RESEARCH ARTICLE

Plants as resource islands and storage units – adopting the mycocentric view of arbuscular mycorrhizal networks

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Abstract

The majority of herbaceous plants are connected by arbuscular mycorrhizal (AM) fungi in complex networks, but how this affects carbon (C) and phosphorus (P) allocation among symbionts is poorly understood. We utilized a monoxenic AM system where hyphae from donor roots colonized two younger receiver roots of varying C status. AM fungal C allocation from donor to receiver compartments was followed by measuring the ^{13}C contents in fungal- and plant-specific lipids, and P movement from a hyphal compartment was traced using ^{33}P. Four times more ^{13}C was translocated from donor to C-limited receiver roots, but C remained in fungal tissue. Root C status did not influence the overall AM colonization, but arbuscule density was twice as high in non-C-limited roots, and they received 10 times more ^{33}P. The number of hyphal connections between compartments did not influence C and P allocation. Interestingly, there were more fungal storage lipids, but fewer structural lipids inside C-limited roots. Our results indicate that AM colonization may poorly reflect host quality as C can be supplied from neighboring roots. A mycocentric view of the symbiosis is proposed where C-delivering hosts are resource islands for the exchange of P for C, and C-limited hosts are storage units.

Introduction

The great majority of land plants form a symbiosis with arbuscular mycorrhizal (AM) fungi in the phylum *Glomeromycota* (Schüßler *et al.*, 2001). Controlled inoculation experiments with single plants have shown that AM fungi can acquire and transfer the majority of phosphorus (P) required by a plant (Smith *et al.*, 2003) in return for up to 20% of the assimilated carbon (C) (Jakobsen & Rosendahl, 1990). However, plants are not isolated from each other in nature, but are linked by AM hyphae in so-called common mycorrhizal networks (CMN). Molecular studies have shown that mycelia of individual clones can cover an area up to 10 m in diameter (Rosendahl & Stukenbrock, 2004). Combined with the apparent low host preference observed in some systems (Öpik *et al.*, 2003; Stukenbrock & Rosendahl, 2005; Santos *et al.*, 2006; but see Vandenkoornhuyse *et al.*, 2003) and the ability of clones to anastomose (Giovannetti *et al.*, 1999; Mikkelsen *et al.*, 2008), individual fungi can form extensive functional networks that connect plants of the same and different species (Giovannetti *et al.*, 2004). Indeed, *in situ* studies have shown that P can transfer between plants up to 0.5 m apart that share little or no taxonomic affinity, and be facilitated by the hyphal connectivity among plants (Chiariello *et al.*, 1982; Walter *et al.*, 1996). Likewise, source–sink relationships may shift an estimated 10% of C in roots into neighboring plants via the CMN (Francis & Read, 1984; Robinson & Fitter, 1999; Lerat *et al.*, 2002).

The fate of transferred C has been debated intensively. Suggestions have been made that C may be translocated from fungus to plant, which could potentially benefit seedling establishment (Lerat *et al.*, 2002), improve the success of invasive plants (Carey *et al.*, 2004), and be essential for epiparasitic plants (Bidartondo *et al.*, 2002). Clearly, if this happens in nature, it could fundamentally change our understanding of how plants interact within communities. This phytocentric view has been challenged by studies indicating that transferred C remains in fungal tissue (Fitter *et al.*, 1998; Pfeffer *et al.*, 2004; Voets *et al.*, 2008).

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Based on this, Robinson & Fitter (1999) proposed a more mycocentric view of the AM where the fungus allocates C within the CMN to optimize its own fitness, with little consequences for plants.

What drives C and P translocations within a single plant–fungus mycorrhiza is better known. For example, the overall P uptake and delivery by AM fungi may depend on the plant C availability (Tester *et al.*, 1985, 1986), and acquired P may accumulate as polyphosphate in hyphal vacuoles in C-limited plants (Gianinazzipearson & Gianinazzi, 1978; Bücking & Shachar-Hill, 2005). Likewise, plants may allocate C to a localized area around P-delivering arbuscules in order to limit the success of potential parasitic fungi (Fitter, 2006). Whether or not the same dynamics regulate the C and P allocation among organisms within the CMN is uncertain, and an improved understanding of the mechanisms involved would allow us to better estimate the functional consequences of CMN for both symbionts. The overall objective of this study, therefore, was to research how the host's ability to deliver C affects C and P allocation among symbionts in a CMN. This is of interest because the ability of plants to deliver C to AM fungi can be greatly reduced by temporary shading and herbivory (Francis & Read, 1984; Nakano *et al.*, 2001), resulting in lower fungal colonization, decreased growth, and P uptake (Tester *et al.*, 1985).

One fundamental problem with network studies is that connections among plants can never be directly observed in soil; they have to be assumed. To circumvent this problem, we utilized a monoxenic AM system with transformed carrot (*Daucus carota*) roots (Bécard & Fortin, 1988), in which roots are colonized by the AM fungus *Glomus intraradices* (or *Glomus irregulare* as recently argued by Stockinger *et al.*, 2009). In the absence of a shoot, C is provided to the root through sucrose additions to the solid medium, and because the fungus is an obligate biotroph, it cannot access this sugar, relying instead on the host root for C delivery (Pfeffer *et al.*, 1999). The absence of photosynthetic tissue in this system may affect the overall symbiotic benefit, and alter hormonal balances and source–sink relationships (Fortin *et al.*, 2002). Furthermore, the sucrose environment of the root–fungus interface differs drastically from that *in vivo*, which could modify the biochemistry of the symbiosis and alter the colonization of vesicles and arbuscules (Fortin *et al.*, 2002). However, in spite of these potential shortcomings, the root-organ cultures have been demonstrated to possess similar C uptake, metabolism and nutrient transfer as whole-plant mycorrhizas (as discussed in Pfeffer *et al.*, 2004), and has significantly increased our understanding of P and C movements between plants and fungi (e.g. Nielsen *et al.*, 2002; Olsson *et al.*, 2002; Bago *et al.*, 2003; Pfeffer *et al.*, 2004; Bücking & Shachar-Hill, 2005).

We utilized a four-compartment Petri-dish system where an air gap allowed hyphae from mycorrhizal roots to cross the barrier and to colonize two newly established roots, one that was C limited and another that was not C limited, and a hyphal compartment (HC) without roots (Fig. 1). C flow from the older 'donor' roots to younger 'receiver' roots was followed by measuring the ^{13}C concentration within plant- and fungal-specific lipids after pulse labeling with ^{13}C-glucose (Olsson *et al.*, 2005), and P delivery from the HC was traced using ^{33}P. We used the concentration of lipids rather than carbohydrates to follow C flows, because the majority of C in AM fungi is in lipids (Bécard *et al.*, 1991). Because hyphae from the donor compartment (DC) colonized all the other compartments, all roots were connected via a CMN. Our experimental setup thus allowed us to make pair-wise comparisons regarding C allocation from donor roots and P transfer from the HC into two receiver roots that differed in C status. Overall, the study was designed to test the following hypotheses: (1) C allocation within the CMN is source–sink driven, and more C will be allocated to the C-limited root; (2) C translocated among roots remain in fungal tissue even when roots are C limited; and (3) cost–benefit relationships drive the P delivery from fungi to roots, and less P will be allocated to the C-limited root.

Materials and methods

Establishment and maintenance of the CMN

Ri-T-DNA-transformed carrot roots (*D. carota* L., line DC1) colonized by the AM fungus *G. intraradices* Schenck & Smith, now *G. irregulare* (DAOM 197198, Biosystematics Research Center, Ottawa, Canada) were maintained at 24 °C

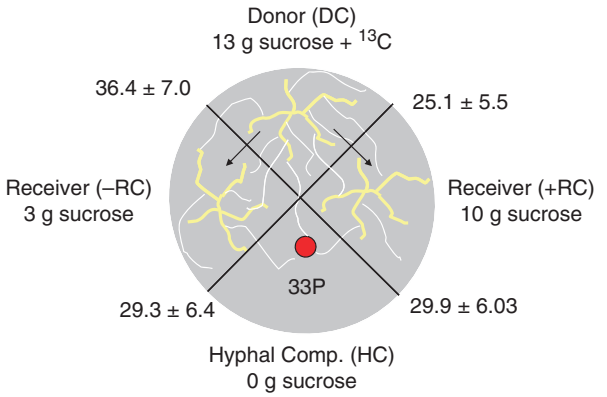

Fig. 1. Experimental outline of the four-compartment Petri dish system with transformed carrot root cultures with the AM fungus *Glomus intraradices*. Thick lines indicate roots and thin lines indicate fungal hyphae. ^{13}C labeling was performed in the DC and ^{33}P labeling in the HC, and transfer was measured to mycorrhizal roots and external mycelia in a receiver compartment containing either 3 g sucrose (−RC) or 10 g sucrose (+RC). Means ± SE of the number of hyphae crossing compartments are indicated on the compartment divider.

on Petri dishes with a minimal nutrient medium containing 0.35% (w/v) phytagel and 10 g L^{-1} of sucrose, and with a pH of 5.5 (Bécard & Fortin, 1988). On January 31, 2008, 2-month-old colonized roots as well as extraradical hyphae and spores were transferred using a sterile 1.5-cm corer to one compartment of a four-compartment Petri dish (Fig. 1) containing 10 mL of minimal medium (hereafter referred to as the DC). Plates were incubated in the dark at 24 °C. After 46 days, when hyphae had crossed into neighboring compartments, 1.5 cm plugs of 2-month-old Ri-T-DNA transformed nonmycorrhizal carrot roots were plated onto neighboring receiver compartments containing either 10 mL of minimal medium with 10 g L^{-1} of sucrose (+RC) or 10 mL of modified minimal medium of only 3 g L^{-1} of sucrose (−RC). We estimated that the reduction of sucrose in the −RC compartment was sufficient to create a C-limited system based on the data presented in Olsson *et al.* (2006). The fourth compartment contained 10 mL of modified minimal medium with no sucrose and no roots (HC). All compartments were separated by plastic barriers that prevented solute transport, but where an air gap between the lid and the barriers allowed hyphae to cross among compartments. Twenty-five plates were prepared to ensure a sufficient number with good hyphal and root development for the labeling. All plates were maintained at 24 °C in the dark and checked weekly to ensure that no roots crossed over the plastic barrier into neighboring compartments. Crossing roots were carefully placed back or burned. At 83 days, 100 μL of a 30% sterile sucrose solution (w/v) was added to DC to ensure that the donor roots did not become C limited. Donor roots continued to grow throughout the experiment, suggesting that they were still active and not severely limited by either C or nutrients.

Isotope labeling

Fourteen Petri dishes showed visible hyphal development in all compartments 98 days after plating. These plates were carefully checked under a dissecting microscope to ensure that no roots had crossed between compartments, and the number of hyphae crossing among compartments was counted as they are easily detected with a dissecting microscope. No separation between living and dead hyphae was possible. ^{13}C labeling was performed by pipetting 100 μL of a sterile-filtered 100 mg D-[^{13}C]glucose mL^{-1} solution (U-$^{13}C_6$ at 99% ^{13}C; Cambridge Isotope Laboratories, Andover, MD) around the original plug near the center of DC. ^{33}P labeling was conducted by pipetting 50 μL water solution, containing 75 KBq of carrier-free $H_3^{33}PO_4$ (Perkin-Elmer, Boston, MA) evenly across HC. Two control plates where hyphae had not crossed from DC were also labeled to verify that hyphae were the only means for isotope transfer between compartments, and two plates were left unlabeled for natural abundance measures of ^{13}C and background levels of ^{33}P. If isotopes were transferred by hyphae alone, the isotope signature of the two receiver compartments in labeled control plates would not differ from those in the unlabeled controls. All plates were checked 3 days after labeling and again at harvest to ensure that no roots had crossed.

Harvest

All plates were harvested 5 days after the labeling event. Roots were carefully picked from −RC and +RC, rinsed in distilled water to remove any remaining solid medium, and checked under a dissecting microscope, where attaching spores and hyphae were carefully picked out and placed back into the respective receiver compartment. No spores or hyphae were found in the labeled control plates where hyphae had not crossed from DC. The external mycelium (containing spores and hyphae) were collected from −RC, +RC, and HC in the remaining plates after dissolving the phytagel in a 10 mM Na-citrate solution. Thus, the roots and internal mycelium (r+im) were analyzed separately from the external mycelium (em) in −RC and +RC, and will hereafter be referred to as −RC(r+im), +RC(r+im), −RC(em), and +RC(em), respectively. No separation of external mycelium and roots was attempted in DC. Fresh weights (FW) of both colonized roots and extraradical mycelium were recorded and part of the sample was ashed at 400 °C before the scintillation counts, and the remaining sample was freeze dried and kept at −20 °C until analyzed for ^{13}C enrichment and fatty acid content and composition. The FW and freeze-dried weights (DW) were used to calculate the water content in the samples and to convert the whole sample FW to DW. Because of the slow processing time, only eight of the 14 labeled plates were randomly chosen and processed for isotope analyses. The remaining six plates were used for the assessment of AM colonization of roots using the gridline intersect method (Giovannetti & Mosse, 1980) after staining with trypan blue (Brundrett *et al.*, 1996), because root material was insufficient for both isotope analyses and AM colonization.

Fatty acid quantification and ^{13}C enrichment

Lipids were extracted from freeze-dried and ground mycelium and colonized roots in a one-phase mixture of citrate-buffer, methanol, and chloroform (0.8 : 2 : 1, v : v : v, pH 4) as described in detail by Olsson *et al.* (2002). The lipids were fractionated into neutral lipids, glycolipids, and phospholipids on silica columns by eluting with chloroform, acetone, and methanol, respectively. The fatty acid residues in neutral lipids and phospholipids were transformed into free fatty acid methyl esters and analyzed by GC using a 50 m HP5 capillary fused silica column (Hewlett Packard, Wilmington,

DE) with H_2 as a carrier gas (Frostegård *et al.*, 1993). The neutral lipid and phospholipid fractions of 16:1ω5 and 18:2ω6,9 were identified from their retention times in relation to that of the internal standard (fatty acid methyl ester 19:0). The neutral lipid fatty acid (NLFA) 16:1ω5 was analyzed as it is a good indication of AM fungal storage lipids, whereas the phospholipid fatty acid (PLFA) 16:1ω5 is an important part of AM fungal membranes and therefore indicates the amount of structural lipids (van Aarle & Olsson, 2003). NLFA 18:2ω6,9 is often dominant in basidiomycetes, ascomycetes, and plants, and because it is almost absent in AM fungi, it can be used as a signature for root lipids in AM-colonized roots when no other types of mycorrhizal colonization occur (Olsson *et al.*, 2005).

In order to determine the C allocation to AM fungi in +RC and −RC, we measured the ^{13}C atom-% in the NLFA 16:1ω5 on the isotope ratio mass spectrometer interphased to a Hewlett Packard gas chromatograph. Furthermore, in order to determine whether, and to what extent, ^{13}C was translocated from fungi to roots in +RC and −RC, we compared ^{13}C enrichment in the plant signature NLFA 18:2ω6,9 of +RC and −RC roots with background values (as discussed in Voets *et al.*, 2008). If no transfer occurs, the values in the +RC and −RC roots would not differ from the background values. The gas chromatograph was equipped with a 50 m HP5 capillary column (Hewlett Packard) with He as a carrier gas. The ^{13}C enrichments were calculated by subtracting the natural ^{13}C abundance of both 16:1ω5 and 18:2ω6,9 (the natural abundance for both lipids was 1.16%) from the measured ^{13}C concentrations in each lipid fraction. The AM fungal excess ^{13}C in the 16:1ω5 was calculated by multiplying the ^{13}C enrichment in the NLFA fraction of 16:1ω5 with the total amount of NLFA 16:1ω5.

^{33}P analyses

The ashed root and mycelial samples were dissolved in 1 mL 1 M HCl and 6 mL Packard Ultima Gold scintillation cocktail, and radioactivity was measured in a Beckman LS 6500 Scintillation Analyzer.

Statistical analyses

The overall C and P allocations to various tissue types and compartments were analyzed using one-way ANOVA (Minitab Inc., Minitab, State College, PA). Paired *t*-tests were used for pair-wise comparisons between +RC(r+im) and −RC(r+im), and between +RC(em) and −RC(em), respectively. The relationships between C and P transfer among compartments and the connectivity (i.e. the number of hyphae connecting two compartments) were investigated through correlation analyses. Data were transformed to fulfill ANOVA assumptions, and when transformations failed, the nonparametric Mann–Whitney and Kruskal–Wallis tests were used, which are equivalent to the *t*-test and one-way ANOVA, respectively.

Results

Biomass and AM colonization

The DW of roots and external mycelia differed drastically among compartments (Table 1). Reducing the sucrose content to 3 g L^{-1} significantly reduced mycorrhizal root biomass in −RC relative to +RC (paired *t*-test: $t = 7.21$, $P < 0.001$), but had no significant effect on the biomass of external mycelium ($t = 1.75$, $P = 0.12$).

The overall AM colonization did not differ significantly between roots in −RC and +RC ($t = 1.10$, $P = 0.32$; Table 2), nor did the vesicule density ($t = 0.09$, $P = 0.93$), but arbuscule density was significantly higher in +RC roots ($t = 3.33$, $P = 0.02$; Table 2). The colonization of donor roots was equivalent to +RC roots (data not shown).

Lipid concentration and content

Receiver roots where no hyphae had crossed from the DC contained no detectable NLFA of PLFA 16:1ω5, indicating that this fatty acid was specific for AM fungi in this system. The PLFA 16:1ω5 concentration, which predominantly measures structural lipids (van Aarle & Olsson, 2003), was significantly higher in +RC(r+im) relative to the −RC(r+im)

Table 1. DW of mycorrhizal roots (r+im) and external mycelium (em) in the DC, receiver compartment supplied with 10 g sucrose L^{-1} (+RC), receiver compartment supplied with 3 g sucrose L^{-1} (−RC), and the HC that contained no roots

Tissue	DW (mg)
DC	23.0 (1.51)
+RC(r+im)	6.65 (1.31)
−RC(r+im)	1.84 (0.42)
+RC(em)	0.99 (0.22)
−RC(em)	1.35 (0.24)
HC	2.94 (0.49)

Dry weight was measured separately for roots (r+im) and external mycelium (em) in +RC and −RC. Mean (± SE), $n = 8$.

Table 2. Percentage AM colonization, vesicles, and arbuscules in roots growing in a medium containing 10 g sucrose L^{-1} (+RC) or 3 g sucrose L^{-1} (−RC)

Treatment	AM (%)	Vesicles (%)	Arbuscles (%)
+RC	46.0 (5.81)[a]	5.21 (1.67)[a]	31.3 (4.91)[a]
−RC	38.9 (3.23)[a]	5.40 (2.84)[a]	16.8 (3.19)[b]

Values with different superscripts differ from each other at $P \leq 0.05$. Mean (SE), $n = 6$.

(Mann–Whitney: $W=76$, $P=0.04$; Table 3), but showed no difference between +RC(em) and −RC(em) ($W=54$, $P=0.16$). The PLFA content in the entire compartment (PLFA concentration × dry mass) showed the same trend and differed significantly between the roots ($W=78$, $P=0.02$), but not between the external mycelium ($W=54$, $P=0.16$). The NLFA 16:1ω5 concentration, which is an indication of fungal storage lipids (van Aarle & Olsson, 2003), showed the opposite trend and was significantly higher in the −RC(r+im) compared with +RC(r+im) ($t=3.31$, $P=0.013$; Table 3), but due to the larger root biomass in +RC, there was no significant difference in the NLFA 16:1ω5 content between +RC(r+im) and −RC(r+im). There were no significant differences in the NLFA 16:1ω5 concentration and content between +RC(em) and −RC(em).

^{13}C allocation to NLFAs 16:1ω5 and 18:2ω6,9

The ^{13}C enrichment in the NLFA 16:1ω5 differed significantly between tissue types and among compartments ($F=26.7$, d.f. = 41, $P<0.001$), and was higher in the external mycelia compared with the roots. Interestingly, it was the highest in mycelia in the HC that contained no roots. Pair-wise comparisons showed that the ^{13}C concentration was significantly higher in both roots ($t=6.93$, $P<0.001$), and external mycelium ($W=91$, $P=0.018$) in −RC relative to +RC (Fig. 2). The ^{13}C content in the NLFA 16:1ω5 mirrored those of the concentration and differed significantly among compartments ($H=34.1$, d.f. = 5, $P<0.001$; Fig. 3a). It was significantly higher in −RC for both mycorrhizal roots ($t=4.40$, $P=0.005$) and external mycelium ($t=3.5$, $P=0.010$) relative to +RC.

^{13}C enrichment was observed in NLFA 18:2ω6,9 in the labeled DC roots (2.82 ± 0.32%, mean ± SE), but not in the receiver roots where neither +RC(r+im) (−0.004) nor −RC(r+im) (−0.014) differed from the background values. This indicates that the ^{13}C transferred from the donor to the receiver roots remained in fungal tissue and was not translocated to the plant. It also suggests that no ^{13}C contamination occurred and that the only means for C transfer among compartments was via hyphae.

^{33}P allocation

The ^{33}P concentration differed significantly among compartments and tissue types ($F=14.36$; $P<0.001$), and approximately 70% of the recovered ^{33}P at harvest appeared to have remained in the HC. Pair-wise comparisons showed that the ^{33}P concentration was significantly higher in +RC(r+im) ($t=3.51$, $P=0.01$) and +RC(em) ($t=3.76$, $P=0.007$) compared with −RC(r+im) and −RC(em), respectively (Fig. 4). The ^{33}P content (^{33}P concentration × dry mass) differed significantly among compartments and tissue types ($F=12.14$; $P<0.001$, Fig. 3b).+RC(r+im) contained 10 times as much ^{33}P relative to −RC (r+im) ($t=3.57$, $P=0.004$), but there were no differences between +RC(em) and −RC(em) ($W=58$, $P=0.32$) due to the higher mycelial biomass in −RC. ^{33}P enrichment in labeled

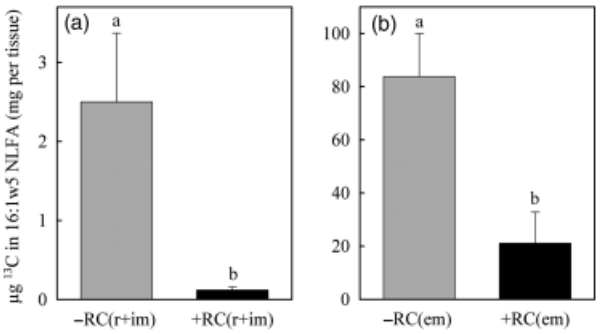

Fig. 2. Concentration of ^{13}C excess in the AM fungal NLFA 16:1ω5 in roots and internal mycelia (a) and external mycelia (b) growing in a medium containing 10 g sucrose L^{-1} (+RC) or 3 g sucrose L^{-1} (−RC). Different letters within tissue types differ from each other at $P\leq0.05$. Mean (SE), $n=8$. Please note the different scales in (a) and (b).

Table 3. Concentration and content of the AM fungal PLFA and NLFA 16:1ω5 extracted from roots and internal mycelium (r+im) or external mycelium (em) growing in a medium containing 10 g sucrose L^{-1} (+RC) or 3 g sucrose L^{-1} (−RC)

Lipid fraction	Treatment	Concentration (nmol per mg tissue)	Content (nmol per compartment)
PLFA	+RC(r+im)	0.045 (0.009)^{a}	0.280 (0.050)^{a}
	−RC(r+im)	0.011 (0.011)^{b}	0.053 (0.053)^{b}
	+RC(em)	0.256 (0.082)^{a}	0.356 (0.119)^{a}
	−RC(em)	0.523 (0.143)^{a}	0.605 (0.138)^{a}
NLFA	+RC(r+im)	1.39 (0.184)^{b}	9.70 (2.54)^{a}
	−RC(r+im)	3.40 (0.834)^{a}	6.90 (2.51)^{a}
	+RC(em)	333 (59.2)^{a}	251 (51.0)^{a}
	−RC(em)	306 (26.8)^{a}	430 (108)^{a}

Statistical comparisons were only made within the same tissue type and lipid fraction, and values with different superscript differs from each other at $P\leq0.05$. Mean (SE), $n=8$.

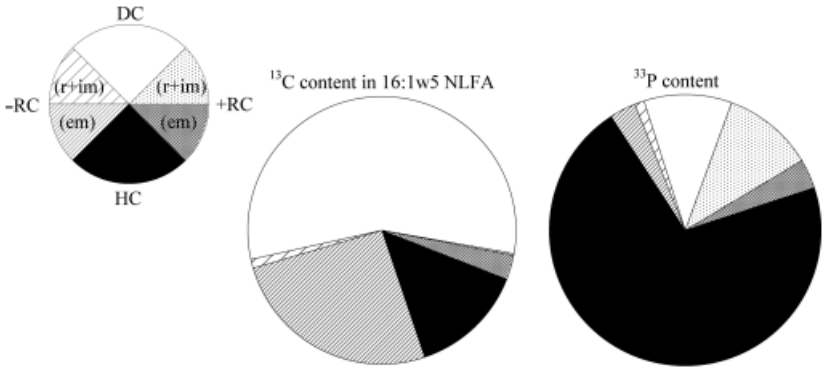

Fig. 3. Mean contents ($n = 8$) of ^{13}C excess in NLFA 16:1ω5 (a) and ^{33}P (b; measured as decay per minute, dpm) in the DC, receiver compartment containing 10 g sucrose L^{-1} (+RC), receiver compartment containing 3 g sucrose L^{-1} (− RC), and the HC that contained no roots. The receiver compartments were separated into a root and internal mycelium (r+im) and an external mycelium (em) fraction.

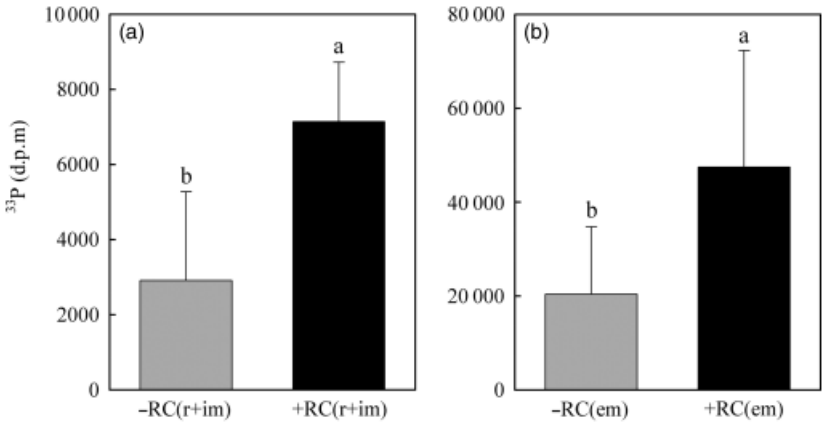

Fig. 4. Concentration of ^{33}P (measured as decay per minute, d.p.m.) in roots and internal mycelia (a) and external mycelia (b) growing in a medium containing 10 g sucrose L^{-1} (+RC) or 3 g sucrose L^{-1} (− RC). Different letters within tissue types differ from each other at $P \leq 0.05$. Mean (SE), $n = 8$. Please note the different scales in (a) and (b).

control plates where no hyphae had crossed into HC [decay per minute (dpm) = 0.44 ± 0.36, average ± SE] was not different from the background values (0.40 ± 0.35), suggesting that hyphae were the only means of ^{33}P transfer among compartments.

Relationship between C and P transfer and hyphal connectivity among compartments

The number of hyphae crossing among compartments did not differ significantly from each other ($F = 0.55$; $P = 0.65$) and averaged 30.2 (7–70, minimum–maximum). However, because the sucrose amount in the media clearly affected the overall flow of both C and P to +RC and − RC, correlations were performed within each receiver compartment type. In terms of C allocations, correlation analyses were conducted between the number of hyphae connecting the DC and either +RC or − RC, and the ^{13}C content in NLFA 16:1ω5 in either mycorrhizal roots or external mycelia. In all four correlation analyses, the *P*-value (which tests whether the slope differs from zero) was > 0.2, and thus there was no significant relationship between the connectivity between the compartments and C flow to either roots or external mycelia. The impact of hyphal connections for P allocation was tested by correlating the number of hyphae connecting HC and either +RC or − RC with the ^{33}P content in either roots or mycelia. Similar to the C allocation, all four *P*-values were > 0.2. Because all *P*-values were nonsignificant, suggesting no relationships between variables, no *r*-values were calculated.

Discussion

The results of this study show that C and P allocations among symbionts in the CMN are very variable and depend strongly on the ability of roots to deliver C. Our first hypothesis was supported: C allocation within the CMN appears to be source–sink driven, because more C was allocated to − RC relative to +RC (Fig. 2). This suggests that both internal and external mycelium were supported by neighboring roots to a greater extent if the host root was C limited. Our results are in accordance with previous findings showing increased C flows to shaded plants (Francis & Read, 1984), tree seedlings that are undergoing leaf expansion

(Lerat *et al.*, 2002), and clipped plants (Waters & Borowicz, 1994; Nakano *et al.*, 2001). The fate of this transferred C has been debated intensively (Robinson & Fitter, 1999; Selosse *et al.*, 2006), and some have argued that C is allocated from fungus to plant in quantities that could be of ecological significance (Bidartondo *et al.*, 2002; Lerat *et al.*, 2002; Carey *et al.*, 2004). Others, adopting a more mycocentric view of the AM symbiosis, claim that transferred C remains in fungal tissue with little or no consequence for the plant (Robinson & Fitter, 1999; Pfeffer *et al.*, 2004; Voets *et al.*, 2008). Our results support the latter view and our second hypothesis, because no ^{13}C was detected in the plant-specific NLFA 18:2ω6,9 lipid fraction in receiver roots, only in the AM fungal NLFA 16:1ω5. One could argue that because we are only measuring ^{13}C in lipids, we are biasing against detecting a potential C transfer from the fungus to the plant via, for example, carbohydrates and amino acids. This is unlikely, because Pfeffer *et al.* (2004) found no labeled C when measuring sucrose in receiver roots when using a similar system, and Fitter *et al.* (1998) detected no C transfer from AM fungi to shoots even when shoots were entirely dependent on root C for regrowth. Thus, allocation of fungal lipids into C-limited roots did not appear to benefit the host directly, and we hypothesize that this is a strategy of the fungus to optimize its fitness. In fact, the lack of a significant difference in the weight of the extraradical mycelium between +RC and −RC (Table 1) suggests that C allocated from the donor root may have compensated for the lower host quality in −RC. It is interesting to note that the overall ^{13}C concentration and content in NLFA 16:1ω5 were higher in the external fungal structures than within roots (Fig. 2). This is in agreement with previous findings obtained by Nakano-Hylander & Olsson (2007) showing that the fungus may not allocate C preferentially to seedlings, but may invest in spores and ERH structures as a strategy to increase the likelihood of encountering new host roots to colonize.

If transferred C from neighboring plants was utilized for extensive colonization of roots and subsequent P delivery, it could result in a subsidized symbiosis. However, the P allocation observed here indicates that there may be no such thing as a 'free lunch,' because the C-limited roots only received one-tenth of the amount of P allocated to the +RC roots. Thus, there appears to be a relationship between P delivery and C acquisition, which supports our third hypothesis, and previous findings that show that reciprocal C delivery from plants affects the uptake and transfer of P (Smith & Gianinazzi-Pearson, 1990; Bücking & Shachar-Hill, 2005). Essentially, if the roots are not delivering C, they will not receive P. Instead, in low C environments, P allocation may shift spatially and metabolically to accumulate in fungal structures (Bücking & Shachar-Hill, 2005). It is important to remember, though, that our analyses of ^{33}P in mycorrhizal roots do not differentiate between P in plant and fungal structures. However, arbuscules have previously been shown to be the primary site for P delivery (Rausch *et al.*, 2001), and arbuscule density was twice as high in the +RC roots relative to the −RC roots (Table 2). This, coupled with the 10 times higher allocation of P to the +RC roots (Fig. 4), suggests that more P was accessible to the plant in the +RC treatment relative to the −RC treatment. The idea that hyphal connectivity is important for P transfer (Walter *et al.*, 1996) was not supported in this study, because we observed no correlation between the number of hyphal connections between compartments and P transfer. Thus, P demand and the ability to deliver C appear to be stronger drivers for both C and P flow within the CMN than the number of hyphae connecting plants.

In experimental, single plant–fungus combinations, a reduction in light levels normally results in reduced AM colonization (Tester *et al.*, 1986 and references therein), presumably due to a lower C assimilation and allocation belowground (Smith & Read, 2008). Similar responses have been recorded when shading whole sections of natural plant communities (Heinemeyer & Fitter, 2004). In our experiment, however, C limitations in the −RC compartment did not result in a reduced AM colonization (Table 2). This raises the possibility that responses to shading may be very different depending on whether or not the fungus is simultaneously connected to an unshaded plant. As a consequence, mycorrhizal colonization within CMN-connected plants may poorly reflect host quality. Why would it make ecological sense for a fungus to colonize a poor host? The poor host may become a good host at some point in the future, whereby the established colonization will confer a competitive advantage. For example, changes in host quality can be drastic and random due to temporary shading and herbivory, as well as predictable because of seasonal changes or succession. In light of this, the little or no host preference observed in some plant communities (Öpik *et al.*, 2003; Stukenbrock & Rosendahl, 2005; Santos *et al.*, 2006) makes ecological sense as it may be a fungal strategy to minimize risk and maximize the likelihood of a continuous C supply.

The NLFA to PLFA ratios have been proposed as a good indicator of the fungal storage status (van Aarle & Olsson, 2003). When we calculated this ratio using average lipid concentrations from this experiment (Table 3), we found it to be 340 in mycorrhizal −RC roots and 28 in mycorrhizal +RC roots. Based on this, our results suggest that the AM fungal strategy in poor hosts may be to store acquired C, and to forage for additional C in good hosts. Likewise, Fitter *et al.* (1998) showed that C transfer was positively correlated with vesicle colonization in neighboring roots, but negatively correlated with hyphal growth, reinforcing the notion that C may be allocated preferentially to storage and not for active proliferation within neighboring roots. It is possible

that C storage within roots offers a more protective environment where fungal-feeding nematodes and collembolans (Finlay, 1985; Bakhtiar *et al.*, 2001) are absent. Surprisingly, there was no significant difference in vesicle colonization between −RC and +RC roots here, but vesicle abundance may correlate poorly with storage lipids (van Aarle & Olsson, 2003).

By adopting the mycocentric view, Robinson & Fitter (1999) argued that C transfer among plants via the CMN might be 'irrelevant to the botanical component of a community.' However, unequal C allocation for the growth and maintenance of the ERH could influence the competitive balance among plants (as discussed by Selosse *et al.*, 2006). Even though the −RC received significantly less P than the +RC roots, they could still benefit from being connected to an extensive network if C limitations were eliminated. Indeed, Jakobsen (2004) argued that the C cost for seedlings connected to a CMN may be negligible, and seedlings' establishment has been improved in the presence of a CMN in some instances (van der Heijden *et al.*, 2003), but not in others (Pietikäinen & Kytöviita, 2007). Most likely, the CMN benefit to seedlings may depend on the size and proximity of neighboring plants, because AM fungi can increase size-assymetric competition (Nakano-Hylander & Olsson, 2007) where soil resources acquired by the hyphae are preferentially allocated to the larger, C-rich host.

Our study system is artificial and the results need to be verified under more realistic conditions to better estimate the ecological significance. We would have preferred to use the *in vitro* system with autotrophic plants utilized by Voets *et al.* (2008) to reduce many of the shortcomings of the root-organ cultures listed in Introduction. However, the level of control required in this experiment would have been difficult to achieve with autotrophic plants. Furthermore, we opened all Petri dishes weekly to control root growth among compartments, and this maintenance could have easily damaged the shoots. By excluding the shoot, one could argue that the C-delivering ability of the donor roots and the P sink strength of the receiver roots were reduced, and our findings may thus be considered a conservative estimate of what may occur in autotrophic systems. Regardless of any potential study system limitations, our findings are in agreement with those using whole plant mycorrhizas (Fitter *et al.*, 1998; Nakano-Hylander & Olsson, 2007; Voets *et al.*, 2008), and we therefore believe that these axenic cultures can be used favorably to enhance our basic knowledge regarding CMN dynamics. We showed here that C allocation within the CMN appears to be source–sink driven and that the fungus moves C in accordance to its own C demand, not that of the host, because none of the transferred C was incorporated into the receiver roots. Furthermore, the fungus may colonize plants indiscriminately, but only allocate P to roots that are delivering C. Acquired C may then primarily be stored in less active roots. By adopting a mycocentric view of AM, the fungus may view a plant community as a dynamic landscape of resource islands and storage units.

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