-urea were 0.369, 0.366, and 3.07, respectively.

A total of 1000 spores of *Gigaspora margarita* Becker & Hall (MAFF520054, National Grassland Research Institute, Japan) was inoculated into each pot, equally distributed among all compartments halfway between the top and bottom. Surface-sterilized (15 min in 0.5% NaClO) seeds of the C_3 species alfalfa (*Medicago sativa L.*) and the C_4 species bahia grass (*Paspalum notatum Fluegge*) were planted in the PC_3 and the PC_4 compartments, respectively. Replication of the treatments was as shown in Table 1. Pots were placed in a glasshouse for 114 days from May to September 1999. Water was given daily to moisten the soil in all compartments equally, taking care to minimize N loss via leaching and N flow among the compartments.

Sample preparation and analysis

After the given culture period, water was withheld from the plants. A preliminary experiment showed that more spores of *G. margarita* are found after soil drying and plant wilting than after well-watered culture (data not shown). Each compartment of each pot was sampled separately.

Newly formed white spores of *G. margarita* (Nakano et al. 1999) were separated from each soil sample by wet sieving and from debris under a microscope. The spores were cleaned at least 10 times with tap water and then three times with deionized water before analysis. About 30 spores were placed into a tin capsule (5 mm diameter×8 mm), which was then closed.

Each plant sample was divided into shoot and root. A part of the root sample was kept for analysis of AM colonization (Kormanik and McGraw 1982). Shoot and root samples were separately cleaned with running water, dried for 48 h at 70°C, and pulverized in a vibrating sample mill (TI-100, Heiko Seisakusho, Ltd., Japan). Approximately 0.5 and 1.5 mg aliquots of the pulverized samples of alfalfa and bahia grass, respectively, were put into tin capsules, which were then closed.

Mineral N in each soil sample was extracted with 2 M KCl, diffused by addition of Devarda alloy and MgO (Keeney and Nelson 1982), and trapped on a glass filter with $KHSO_4$ (Jensen 1991). Each filter was put into a tin capsule, which was then closed.

$AP^{15}N$ values were determined using an isotope ratio mass spectrometer ($Delta^{plus}$, Finnigan MAT GmbH, Bremen, Germany) coupled with an elemental analyzer (NC 2500, ThermoQuest Italia S.p.A., Milan, Italy) by an interface (ConFlo II, Finnigan MAT GmbH, Bremen, Germany). The $AP^{15}N$ data, comparing between ^{14}N-labeled reference and ^{15}N-labeled treatment, were arcsine-square-root-transformed for statistical analysis.

Spore $AP^{15}N$ values for the five treatments (S1–S5) were determined as above. For each treatment, spore $AP^{15}N$ values are given by the following equations (as illustrated in Fig. 2):

$$S1=fm\times M+fs\times F(14)+fa\times F(14)+fb\times F(14) \quad (1)$$

$$S2=fm\times M+fs\times F(15)+fa\times F(14)+fb\times F(14) \quad (2)$$

$$fm+fs+fa+fb=1 \quad (3)$$

$$S3=fm'\times M+fa'\times F(14)+fb'\times F(14) \quad (4)$$

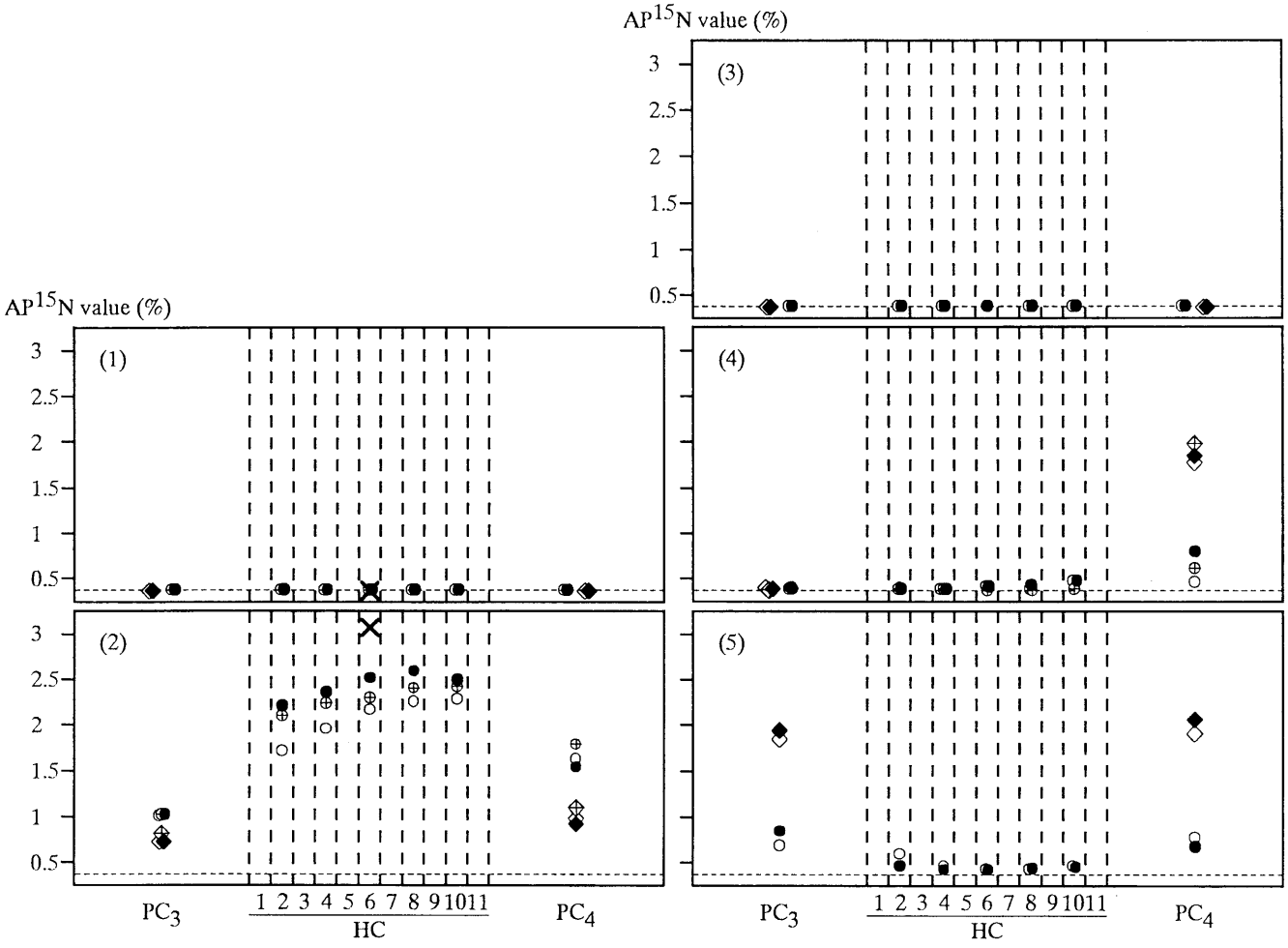

Fig. 3 Atom % ^{15}N ($AP^{15}N$) of *Gigaspora margarita* spores in different compartments of pots given ^{14}N- or ^{15}N-urea to soil in a hyphal compartment and plant shoots in plant compartments. Treatment numbers are given in parentheses. See Table 1 for sites of N application and number of replicate pots per treatment (--- $AP^{15}N$ of soil, × soil-applied urea, ♦, ⧫, ◊ plant roots, ●, ⊕, ○ spores)

$$S4=fm'\times M+fa'\times F(14)+fb'\times F(15) \quad (5)$$

$$S5=fm'\times M+fa'\times F(15)+fb'\times F(15) \quad (6)$$

$$fm'+fa'+fb'=1 \quad (7)$$

where fm, fs, fa, and fb are fractional contributions of the soil (subsoil-sand mixture) and the fertilizers applied to the soil, alfalfa shoots, and bahia grass shoots, respectively, in treatments 1 and 2; fm', fa', and fb' are fractional contributions of the soil and the fertilizers applied to alfalfa shoots and bahia grass shoots, respectively, in treatments 3–5; S*n* is spore $AP^{15}N$ value in treatment *n*, and M, F(14), and F(15) are the $AP^{15}N$ values of the soil, ^{14}N-urea, and ^{15}N-urea, respectively. Contributions of plant seeds and *G. margarita* spores introduced into the pot were not taken into account in this study.

The values for fs, fa', and fb' are calculated from equations 1 and 2, 5 and 6, and 4 and 5, respectively, as follows:

$$fs=(S2-S1)/(F(15)-F(14)) \quad (8)$$

$$fa'=(S5-S4)/(F(15)-F(14) \quad (9)$$

$$fb'=(S4-S3)/(F(15)-F(14) \quad (10)$$

Because fs, fa', and fb' are determined from differences, the value of M is canceled. Equations 8, 9, and 10 are in accordance with an equation used to calculate the contribution of fertilizer to soil or plant by a ^{15}N-dilution technique (Hauck and Bremner 1976; Marison 1987). Each compartment was considered a replicate. The mean for each compartment was calculated in the 2–3 pots of each treatment.

AM colonization of root systems was determined using the gridline-intersect method after staining with trypan blue (Giovannetti and Mosse 1980) according to the following equation:

$$\text{Colonization (\%)}=(\text{intersections with colonized root})\div(\text{total intersetions with root})\times 100 \quad (11)$$

The AM colonization data were arcsine-square-root-transformed for statistical analysis. Dry weight of colonized roots was calculated by multiplying percentage of root colonization by root dry weight (Rajapakse and Miller 1994).

Results and discussion

$AP^{15}N$ values of *G. margarita* spores in different compartments of pots given ^{14}N- or ^{15}N-urea are shown in Figure 3. In treatment 1, spore $AP^{15}N$ values were in the range 0.368–0.372 and were thus similar to those of the soil, ^{14}N-urea, alfalfa roots, and bahia grass roots. In the central labeling compartment (HC6), $AP^{15}N$ of soil mineral N (0.369±0.001) was similar to those of the soil, ^{14}N-urea,

Table 2 Atom % ^{15}N ($AP^{15}N$) values of plant shoots and roots, soil mineral N, and *Gigaspora margarita* spores in plant compartments of pots given ^{14}N- or ^{15}N-urea to soil in a hyphal compartment and plant shoots in plant compartments. Standard deviations are shown in parentheses. For abbreviations, see Table 1

Treatment	Number of replicates	Alfalfa (PC_3)				Bahia grass (PC_4)			
		Shoot	Root	Mineral N	Spores	Shoot	Root	Mineral N	Spores
1	2	0.370 (0.000)	0.368 (0.001)	0.367 (0.001)	0.368 (0.000)	0.372 (0.003)	0.371 (0.002)	0.368 (0.001)	0.368 (0.000)
2	3	0.585 (0.016)	0.764 (0.053)	0.479 (0.031)	1.01 0.01	0.888 (0.058)	1.01 (0.10)	0.455 (0.026)	1.64 (0.13)
3	2	0.372 (0.001)	0.370 (0.000)	0.380 (0.016)	0.369 (0.001)	0.377 (0.001)	0.374 (0.001)	0.375 (0.012)	0.370 (0.001)
4	3	0.408 (0.018)	0.398 (0.013)	0.381 (0.004)	0.390 (0.003)	1.94 (0.08)	1.87 (0.11)	0.523 (0.081)	0.620 (0.161)
5	2	2.38 (0.06)	1.90 (0.06)	0.643 (0.030)	0.761 (0.103)	2.04 (0.12)	1.98 (0.11)	0.885 (0.180)	0.717 (0.074)

Table 3 Correlation coefficients among $AP^{15}N$ values of plant shoots and roots, soil mineral N, and *Gigaspora margarita* spores in plant compartments of pots for all treatments (1–5), and for treatments with (1–2) and without (3–5) soil-applied fertilizer (see Table 1) (***, **, and * significant correlation at $P<0.001$, $P<0.01$, and $P<0.05$, respectively)

Treatment	Alfalfa (PC_3)				Bahia grass (PC_4)			
	Shoot × Root	Root × Mineral N	Root × Spores	Mineral N × Spores	Shoot × Root	Root × Mineral N	Root × Spores	Mineral N × Spores
1–5	0.98***	0.97***	0.54	0.64*	1.00***	0.73**	0.12	0.03
1–2	0.99**	0.98**	0.99**	0.94*	1.00***	0.92*	1.00***	0.93*
3–5	1.00***	0.99***	0.98***	0.95**	1.00***	0.65	0.81*	0.65

and spores (0.371±0.002) were similar. In treatment 2, spore $AP^{15}N$ ranged from 1.00 to 2.57 and was thus higher than for the soil, alfalfa roots, and bahia grass roots, but lower than for ^{15}N-urea. Spore $AP^{15}N$ increased with proximity to the ^{15}N-urea. In the central labeling compartment (HC6), $AP^{15}N$ of soil mineral N (2.70±0.12) was lower than that of ^{15}N-urea, but higher than the soil and thus slightly higher than spores (2.31±0.17). In treatment 3, spore $AP^{15}N$ ranged from 0.368 to 0.370 and was thus similar to the soil, alfalfa roots, and bahia grass roots. In treatment 4, spore $AP^{15}N$ increased slightly from alfalfa roots to bahia grass roots in the range 0.379–0.789. In treatment 5, spore $AP^{15}N$ increased with proximity to alfalfa roots or bahia grass roots in the range 0.419–0.833.

Table 2 shows $AP^{15}N$ values of plant shoots and roots, soil mineral N, and *G. margarita* spores in the plant compartments. In all but treatment 2, root $AP^{15}N$ was slightly lower than for shoots. In treatment 2, root $AP^{15}N$ was higher than for shoots ($P<0.01$ and $P=0.14$ for alfalfa and bahia grass, respectively). In treatment 1, $AP^{15}N$ of soil mineral N was similar to roots and spores. In treatment 2, $AP^{15}N$ of soil mineral N was lower than roots ($P<0.01$) and spores ($P<0.01$). In treatments 3 and 4, $AP^{15}N$ of soil mineral N in the compartments of ^{14}N-labeled plants was similar to roots and spores. In treatments 4 and 5, $AP^{15}N$ of soil mineral N in the compartments of ^{15}N-labeled plants was lower than roots ($P<0.001$) and similar to spores.

For all treatments, there was significant correlations between $AP^{15}N$ of shoots and roots in both the PC_3 and PC_4 compartments (Table 3). For treatments with soil-applied fertilizer, there was significant correlations among $AP^{15}N$ of roots, soil mineral N, and spores in both the PC_3 and PC_4 compartments. For treatments without soil-applied fertilizer, there was significant correlations among $AP^{15}N$ of roots, soil mineral N, and spores in the PC_3 compartment, but less so in the PC_4 compartment.

In all compartments, no differences in spore density of *G. margarita* were found among treatments (Fig. 4). There were more spores in the HC10 than in all other compartments (Fisher's PLSD, $P<0.01$).

For all treatments, dry weights of total, shoot, root, and colonized root of bahia grass were higher than those of alfalfa ($P<0.001$), while colonization of bahia grass was lower than that of alfalfa ($P<0.001$) (Table 4). For all tested characteristics of both alfalfa and bahia grass, no significant differences were found among the five treatments, but shoot dry weight of bahia grass was higher in treatments with soil-applied fertilizer ($P<0.05$).

Plant $AP^{15}N$ values change with N_2 fixation (Marison 1987; Handley and Raven 1992). However, in this study, the possible contribution of atmospheric N_2 to spore N was not taken into account because no nodules were found on alfalfa roots.

Comparing between treatments 1 and 2, the soil-applied fertilizer had significant effects on spore $AP^{15}N$ in all compartments (ANOVA, $P<0.01$). The contribution of the soil-applied fertilizer to spore N was in the range 24–75% (mean 60±19%). This agrees with the findings on AM hyphae by Johansen et al. (1992, 1993a, 1994). In pot

Table 4 Dry weight of shoots, roots, and colonized roots and colonization of plants in pots given ^{14}N- or ^{15}N-urea to soil in a hyphal compartment and plant shoots in plant compartments. For treatments, see Table 1

Treatment	Alfalfa					Bahia grass				
	Total (g)	Shoot (g)	Root (g)	Colonized root (g)	Colonization (%)	Total (g)	Shoot (g)	Root (g)	Colonized root (g)	Colonization (%)
1	1.9	1.1	0.8	0.18	23	8.6	6.0	2.6	0.47	18
2	1.6	1.0	0.6	0.13	23	10.3	6.6	3.6	0.61	17
3	1.5	0.9	0.6	0.13	22	7.2	4.8	2.4	0.36	15
4	2.0	1.0	0.9	0.23	27	8.9	5.7	3.1	0.52	18
5	1.9	1.2	0.7	0.24	36	8.7	5.8	2.9	0.44	15

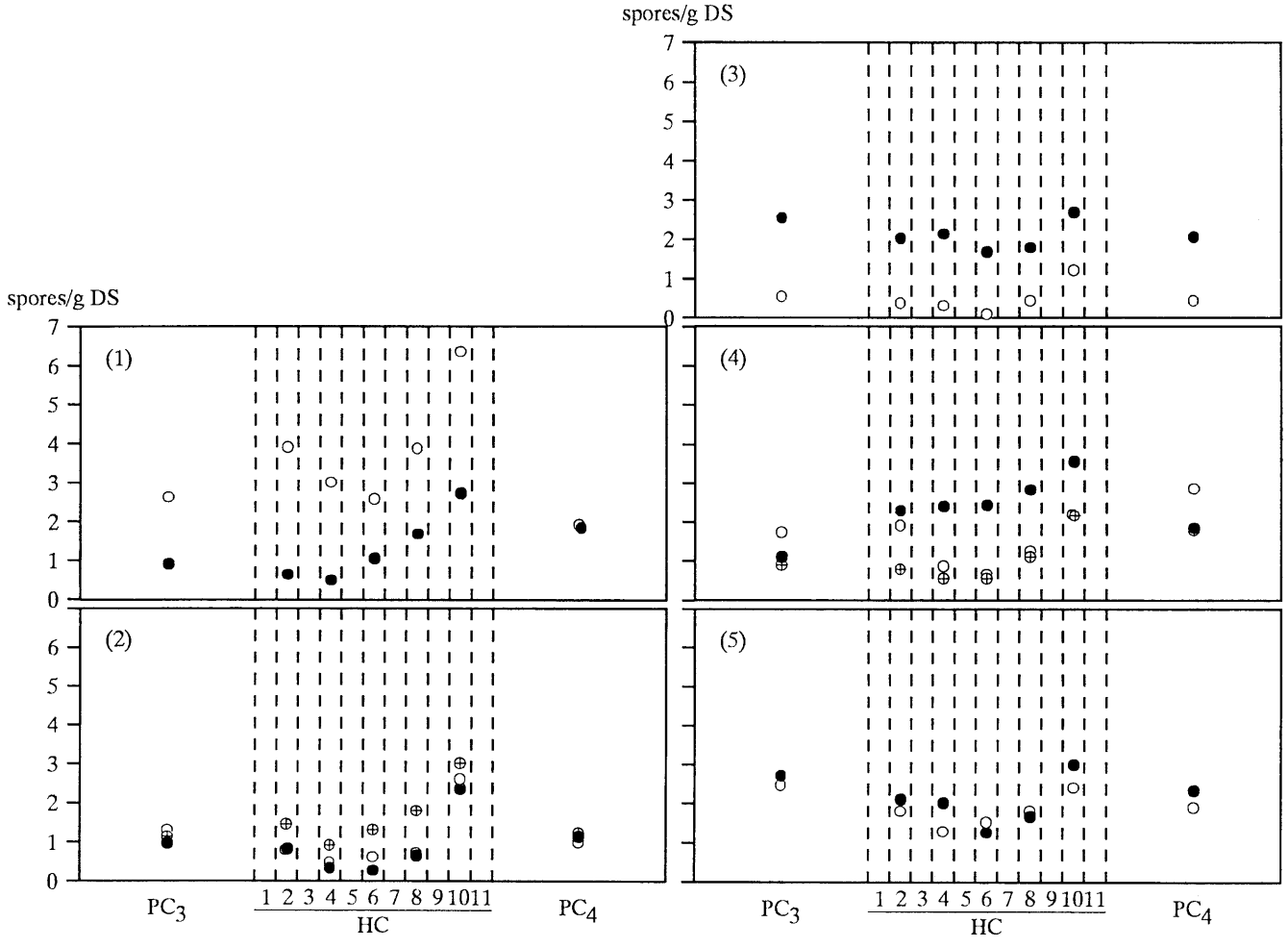

Fig. 4 Density of *Gigaspora margarita* spores per g dry soil (DS) in different compartments of pots given ^{14}N- or ^{15}N-urea to soil in a hyphal compartment and plant shoots in plant compartments. Treatment numbers are given in parentheses. See Table 1 for sites of N application and number of replicate pots per treatment (●, ⊕, ○ spore density)

experiments in which $^{15}NH_4^+$ was given to a hyphal compartment separated from the root compartment by a mesh, ^{15}N enrichment of *Glomus intraradices* hyphae in the hyphal compartment was 3–8 times higher than that of the root and higher when there was more ammonium than nitrate (Johansen et al. 1992). The enrichment was similar to that of the root adjacent to the hyphal compartment and tended to decrease with increasing supply of N to the root compartment (Johansen et al. 1994). The enrichment decreased with increasing distance from both the ^{15}N source and the plant (Johansen et al. 1993a).

Although the soil-applied fertilizer had a significant effect on $AP^{15}N$ of soil mineral N in the plant compartments ($P<0.05$), the contribution to soil mineral N was small according to calculations similar to those applied to spores (4 and 3% of soil mineral N for the PC_3 and the PC_4 compartments, respectively). On the other hand, considerable increases in spore $AP^{15}N$ were detected in

the plant compartments. These results suggest that *G. margarita* hyphae took up N from soil containing the highest concentrations and transferred it to hyphae in soil at lower N concentrations, where there was some mass flow or diffusion of soil N from the central compartment to the plant compartments, or some leakage of N from hyphae to soil. In pot experiments similar to those of Johansen et al. (1992, 1993a, 1994) described above, Frey and Schüepp (1993) and Johansen et al. (1993b) monitored ^{15}N enrichment of soil N in the hyphal compartment. Frey and Schüepp (1993) detected significant depletion of soil total ^{15}N in the undisturbed mycorrhizal treatment compared with the hyphae-disturbed mycorrhizal and the non-mycorrhizal treatments. This suggests that ^{15}N was taken up by mycorrhizal plants with access to the hyphal compartment. As a form of N potentially available to AM fungi, soil mineral N may be correlated more with N taken up and transferred by hyphae than with soil total N. Johansen et al. (1993b) suggested that hyphae depleted soil mineral N because the concentration of soil mineral N was lower in the mycorrhizal than in the non-mycorrhizal treatments. They also suggested that N transport could occur by mass flow and diffusion because of the high ^{15}N enrichment of soil mineral N.

Comparing between treatments 4 and 5 or treatments 3 and 4, the fertilizer applied to either the alfalfa shoots or the bahia grass shoots had no significant effect on spore $AP^{15}N$, except in the PC_3 ($P<0.01$) and the HC4 compartments ($P<0.05$). This variability may have been due to variation of spore $AP^{15}N$ in treatments 3, 4, and 5. The contributions to spore N of the fertilizer applied to the alfalfa shoots and the bahia grass shoots were in the range 0–14% (mean 4±5%) and 1–9% (mean 2±3%), respectively, and were thus lower than that of the soil-applied fertilizer. This agrees with the findings on AM hyphae by Hamel et al. (1991). Using ^{15}N-labeled soybean and unlabeled maize, they found that ^{15}N enrichment of *Glomus versiforme* hyphae was much lower than that of the soybean root and increased with increasing soil total ^{15}N.

For the treatments without soil-applied fertilizer, spore $AP^{15}N$ was similar to soil mineral N in the plant compartments (Table 2). Because of the high contribution of the soil-applied fertilizer to spore N described above, it seems reasonable to suggest that *G. margarita* took up N exuded from roots to soil. $AP^{15}N$ of soil mineral N in the plant compartments was affected significantly by the fertilizer applied to the alfalfa shoots ($P<0.001$ for the PC_3 compartment), but the effect was less significant when the fertilizer was applied to the bahia grass shoots ($P=0.09$ for the PC_4 compartment). The correlations among $AP^{15}N$ of roots, soil mineral N, and spores were high in the PC_3 compartment, but lower in the PC_4 compartment (Table 3). This may have been due to difference in root exudation between alfalfa and bahia grass.

N transfer from AM fungi to plants is known to vary with the plant–fungus combination (Frey and Schüepp 1993; Johansen 1999), distance from the N source (Johansen et al. 1992, 1993a, b), N quantity (Johansen et al. 1993b, 1994; Hawkins and George 1999; Mäder et al. 2000), N quality (Ames et al. 1983; Frey and Schüepp 1993), soil water content (Tobar et al. 1994), and soil P concentration (Barea et al. 1989; Azcón-Aguilar et al. 1993). In this study, the contribution of the soil-applied fertilizer to spore N was higher in the PC_4 than in the PC_3 compartment. On the other hand, the contributions to soil mineral N were similar in the two compartments. This suggests that *G. margarita* made more hyphal links from the labeling compartment to bahia grass than to alfalfa, especially considering the high dry weight of bahia grass root colonized (Table 4) and the large contribution of bahia grass to spore C (97±18%) in the labeling compartment as estimated from $\delta^{13}C$ data according to the equations of Nakano et al. (1999). Because of the increased $AP^{15}N$ of both shoots and roots (Tables 2 and 3) in treatments with soil-applied fertilizer, it seems reasonable to suppose that the hyphae transferred some N from the central compartment to plants. This may have produced the high shoot dry weight of bahia grass (Table 4).

In conclusion, this study indicates that AM fungi obtain spore N mostly from the soil, although they may also obtain spore N from plants, possibly through exudation from roots.

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