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The carbon origin of arbuscular mycorrhizal fungi estimated from $\delta^{13}C$ values of individual spores

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Abstract The origin of carbon in the spores of arbuscular mycorrhizal (AM) fungi was quantified based on their obligate symbiosis with C_3 and C_4 plants showing clearly different $\delta^{13}C$ values. The $\delta^{13}C$ values of individual spores of the AM fungus *Gigaspora margarita* were analyzed. In monoculture pots of a C_3 or a C_4 plant species, spore $\delta^{13}C$ values were ca. 3.5‰ lower than those of host roots. In coculture pots of a C_3 and a C_4 plant species, spore $\delta^{13}C$ values varied between those of the roots of C_3 and C_4 plants, and increased linearly from the C_3 to the proximity of the C_4 plant ($P<0.01$). This reflects the higher $\delta^{13}C$ values in C_4 plants than in C_3 plants. Thus the carbon origin of *G. margarita* spores changed with growth state and combination of host plants. In the presence of fresh plant residue instead of living host plants, spore $\delta^{13}C$ values did not vary with distance from the residue. This finding supports the current view that AM fungi are obligate symbionts.

Key words Arbuscular mycorrhizal fungi · Carbon origin · $\delta^{13}C$ · C_3 plant · C_4 plant

Introduction

Arbuscular mycorrhizal (AM) fungi are known to develop hyphal networks among plants and to transfer materials from one plant to another via hyphae. Such linkages between plants were demonstrated by direct observations (Heap and Newman 1980) or by radioactive isotope tracer experiments (Chiariello et al. 1982; Francis and Read 1984; van Kessel et al. 1985). AM-colonized plants are autotrophic and can grow without AM colonization provided nutrient supplies are adequate. In contrast, AM fungi have little or no saprophytic ability and cannot form propagules without colonization of plants. Thus AM fungi are considered to be ecologically obligate symbionts dependent on host plants for carbon (Smith and Read 1997).

Although tracer experiments demonstrate material transfer via AM hyphae, the pulse-labeling techniques are generally too transitory to label uniformly all carbon pools of either plant or AM fungus, and the continuous labeling experiments can not be extrapolated to natural ecosystems (Lynch and Whipps 1990; Watkins et al. 1996). The natural abundance of stable isotopes, expressed as an δ value, has great potential for the study of nutrient cycling in ecosystems. Based on the fact that C_3 and C_4 plants have different $\delta^{13}C$ values owing to their different photosynthetic mechanisms (Edwards and Walker 1983), Rochette and Flanagan (1997) monitored the seasonal variation in CO_2 flux and $\delta^{13}C$ values in a corn (C_4 plant) field which had a history of C_3 plant cultivation. They estimated that the rhizosphere respiration was equivalent to 18–25% of crop net photosynthesis.

Although $\delta^{13}C$ analysis has been utilized rarely in studies of AM fungi, it has been applied recently to quantify materials transferred via AM networks. Watkins et al. (1996) used the difference in $\delta^{13}C$ value between root and shoot to estimate carbon transfer between *Plantago lanceolata* (C_3 plant) and *Cynodon dactylon* (C_4 plant). They calculated that 0–41% of *C. dactylon* root carbon was derived from *P. lanceolata*.

While many studies on AM symbiosis have focused on the effects of nutrient transfer via AM hyphae on host plant growth (Sanders et al. 1995; Smith and Read 1997), the effects of plants on AM fungi have been neglected. Studies on AM fungal ecology are essential for a better understanding of the AM symbiosis. Spore formation of AM fungi is important for survival and dispersal. Under a mixed vegetation of C_3 and C_4 plants,

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Allen and Allen (1990) concluded that the AM fungal spores contained carbon originating from both plant types because the $\delta^{13}C$ values of the spores lay between those of the C_3 and C_4 plants.

Our objective was to determine the origin of the carbon within AM fungal spores. We conducted experiments with monocultures of either C_3 or C_4 plants, cocultures of C_3 and C_4 plants, taking into consideration the distance from both plants and monocultures of C_3 or C_4 plants with fresh plant residue. Plants were selected from pasture fields, where many C_3 and C_4 plant species occur and where the low level of soil disturbance increases the potential of material transfer via AM networks. *Gigaspora margarita* spores, with the relatively large diameter of 260–480 μm (Becker and Hall 1976), were inoculated and subsequently analyzed individually for $\delta^{13}C$.

Materials and methods

Determination of $\delta^{13}C$ for individual *G. margarita* spores

Onion (*Allium cepa* L. cv. Sensyuchukouki) pot cultures of the AM fungus *G. margarita* Becker & Hall were obtained from the National Grassland Research Institute, MAFF920054, Japan. Spores were collected using the sucrose density gradient centrifugation method (Sano and Saito 1996). A preliminary experiment showed that sucrose used for spore collection does not influence spore $\delta^{13}C$ values (data not shown). To determine whether it is possible to assay individual spores, samples of one, 50, 100, and 200 spores were placed into tin capsules (5 mm diameter × 8 mm) and the capsules were then closed. In the capsule containing 1-spore sample 2.5 μl of 100 g l^{-1} L alanine solution (100 μg C) was added to obtain the minimal carbon content required for carbon analysis. $\delta^{13}C$ value and carbon mass were determined using an automated nitrogen and carbon analyzer – mass spectrometer (ANCA–MS, Europa Scientific Ltd., UK). Its precision at natural abundance was 0.1‰ $\delta^{13}C$ values for >100 μg C (Barrie et al. 1995). $\delta^{13}C$ value for 1-spore samples was calculated from:

$$\text{Total (‰)} \times \text{total (μg C)} = \text{L alanine (‰)} \times \text{L alanine (μg C)} + \text{spore (‰)} \times \text{spore (μg C)} \quad (1)$$

The mass of spore carbon was calculated from:

$$\text{Spore (μg C)} = \text{total (μg C)} - \text{L alanine (μg C)} \quad (2)$$

Experiment 1: $\delta^{13}C$ of *G. margarita* spores formed in monocultures of a C_3 or a C_4 plant species

Plants of one species were grown in a polystyrene cylindrical pot (113 mm diam. × 140 mm) filled with 1.2 l of a mixture of equal volumes subsoil and sand. The subsoil was collected from the Nagoya University Farm (Red-Yellow soil, Typic Hapludult) and had a pH of 4.9 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 4-mm screen before mixing. The mixture was autoclaved for 1 h at 120 °C and amended with 0.74 g of 14:8:13 ($N:P_2O_5:K_2O$) fertilizer and 3.60 g of lime. The amount of each nutrient in the pot corresponded to less than half of conventional application levels at the farm. Lime application raised the pH to 6.5–7.0.

Three hundred spores of *G. margarita*, prepared as described above, were inoculated into the middle of the pot. The $\delta^{13}C$ value of the spores was –31.1 ± 2.0‰.

The C_3 species red clover (*Trifolium pratense*), alfalfa (*Medicago sativa*), and perennial ryegrass (*Lolium perenne*), and the C_4 species sorghum (*Sorghum bicolor*), guinea grass (*Panicum maximum*), and bahia grass (*Paspalum notatum*) were chosen for this experiment. The number of replicate pots for each species is shown in Table 1. Cultivation was carried out in a glasshouse for 3 months from July to October 1995.

Experiment 2: $\delta^{13}C$ of *G. margarita* spores formed in cocultures of a C_3 and a C_4 plant species

Each PVC square pot (188 × 126 × 150 mm) used for cultivation was separated into seven compartments divided by screens of stainless steel mesh (106 μm): the C_3 plant compartment (P_3C), the C_4 plant compartment (P_4C), and five central AM hyphae compartments (HC1–5) (Fig. 1). The fungal hyphae passed freely through the screens. Most plant roots were restricted to their respective plant compartments by the screens but a few fine roots were found in all hyphae compartments; the species could not be distinguished. The pots were filled with 3 l of the same subsoil-sand mixture used in experiment 1 after sieving through a 2-mm screen. The mixture was autoclaved for 1 h at 120 °C and amended with 2.78 g of the fertilizer described above and 3.93 g of lime. One litre of soil suspension (soil : water 1 : 100, sieved through a 38-μm screen) was added 10 days before pot preparation.

Six hundred spores of *G. margarita*, prepared as described above, were inoculated into the middle of the central compartment (HC3). The $\delta^{13}C$ value of the spores was –31.3 ± 1.3‰.

The C_3 species alfalfa and perennial ryegrass, and the C_4 species bahia grass were selected from the plants used in experiment 1, taking into consideration spore productivity. Two kinds of pots were prepared: alfalfa (C_3) versus bahia grass (C_4) (pots A and B) and perennial ryegrass (C_3) versus bahia grass (C_4) (pot C) (Table 3). Cultivation was carried out in a glasshouse for 3 months from April to July 1996.

Experiment 3: $\delta^{13}C$ of *G. margarita* spores formed in monocultures with plant residue

In this experiment either the C_3 or the C_4 plant was replaced with fresh plant residue. Each pot was prepared as described for experiment 2, except that in either the P_3C or the P_4C compartment 0.9% of pulverized plant residue was incorporated into the subsoil-sand mixture before autoclaving. No plant seeds were sown in those compartments. The plant residue was from mature plants. The screens dividing the plant compartments from hyphae compartments were of finer mesh (38 μm) than in experiment 2 to prevent passage of roots. Two kinds of pots were prepared:

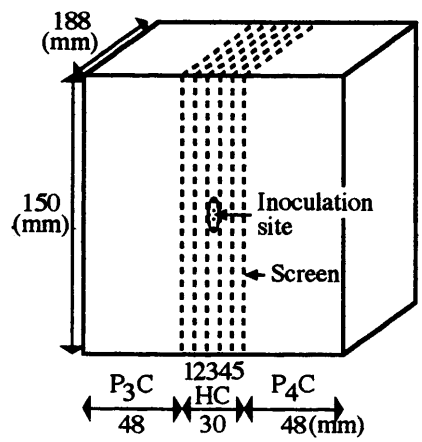

Fig. 1 PVC square pot. Each pot was divided into 7 compartments by 106-μm (experiment 2) or 38-μm (experiment 3) mesh screens (*P_3C* C_3 plant compartment, *P_4C* C_4 plant compartment, *HC* hyphal compartment)

Table 1 $\delta^{13}C$ values (‰) of plant roots and *Gigaspora margarita* spores in monoculture pots (*n* number of spores assayed individually)

Plant species		Pot	Root		Spore		
			Pot value	Treatment average ± SD	Pot average ± SD	(n)	Treatment average ± SD
C_3	Red clover	1	−28.9	−29.7 ± 0.6	−31.5 ± 2.6	(20)	−33.0 ± 1.5
		2	−29.3		−34.2 ± 2.2	(20)	
		3	−29.4		−31.6 ± 2.8	(20)	
		4	−30.1		−35.2 ± 4.1	(20)	
		5	−30.2		−32.6 ± 2.4	(17)	
		6	−30.5		−32.7 ± 1.9	(17)	
	Alfalfa	7	−28.8	−30.5 ± 1.0	−34.4 ± 1.8	(19)	−35.7 ± 1.2
		8	−29.8		−35.4 ± 2.1	(19)	
		9	−30.6		−36.4 ± 2.6	(14)	
		10	−31.2		−34.4 ± 1.9	(22)	
		11	−31.4		−37.4 ± 2.0	(20)	
		12	−31.5		−36.1 ± 1.8	(19)	
	Perennial ryegrass	13	−30.6	−30.6	−33.7 ± 2.8	(4)	−33.7
C_4	Sorghum	14	−12.2	−12.6 ± 0.3	−17.1 ± 1.8	(9)	−16.2 ± 1.3
		15	−12.6		−17.4 ± 2.4	(8)	
		16	−12.7		−15.8 ± 1.5	(11)	
		17	−13.0		−14.6 ± 2.3	(9)	
	Guinea grass	18	−13.3	−13.6 ± 0.3	−17.0 ± 3.0	(4)	−16.3 ± 0.6
		19	−13.8		−15.8 ± 2.2	(20)	
		20	−13.8		−16.2 ± 2.8	(19)	
	Bahia grass	21	−12.1	−12.8 ± 0.6	−15.9 ± 3.2	(26)	−15.9 ± 0.8
		22	−12.5		−17.2 ± 1.2	(21)	
		23	−12.8		−15.6 ± 2.8	(20)	
		24	−13.3		−15.5 ± 0.7	(20)	
		25	−13.4		−15.1 ± 0.8	(20)	

alfalfa (C_3) plant versus bahia grass (C_4) residue (–12.3 ± 0.2‰) (pots D1 and D2) and a mixture of alfalfa (C_3) and perennial ryegrass (C_3) residue (–30.5 ± 0.4‰) versus bahia grass (C_4) plant (pots E1 and E2) (Table 3). Cultivation was carried out in a glasshouse for 6 months from May to November 1997.

Sample preparation and analysis

After the respective culture periods, water was withheld from the plants. The soil and plant samples were then taken from each cylindrical pot (experiment 1) or from each compartment (experiments 2 and 3).

Newly formed, white spores of *G. margarita* were collected from each soil sample using a sucrose density gradient. Individual spores were placed into tin capsules containing added L alanine solution as described above.

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, Japan). About 1 mg of the pulverized sample was put into a tin capsule, which was then closed.

Analysis of $\delta^{13}C$ values and carbon masses was carried out by ANCA-MS and $\delta^{13}C$ values of spore samples calculated using the equation above.

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:

Colonization (%) = (intersections with colonized root) ÷ (total intersections with root) × 100 (3)

Dry weight of colonized root was calculated by multiplying the percentage of root colonization by the dry weight of root (Rajapakse and Miller 1994). Spore density was expressed as spore number per g of total plant dry weight.

Results and discussion

Determination of $\delta^{13}C$ for individual *G. margarita* spores

For the strict determination of carbon origin in AM fungal spores formed under a mixture of plants, information on individual spores is indispensable. $\delta^{13}C$ values of individual spores of *G. margarita* (–31.1 ± 2.0‰, n = 5), analyzed after the addition of L alanine (100 μg C) to ensure the minimal carbon content for ANCA-MS, were not significantly different from those of 50, 100, and 200 spore batches (–32.9 ± 0.2‰, n = 3). Thus the method can be used for the determination of $\delta^{13}C$ values of individual *G. margarita* spores.

Experiment 1: $\delta^{13}C$ of *G. margarita* spores formed in monocultures of a C_3 or a C_4 plant species

$\delta^{13}C$ values of roots of C_3 and C_4 plants were clearly different, averaging –30.3 ± 0.5‰ for C_3 plants and –13.0 ± 0.5‰ for C_4 plants (Table 1). No differences were found between $\delta^{13}C$ values of roots and shoots except for red clover (–29.7 ± 0.6‰ for roots and –30.6 ± 0.7‰ for shoots, $P < 0.05$). This is in accordance with the low variation reported for plant organs, except storage organs (O'Leary 1981).

Table 2 Height, dry weight (dwt.), colonization of host plants and spore density of *G. margarita* in monoculture pots

Plant species	Height (cm)	Total dwt. (g)	Shoot dwt. (g)	Root dwt. (g)	Colonized root dwt. (g)	Colonization (%)	Spore density (per g total dwt.)
C_3 Alfalfa	23.1	2.09	0.89	1.21	0.54	49	500
Red clover	13.7	1.22	0.73	0.49	0.32	66	460
Perennial ryegrass	17.0	1.67	0.82	0.85	0.31	36	27
C_4 Sorghum	93.1	21.0	14.8	6.21	3.7	56	1.7
Guinea grass	133	29.3	19.4	9.84	4.8	47	17
Bahia grass	27.0	4.95	2.71	2.24	0.73	33	84

$\delta^{13}C$ values of individual spores of *G. margarita* associated with C_3 and C_4 plants were -34.1 ± 1.4‰ and -16.1 ± 0.3‰, respectively, and thus 2.7–5.2‰ (mean 3.5 ± 0.9‰) lower than those of host roots (Table 1). The differences between the $\delta^{13}C$ values of spores and host roots did not vary significantly among the plants tested.

The lower spore values were probably due to lipid accumulation. The lipid fraction has lower $\delta^{13}C$ values than the whole organism and other major constituents, such as proteins and carbohydrates, due to isotopic fractionation in the lipid synthetic pathway (DeNiro and Epstein 1977). Oil droplets are frequently observed in AM fungi, especially in spores, and the lipid content is ca. 45% spore dry weight (Smith and Gianinazzi-Pearson 1988).

Table 2 shows height, dry weight, colonization of host plants and spore density of *G. margarita* in monoculture pots. C_4 plant growth was higher than C_3 the colonized root weights were higher but spore densities lower than in pots with C_3 plants. Total plant weight, colonized root weight and spore density were not correlated for any of the tested plants.

Experiment 2: $\delta^{13}C$ of *G. margarita* spores formed in cocultures of a C_3 and a C_4 plant species

Figure 2 shows frequency distributions of $\delta^{13}C$ values for *G. margarita* spores in different compartments of

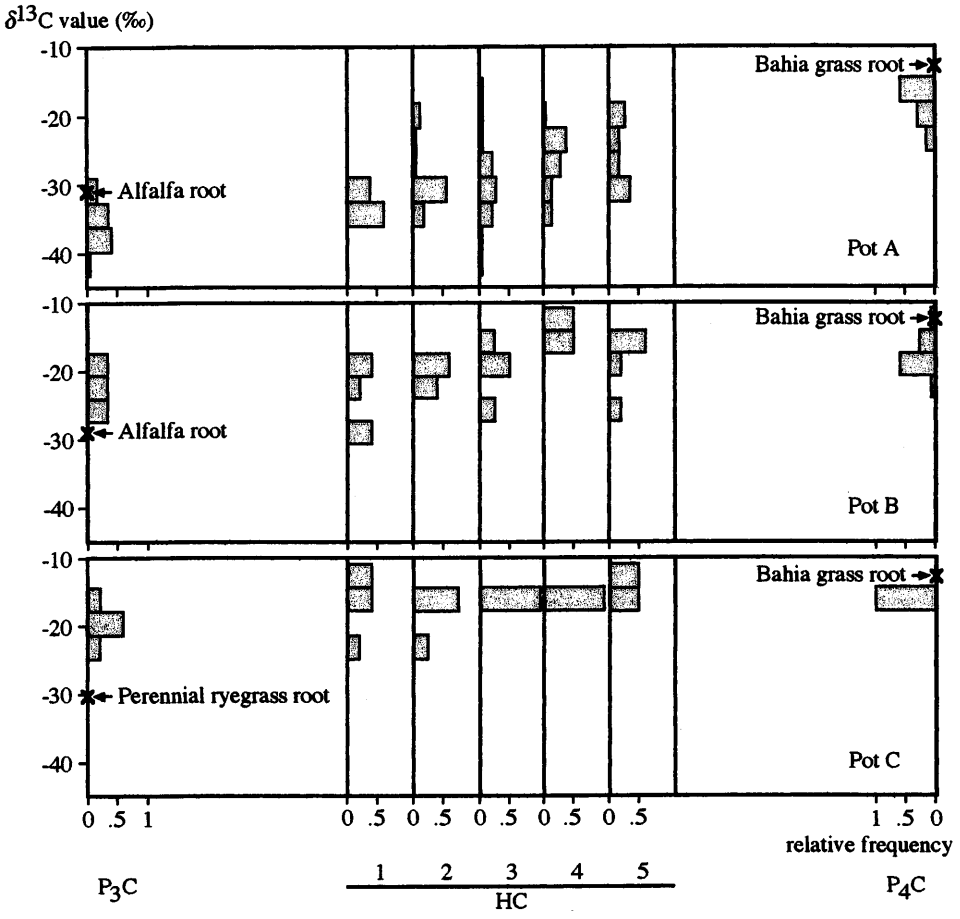

Fig. 2 Frequency distribution of $\delta^{13}C$ values for *Gigaspora margarita* spores in different compartments of coculture pots (experiment 2). $\delta^{13}C$ values of plant roots (x) are shown together

Table 3 $\delta^{13}C$ values of *G. margarita* spores

Treatment	C_3 species	C_4 species	Pot	Spores assayed	mean $\delta^{13}C$ value (‰)	SD (‰)	Coefficient of variation	Regression slope (‰/mm)	Correlation coefficient	*P*
Experiment 2: coculture										
	Alfalfa	Bahia grass	A	110	−28.8	6.4	22.3	0.22	0.76	<0.001
	Alfalfa	Bahia grass	B	42	−20.0	3.8	19.0	0.07	0.48	0.001
	Perennial ryegrass	Bahia grass	C	28	−16.7	2.8	16.6	0.06	0.49	0.008
Experiment 3: monoculture with plant residue										
	Alfalfa	Bahia grass residue	D 1	32	−16.8	1.0	6.2	0.02	0.20	0.32
			D 2	42	−16.6	1.5	9.3	0.01	0.04	0.83
	Perennial ryegrass & alfalfa residue	Bahia grass	E 1	28	−31.5	2.1	6.5	0.00	0.07	0.68
			E 2	28	−29.0	3.4	11.6	−0.02	0.23	0.14

Table 4 Dry weight of host plants, colonization, and estimated contribution to spore carbon

Pot	Plant species	Total dwt. (g)	Shoot dwt. (g)	Root dwt. (g)	Colonized root dwt. (g)	Colonization (%)	Contribution to spore carbon (%)
A	C_3 Alfalfa	1.8	1.0	0.7	0.5	65	107
	C_4 Bahia grass	11.8	8.3	3.5	0.8	22	88
B	C_3 Alfalfa	0.7	0.5	0.2	<0.1	27	41
	C_4 Bahia grass	10.7	7.4	3.3	0.7	20	86
C	C_3 Perennial ryegrass	4.8	3.0	1.8	0.9	49	20
	C_4 Bahia grass	16.7	11.8	4.9	1.5	30	105

the coculture pots. The $\delta^{13}C$ values of individual spores varied between those of the roots of C_3 and C_4 plants. Spore $\delta^{13}C$ values increased linearly from the P_3C to the P_4C compartment in all three pots (Fig. 2, Table 3). This suggests that *G. margarita* obtained carbon for spore formation from host plants in inverse proportion to the distance from the plants.

Assuming that the difference (3.5‰) in $\delta^{13}C$ values of spores and host roots was the same as in experiment 1, contribution of each plant to spore carbon was estimated by:

$$\text{Contribution of } C_3 \text{ plant to spore carbon (\%)} = \{[\delta^{13}C\ (C_4\ \text{plant})–3.5]–\delta^{13}C\ (\text{spore})\} \div \{[\delta^{3}C(C_4\ \text{plant})–3.5]–[\delta^{13}C(C_3\ \text{plant})–3.5]\} \times 100 \quad (4)$$

$$\text{Contribution of } C_4 \text{ plant to spore carbon (\%)} = 100–[\text{contribution of } C_3 \text{ plant to spore carbon (\%)}] \quad (5)$$

Table 4 shows dry weight of host plants, colonization, and estimated contribution to spore carbon. In bahia grass compartments (P_4C) of pots A and B, the dry weights of both total plant and colonized root and the contribution of bahia grass to spore carbon were similar. On the other hand, in alfalfa compartment (P_3C) of pot B, dry weights of both total plant and colonized root and the contribution of alfalfa to spore carbon were less than half of that in pot A. In bahia grass compartment (P_4C) of pot C, dry weights of both total plant and colonized root were higher than in pots A and B, and all spore carbon was calculated to be derived from bahia grass. On the other hand, in perennial ryegrass compartment (P_3C), although dry weights of both total plant and colonized root were higher than those of alfalfa in pots A and B, the contribution of perennial ryegrass to spore carbon was less than that of alfalfa in pots A and B.

Thus the contributions of host plants to spore carbon of *G. margarita* changed with growth state (pot A versus pot B) and combination of host plants (pots A and B versus pot C). Colonization by an AM fungus, however, may not be related simply to contribution to spore carbon. It seems reasonable to suppose that perennial ryegrass and bahia grass had more colonized root than alfalfa due to their much larger root systems.

Although variables such as spore density, hyphal length and weight, and colonization have been considered to affect plant growth (Tommerup 1994), they are known to fluctuate with the fungal species, plant-fungus combination, and environmental conditions. Thus, these characteristics of an AM fungus may not be correlated with plant response (Smith and Read 1997). AM fungi probably form spores according to the growth state or the identity of host plant. Our results suggest this variability may be caused by variation in carbon transfer.

Watkins et al. (1996) indicated that $\delta^{13}C$ values of plants change when carbon transfer occurs between C_3 and C_4 plants via an AM network, and that the differences in the $\delta^{13}C$ values between shoots and roots could be used to estimate the percentage of carbon transfer. However, it was not evident whether transferred carbon remained in the fungal tissue or moved

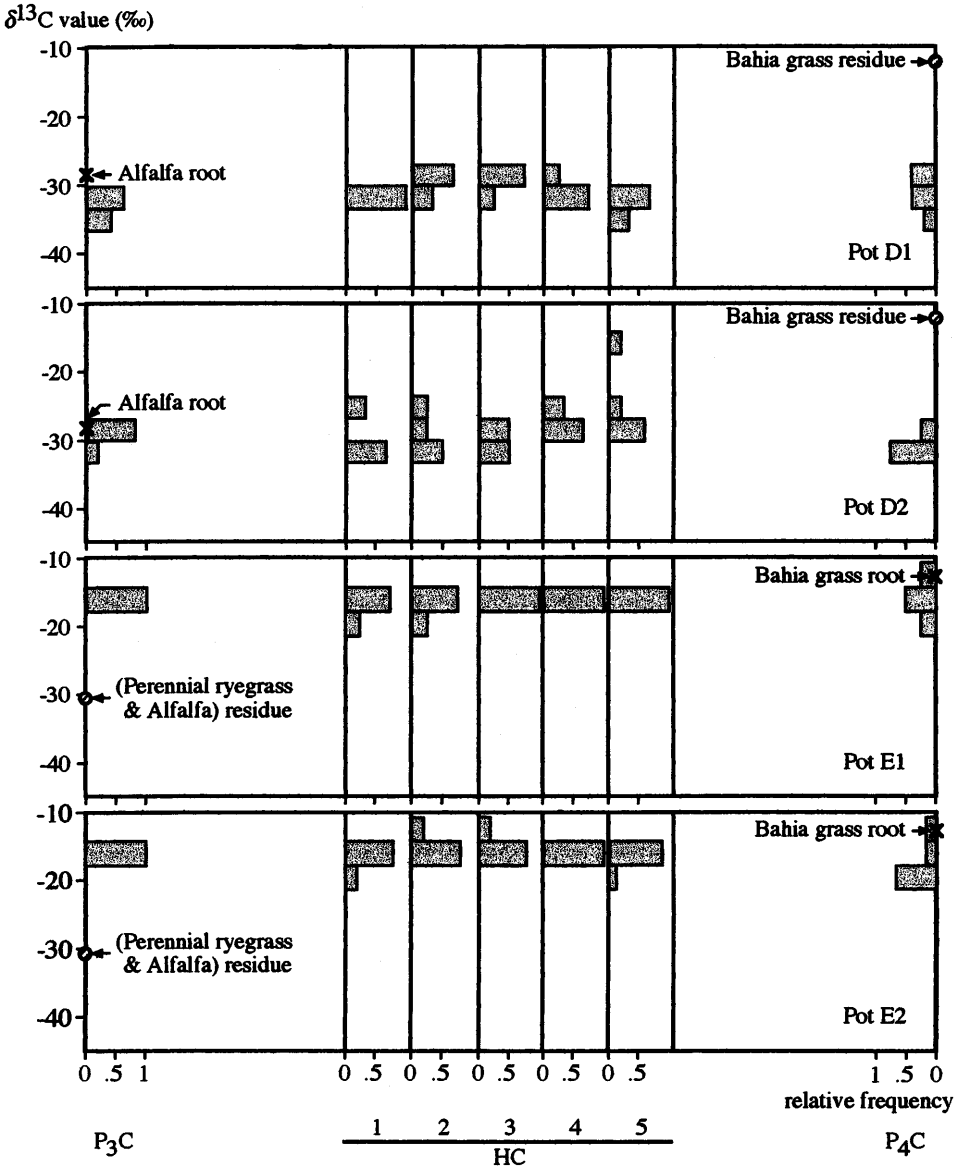

Fig. 3 Frequency distribution of $\delta^{13}C$ values for *G. margarita* spores in different compartments of monoculture pots with plant residue (experiment 3). $\delta^{13}C$ values of plant roots (*x*) and the plant residues (*o*) are shown together

into the plant tissue. In our study, the root–shoot differences were 0.1–1.2‰ (mean 0.6 ± 0.4‰) for all tested plants in monocultures (experiment 1). The differences were greater for alfalfa in cocultures (experiment 2) and the increases in $\delta^{13}C$ values of roots over shoots were 1.3‰ in pot A and 1.9‰ in pot B. On the other hand, the differences for bahia grass (0.2 ± 0.1‰) and perennial ryegrass (0.5‰) were negligible. These results may suggest that carbon transfer in the AM network occurred in pot B from bahia grass to alfalfa, which showed less growth and a lower contribution to spore carbon.

Experiment 3: $\delta^{13}C$ of *G. margarita* spores formed in monocultures with plant residue

Figure 3 shows the frequency distributions of $\delta^{13}C$ values for *G. margarita* spores in different compartments of monoculture pots with plant residue. The $\delta^{13}C$ values of spores were in the range –25.3 to –33.3‰ (alfalfa plant: –28.3 ± 0.2‰) in pots D1 and D2, and –15.0 to –17.9‰ (bahia grass plant: –12.6 ± 0.0‰) in pots E1 and E2. All coefficients of variation were lower than those in the coculture experiment (Table 3). Regression analysis showed that the $\delta^{13}C$ values of spores did not vary with distance from the residue compartment. This finding supports the current understanding that AM fungi are obligate symbionts (Pfeffer et al. 1998; Smith and Read 1997).

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