

Phosphorus Effects on Metabolic Processes in Monoxenic Arbuscular Mycorrhiza Cultures¹

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The influence of external phosphorus (P) on carbon (C) allocation and metabolism as well as processes related to P metabolism was studied in monoxenic arbuscular mycorrhiza cultures of carrot (*Daucus carota*). Fungal hyphae of *Glomus intraradices* proliferated from the solid minimal medium containing the colonized roots into C-free liquid minimal medium with different P treatments. The fungus formed around three times higher biomass in P-free liquid medium than in medium with 2.5 mM inorganic P (high-P). Mycelium in the second experiment was harvested at an earlier growth stage to study metabolic processes when the mycelium was actively growing. P treatment influenced the root P content and [¹³C]glucose administered to the roots 7 d before harvest gave a negative correlation between root P content and ¹³C enrichment in arbuscular mycorrhiza fungal storage lipids in the extraradical hyphae. Eighteen percent of the enriched ¹³C in extraradical hyphae was recovered in the fatty acid 16:1ω5 from neutral lipids. Polyphosphate accumulated in hyphae even in P-free medium. No influence of P treatment on fungal acid phosphatase activity was observed, whereas the proportion of alkaline-phosphatase-active hyphae was highest in high-P medium. We demonstrated the presence of a motile tubular vacuolar system in *G. intraradices*. This system was rarely seen in hyphae subjected to the highest P treatment. We concluded that the direct responses of the extraradical hyphae to the P concentration in the medium are limited. The effects found in hyphae seemed instead to be related to increased availability of P to the host root.

Arbuscular mycorrhizal (AM) association is the only way for fungi in the order Glomales to proliferate and reproduce (Bécard and Fortin, 1988; Smith and Read, 1997; Bago et al., 2000). It is well known that C is transferred from colonized plants to AM fungi (Ho and Trappe, 1973), whereas the plants in many cases receive most of their P through hyphal uptake and fungal transfer to the host root (Pearson and Jakobsen, 1993). Colonization by AM fungi increases the C sink strength of roots (Douds et al., 1988). The fungal C demand upon root colonization can constitute a significant cost to the host plant, as indicated by reduced growth at high P levels (Peng et al., 1993). This also implies that there is an important connection between external P supply and the regulation of C allocation to the fungal partner in the symbiosis.

C metabolism of the AM fungus *Glomus intraradices* has been studied using ¹³C-NMR in monoxenic cultures (Pfeffer et al., 1999). Although no hexose uptake occurs in the extraradical mycelium, intraradical AM fungal mycelium takes up C supplied as hexoses to

the root. Triacylglycerols are synthesized from this C in the fungus and transported to the extraradical mycelium. These triacylglycerols are substantial sinks for C in the AM fungal mycelium (Bago et al., 2000; Olsson and Johansen, 2000).

Formation of AM is important for P acquisition in most plants. The adverse effect of high soil P levels on AM formation is well documented (Mosse, 1973; Menge et al., 1978; Jasper et al., 1979; Abbott et al., 1984; Bååth and Spokes, 1989), and is mainly caused by P concentrations being higher in the roots (Sanders, 1975). It has, however, also been shown that high P levels inhibit AM fungi more directly by reducing spore germination and hyphal growth from the germinated spores (Miranda and Harris, 1994; Nagahashi et al., 1996). P-regulated gene expression in fungi includes phosphatases (Kaffman et al., 1994), P transporters (Versaw, 1995), and proteins related to polyphosphate metabolism (Ogawa et al., 2000). A high-affinity P transporter is expressed in the extraradical mycelium of the AM fungus *Glomus versiforme* (Harrison and Van Buuren, 1995). In *G. intraradices*, a similar P transporter is regulated by P availability in the external medium and possibly also by the P status of the host root (Maldonado-Mendoza et al., 2001). The P-related processes of polyphosphate accumulation and intracellular phosphatase activities are mainly localized to the vacuolar compartment (Gianinazzi and Gianinazzi-Pearson, 1978; Smith and Read, 1997). The vacuolar compartment in

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Table I. Influence of P treatment on AM fungal growth

Experiment 1. Growth and fatty acid content of the *G. intraradices* mycelium in P-free medium and in 2.5 mM KH₂PO₄ (high-P). Total amounts of PLFAs and NLFAs extracted from mycelia in liquid medium (see Table II) are given together with the proportion of unsaturated PLFAs and NLFAs (mean \pm SE, $n = 4$). The effect of P treatment was tested with Student's *t* test.

Treatment	Biomass of Mycelium <i>mg dry wt</i>	Mycelium Fatty Acid Content <i>nmol</i>		Proportion Unsaturated <i>%</i>	
		PLFAs	NLFAs	PLFAs	NLFAs
P-free	3.56 \pm 0.36	6.85 \pm 0.76	2400 \pm 327	45.3 \pm 3.3	81.1 \pm 0.2
High-P	1.23 \pm 0.23	3.52 \pm 0.47	782 \pm 345	34.4 \pm 2.1	76.4 \pm 1.0
<i>P</i> value	0.002	0.001	0.015	0.03	0.004

fungi may consist of separate spherical vacuoles, but there is increasing evidence for functioning vacuoles in fungi having the shape of dynamic tubular networks (Cole et al., 1998). This has recently also been shown in the AM fungus *Gigaspora margarita* (Uetake et al., 2002).

We studied the influence of external P availability on C allocation and metabolism as well as P metabolism in a monoxenic system with carrot (*Daucus carota*) root-organ cultures in symbiosis with the AM fungus *G. intraradices*. This system has proved suitable for study of growth strategies of the fungus (Bago et al., 1998) and is now well established as a model system for metabolism and transport processes in the AM symbiosis (Bago et al., 2000; Fortin et al., 2002). We used a two-compartment petri dish system where the root grew in a solid medium in one compartment and extraradical AM fungal mycelium proliferated over a barrier into a second compartment containing liquid medium subjected to different P treatments. The method is described in more detail by Maldonado-Mendoza et al. (2001).

RESULTS

Growth of Mycelium and Roots and Fungal Fatty Acid Composition

The high-P treatment (containing 2.5 mM inorganic P) reduced the growth of extraradical mycelium in the liquid medium in experiment 1, and fungal biomass was 3-fold higher in the P-free medium (Table I).

Mycelium of both treatments sporulated, but finely branched absorption hyphae were rarely seen. Mycelium covered 100% of the area of the liquid medium compartment. The high-P treatment had a similar effect on the total amounts of phospholipid fatty acids (PLFAs) and neutral lipid fatty acids (NLFAs) of the mycelium (Table I). The fatty acid composition of the mycelium was influenced by the high-P treatment in that a higher proportion of unsaturated fatty acids was found in mycelium growing in P-free medium (Table I). The fatty acid 16:1 ω 5 dominated the neutral lipid fraction. All saturated NLFAs occurred at a higher proportion after the high-P treatment than the P-free treatment (Table II).

Between 15% and 80% of the area of the liquid medium compartment was covered by mycelium at the time of harvest in experiment 2. Mycelia all sporulated, and finely branched hyphae occurred frequently. The addition of P increased the biomass of the roots and influenced the root P concentration in experiment 2 (Table III) in a fashion that could be expected from the amounts of orthophosphate in the liquid medium. The P treatment did not influence the N concentration of the roots. There was no significant difference in mycelium biomass between the P-free, low-P (containing 25 μ M inorganic P), or high-P treatment in experiment 2 (ranging from 0.89 to 1.13 mg dry weight), and the results were similar for total amounts of PLFAs and NLFAs. A higher mycelium dry weight was found in the organic P (org-P) medium (containing 2.5 mM organic P as sodium-

Table II. Fatty acid composition of AM fungal mycelium

Experiment 1. Mean fatty acid composition ($n = 4$) of PLFAs and NLFAs in mycelium of *G. intraradices* in P-free medium and with 2.5 mM KH₂PO₄ (high-P). The nomenclature for fatty acids follows that of Tunlid and White (1992).

Lipid Fraction	Treatment	Unsaturated Fatty Acids				Saturated Fatty Acids		
		16:1 ω 5	18:2 ω 6,9	18:1 ω 9	18:1 ω 7	16:0	18:0	20:0
		%						
PLFAs	P-free	23.2	1.8	6.2	15.1	31.0	11.4	11.2
	High-P	14.7	0.0	6.2	13.8	40.0	18.1	7.1
NLFAs	P-free	77.6	0.4	1.1	2.1	17.7	0.7	0.5
	High-P	74.8	0.3	0.9	1.7	19.8	1.4	1.1

Table III. Influence of P treatment on root biomass and nutrient content

Experiment 2. The effect of P treatment was tested with one-way ANOVA (mean values \pm SE, $n = 4$), and different letters indicate significantly different values ($P < 0.05$, Fischer's LSD).

Treatment	Root P Concentration	Total Root P	Root N Content	Root Dry Wt
	$mg\ g^{-1}$	μg	$mg\ g^{-1}$	mg
P-free	1.5 ± 0.12 a	62 ± 4.5 a	12.3 ± 1.4	41.5 ± 1.2 a
Low-P	1.7 ± 0.16 ab	80 ± 8.7 ab	13.5 ± 1.7	47.7 ± 2.1 ab
Org-P	1.9 ± 0.06 bc	91 ± 8.2 bc	15.2 ± 1.0	47.8 ± 4.1 ab
High-P	2.0 ± 0.05 c	104 ± 8.0 c	13.1 ± 0.6	51.6 ± 3.4 b
P value	0.020	0.012	0.45	0.16

phytate). This was presumably attributable to a precipitate (probably containing sodium-phytate) on the hyphae, and the total amounts of PLFAs and NLFAs were not influenced by this treatment.

Although in the extraradical hyphae, the total NLFA to PLFA ratio (indicating the relative amount of C allocated to storage lipids) was lower after the high-P treatment than the other kinds of treatment, this difference was not statistically significant (Fig. 1). The relative amount of C allocated to storage lipids of the AM fungus inside the roots was estimated from the amount of the AM fungal fatty acid 16:1 ω 5. The ratio between NLFA 16:1 ω 5 and PLFA 16:1 ω 5 was significantly lower after the high-P treatment than the low-P and P-free treatment, indicating less allocation to storage lipids at high P.

Changes in the Liquid Medium during Experiment 2

Mycelia proliferated into the liquid medium after all kinds of P treatment. Most roots that crossed the barrier between the compartments were cut, but at

the time of harvest, some roots had entered the liquid medium in most of the replicates of each P treatment. The orthophosphate in the low-P medium was almost depleted by roots and hyphae after the experimental period, whereas no depletion could be observed in the high-P treatment (Table IV). Only about 60% to 70% of the originally added liquid remained after the experimental period because of evaporation, meaning that the orthophosphate concentration in the remaining liquid medium would have increased. This was also the reason for an increase in orthophosphate concentration in the control dishes incubated without cultures (Table IV). The org-P medium contained 88 μM orthophosphate in addition to the 2.5 mM sodium-phytate at the commencement of the experiment. This orthophosphate had to a large extent been depleted by the end of the experiment. In all kinds of P treatment, the growth of the AM fungal mycelia raised the pH compared with that of the control dishes (Table IV). At the end of the experiment, the pH of the P-free and low-P treatments was higher than that of the high-P treatment and the org-P treatment.

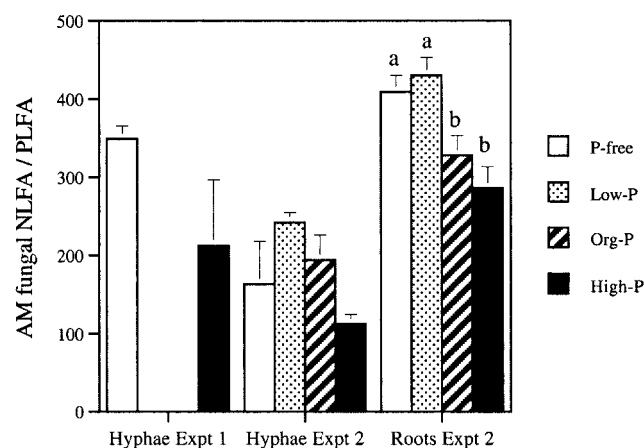


Figure 1. The relative allocation of C to storage lipids is indicated by the ratio of NLFAs to PLFAs (average \pm SE, $n = 4$; for hyphae in experiment 2, $n = 3$). For hyphae, the ratio was calculated using all detected fatty acids (see Table II), whereas the AM fungal signature fatty acid 16:1 ω 5 (Olsson et al., 1997) was used for the intraradical mycelium because of a background from other fatty acids in the root. The P treatment significantly influenced the NLFA to PLFA ratio in the root ($P = 0.004$; one-way ANOVA) but not in hyphae. Different letters indicate significantly different values ($P < 0.05$, Fischer's LSD).

^{13}C Enrichment in Hyphae and Roots

^{13}C enrichment in the extraradical mycelium was low and variable in experiment 1 ($0.021\% \pm 0.015\%$ for P-free and $0.030\% \pm 0.027\%$ for high-P treatment, averages \pm SE) after a 4-d chase period, and there was no significant difference between various P treatments. The results were similar for ^{13}C enrichment in NLFA 16:1 ω 5. ^{13}C enrichment in PLFA 16:1 ω 5 ($0.27\% \pm 0.17\%$ for P-free and $0.58\% \pm 0.30\%$ for high-P treatment) was higher than both total ^{13}C enrichment ($P = 0.04$, paired t test, $n = 8$) and ^{13}C enrichment in NLFA 16:1 ω 5 ($P = 0.04$, paired t test, $n = 8$).

High initial orthophosphate content of the liquid medium gave high Glc uptake in the root in experiment 2, as shown by high ^{13}C enrichment, whereas the opposite trend was found for the extraradical mycelium, although this was not statistically significant (Fig. 2). The P content of the roots correlated negatively with ^{13}C enrichment in NLFA 16:1 ω 5 ($P < 0.01$) in extraradical hyphae, indicating that neutral lipids accumulate in particular in mycelia growing

Table IV. Orthophosphate concentration and pH in liquid medium with *G. intraradices*

Experiment 2. Final values were measured at the end of the experimental period (mean \pm SE, $n = 4$). Initial values were measured in media stored at -20°C . Liquid medium incubated in petri dishes without cultures was used as control ($n = 2$). The pH of the medium was set at 5.5 before incubation, but because the organic P was added after autoclaving, the pH could not be adjusted after this addition. The effect of P treatment on pH was tested with one-way ANOVA and different letters indicate significantly different values ($P < 0.05$, Fischer's LSD).

Treatment	PO ₄ ⁻ concentration		Control	pH	
	Initial	Final		Final	Control
		μM			
P-free	0.06	0.36	0.06	7.4 \pm 0.18 a	5.0
Low-P	23	1.0	29	7.6 \pm 0.14 a	5.5
Org-P	88	16	114	6.6 \pm 0.22 b	4.7
High-P	2.5*10 ³	2.6*10 ³	2.9*10 ³	6.8 \pm 0.03 b	5.5
ANOVA				$P = 0.002$	

with root organs with limited P availability (Fig. 3). The ¹³C enrichment in hyphae in the liquid medium was higher than in roots (Table V). The ¹³C enrichment could be determined in the AM fungal fatty acid 16:1 ω 5 and in fatty acid 18:2 ω 6,9 which is a common plant fatty acid. Fatty acid 16:0 is common both in plants and AM fungi, whereas in roots, PLFA 16:0 mainly reflects plant origin; NLFA 16:0 in roots normally also represents AM fungal lipids to a large extent. The ¹³C enrichment in fatty acids indicated higher C accumulation in the fungus than in the root (Table V).

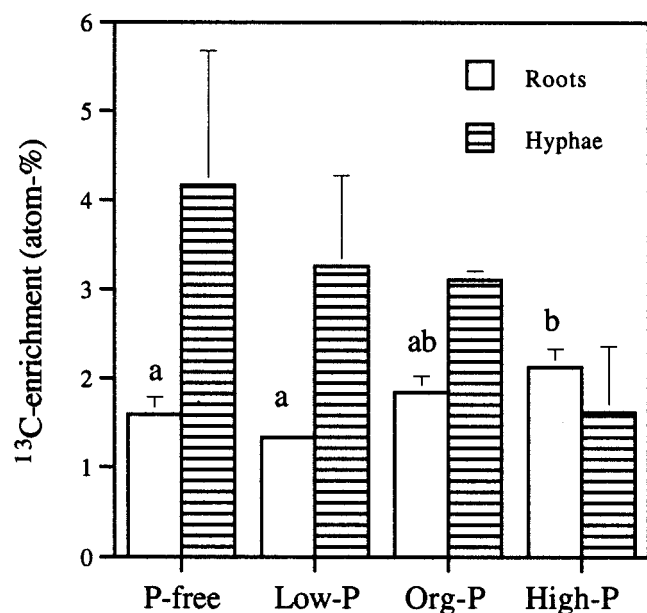


Figure 2. Experiment 2. ¹³C enrichment expressed as excess atomic percent in roots and hyphae (average \pm SE, $n = 4$ for roots, $n = 3$ for hyphae). The background attributable to the natural abundance of ¹³C (1.14%) has been subtracted. P treatment influenced ¹³C enrichment significantly in roots ($P = 0.038$; one-way ANOVA) but not in hyphae. Different letters indicate significantly different values ($P < 0.05$, Fischer's LSD).

The total amount of ¹³C transported to the mycelium in the liquid medium and incorporated into NLFA 16:1 ω 5 was lower after the high-P treatment than for the other kinds of P treatment (Fig. 4). Mycelia growing in org-P medium exhibited the highest incorporation in PLFA 16:1 ω 5. In relation to incorporation into PLFA 16:1 ω 5, mycelia growing in P-free and low-P media allocated more C to neutral lipids than fungi growing in high-P and org-P media. In total, 18% (average for all treatments) of the excess ¹³C in extraradical mycelium was recovered in NLFA 16:1 ω 5. The corresponding figure for the roots was 7.1% of the excess ¹³C in NLFA 16:1 ω 5.

Metabolic Processes Related to Fungal P Uptake

The proportion of hyphal length with an intact cytoplasm (Fig. 5) was reduced by 40% in solutions

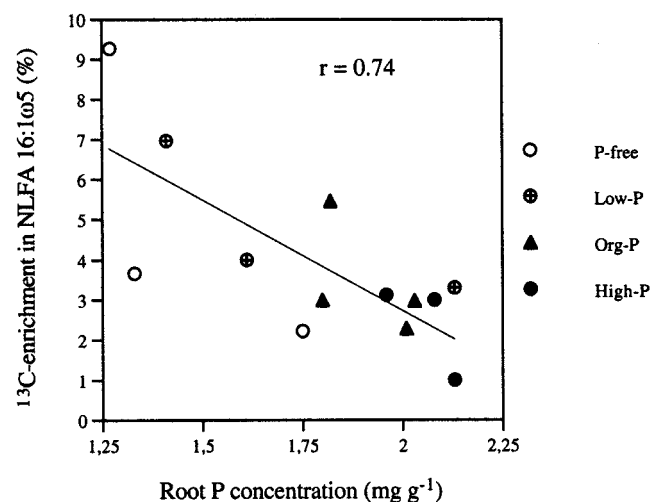


Figure 3. Experiment 2. The relationship between root P concentration and ¹³C enrichment in NLFA 16:1 ω 5 in hyphae in liquid medium after different kinds of P treatment ($P < 0.01$). The background attributable to the natural abundance of ¹³C (1.14%) has been subtracted.

Table V. ^{13}C enrichment in signature fatty acids and total root and hyphal C

Experiment 2. Values from NLFA-C and PLFA-C in roots and hyphae together with the total ^{13}C enrichment in roots and hyphae are given (mean \pm SE). The background attributable to natural abundance of ^{13}C (1.14%) has been subtracted. Differences between roots and hyphae were tested with the paired t test. In some cases, it was not possible to determine the ^{13}C enrichment (nd).

	^{13}C Enrichment						Total C
	PLFA-C			NLFA-C			
	16:1 ω 5	16:0	18:2 ω 6,9	16:1 ω 5	16:0	18:2 ω 6,9	
	excess atom % ^{13}C						
Roots ($n = 16$)	nd	3.0 \pm 0.20	2.7 \pm 0.22	1.7 \pm 0.18	3.7 \pm 0.38	1.3 \pm 0.10	1.7 \pm 0.11
Mycelium ($n = 12$)	4.2 \pm 0.58	4.5 \pm 0.64	nd	3.9 \pm 0.61	5.3 \pm 0.72	nd	3.0 \pm 0.50
P value		0.031		0.006	0.051		0.029

subjected to high-P and org-P treatment compared with P-free and low-P treatment (Fig. 5). Polyphosphate accumulated in the high-P-treated hyphae, as indicated by the proportion of hyphae containing cytoplasm with metachromasy (see Fig. 6A). One of the replicates of the org-P treatment also showed high polyphosphate accumulation, but no significant difference was found compared with the other treatments. Polyphosphate was not detected in hyphae without cytoplasm. Cross-walls were formed in empty hyphae and polyphosphate was detected on the cytoplasmic side of the cross-walls (Fig. 6A). Low levels of polyphosphate were detected after the P-free and low-P treatment.

The proportion of acid-phosphatase-active length of hyphae was not significantly influenced by the P treatment for hyphae with or without cytoplasm. Both the acid phosphatase activity and the alkaline

phosphatase activity was, in many cases, associated with the vacuoles (Fig. 6B). The proportion of alkaline-phosphatase-active length of hyphae containing cytoplasm was, however, increased in the high-P treatment and decreased in the org-P treatment compared with other kinds of P treatment. The high-P treatment also increased the proportion of alkaline-phosphatase-active hyphal length in hyphae without cytoplasm compared with other kinds of P treatment. In the empty hyphae, the alkaline phosphatase activity was associated with cell walls.

Phosphatase activity in the liquid medium was below the usual detection limit of 0.01 unit mL^{-1} in both experiments 1 and 2. Phosphatase activity was also assayed after decreasing the detection limit to 0.001 unit mL^{-1} in experiment 2. Still, only occasional acid phosphatase activity and no alkaline phosphatase activity was found. The highest value recorded was 0.0023 unit acid phosphatase activity mL^{-1} in one of the org-P-treated replicates. This could, however, be attributable to phosphatases released by the few roots observed in the liquid medium.

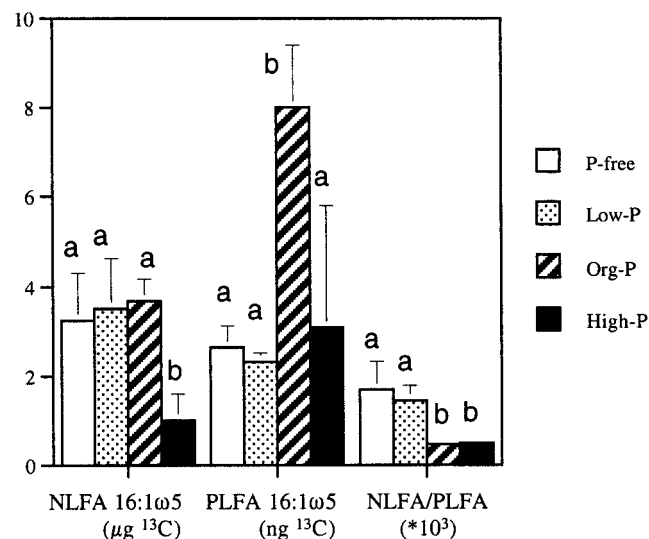


Figure 4. Experiment 2. Amount of enriched ^{13}C in NLFA and PLFA 16:1 ω 5 in hyphae of *G. intraradices* in the liquid medium calculated after the background attributable to natural abundance of ^{13}C (1.14%) had been subtracted (average \pm SE, $n = 3$). P treatment influenced the [^{13}C]NLFA to [^{13}C]PLFA ratio significantly ($P = 0.016$; one-way ANOVA). There was also an indication of effects on NLFA 16:1 ω 5- ^{13}C ($P = 0.068$) and PLFA 16:1 ω 5- ^{13}C ($P = 0.055$). Different letters indicate significantly different values ($P < 0.05$, Fischer's LSD).

The Tubular Vacuolar System in *G. intraradices*

The medium originally containing 35 μM P was almost depleted of P at the time of investigation of the vacuolar structures and contained only 1.1 ± 0.38 μM P. No depletion was detected in the medium initially containing 350 μM P. Tubular vacuoles were seen only in live hyphae with cytoplasmic streaming. The cytoplasmic streaming was bidirectional. In the healthiest of cells, in the 35 μM P medium, the tubular vacuoles often formed a finely branched network (Fig. 7A) and there were few rounded vacuoles. In the medium originally containing 350 μM P, there were more empty hyphae. In living hyphae, cytoplasmic streaming was slower, and in this medium, the hyphae mostly contained rounded vacuoles, sometimes interconnected with tubular vacuoles (such as shown for hyphae in 35 μM P medium; Fig. 7B). Both tubules and rounded vacuoles varied in diameter (Fig. 7B). In P-free medium, the shape and organization of the vacuoles were intermediate between that of the vacuoles in the other two media, although they

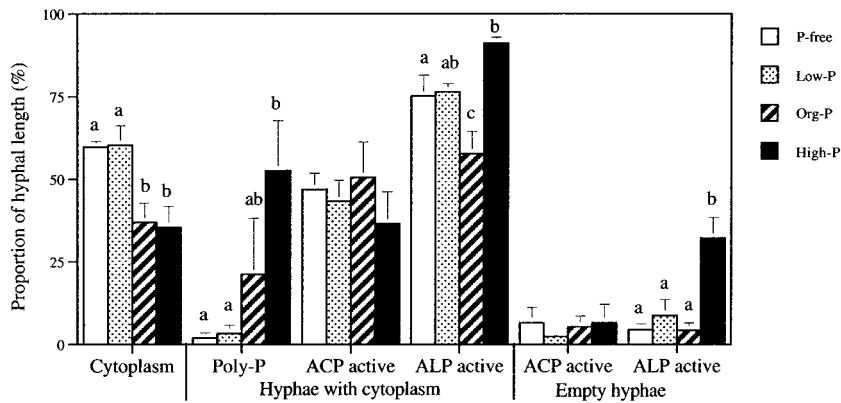


Figure 5. Microscopically investigated processes in the hyphae of *G. intraradices*. The proportion of hyphal length with polyphosphate (Poly-P) and hyphae with acid phosphatase (ACP) and alkaline phosphatase (ALP) activity was determined both in hyphae with cytoplasm and in empty hyphae (average \pm SE, $n = 4$). No polyphosphate was detected in empty hyphae. The P treatment significantly influenced the proportion of hyphae containing cytoplasm ($P = 0.007$; one-way ANOVA), hyphae containing cytoplasm with polyphosphate ($P = 0.03$) and ALP-active cytoplasmic ($P = 0.004$) and ALP-active empty hyphae ($P = 0.002$). Different letters indicate significantly different values ($P < 0.05$, Fischer's LSD).

resembled more closely that of the hyphae in the medium containing $35 \mu\text{M}$ P.

In hyphae after all treatments, tubular vacuoles seemed fragile. It was easiest to make observations very soon after transfer of the hyphae to the slide, and after approximately 30 min, the tubular network tended to coalesce (Fig. 7C), increased numbers of rounded vacuolar structures appeared, and streaming was reduced.

DISCUSSION

External P Influences C Metabolism of the AM Fungus

In experiment 1, we found a negative feedback of high P availability on mycelium growth of the AM

fungus. Local P sources that can only be accessed by AM fungal hyphae have been shown to reduce root colonization by the fungus (Boddington and Dodd, 1998) and with this possibly also the development of extraradical mycelium, because root colonization and the amount of extraradical mycelium are related (Olsson et al., 1997). A good P status of plants usually inhibits colonization by AM fungi (Sanders, 1975) and prevents the accumulation of AM fungal neutral lipids (Peng et al., 1993; Olsson et al., 1997). In the present study, root colonization was already established when we applied the P treatment. AM fungal

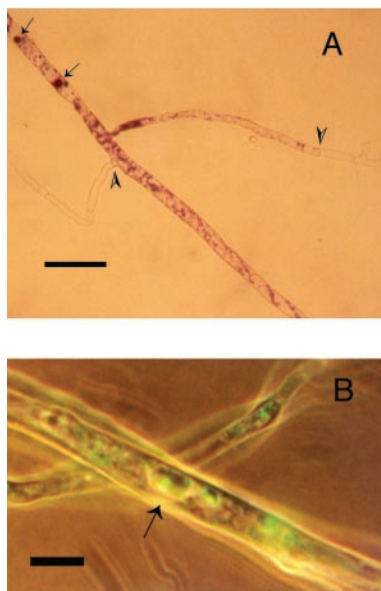


Figure 6. A, Hyphae after low-P treatment with metachromasy, indicating the localization of polyphosphate (arrows). Metachromatic hyphae with cytoplasm separated from empty hyphae by cross-walls (arrow heads) can be seen. B, Localization of alkaline phosphatase activity by enzyme-labeled fluorescence substrate (ELF) staining (arrow) of a sample from the low-P treatment. Transmitted long-wavelength light was combined with UV light. Scale bar in A = $25 \mu\text{m}$ and in B = $9 \mu\text{m}$.

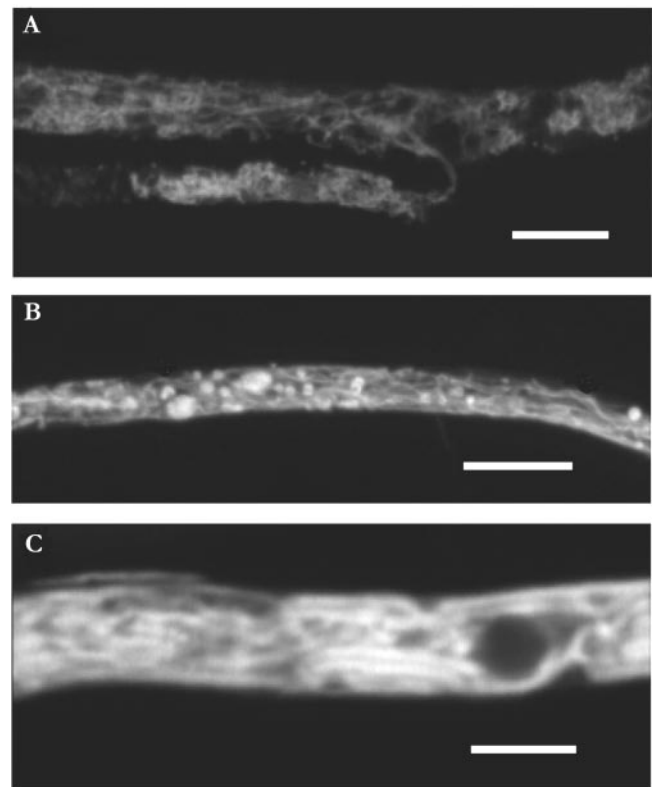


Figure 7. Tubular vacuole system of *G. intraradices* when growing in a medium originally containing $35 \mu\text{M}$ P. A and C, Digital images of a single focus plane. B, Maximum intensity projection of multiple scan (20 images). Scale bar in A = $8 \mu\text{m}$, in B = $10 \mu\text{m}$, and in C = $4 \mu\text{m}$.

hyphae, and later also some roots, reached the liquid medium. The reduced hyphal growth in medium with a high P content indicates that the root may reduce the C flow to the fungus under improved P conditions. Considering the proposed C metabolism in the symbiosis, this would result in reduced lipid transport (mainly triacylglycerols) from intraradical mycelium to the extraradical mycelium (Bago et al., 2000).

The hyphae in experiment 1 had stopped expanding because the whole area of the liquid compartment was covered at the time of harvest. The proportion of unsaturated fatty acids in *G. intraradices* was higher in neutral lipids than in phospholipids, which is in contrast to many other fungi (Lösel, 1988). The lower proportion of unsaturated fatty acids after the high-P treatment could be a response to a nutritional factor such as the C supply from the host. Plasticity in fatty acid synthesis attributable to nutritional factors is evident in filamentous fungi (Lösel, 1988). Earlier studies have shown that P application influences the lipid metabolism of AM fungi. Graham et al. (1997) found little effect of P application on AM colonization in citrus, but the content of the AM fungal fatty acid 16:1 ω 5 (from all types of lipids) in roots was reduced at high P. Using the same fatty acid as a signature, Olsson et al. (1997) showed that high-P level in the colonized root particularly reduced the AM fungal storage lipids. Similar effects were shown in this study by a reduced NLFA to PLFA ratio at high P, and coherent with this was the reduced ^{13}C enrichment in NLFA 16:1 ω 5 at high P (Fig. 3).

In experiment 2, we harvested mycelium while it still was expanding, and a considerable C flow to the mycelium in the liquid medium occurred. The flow of ^{13}C reflects the total flow of C, because there was no C source in the liquid medium. There is normally a net movement of lipids from intraradical mycelium to extraradical in AM fungi, although significant bidirectional translocation occurs (Bago et al., 2002). The higher ^{13}C enrichment in AM fungal signature fatty acids in the extraradical mycelium than in the root compartment (Table V) indicates that the extraradical mycelium had a higher growth rate. It is proposed that triacylglycerols are the major C compounds translocated from the intraradical mycelium to the extraradical mycelium (Bago et al., 2000). We found that 18% of the excess ^{13}C in extraradical mycelium was present in NLFA 16:1 ω 5, a dominant constituent of the AM fungal triacylglycerols. Calculations based on the assumption that at least 75% of NLFA is 16:1 ω 5 (see Table II) and that NLFAs constitute about 75% of triacylglycerols (see Olsson and Johansen, 2000) give an estimate that 32% of ^{13}C enrichment in the extraradical mycelium is found in the triacylglycerols. This result supports the suggestion that these lipids are important for AM fungal C metabolism.

P Translocation in the Fungus

The mycelium of *G. intraradices* grew very well in the P-free medium, which indicates a good allocation ability of P in the mycelium because P could only have been taken up on the root side of the cultures. Polyphosphate with an approximate average chain length of 17 accumulates in the vacuoles of *G. intraradices* (Rasmussen et al., 2000) and may be important in reducing osmotic stress at high internal P concentrations but is probably also important for the translocation of P (Smith and Read, 1997). Incubation of extracted intraradical mycelium of *G. margarita* in Glc increased the efflux of P and at the same time decreased the polyphosphate content in the hyphae, indicating a role for polyphosphate in the exchange of C and P between the symbionts (Solaiman and Saito, 2001). Up to 17% of the P in the extraradical mycelium of *G. margarita* may be stored as polyphosphate (Solaiman et al., 1999) and 37% in Brewer's yeast (*Saccharomyces cerevisiae*; Ogawa et al., 2000). The detection of polyphosphate in mycelium growing in P-free medium supported a role for polyphosphate in the transport of P taken up in other parts of the mycelium. This means that bidirectional transport of P, possibly as polyphosphate, may occur in AM fungal hyphae. Ezawa et al. (2001) did not detect polyphosphate in the extraradical hyphae of *Glomus coronatum* growing in P-free sand. However, they used a slightly different staining method not including the precipitation of polyphosphates with ethanol.

The presence of a motile tubular vacuole system has been demonstrated in a wide range of fungi (Ashford, 1998), and the system is found in the mycelium of *Pisolithus* sp. when growing in ectomycorrhizal symbiosis (Allaway and Ashford, 2001). The first AM fungus in which this kind of system was demonstrated was *G. margarita* (Uetake et al., 2002). In *G. intraradices*, individual spherical vacuoles have been described (Bago et al., 1998). The tubular components of the vacuolar system are, however, not retained in conventional microscopy preparation methods (Cole et al., 1998), and here, we show the presence of a mobile tubular vacuolar system in *G. intraradices*. Tubular vacuole systems may be important in polyphosphate storage and transport in fungi (Ashford and Allaway, 2002).

Fungi typically respond to P deficiency by increasing the production of acid phosphatases (Kaffman et al., 1994) and, at least in some cases, also by increased production of alkaline phosphatases (Grotelueschen et al., 1994). The induction of phosphatase activity in fungal mycelium because of P starvation can easily be detected by both the methods used in this study, as shown for *Aspergillus fumigatus* (Van Aarle et al., 2001). However, we observed no such effect in *G. intraradices* when growing in P-free medium. This is consistent with high-P translocation ability within the mycelium, with the result that external P avail-

ability did not limit the growth of mycelium. Instead, the results indicate that C provided by the root limits mycelium growth at high-P levels. In an earlier study, however, Maldonado-Mendoza et al. (2001) observed a response of the mycelium of *G. intraradices* in a similar system that could indicate P limitation. A high-affinity P transporter gene was expressed in a medium similar to our low-P medium but was repressed at high P and in a medium without P.

Another explanation of the lack of induction of phosphatase activity in the AM fungus could be that acquisition of external organic P is not the major role of AM fungal phosphatases, although at least some easily mineralized organic P compounds can be used by AM fungi (Joner et al., 2000; Koide and Kabir, 2000). In our study, there seemed to be no special response to the applied organic P (sodium-phytate). Instead, the effect of this treatment seemed to be more related to the orthophosphate that was present in this P source because, in most cases, the response to this treatment seemed to be intermediate to responses to the low-P and the high-P treatment. The proportion of alkaline-phosphatase-active hyphae was highest after the high-P treatment, which indicates a role of this enzyme either in polyphosphate metabolism or in the degradation of P compounds in dying hyphae.

CONCLUSIONS

We propose that the response of AM fungi to high external P availability depends, to a large extent, on the influence on the P status of the colonized root. Although the hyphae experienced extreme P levels (no P or P concentration 70 times more than the initial root medium), the influence on the hyphal processes seemed rather to be an effect of the subsequent regulation of the C flow in the symbiosis. This was evident because the variation within each treatment was large, and this variation was explained by variation in root P content (Fig. 3). For an AM fungal mycelium, the sink region for P is normally the intraradical mycelium, because large quantities of P are transferred to the host plant. Here, we showed that extraradical mycelium deprived of a P source may just as well act as a P sink.

MATERIALS AND METHODS

Monoxenic AM Cultures

The AM fungus *Glomus intraradices* Schenck & Smith was grown monoxenically in mycorrhizal association with root-organ cultures of carrot (*Daucus carota*). The cultures are clones of carrot roots (line DC1) that were initially transformed using the bacterium *Agrobacterium rhizogenes* as transformation system with the T-DNA of the root-inducing plasmid transferred to the plant genome (Bécard and Fortin, 1988). AM-colonized cultures were maintained at a constant temperature of 24°C on petri dishes with 0.3% (w/v) Phytigel (Sigma-Aldrich, St. Louis) as the gelling agent and with a minimal nutrient medium (Bécard and Fortin, 1988) containing 10 g Suc L⁻¹ as the C source and 35 μM P (as 4.8 mg KH₂PO₄ L⁻¹).

Experimental Setups

Plugs of solid medium containing carrot roots and mycelium and spores of *G. intraradices* were transferred at the start of each experiment from 3- to 4-month-old cultures to the experimental two-compartment petri dishes (Maldonado-Mendoza et al., 2001). One plug was transferred to each new dish and inserted into a hole in the solid minimal nutrient medium (about 20 mL) on the root side. The cultures were sealed with Parafilm "M" (American National Can, Chicago). The second compartment of the petri dishes was at this time still empty. At the start of the experimental treatment, the empty compartment was filled with liquid minimal medium lacking Suc and with the 35 μM P replaced by a specific amount of P for each treatment (see details below). Mycelia from experiment 1 were harvested when they had covered the liquid medium compartment and stopped expanding, whereas those in experiments 2 and 3 were harvested while still under expansion.

Experiment 1

Liquid medium was added 21 d after the root side of two-compartment dishes had been inoculated. Roots passing over the barrier between the two compartments were removed at this time. Two P treatments were applied to the liquid medium, one with no P (P-free treatment) and one with 2.5 mM P as KH₂PO₄ (high-P treatment). The root side was supplied with 10 mg of [¹³C]D-Glc (U-¹³C₆, 99% [w/w] ¹³C, Cambridge Isotope Laboratories, Andover, MA) in four replicate dishes 106 d after adding the liquid medium. One dish with P-free treatment was not labeled and was kept as a control to measure the natural abundance of ¹³C in roots and AM fungal mycelium. The systems were harvested 4 d after labeling, and the systems were at that time 131 d old. Mycelium from the liquid medium was collected and freeze-dried, the dry weight was determined, and the dry mycelium was stored at -20°C until determination of ¹³C enrichment and lipid composition.

Experiment 2

Liquid medium was added to the second compartment 54 d after inoculation in the root compartment. Four treatments were applied to the liquid medium: no P added (P-free), 25 μM P as KH₂PO₄ (low-P), 2.5 mM P as KH₂PO₄ (high-P), and 2.5 mM P as inositol hexaphosphoric acid (Na salt, from corn, Sigma-Aldrich; org-P). Nutrient solutions incubated in sterile petri dishes without cultures were used as controls. One replicate from each treatment was labeled on each of 4 subsequent d. This was done to be able to harvest over 4 d and still have the same period of labeling for all four replicates. The root side was supplied with [¹³C]D-Glc (10 mg Glc dish⁻¹) 28 to 31 d after adding the liquid medium (roots close to the barrier to the hyphal side were removed twice during this period). The systems were harvested 7 d after labeling, and were at that time 89 to 92 d old. The mycelium from each dish was collected and transferred to liquid medium containing the same treatment, and subsamples of mycelium were taken for microscopical assessment of acid- and alkaline-phosphatase-active hyphal length on the same day. Another subsample of mycelium was fixed in 96% (v/v) ethanol for microscopical determination of polyphosphate accumulation. The remaining mycelium was stored at -20°C until required for freeze-drying, for determination of ¹³C enrichment, and for lipid extraction and analysis. The liquid growth medium was collected for determination of pH, orthophosphate content, and external phosphatase activity on the same day. The solid medium of the root compartment was dissolved in 250 mL of 10 mM sodium citrate by mixing on a magnetic stirrer for 1 h at low speed. The roots were collected, freeze-dried, weighed, and stored at -20°C for nutrient analysis, for determination of ¹³C enrichment, and for lipid analysis.

Experiment 3

The liquid medium was added 75 d after inoculation and included three kinds of P treatment (P-free, 35 μM P, and 350 μM P as KH₂PO₄). The 350 μM P was chosen to obtain a treatment where the P was not depleted at the time of harvest (see Table III), but avoiding a reduced proportion of hyphae with cytoplasm such as caused by the high P in experiment 2 (see Fig. 5). Mycelia were harvested 30 d after adding the liquid medium, and the amount of

orthophosphate remaining in the liquid medium was measured. The formation and morphology of the vacuolar system of extraradical hyphae were studied in three replicates each of the 35 μM P and 350 μM P treatments and 1 replicate of the P-free treatment.

Lipid Analysis

Mycelium samples were milled with iron balls (7 mm diameter) in 50-mL Teflon tubes, and roots were ball-milled in iron beakers. The lipids from mycelium and mycorrhizal roots were then extracted by vortex mixing (1 min) in a one-phase mixture of citrate buffer, methanol, and chloroform (0.8:2:1, v/v, pH 4.0; Bligh and Dyer, 1959). The lipids were fractionated into neutral lipids, glycolipids, and phospholipids on prepacked silica columns (100 mg of sorbent mass, Varian Medical Systems, Palo Alto, CA) by eluting with 1.5 mL of chloroform, 6 mL of acetone, and 1.5 mL of methanol, respectively. The fatty acid residues in neutral lipids and phospholipids were transformed into free fatty acid methyl esters and analyzed by gas chromatography using a 50-m HP5 capillary fused silica column (Hewlett Packard, Palo Alto, CA) with H_2 as carrier gas (Frostegård et al., 1993). The fatty acids were identified from their retention times in relation to that of the internal standard (fatty acid methyl ester 19:0). These were compared with those identified earlier by gas chromatography-mass spectrometry.

Determination of ^{13}C Enrichment in Solid Samples and Fatty Acids

Freeze-dried mycelium (approximately 20 μg) or ball-milled root material (approximately 100 μg) was enclosed in tin capsules, and ^{13}C atomic percent was determined on an isotope ratio mass spectrometer (20–20 Stable Isotope Analyser, PDZ Europa Scientific Instruments, Crewe, UK) interfaced to a combustion module (ANCA-NT). Fatty acid methyl esters (prepared as described above) were analyzed on the isotope ratio mass spectrometer, interfaced to a Hewlett Packard gas chromatograph to determine the ^{13}C atomic percent in NLFAs and PLFAs. The gas chromatograph was equipped with a 30-m HP5MS capillary column (Hewlett Packard) with He as carrier gas. The ^{13}C enrichment (excess atomic percent ^{13}C) was calculated by subtracting the natural abundance of ^{13}C (1.14%).

Microscopical Investigation of Hyphal Polyphosphate Accumulation and Phosphatase Activity

Subsamples of the extraradical hyphae were placed in 96% (v/v) ethanol at 5°C to precipitate polyphosphate. After 5 d, the samples were washed for 10 min in water, and then stained for 5 min in 0.05% (w/v) toluidine blue O in 25 mM sodium acetate buffer at pH 4.4 (Ashford et al., 1975). Improvement in the penetration of the stain was obtained by spreading the extraradical hyphae out in the staining solution and shaking this suspension continuously. Extraradical hyphae were then washed for 1 min in 1% (v/v) HCl to remove excess staining of compounds other than polyphosphate. The extraradical hyphae were mounted in water on a microscope slide, and a coverslip was sealed immediately with nail varnish. The polyphosphate accumulation was assessed within 2 h of mounting at 500 \times magnification. For each sample, approximately 200 hyphal intersections were assessed to determine polyphosphate accumulation, which were classified as containing polyphosphate or not containing polyphosphate. The proportion (%) of hyphal length with polyphosphate was determined for hyphae with and without cytoplasm (Fig. 6A).

Extraradical mycelium was subjected to histochemical assessment of acid phosphatase and alkaline phosphatase activity. Collected samples were incubated with a fluorogenic phosphatase substrate (ELF; Molecular Probes, Leiden, The Netherlands), buffered at either pH 4.8 or 8 (Van Aarle et al., 2001). The substrate-buffer solution was filtered through a 0.22- μm filter (Millex-GV, Millipore, Bedford, MA) before use to remove any aggregates of the substrate that may have formed during storage. The samples were mounted on microscope slides with the ELF mounting medium. For each sample, approximately 200 hyphal intersections were assessed at 300 \times magnification for ELF precipitation, which were classified as active or nonactive (see Fig. 6B). The proportion (%) of acid- and alkaline-phosphatase-active hyphal length was determined for hyphae with cytoplasm and empty hyphae.

Micrographs were recorded on Ultra Gold film (Eastman Kodak, Rochester, NY) with a microscope camera (Zeiss, Welwyn Garden City, UK), using either transmitted long-wavelength light alone or transmitted long-wavelength light combined with UV light.

External Phosphatase Activity

To measure acid phosphatase and alkaline phosphatase activity of the liquid medium, a modified procedure based on that of Tabatabai and Bremner (1969) was used. Phosphatase activities were determined spectrophotometrically using *p*-nitrophenyl phosphate as substrate (Sigma-Aldrich). Samples of 100 μL were incubated with 100 μL of *p*-nitrophenyl phosphate solution (4 mg *p*-nitrophenyl phosphate mL^{-1} in water) and 100 μL of buffer solution. Tris-HCl buffer (50 mM Tris, pH 9.0) was used for the alkaline phosphatase activity determination and citrate buffer (90 mM citrate and 10 mM chloride, pH 4.8) for the acid phosphatase activity determination. Adding 1 mL of 0.1 M NaOH after 3 h stopped the reaction, and A_{420} was measured. One unit of phosphatase activity, under the specified conditions, was defined as the amount of enzyme activity that had liberated 1 μmol of *p*-nitrophenol in 1 h. Liquid media from each kind of treatment, from control dishes without cultures, were used as controls for background A_{420} .

P and N Content in Medium and Roots

The content of orthophosphate ($\text{PO}_4\text{-P}$) in the liquid medium was analyzed spectrophotometrically using the molybdate blue method of Murphy and Riley (1962). The P content of nutrient solutions stored at -20°C from the start of the experiment, was measured to assess actual starting levels of orthophosphate. One milliliter of sample (diluted if necessary to contain less than 870 μg $\text{PO}_4\text{-P L}^{-1}$) was mixed with 200 μL of reaction mixture (H_2SO_4 , ammonium molybdate, ascorbic acid, and potassium antimony tartrate), and A_{882} was measured after 1.5 h of incubation.

Total P and N concentrations in roots were determined after Kjeldahl combustion. Freeze-dried root samples (20 mg) were heated for 1 h at 337°C in H_2SO_4 with a catalytic mix of K_2SO_4 and CuSO_4 . Ammonia and orthophosphate were analyzed with a flow injection analysis system.

Confocal Visualization of the Fungal Vacuolar System

Extraradical mycelia were stained with Oregon green 488 carboxylic acid diacetate (20 μM). The stain solution was prepared with 10 mM MES buffer at pH 5.5. Ten milliliters of the staining solution was added to the petri dish after removing the liquid medium and incubated in the dark at 20°C . After 8 h of incubation, the staining solution was removed, and the mycelium was washed three times with equivalent amounts of buffer. Part of the extraradical mycelium was carefully removed from the dish and mounted in MES buffer. Samples were immediately observed with a confocal laser scanning microscope (TCS/NT, Leica, Wetzlar, Germany). An Ar/Kr laser (488-nm excitation wavelength) was used for illumination and a 63 \times water immersion (numerical aperture 1.20, free working distance 220 μm) was used. Confocal images (resolution 512 \times 512 pixels) were captured at a green wavelength through a long-pass filter LP 495. The digital single image (Fig. 7, A and C) and the reconstructed projection (Fig. 7B) were processed using Corel Photo-Paint (Corel Corporation, Ottawa).

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