

Phosphate uptake, transport and transfer by the arbuscular mycorrhizal fungus *Glomus intraradices* is stimulated by increased carbohydrate availability

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Summary

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- The influence of carbohydrate availability to mycorrhizal roots on uptake, metabolism and translocation of phosphate (P) by the fungus was examined in axenic cultures of transformed carrot (*Daucus carota*) roots in symbiosis with *Glomus intraradices*.
- ¹⁴C-labelled carbohydrates, ³³P-phosphate and energy dispersive X-ray microanalysis were used to follow the uptake and transfer of C and P in the arbuscular mycorrhizal (AM) symbiosis.
- The uptake of P by the extraradical mycelium (ERM) and its translocation to the mycorrhizal roots was stimulated and the metabolic and spatial distribution of P within the fungus were altered in response to increased carbohydrate availability. Sucrose supply resulted in a decrease of polyphosphates and an increased incorporation into phospholipids and other growth-related P pools and also caused elevated cytoplasmic P levels in the intraradical mycelium (IRM) within the root and higher cytoplasmic P levels in the root cortex.
- These findings indicate that the uptake of P by the fungus and its transfer to the host is also stimulated by the transfer of carbon from plant to fungus across the mycorrhizal interface.

Key words: arbuscular mycorrhiza, *Glomus intraradices*, interface, nutrient exchange, phosphate, polyphosphate.

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Introduction

Arbuscular mycorrhizas are the most widespread underground symbiosis and are formed between a wide variety of plants and obligately symbiotic fungi of the phylum Glomeromycota. Arbuscular mycorrhizal (AM) fungi are known to stimulate host plant growth, mainly by enhancing soil nutrient uptake, particularly P, but also by increasing the resistance of host plants to biotic and abiotic stresses. The positive effect on P uptake has been attributed to: (i) an exploration of a larger soil volume by the extraradical mycelium (ERM); (ii) the small hyphal diameter leading to an increased P absorbing surface area and, compared to nonmycorrhizal roots, higher P influx rates

per surface unit; (iii) the formation of polyphosphates (polyP) by mycorrhizal fungi and thus lower internal inorganic P (P_i) concentrations; and (iv) the production of organic acids and phosphatases that catalyse the release of P from organic complexes (Marschner & Dell, 1994).

However, the benefit of a mycorrhizal infection for the P nutrition of a host plant is not due to higher P absorption under all supply conditions, but also to the capability to accumulate P under high external supply and to remobilize this storage pool under P stress and to maintain a continuous flux of P to the mycorrhizal host (Harley & Smith, 1983). The benefits of the host plant do not always outweigh the costs and under conditions of sufficient and supraoptimal P supply,

a mycorrhizal infection may have no positive effect on the P absorption (Amijee *et al.*, 1993) and a reduction of plant growth may be observed. This can be explained by the carbon (C) costs of a mycorrhizal infection for the host plant (Peng *et al.*, 1993). In return for the improved nutrient supply, up to 20% of the assimilated C from the plant is translocated to the fungal symbiont for the formation, maintenance and function of mycorrhizal structures (Wright *et al.*, 1998).

Since there is no direct symplastic continuity between the partners, nutrients must pass an interfacial apoplast before they can be absorbed (Peterson & Bonfante, 1994). Models of transfer processes across the mycorrhizal interface generally involve the passive efflux of P_i and carbohydrates through the fungal and plant plasma membranes into the interfacial apoplast and then the active absorption of nutrients by both partners driven by H^+ -ATPase(s) (Smith *et al.*, 1994b). Although C allocation to the roots increases during mycorrhizal association, the amounts of C estimated to leak out of intact root cells into the interfacial apoplast are thought to be insufficient for the extensive hyphal growth that is observed (for review see Harrison, 1999). In addition, the flows of P across the interface and the estimated P influx rates into plant cells have been observed to be considerably larger than the efflux from fungal hyphae in axenic cultures (Smith *et al.*, 1994a). Therefore, conditions in the interface that cause an enhanced efflux and/or a decrease in the level of competing uptake systems have been suggested (e.g. Smith *et al.*, 1994b).

Several authors have shown that a reduction of photon irradiance leads to a reduction of the mycorrhizal growth response and of the P uptake by AM systems (Hayman, 1974; Tester *et al.*, 1985; Son & Smith, 1988). It was postulated that the reduced P influx in mycorrhizal systems under photosynthetic limitation was due to a higher competition between the symbionts for carbohydrates leading to lower colonization rates of roots (Son & Smith, 1988) and to a decrease in the number of functional arbuscules (Hayman, 1974).

Presently we know very little about the regulation of exchange processes occurring in a mycorrhiza and the mechanisms involved in polarizing the transfers. Woolhouse (1975) and Schwab *et al.* (1991) suggested that the exchange processes may be coupled by carbohydrate-phosphate transporters, similar to those located in the chloroplast envelope. Based on molecular techniques it has been proposed, however, that at the plant–fungus interface separate membrane transport systems operate for the uptake of P and carbohydrates (Harrison, 1999; Nehls *et al.*, 2001; Rausch *et al.*, 2001).

Since the exchange of C for P is central to the AM symbiosis, our lack of knowledge of its regulation limits our physiological and ecological insight into the most abundant and most important mutualistic symbiosis of land plants. The goal of this study was therefore to determine whether the carbohydrate availability regulates the uptake of P by an AM fungus and its transfer to mycorrhizal roots.

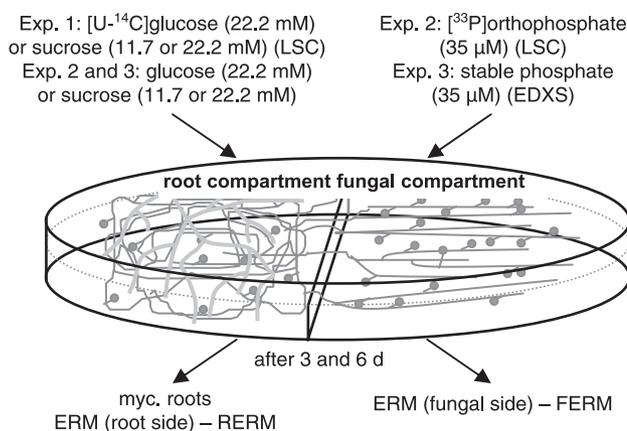


Fig. 1 Experimental design. EDXS, energy-dispersive X-ray spectroscopy; exp, experiment; ERM, extraradical mycelium; FERM, ERM from the fungal compartment; LSC, liquid scintillation counting; myc, mycorrhizal; RERM, ERM from the root compartment.

Materials and Methods

Culture of plant and fungal material

Ri T-DNA-transformed carrot (*Daucus carota* clone DCI) roots colonized by *Glomus intraradices* Schenck & Smith (DAOM 197198, Biosystematics Research Center, Ottawa, Canada) were grown in Petri plates with two compartments as previously described (St-Arnaud *et al.*, 1996; Pfeffer *et al.*, 1999). The mycorrhizal roots were confined to one compartment (root compartment) filled with 25 ml solidified medium containing mineral nutrients (M medium) (Chabot *et al.*, 1992) containing 10 g l^{-1} sucrose. The extraradical mycelium of the fungus (ERM) was allowed to grow over the divider into the fungal compartment, which was filled with 25 ml solidified M medium without sucrose addition (Fig. 1). After 6 wk of root growth and colonization on the plates and a further 2 wk for cross over of the ERM into the fungal compartment, the plates were ready for the experiments. Based on the ERM development (assessed stereomicroscopically) the plates were separated into four groups according to the extent of ERM development in the fungal compartment. The plates were then distributed equally among the different experimental treatments so that each treatment had the same variability in the amounts of fungal biomass in the fungal compartment.

Sucrose and phosphate analysis

To measure the sucrose and phosphate residue of the medium after 8 wk of mycorrhizal root growth on the plates, the medium from the root compartment of five plates was removed and solubilized in 20 ml Na citrate buffer (10 mM, pH 6). After centrifugation aliquots of the liquid medium were taken and the sucrose concentration was analysed using the sucrose assay kit by Sigma-Aldrich (St. Louis, USA). The phosphate concentration was colorimetrically determined by adding the

ammonium molybdate-vanadate mixture by Ricca Chemicals (Arlington, USA). After 8 wk $15.4\% \pm 2.3$ or $11.5\% \pm 1.0$ (mean \pm SE, $n = 5$) of the initial phosphate ($35 \mu\text{M}$) and sucrose concentration (10 g l^{-1}), respectively, was detectable in the medium of the root compartment.

Growth response

To examine the effect of various carbohydrate additions on growth of Ri T-DNA-transformed carrot roots, mycorrhizal roots previously cultured for 8 wk on plates with solidified M medium (Chabot *et al.*, 1992) were carefully removed, weighed and transferred into plates filled with liquid M medium containing 15% of the standard nutrient concentration and 11.5% of the standard sucrose concentration (1.15 g l^{-1}) ($n = 10$). To these plates H_2O (control), 22.2 mM glucose, 11.7 mM or 22.2 mM sucrose was added. After 3 and 6 d the roots were harvested and the f. wt and d. wt of these roots was determined. The initial d. wt of these roots was estimated based on a f. wt to d. wt ratio of previously harvested comparable roots ($n = 10$).

Experimental design

The uptake of carbohydrates by mycorrhizal roots and the transport of ^{14}C -labelled compounds from the root to the fungal compartment was examined by adding [^{14}C]glucose or [^{14}C]sucrose (Sigma-Aldrich, St. Louis, USA) to the root compartment (experiment 1 in Fig. 1). The final concentration added to the root compartment was 22.2 mM glucose (1 : 50 000, v/v as [^{14}C]glucose with a specific activity of 245 mCi mm^{-1}) or 11.7 mM or 22.2 mM sucrose (1 : 75 000 or 1 : 120 000, v/v, respectively, as [^{14}C]sucrose with a specific activity of 643 mCi mm^{-1}). After 3 and 6 d eight plates of each treatment were harvested and of each plate two root aliquots and the ERM samples ($n = 8$, see Harvest of medium and ERM samples) were collected and prepared for liquid scintillation counting.

P uptake by the ERM and the transfer to the host root was examined under various carbohydrate supply conditions (experiment 2 in Fig. 1). Simultaneously, the mycorrhizal roots were supplied with 22.2 mM glucose, 11.7 mM or 22.2 mM sucrose (H_2O in the controls) and $1.4 \mu\text{M}$ phosphate (as KH_2PO_4 , 1 : 100 000, v/v as [^{33}P]orthophosphate) was added to the fungal compartment. After 3 and 6 d eight plates of each treatment were harvested and of each plate three root aliquots were taken. The ERM samples ($n = 8$) were harvested as described in Harvest of medium and ERM samples and prepared for liquid scintillation counting ($n = 5$) or phosphate fractionation followed by liquid scintillation counting ($n = 3$).

Additionally, the P distribution in the interface region of mycorrhizal roots dependent on different carbohydrate supply conditions was examined with energy-dispersive X-ray spectroscopy (EDXS). For this purpose, instead of the [^{33}P]orthophosphate added in experiment 2, $1.4 \mu\text{M}$ of nonlabelled KH_2PO_4 was added to the fungal compart-

ment (conditions on the root side were as described above, experiment 3 in Fig. 1). After 6 d mycorrhizal roots were harvested, cryofixed in subcooled nitrogen and the subcellular element distribution in these roots was then analysed by EDXS.

Harvest of medium and ERM samples

To harvest ERM samples from both sides of the plates, the medium was transferred into centrifuge tubes and solubilized in 20 ml Na citrate buffer (10 mM, pH 6.0). The ERM samples from the fungal compartment (FERM) were collected after several washes (in buffer) and centrifugation steps. Since a complete removal of the mycorrhizal roots from the root compartment was not possible, the ERM samples from this side (RERM) were collected by hand under a stereomicroscope. After harvest the ERM samples were dried in an oven (70°C) and prepared for liquid scintillation counting. An aliquot of the dissolved medium was taken to determine the ^{14}C or ^{33}P residue within the medium.

Extraction of various phosphate pools

The phosphate fractionation of the ERM ($n = 3$) was carried out according to the method described by Aitchison & Butt (1973). The following phosphate fractions were differentiated: (i) phosphates with a low molecular weight and acid soluble polyP after extraction with 10% TCA (w/v) at 4°C (two times); (ii) phospholipids after extraction with first 100% ethanol (first step) and then ethanol:ether (3 : 1, v/v); (iii) acid insoluble polyP after extraction with 1 M KOH at room temperature (two times); and (iv) DNA-, RNA- and protein-phosphates (residue). Acid soluble (short chain length) and acid insoluble polyP (long chain length) within the supernatants were precipitated by BaCl_2 (saturated) overnight at 4°C . The ^{33}P content in all fractions was determined by liquid scintillation counting.

Liquid scintillation counting

The samples were dried in an oven at 70°C , weighed and digested with a tissue solubilizer (TS-2, rpi corp., Mount Prospect, USA) or measured directly without solubilization (phosphate fractions, medium samples). After digestion the samples were suspended in a scintillation cocktail (Biosafe II, rpi corp., Mount Prospect, USA). The radioactivity was determined by liquid scintillation counting (LS 6500, Beckman Coulter, Fullerton, USA) with a correction of the counting accuracy by use of an internal standard.

Energy dispersive X-ray spectroscopy (EDXS)

The root samples were cryofixed by plunging in subcooled nitrogen and were slowly cryo-substituted with 100% ether (dried with molecular sieve) successively at -80°C for 4 wk, at -20°C for 1 wk and at room temperature for 1 d with regular changes of the substitution medium. The samples

were then embedded in Spurr's epoxy resin (Spurr, 1969) by transfer to progressively higher levels of resin in ether (1 : 4, 2 : 1, 4 : 1 v/v). After polymerization of the resin, dry sections of 500 nm were made with glass knives and transferred to filmed (Pioloform) folding Cu-grids and carbon coated (20 nm). Cryo-substitution has been shown to minimize losses and dislocations of diffusible elements (Kuhn *et al.*, 2000).

The X-ray microanalytical studies were carried out under standardized conditions using an electron microscope Philips CM 30 (Eindhoven, the Netherlands) equipped with the DX4 system of EDAX (Taanusstein-Neuhof, Germany). The spectra were collected between 0 and 20 keV for 50 live seconds with a Si(Li) X-ray detector with a thin beryllium window and an acceleration voltage of 300 kV. The element distribution is documented as a peak to background ratio (P/B) in order to diminish the effects of surface irregularities of the sections during analysis. Out of the harvested roots, three were dry cut and within each five spectra of every cellular compartment of different randomly chosen cells were analysed ($n = 15$).

The following subcellular compartments were analysed in the interface region of the mycorrhizal roots: cytoplasm and vacuole of arbuscular hyphae, interface between arbuscular hyphae and cortical cells, cytoplasm and vacuole of cortical cells, and the apoplast between cortical cells.

Statistical treatment

In the figures mean values and vertical bars representing SE (Figs 2, 3, 4 and 6) or data ranges (Fig. 5) are shown. The results are only discussed if a statistically significant difference ($P \leq 0.05$) between the treatments was found by ANOVA and Fisher's LSD test (Unistat Software, London, UK). In Fig. 5 the median ($n = 3$) and data range is shown, since the median is an unbiased estimator of the population mean for normal distributions and is more resistant to outliers when a smaller number of biological replicates is used (Quinn & Keough, 2003). In this case results are only discussed if no overlap of the range of values was observed (phosphate fractions; significance at $P = 0.0495$ according to the Kruskal–Wallis test followed by u -test; Unistat Software). Significance is indicated in figures and tables by different letters.

Results

Uptake and transfer of carbohydrates by mycorrhizal carrot roots

Mycorrhizal roots took up carbohydrates, with the uptake of sucrose being much faster than that of glucose. After 3 d $33.2 \pm 3.9\%$ or $39.0 \pm 1.2\%$ of the supplied sucrose had been absorbed depending on the concentration provided, as compared to $10.2 \pm 3.4\%$ of the glucose (means \pm SE,

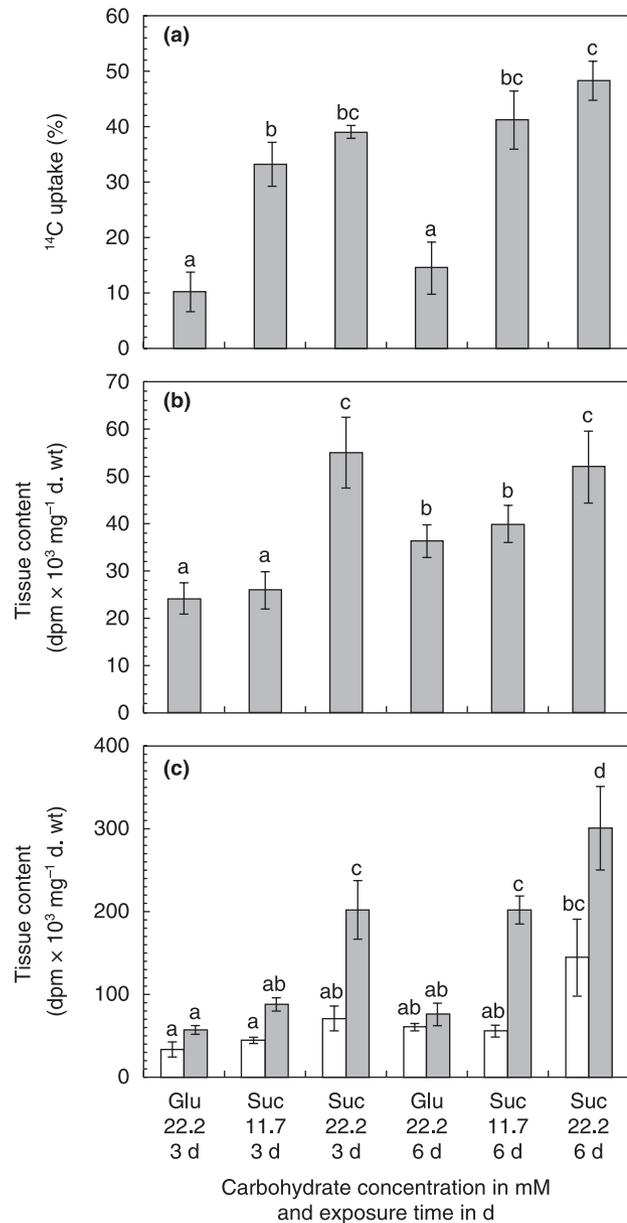


Fig. 2 Uptake of the ^{14}C -labelled carbohydrates from the medium (a) and content of ^{14}C -labelled compounds in roots (b) and the extraradical mycelium (ERM) (c) in the root compartment (RERM, open bars) and in the fungal compartment (FERM, grey bars) as affected by the carbohydrate supply in the root compartment and the application time (experiment 1). Bars represent mean \pm SE ($n = 8$). Glc, glucose; Suc, sucrose. Statistically significant differences ($P \leq 0.05$, ANOVA followed by Fisher's LSD test) are indicated by different letters above the bars.

Fig. 2a). Supplying 22.2 mM sucrose led to higher ^{14}C contents in the mycorrhizal roots than when an equimolar concentration of glucose or a lower sucrose concentration was provided (Fig. 2b). Between 3 and 6 d after supplying the higher sucrose concentration the ^{14}C content remained the same on a per tissue d. wt basis, whereas when glucose or

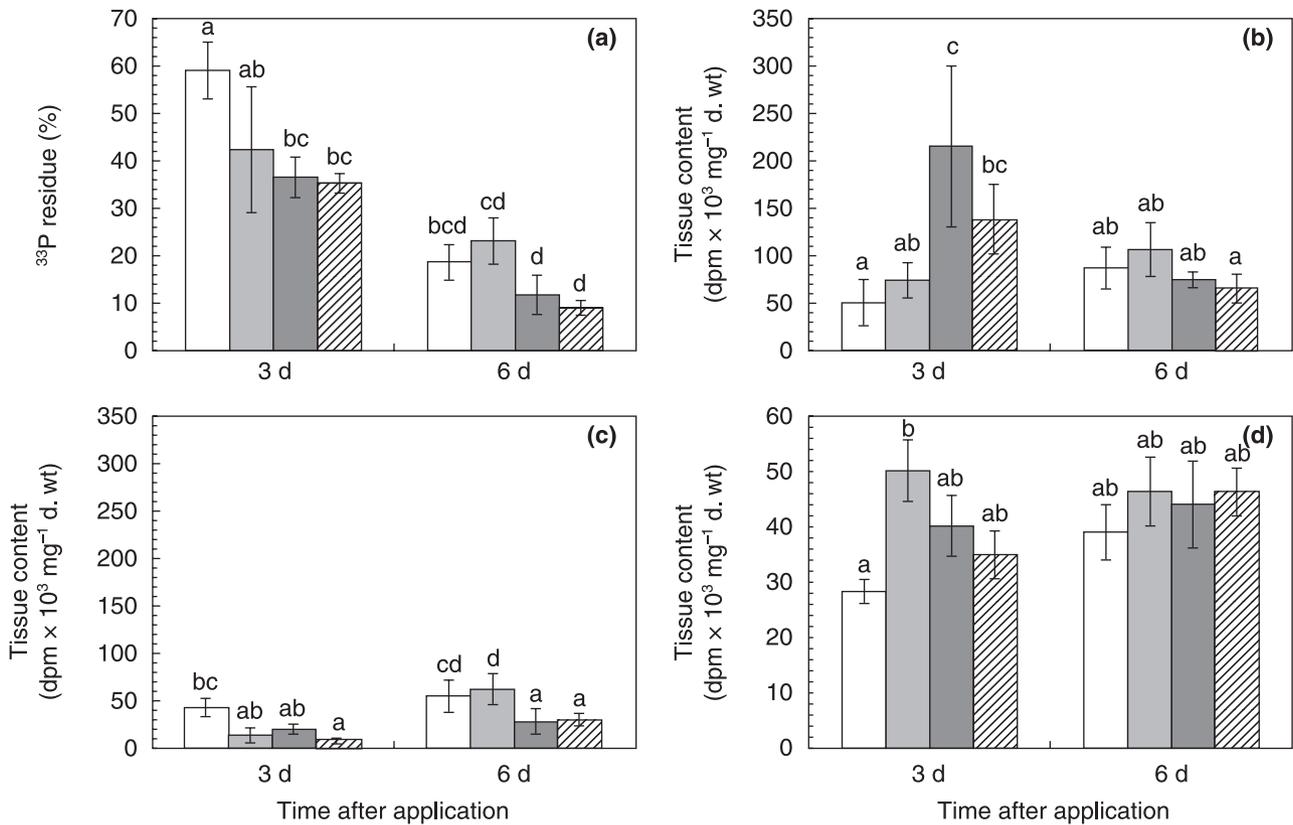


Fig. 3 Proportion of the ³³P supplied remaining in the medium (a) and tissue contents of the extraradical mycelium (ERM) in the fungal compartment (FERM) (b), and in the root compartment (RERM) (c), and roots (d) as affected by the carbohydrate supply added to the root side and the application time (experiment 2; control, open bars; 22.2 mM glucose, light grey bars; 11.7 mM sucrose, dark grey bars; 22.2 mM sucrose, hatched bars). Bars represent mean ± SE (a, n = 8; b and c, n = 5; d, n = 8 (× 3)). Statistically significant differences (P ≤ 0.05, ANOVA followed by Fisher's LSD test) within one figure are indicated by different letters above the bars.

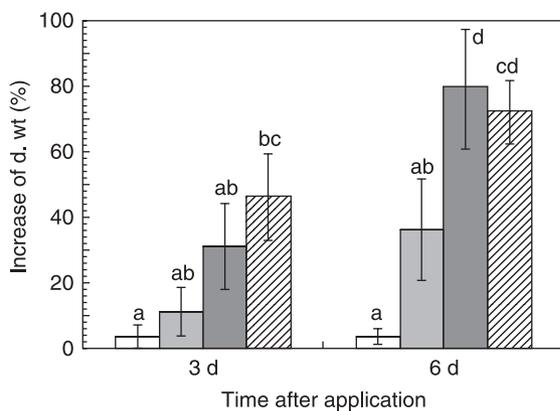


Fig. 4 Growth response of mycorrhizal roots under various carbohydrate supply conditions (experiment 4; control, open bars; 22.2 mM glucose, light grey bars; 11.7 mM sucrose, dark grey bars; 22.2 mM sucrose, hatched bars). Bars represent mean ± SE (n = 10). Statistically significant differences (P ≤ 0.05, ANOVA followed by Fisher's LSD test) are indicated by different letters on the bars.

the lower sucrose concentration were added, the ¹⁴C content continued to increase between 3 and 6 d.

The greater uptake of sucrose by mycorrhizal roots was also associated with a higher flux of ¹⁴C from the mycorrhizal roots to the ERM of the fungal symbiotic partner. Three days after adding 11.7 mM or 22.2 mM sucrose, 0.28% or 0.36%, respectively, of the radioactivity supplied was detectable in the ERM of the fungal compartment (FERM), but after glucose addition only 0.07% was exported to the FERM (means). The increased ¹⁴C transfer to the fungal symbiont after sucrose addition led to significant higher tissue contents in the ERM, especially in the fungal compartment (Fig. 2c). After sucrose supply the ¹⁴C contents of the FERM were up to four times higher than after glucose addition. Between 3 and 6 d there was a continued increase of the ¹⁴C content per d. wt in the FERM after sucrose supply, but not after glucose addition. The ¹⁴C contents in the ERM of the root compartment (RERM) were similar for the different carbohydrate additions and remained the same between 3 and 6 d after addition except for a slight increase at the higher sucrose level. After sucrose supply, the ¹⁴C levels were significantly lower in the RERM than in the FERM.

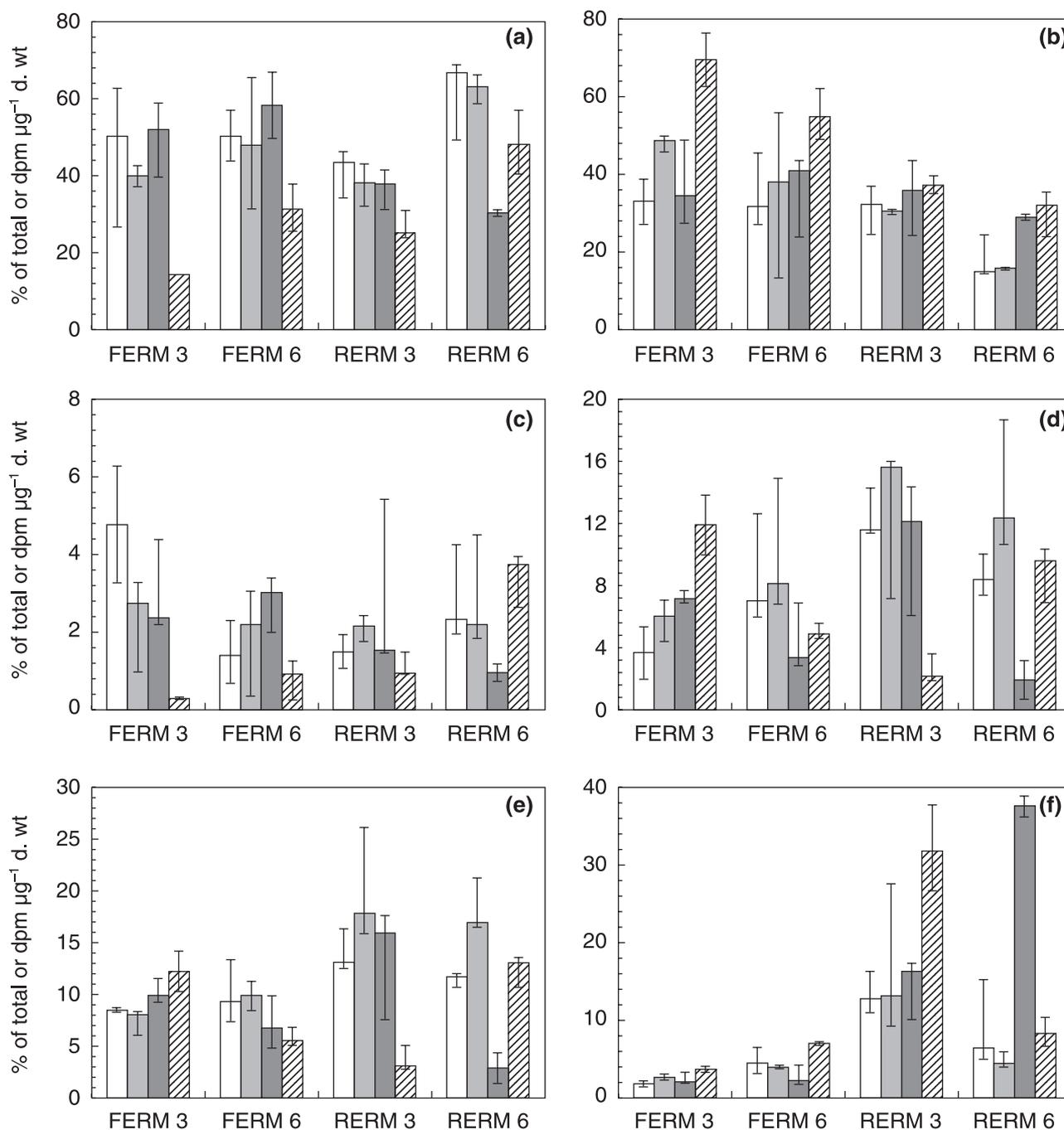


Fig. 5 Phosphate fractions (in percentage of total absorbed P) in the extraradical mycelium (ERM) in the fungal compartment (FERM) and in the root compartment (RERM) as affected by the carbohydrate supply to the host roots (experiment 2; control, open bars; 22.2 mM glucose, light grey bars; 11.7 mM sucrose, dark grey bars; 22.2 mM sucrose, hatched bars). In the figure median ($n = 3$) and range of the data (vertical bars) are given (see also the Material and Methods section). If there is no overlap of the range, this is significant at $P = 0.0495$ according to Kruskal–Wallis test and u -test. (a) Low molecular weight phosphate (b) phospholipids (c) acid soluble polyP (d) acid insoluble polyP (e) total polyP pool (f) residue (nucleic acid and protein-phosphates).

Uptake and transfer of ^{33}P as affected by the carbohydrate supply

The uptake of P by the extraradical hyphae of *G. intraradices* and its transfer and distribution between the various tissues

were affected by the carbohydrate levels supplied to the roots. ^{33}P uptake by the hyphae from the fungal compartment was stimulated by supplying sucrose to the root compartment (Fig. 3a). Three days after adding 11.7 mM or 22.2 mM sucrose to the root compartment a mean of 63.4% or

Table 1 Quotient between the ^{33}P contents ($\text{dpm mg}^{-1} \text{ d. wt}$) in various tissues as affected by the carbohydrate supply to the root compartment (experiment 2)

Treatment	Time (d)	Ratio		
		RERM/FERM	RERM + Root/FERM	Root/RERM
Control	3	1.49 ± 0.75a	2.49 ± 0.6a	0.76 ± 0.17a
	6	0.71 ± 0.32a	1.22 ± 0.18ab	0.80 ± 0.24a
22.2 mM Glucose	3	0.16 ± 0.08a	0.56 ± 0.12b	3.85 ± 1.55b
	6	0.89 ± 0.4a	1.42 ± 0.35ab	0.51 ± 0.10a
11.7 mM Sucrose	3	0.14 ± 0.07a	0.42 ± 0.08b	2.50 ± 0.97abc
	6	0.38 ± 0.17a	0.96 ± 0.25ab	2.23 ± 1.00ab
22.2 mM Sucrose	3	0.09 ± 0.04b	0.32 ± 0.06b	5.23 ± 2.42c
	6	0.53 ± 0.24a	1.52 ± 0.18ab	2.04 ± 0.36ab

Values are means ± SE ($n = 5$). Statistically significant differences ($P \leq 0.05$) within one column are indicated by different letters.

FERM, extraradical mycelium (ERM) samples from the fungal compartment; RERM, ERM samples from the root compartment.

64.7% of the ^{33}P supplied, respectively, was taken up by the FERM, compared with 40.9% under control conditions – this represents a stimulation of uptake by over 50%. The ^{33}P contents in the FERM after 3 d were up to three times higher after supply of sucrose than after water or glucose addition. After 6 d no differences in the ^{33}P content in the FERM between the various treatments were observed (Fig. 3b). In contrast to the effect on levels in the FERM, the ^{33}P contents of the RERM were significantly reduced by supplying sucrose to the root compartment (Fig. 3c). By contrast, the ^{33}P contents in aliquots of mycorrhizal roots calculated on a d. wt basis were unaffected by sucrose addition (Fig. 3d) and only roots supplied with glucose for 3 d showed significantly higher ^{33}P contents in their tissues than control roots.

Figure 4 shows the growth response of mycorrhizal roots under various carbohydrate supply conditions. Control roots were cultured on medium containing the level of sucrose measured in the root compartment of plates at the start of the experimental treatments, which was 11.5% of the initial sucrose content of the medium. These controls showed no significant root growth after 6 d. In contrast, the addition of carbohydrates, particularly sucrose, resulted in substantial root growth. Taking this root growth response into account the total amount of ^{33}P translocated from the FERM to the mycorrhizal roots was higher after sucrose supply than after control or glucose treatment.

The greater transfer of ^{33}P from the fungal to the root compartment after supplying carbohydrates can also be seen in the relative allocation of ^{33}P to different tissues (Table 1). In the controls the ratio of the ^{33}P contents between RERM and FERM decreased from day 3 to day 6 as did the ratio between the RERM + root and the FERM; so that after 6 d a higher proportion of ^{33}P was kept in the FERM and was not transferred to the root compartment. By contrast, in all carbohydrate treatments these ratios increased from day 3 to day 6. Additionally, the low ratio of RERM to root contents demonstrated that under control conditions a relatively high pro-

portion of the ^{33}P transferred from the FERM to the root compartment was retained in the RERM, whereas when sucrose was provided a higher proportion of ^{33}P is translocated from the RERM to the mycorrhizal root.

The distribution of P among fungal metabolic pools in the ERM as a function of the carbohydrate supply to the root system

Adding 22.2 mM sucrose to the root compartment led to a decrease in the proportion of ^{33}P incorporated into the soluble low molecular weight P pool in both FERM and RERM (Fig. 5a), but to an increased incorporation of ^{33}P into phospholipids (Fig. 5b) and into the pool containing nucleic acids and protein-phosphates (Fig. 5f). The increase of these pools, especially in the FERM, indicates a higher allocation of P into growth related P pools. Phospholipids are an important component of membranes and greater spore production might explain the higher incorporation of ^{33}P into nucleic acids and protein-phosphates.

Three days after supplying 22.2 mM sucrose to the root compartment the acid soluble polyP pool (containing shorter chain length polyphosphates) contained substantially less of the P taken up by the FERM than after control conditions, but no differences were found in the RERM (Fig. 5c). In contrast, the incorporation of ^{33}P into the acid insoluble polyP pool (containing polyP with a longer chain length) was increased in the FERM by the high sucrose supply after 3 d, but reduced in the RERM (Fig. 5d). After 6 d the incorporation into this pool in the FERM was lowered by the sucrose treatment. Supplying sucrose led to an initial increase of total polyP in the FERM after 3 d, but after 6 d the content in the FERM was reduced (Fig. 5e). Whereas in the FERM after control and glucose treatment, a mean of 10.1 or 11.9% of the radioactivity, respectively, was incorporated into polyP, the allocation into this pool was significantly lower after the high sucrose treatment (5.9% for 22.2 mM sucrose). After control

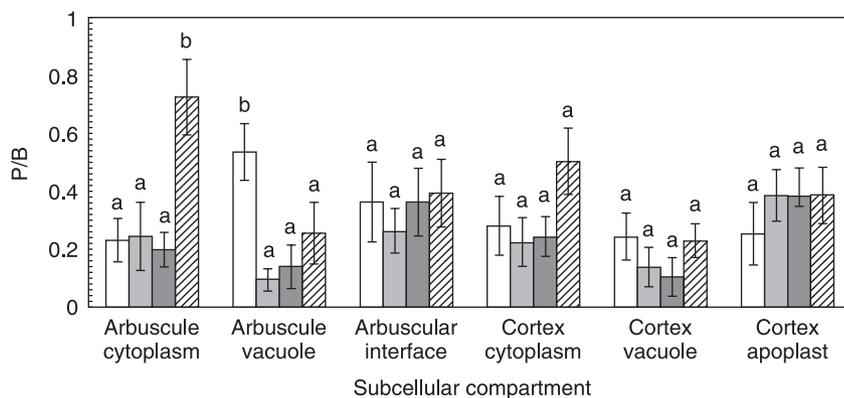


Fig. 6 The elemental phosphorus content as peak to background ratio (P/B) in different subcellular compartments of mycorrhizal roots as a function of the carbohydrate supply to the root compartment (experiment 3; control, open bars; 22.2 mM glucose, light grey bars; 11.7 mM sucrose, dark grey bars; 22.2 mM sucrose, hatched bars). Bars represent mean \pm SE (of 15 cells; $n = 15$). Statistically significant differences ($P \leq 0.05$, ANOVA followed by Fisher's LSD test) within one subcellular compartment are indicated by different letters on the bars.

or glucose treatment a higher ^{33}P incorporation into polyP was found in the RERM than in the FERM (14.4%; mean after 3 and 6 d). In the RERM the sucrose supply (11.7 mM sucrose for 6 d or 22.2 mM sucrose for 3 d) caused a reduction of approximately 75% in the proportion of P incorporated into polyP.

P was not equally distributed among various P pools in FERM and RERM. The low molecular weight soluble P pool was the same in FERM and RERM (Fig. 5a), whereas higher proportions of phospholipids were generally found in the FERM than in the RERM (Fig. 5b). In contrast, the proportion of ^{33}P in the fraction containing nucleic acids and protein-phosphates was higher in the RERM (Fig. 5f).

After 6 d the proportion of low molecular weight soluble phosphates in the RERM was generally higher than after 3 d (Fig. 5a), whereas the proportion of phospholipids and nucleic acids and protein-phosphates was reduced (Fig. 5b,f). In contrast, in the FERM an increase of the nucleic acid and protein-phosphate pool after 6 d was found (Fig. 5f). In the FERM the effect of the application time on P distribution was affected by the carbohydrate supply. An extension of the application time from 3 to 6 d after control or glucose treatment led in the FERM to an increase of the acid insoluble polyP pool, but after sucrose treatment this pool decreased (Fig. 5d). The pools of low molecular weight soluble phosphates (Fig. 5a) and phospholipids (Fig. 5b) in the FERM after control, glucose or low sucrose treatment were not affected by the application time, but an increase of low molecular weight soluble phosphates and a decrease of phospholipids after supply of the high sucrose concentration were observed.

Effect of carbohydrate supply on P distribution in fungal and plant cells within mycorrhizal roots

The distribution of P among different fungal subcellular compartments was significantly altered in response to increased carbohydrate supply (Fig. 6). When no carbohydrates were supplied, and the transfer of C from the host root to the mycorrhizal fungus can be assumed to be low, the P content in the fungal vacuoles was higher than in the cytoplasm of the

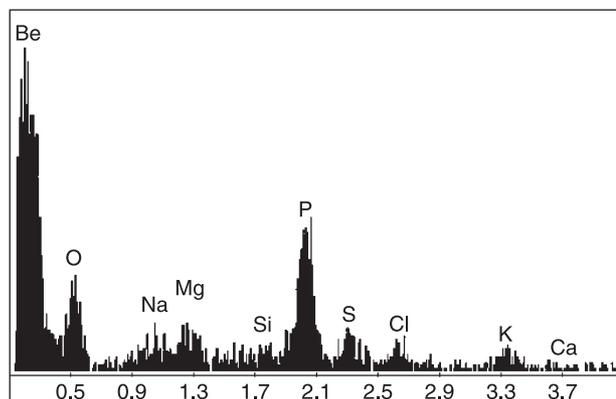


Fig. 7 Energy-dispersive X-ray microanalytical spectrum (between 0 and 4 keV) of a typical polyphosphate granule located in the vacuole of an arbuscular hyphae after control treatment (experiment 3). The spectrum shows element-specific peaks of Be ($K\alpha$ 0.11 keV), O ($K\alpha$ 0.52 keV), Na ($K\alpha$ 1.04 keV), Mg ($K\alpha$ 1.25 keV), Si ($K\alpha$ 1.74 keV), P ($K\alpha$ 2.01 keV), S ($K\alpha$ 2.31 keV), Cl ($K\alpha$ 2.62 keV), K ($K\alpha$ 3.31 keV). The Be and Si peaks are caused by the detector system, the Cl peak is due to the epoxy resin used.

arbuscular hyphae. An increased carbohydrate supply to the mycorrhizal roots and the transfer of C from the host root to the fungus (Fig. 2c) was accompanied by a significant reduction in the fungal vacuolar P levels and, in the case of supplying 22.2 mM sucrose by an increase of the cytoplasmic P levels of the arbuscular hyphae (Fig. 6). The P levels in the cytoplasm of cortical cells also tended to increase after supplying the higher sucrose concentration to the root compartment (significant at $P \leq 0.05$ using the nonparametric u -test, but not for Fisher's LSD test, see Material and Methods). Bearing in mind the substantial stimulation of root growth by sucrose (Fig. 4), the total plant cell contents increased following sucrose supply.

The energy-dispersive X-ray microanalysis of electron-opaque granules within the vacuole of arbuscular hyphae revealed high P contents and elements such as Na, Mg and K (Fig. 7). Ca was also detected in some granules. Signals from the elements Be, Si and Cl (Fig. 7), were due to the detector system used (Be and Si) and to the epoxy resin in which samples were embedded (Cl). Significant contents of the

Table 2 Element contents (peak to background ratio, P/B) in the vacuole of arbuscular hyphae within mycorrhizal roots dependent on the carbohydrate supply added to the root compartment (experiment 3)

Treatment	Element contents in P/B			
	Na	Mg	K	Ca
Control	0.71 ± 0.17a	0.84 ± 0.22a	2.03 ± 0.78ab	1.09 ± 0.57a
22.2 mM Glucose	0.34 ± 0.23a	0.49 ± 0.32a	3.07 ± 1.50a	0.72 ± 0.17a
11.7 mM Sucrose	0.27 ± 0.15b	0.53 ± 0.21a	1.37 ± 0.54b	0.63 ± 0.22a
22.2 mM Sucrose	0.52 ± 0.08a	0.38 ± 0.15a	1.01 ± 0.41b	0.39 ± 0.10b

Values are mean (of 15 cells; $n = 15$) ± SE.

cations Na, Mg, K and Ca were also detectable in the electron lucent parts within the vacuole of the arbuscular hyphae and after supplying carbohydrates to the root compartment these element levels tended to be lower than in control roots (Table 2).

Discussion

The *in vitro* mycorrhizal system and its suitability for these investigations

The *in vitro* AM split plate culture system (St-Arnaud *et al.*, 1996) allows control of the forms and quantities of C sources and mineral nutrients, complete separation of the medium surrounding mycorrhizal roots and fungal ERM, exclusion of other microorganisms, standardization of the amounts of ERM present, and complete but separate harvest of different tissues. It has therefore become the system of choice for labelling and molecular studies of C metabolism (e.g. Pfeffer *et al.*, 1999; Lammers *et al.*, 2001; Bago *et al.*, 2002), of uptake and transfer of P and N to the host roots (Hawkins *et al.*, 2000; Nielsen *et al.*, 2002), and the uptake of heavy metals by an AM symbiosis (Rufyikiri *et al.*, 2002). For these investigations this system was particularly chosen because it allowed control over quantities of carbohydrates supplied to the roots and thereby also to the fungus without dramatically changing the physiology of a whole plant by shading or dark treatment. However, potential differences in mutual regulation by the host and fungal partners will make it desirable to investigate the interaction of P and C nutrition further in monoxenic whole plant split-chamber systems when they become available.

The effect of carbohydrate on uptake and export of carbon to the fungus and its growth

Interestingly, the uptake of carbohydrates by the mycorrhizal roots and the C export from the mycorrhizal root compartment to the FERM was more stimulated by sucrose than by glucose. It is known that mycorrhizal fungi are unable to use sucrose as a C source (Solaiman & Saito, 1997) and that in mycorrhizas sucrose has to be hydrolysed by an acid invertase of plant origin and the hexoses resulting are absorbed by

the mycorrhizal fungus (Schubert *et al.*, 2004). On the other hand, glucose supplied exogenously to mycorrhizal roots can also reach the fungus (this study and Shachar-Hill *et al.*, 1995) and can be taken up by the intraradical mycelium (IRM) but not by the ERM (Pfeffer *et al.*, 1999). This shows that the uptake of sucrose by mycorrhizal roots and its transfer to the fungal symbiont was faster and higher than the uptake of glucose, which can be taken up directly by the IRM from the apoplast of mycorrhizal roots. Sucrose is the main carbohydrate translocated in higher plants, and transformed carrot roots are able to translocate it along their axis (Hernandez-Sebastian, unpublished results). These observations suggest that the higher uptake of sucrose by the mycorrhizal roots may be associated with a high transfer of sucrose within the root system to the mycorrhizal root parts acting as sinks, and with active hydrolysis of the sucrose at the sites of fungal colonization (Blee & Anderson, 2002). In addition, since plant root cells also take up hexose there may be competition for its uptake between plant and fungus after glucose supply (Harrison, 1996). On the other hand the higher uptake of sucrose by colonized roots may be due to the fact that the roots are cultured with sucrose as the carbon source (St-Arnaud *et al.*, 1996) and may therefore be preferentially adapted to this substrate.

The stimulation of P incorporation into growth related fungal P pools (phospholipids and nucleic acids and protein-phosphates) indicates that supplying sucrose promotes fungal development. However, no significant effects on the d. wt of the FERM were found, probably because the variability in the amount of FERM was larger than any fungal growth during the 3- and 6-d treatment. Douds (2002) reported that spore production in the fungal compartment can be increased by replenishing carbohydrates in the root compartment. This is consistent with several reports that AM fungal growth and mycorrhizal colonization are affected by altered light conditions that change the C availability for the fungus (Hayman, 1974; Son & Smith, 1988; Smith & Gianinazzi-Pearson, 1990). However, it is likely that in the present study the mycorrhizal colonization of the roots was not dramatically altered by the C availability for the fungus since root colonization was already established when the various carbohydrate sources were added and the increased carbohydrate supply was for 3 or 6 d, which is short compared to changes in colonization

under these conditions. Thus the effects of carbohydrate supply on P handling (see The effect of C availability on P uptake and transfer within the symbiosis) are likely to have been due to regulation of metabolic and transport processes with a modest or negligible contribution from increased fungal biomass.

The effect of C availability on P uptake and transfer within the symbiosis

Increasing the availability of C to the mycorrhizal fungus by supplying sucrose to the AM roots was associated with: (i) an increase in the ^{33}P uptake by the FERM; (ii) reduced ^{33}P levels within the RERM; and (iii) a stimulation of ^{33}P transfer from the ERM to the mycorrhizal roots. The root growth was clearly stimulated by supplying sucrose to the root compartment. Thus although the P content per tissue d. wt was not significantly increased by the sucrose supply, the total ^{33}P transferred from the ERM to the mycorrhizal roots was significantly stimulated by supplying sucrose. Several studies have reported that when the photosynthetic activity of a host plant is reduced, P uptake by AM decreased (e.g. Tester *et al.*, 1985; Son & Smith, 1988; Smith & Gianinazzi-Pearson, 1990). Son & Smith (1988) suggested that this effect may be due to a reduction of the colonization rate of the root system, leading to a lower contribution of the hyphal pathway to uptake. A reduction of fungal activity as a result of competition between the symbionts for C supply, with the allocation of photosynthate to fungal growth rather than to nutrient transport processes, could also be an explanation for this effect (Son & Smith, 1988). In the present experiments the former effect is unlikely to have had a major effect (see The effect of C availability on P allocation within the ERM) and although the uptake of ^{33}P by the fungus was lower when less carbohydrate was available to the fungus this was not the only or even the dominant effect on P handling observed – the relative allocation of P both spatially and metabolically was much altered.

The P demand of the host plant has been thought to regulate P uptake by the fungus (Thomson *et al.*, 1990). Regulation by the host would ensure that the mycorrhizal root absorbs P with the greatest rate when the plant is P limited. According to this hypothesis, the observation in this study that the addition of carbohydrates increased P uptake and translocation by the fungus would be explained by a stimulation of root growth and thereby an increased P demand of the host plant. However, this does not seem to have been the only regulatory mechanism since for example glucose supply had an effect on root growth after 6 d but did not stimulate P uptake by the FERM at that time. Also opposing this idea is the report that in ECM associations, even if the plant is P deficient, the P uptake and transfer by the fungus to its host plant is reduced when the transfer of carbohydrates to the fungal symbiotic partner is low (Bücking & Heyser, 2003). Thus we

conclude that C supply to the fungus is more influential than P demand by the host in P uptake and translocation by the fungus.

The effect of C availability on P allocation within the ERM

P_i absorbed by the ERM can: (i) replenish the cytoplasmic, metabolically active P_i pool; (ii) be incorporated into phospholipids, RNA-, DNA- and protein-phosphates; (iii) be transferred to the P releasing hyphae within the symbiosis in the form of short chain polyP; or (iv) be accumulated in vacuoles as short- or long-chained polyP (Beever & Burns, 1980; Bücking & Heyser, 1999). In the FERM of *G. intraradices* low molecular weight soluble phosphates and phospholipids represented the most abundant ^{33}P pools with 53.5% and 34%, respectively (mean of controls after 3 and 6 d). The proportions of the other P pools that were distinguished: short-chained polyP (3.2%), long chained polyP (6.2%) and RNA-, DNA- and protein-phosphates (3.3%) were much lower. The contribution of polyP to the total P was in the same range as reported for the AM fungus *Gigaspora margarita* by Solaiman *et al.* (1999). The functions of polyP are believed to be: P storage, regulation of the levels of ATP and other nucleoside triphosphates, homeostasis of cations, P transport through fungal hyphae, and participation in membrane transport processes, cell wall formation and gene expression (Kulaev *et al.*, 1999; Ezawa *et al.*, 2002).

An increase of the C flux from mycorrhizal roots to the FERM of *Glomus intraradices* was associated with a decreased labelling of low molecular weight soluble phosphates, and an increased labelling in phospholipids and in the nucleic acid and protein-phosphate containing pool (see Discussion of growth-related P pools above). Additionally, when more C is provided by the mycorrhizal roots to *G. intraradices*, the total polyP contents in FERM and RERM decreased. Solaiman & Saito (2001) found, using IRM of *G. margarita* digested out of the host roots that the total polyP contents decreased after the addition of glucose or 2-deoxyglucose. P efflux from the IRM increased under these conditions and the authors deduced that the P efflux from the IRM is coupled to the hydrolysis of polyP within the hyphae.

The effect of C availability on the subcellular distribution of P within the roots

Under control conditions when the C availability to the fungus may be assumed to have been low, P was mainly accumulated in the fungal vacuole of the IRM. After supplying carbohydrates to the roots the P contents within the fungal vacuole of the IRM decreased. By contrast, when mycorrhizal roots were provided with the higher sucrose concentration, an increase was observed in the cytoplasmic P contents in the IRM. This is consistent with the results of

Bücking & Heyser (2003), who reported that in the ECM symbiosis the cytoplasmic P content in the P releasing hyphae of the Hartig net decreased and the vacuolar polyP levels rose when the flux of C across the plant fungal interface is reduced. It has been suggested that P transfer to the host plant is regulated by the cytoplasmic P concentration in hyphae of the Hartig net (Bücking & Heyser, 2000). According to this model, transfer of P across the interface would: (i) reduce the intracellular P concentration in the IRM; (ii) stimulate the flux of P from the ERM to the IRM; and (iii) therefore increase the P absorption by the ERM. This is consistent with results of Maldonado-Mendoza *et al.* (2001), who reported, that the P status of the IRM influences the expression of P transporters and P uptake by the ERM.

The higher P contents in the vacuoles of the IRM were associated with higher contents of cations. In the IRM of AM roots a P concentration between 60 and 170 mM was detected, which was closely correlated with the levels of K and Mg (Ryan *et al.*, 2003). These cations have been shown to be highly abundant in P-rich electron-opaque granules in ECM fungi (Bücking & Heyser, 1999) and were also detectable in vacuolar granules of *G. intraradices* besides Na and Ca. It has been concluded, that these P-rich granules with cations associated are polyP granules (White & Brown, 1979), although their state *in vivo* (granules vs soluble) has been disputed (Orlovich & Ashford, 1993; Bücking & Heyser, 1999). EDXS does not identify the chemical forms of P, but based on the accumulation of polyP in FERM and RERM, it can be assumed that the accumulation of P in the fungal vacuole involves an accumulation of polyP within the IRM. PolyP may be important in reducing the osmotic stress at high internal P concentrations and for the regulation of the cytoplasmic P in releasing hyphae of the IRM and thereby also of the P flux across the mycorrhizal interface. Supplying sucrose caused a lower incorporation of P into polyP, and increased the cytoplasmic P content in the IRM. This would be expected to promote P efflux from the hyphae into the interface. This is consistent with results of Solaiman & Saito (2001), who showed that polyP breakdown in hyphae of the IRM and the P efflux from hyphae can be increased by an external supply of glucose or 2-deoxyglucose. Bücking (2004) found that the P efflux from hyphae of ECM fungi can be stimulated by exogenously supplied sucrose and suggested that even though sucrose cannot be used as a C source by the hyphae, it might better represent the release of carbohydrates from the plant cell into the interfacial apoplast than glucose or fructose, whose concentrations varied depending on the activity of the acid invertase and fungal hexose transporters.

A model for the interaction between C and P fluxes in the AM symbiosis

The results showed that increased carbohydrate availability stimulated C flux across the mycorrhizal interface and altered

P uptake, P allocation and P transfer within the symbiosis. These findings combined with the results of other studies (Solaiman & Saito, 2001; Bücking & Heyser, 2003) suggest that the exchange of C for P may be coupled. A model for a possible interaction between the carbohydrate and P flux in a mycorrhiza is shown in Fig. 8. According to this scheme P_i is actively absorbed by the ERM, enters the cytoplasmic metabolically active P pool, is translocated to the mycorrhizal root in form of short chain polyP, and is accumulated in fungal vacuoles in form of short- and long-chained polyP. P uptake by the ERM (Maldonado-Mendoza *et al.*, 2001) and P efflux into the interfacial apoplast (Bücking & Heyser, 2000) are regulated by the concentration of intracellular metabolically active P_i within the hyphae. A continuous flow of P_i across the interfacial apoplast to the mycorrhizal host plant and its transfer to the sinks will: (i) improve plant growth and photosynthetic activity of the plant; (ii) enhance the transfer of carbohydrates to the mycorrhizal roots and; (iii) promote sucrose efflux through the plant plasma membrane into the interfacial apoplast. Plant acid invertase in the interfacial apoplast hydrolyzes the sucrose into glucose and fructose, which can be absorbed by the IRM. The invertase activity is regulated by the pH and thereby stimulated by the activity of the H^+ -ATPases, whose activity and gene expression is increased by an AM infection and by the sucrose concentration (Murphy *et al.*, 1997; Blee & Anderson, 2002). The uptake of hexoses by the mycorrhizal fungus and the consequent conversion of these hexoses to fungal carbohydrates such as trehalose and glycogen (Shachar-Hill *et al.*, 1995; Bago *et al.*, 2003) via hexose-phosphates driven by a polyP-hexokinase (Cappacio & Callow, 1982) or other hexokinases will enhance the remobilization of polyP (Solaiman & Saito, 2001). The remobilization of polyP will increase the intracellular P_i concentration in the hyphae (Bücking & Heyser, 2003), and thereby promote P_i efflux through the fungal plasma membrane into the interfacial apoplast. The increased transfer of TAG and glycogen to the ERM would provide the necessary energy for active uptake processes from the soil and the C skeletons for an extension of the ERM to get access to new P resources.

The results of the present investigation show that P uptake by the ERM, P allocation within the hyphae and P transfer within the symbiosis are affected by the availability of C to the mycorrhizal root and thereby also for the fungal symbiotic partner. However, to test the proposed model further transport and localization studies will be needed. In particular, it is important to find out where the exchange processes are located and what the molecular mechanisms involved in polarizing and regulating the transfer processes are. This will lead to a better understanding of the exchange processes that form the nutritional basis of this ecologically important plant/fungus association.

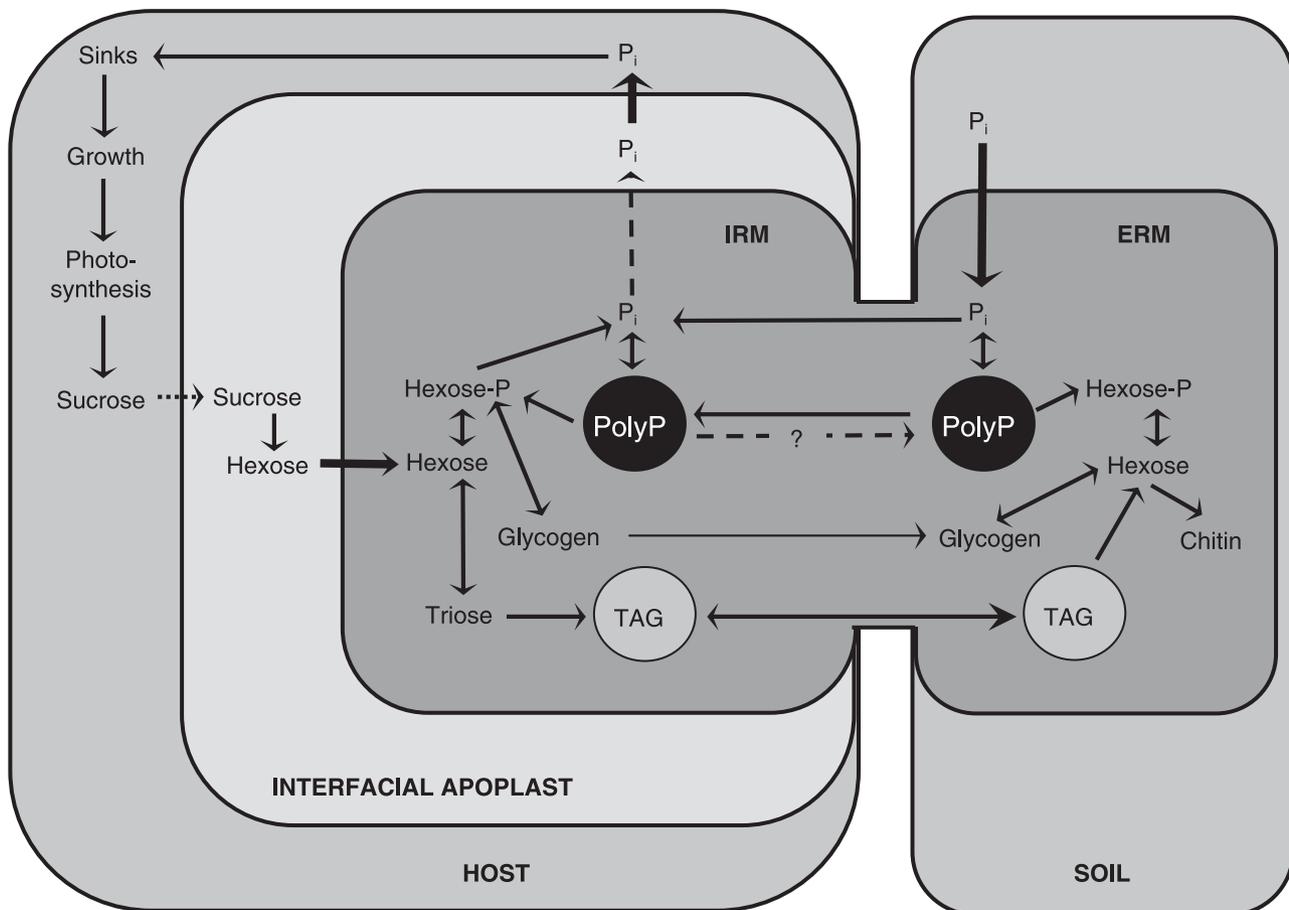


Fig. 8 Illustration of a model for the interaction between phosphate (P) and carbohydrate exchange in the arbuscular mycorrhizal (AM) symbiosis. Bold arrows, active uptake processes; broken arrows, passive efflux processes. ERM, extraradical mycelium; IRM, intraradical mycelium; polyP, polyphosphates; TAG, triacylglycerol.

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