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P DEPLETION AND ACTIVITY OF PHOSPHATASES IN THE RHIZOSPHERE OF MYCORRHIZAL AND NON-MYCORRHIZAL CUCUMBER (*CUCUMIS SATIVUS* L.)

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Summary—An experiment was set up to test the ability of arbuscular mycorrhizal (AM) roots and hyphae to produce extracellular phosphatases and to study the relationship between phosphatase activity and soil organic P (P_o). Non-mycorrhizal cucumber and cucumber in symbiosis with either of two mycorrhizal fungi were grown in a sandy loam–sand mixture in three-compartment pots. Plant roots were separated from two consecutively adjoining compartments, first by a 37 m mesh excluding roots and subsequently by a 0.45 m membrane excluding mycorrhizal hyphae. Soil from the two root-free compartments was sectioned in a freezing microtome and analyzed for extracellular acid (pH 5.2) and alkaline (pH 8.5) phosphatase activity as well as depletion of $NaHCO_3$-extractable inorganic P (P_i) and P_o. Roots and mycorrhizal hyphae depleted the soil of P_i but did not influence the concentration of P_o in spite of increased phosphatase activity in soil influenced by roots. Phosphatase activity at both pH values was highest in soil influenced by uncolonized roots, but this was attributed to higher root length densities as compared to mycorrhizal roots. Mycorrhizal hyphae showed no influence on soil phosphatase activity in spite of high hyphal length densities (>22 m cm^{-3}). Hyphae were also able to deplete soil of P_i beyond the membrane interface.

INTRODUCTION

Organic P (P_o) in soil may be utilized by plants after mineralization and subsequent release of inorganic P (P_i). Hydrolytic cleavage of P_o by extracellular phosphatases of microbial or root origin is one mechanism of such mineralization (Eivazi and Tabatabai, 1977). Phosphatases have been detected on root surfaces (Dracup *et al.*, 1984), in rhizosphere soil (Hedley *et al.*, 1982) and in soil without plant root influence (Eivazi and Tabatabai, 1977). In some instances the activity of phosphatases has been positively correlated with P_o depletion (Tarafdar and Jungk, 1987; Tarafdar and Claassen, 1988), while others have found no relationship between phosphatase activity and depletion of P_o (Thompson and Black, 1970; Hedley *et al.*, 1982).

Arbuscular mycorrhiza (AM) increases the P uptake of plants through extraradical hyphae. Few investigators have considered the utilization of P_o by AM plants (Jayachandran *et al.*, 1992; Joner and Jakobsen, 1994), though it has been suggested that increased phosphatase release from AM roots or even from fungal hyphae may be a potential mechanism for mycorrhizal utilization of P_o (Gianinazzi-Pearson and Gianinazzi, 1989; Jungk and Claassen, 1989). Increased activity of acid phosphatase has been found in the roots (Gianinazzi *et al.*, 1979; Thiagarajan and Ahmad, 1994) and in the rhizosphere (Dodd *et al.*, 1987; Tarafdar and Marschner, 1994) of plants colonized by AM fungi. In contrast, Azcon *et al.* (1982) found reduced activity of acid phosphatase in mycorrhizal compared to non-mycorrhizal lavender roots and no differences in wheat. Comparisons between phosphatase activity of mycorrhizal and non-mycorrhizal roots and adhering soil give only limited information on the origin of the detected phosphatases as contributions by roots and hyphae cannot be distinguished from each other.

We report an experiment where mycorrhizal and non-mycorrhizal plants were grown in a sandy loam–sand mixture where the roots were separated from two consecutively adjoining compartments, first by a 37 μm mesh excluding the roots and subsequently by a 0.45 μm membrane excluding mycorrhizal hyphae. The two compartments were detached after a period of plant growth and analyzed for acid (pH 5.2) and alkaline (pH 8.5) phosphatase activity as well as depletion of P_i and P_o at increasing distances from the roots after slicing the soil in a freezing microtome.

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MATERIALS AND METHODS

Pots

Plants were grown in vertical PVC tubes (200 mm, 72 mm dia) each having one lateral compartment (tubes; 40 mm, 52 mm dia) separated by a 37 μm nylon mesh from the root compartment (RC) (Fig. 1). The lateral compartment was subdivided into a 20 mm wide hyphal compartment (HC) closest to the RC and a 20 mm wide bulk soil compartment (BSC) furthest away from the roots. The interface between HC and BSC was made up by a membrane (Durapore HV 0.45 μm; Polyvinylidene difluoride, Millipore) and the two compartments were held together with adhesive tape after adding soil. To facilitate sectioning of the soil in HC and BSC at harvest each 20 mm PVC tube was made up by two 10 mm tubes held together at two points with a drop of glue. The part of each 20 mm tube being closest to the roots was cut radially at one point so that it could be widened and easily removed.

Soil and fungal inoculum

The soil consisted of a sieved (1 mm) partially sterilized (10 kGy, 10 MeV electron beam) 1:1 (w/w) mixture of sandy loam (Jakobsen and Nielsen, 1983) and quartz sand. The soil–sand mixture had a pH(H_2O) of 6.1, a clay content of 9% and contained 0.54% organic C. Side compartments were filled with 71 g soil (4.5% water content) and were packed individually on a vibrating plate to obtain a uniform bulk density (1.4 g cm^{-3}) and a good contact between soil and the mesh–membrane interfaces. Water (7 ml) and a freshly prepared soil filtrate (1 ml; 2 g soil in 100 ml water, no. 40 Whatman filter) to provide microbial inoculum except AM fungi was added carefully to obtain 60% of water holding capacity (WHC) prior to the assembly of one HC and one BSC with each RC. Soil (1000 g, 5% water content) including mycorrhizal inoculum was then filled into RC, where the soil was gently packed towards the mesh interface with a spatula. Nutrients except N, P and Cu were added at optimum concentrations for plant growth (Joner and Jakobsen, 1994). N (NH_4NO_3) was added weekly to a total amount of 125 mg N per pot. The RC of non-mycorrhizal controls received 35 mg KH_2PO_4 kg^{-1} dry soil (8 mg P kg^{-1}) while RC of mycorrhizal plants and all HC and BSC received no P. Cu was omitted to reduce inhibition of phosphatase activity by Cu^{2+} ions.

Mycorrhizal treatments received 100 g mycorrhizal inoculum mixed with 300 g soil placed in RC adjacent to the lateral compartment. Mycorrhizal inoculum consisted of 100 g of the described soil from pot cultures containing colonized roots of *Trifolium subterraneum* L. and spores of either *Glomus invermaium* Hall (isolate WUM 10, obtained from L.K. Abbott, Univ. of Western Australia) or *G. caledonium* (Nicol. and Gerd.) Trappe and Gerdemann (isolate RIS 42). Controls received 5 ml filtrate (Whatman no. 40 filter, 10 g mixed inoculum in 250 ml water) of the two inocula and 100 g of the original soil without added nutrients.

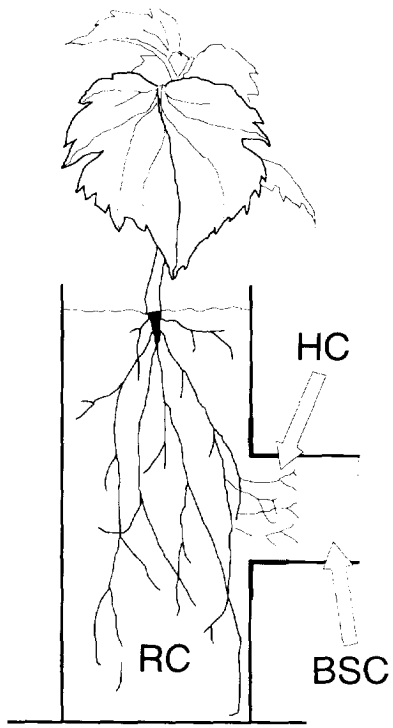

Fig. 1. Schematic diagram of pot showing subdivision into root compartment (RC), hyphal compartment (HC) and bulk soil compartment (BSC).

Plants and growth conditions

Five replicate pots of each treatment were prepared. The soil was watered to 60% of WHC and kept for 8 days for mycorrhizal spores to germinate. Two germinating seeds of cucumber (*Cucumis sativus* L., cv. Aminex) were planted in each pot and thinned to one per pot after seedling emergence. The plants were placed in a growth chamber with a 16–8 h light–dark cycle (500 μmol m^{-2} s^{-1}, Osram HQI-T 250 W/D) at 20–15 C and watered daily by weight.

Sectioning of soil

Thirty-two days after seedling emergence the side compartments were detached from RC and divided at the membrane interface. A 10 mm dia × 20 mm soil core was taken from the periphery of each HC and BSC for measurements of hyphal length. PVC plates were clamped firmly on to both sides of each HC or BSC before submerging them into liquid N_2 for 2 min. Soil cores inside their respective PVC tubes were individually packed in plastic bags and stored at −80 C. Soil cores were cut in a freezing microtome (Leitz 1512) at −16 to −24 C after removing half the PVC tube, fixing the soil to the remaining PVC tube with water and fitting this unit directly into the microtome. Each cut was 25 μm thick, the maximum

cutting width of the microtome. Combining 10 (0–1.5 mm) or 20 (1.5–5 mm) cuts gave a soil mass of ca. 0.7 or 1.4 g moist soil, representing sections of 0.25 and 0.5 mm, respectively. Sectioned soil was stored at −18 C in airtight glass vials until samples were taken for phosphatase measurements. Additional samples were taken with a spatula from the remaining soil 10 mm from the mesh to check if the cutting resulted in increased phosphatase activity due to leaking of fungal cytoplasm into the soil. Two samples were also taken from inside each RC.

Phosphatase measurements

Phosphatase activity was measured by a procedure modified after Tabatabai and Bremner (1969) using 0.2 g moist soil and 4 ml buffered substrate per sample. Acid phosphatase was measured at pH 5.2 using a 0.1 M acetate–acetic acid buffer containing 1 mg *p*-nitrophenyl phosphate (PNPP) ml^{-1}. Buffered substrate with soil was kept for 1 h at 37 C in a shaking water bath before adding 2.4 ml 0.5 M NaOH and 1 ml 0.5 M $CaCl_2$. Alkaline phosphatase was measured similarly at pH 8.5 using 0.1 M $NaHCO_3$ as buffer. Blanks, where enzyme activity was blocked by adding NaOH to buffer and soil from the 4.5–5 mm section prior to substrate addition, were run together with samples of all soil cores. Soil was centrifuged in the sample tubes at $2000 \times g$ for 10 min before *p*-nitrophenol in the supernatant was measured spectrophotometrically at 405 nm. Enzyme activity was expressed as enzyme units (EU: 1 μmol PNPP hydrolysed min^{-1} g^{-1} dry soil) after subtracting blanks.

P measurements

Soil P was measured from 0.2 g air dried soil extracted in 4 ml 0.5 M $NaHCO_3$ at pH 8.5 for 2 h at 20 C at 40 rev min^{-1}. Inorganic P was analyzed using ammonium molybdate–ascorbic acid (Murphy and Riley, 1962). Total P in the same extracts was measured after oxidizing an aliquot in alkaline 0.5 M $K_2S_2O_8$ in an autoclave at 140 C for 15 min (Ebina *et al.*, 1983). $NaHCO_3$-extractable organic P was calculated as the difference between total and inorganic P. Dried roots and tops were ground and subsamples digested in nitric acid:perchloric acid (4:1) (Johnson and Ulrich, 1959). The digests were analyzed for P in an autoanalyzer system using ammonium molybdate–ascorbic acid.

Measurements of roots and hyphae

Subsamples of roots from RC taken adjacent to the nylon mesh were used to determine total and colonized root length (Newman, 1966) after staining with trypan blue (Kormanik and McGraw, 1982). Length of mycorrhizal hyphae was measured using a membrane filter–grid intersect method modified after Abbott *et al.* (1984). Modifications included the use of 2 ml aliquots and 20 mm dia filter area. Duplicate filters were made from one sample of each HC and BSC, and hyphae were counted in 25 fields of vision at 200 × magnification. A common hyphal background was calculated from the 15 BSC and subtracted from the values measured in HC.

Treatment effects were tested by one-way or factorial analysis of variance and means were compared by the Newman-Keuls test for unplanned comparisons.

RESULTS

Inoculation with mycorrhizal fungi resulted in an average colonization of 74 and 66% of the root length by *Glomus invermaium* and *G. caledonium*, respectively (Table 1). Uninoculated controls remained uncolonized. The combined effect of mycorrhizal colonization and no P fertilization resulted in lower dry weights of mycorrhizal plants compared to controls. Similarly, total root length density was highest in uninoculated plants. P concentration, however, was highest in plants inoculated with *G. caledonium* while there was no difference between plants inoculated with *G. invermaium* and uncolonized plants (Table 1). Mean hyphal length density in HC was 22.4 and 22.9 m cm^{-3} for *G. invermaium* and *G. caledonium*, respectively (Table 1). These numbers were obtained after subtracting an average background of 3.0 m cm^{-3} measured in 15 samples from BSC.

At harvest soil in RC contained 5.8–6.2 mg $NaHCO_3$-extractable P_i kg^{-1} when plants were inoculated with *G. caledonium* or left uninoculated and 4.5 mg $NaHCO_3$-extractable P_i kg^{-1} when inoculated with *G. invermaium* [Fig. 2(a)]. Inorganic P was strongly depleted in HC close to the roots as a root mat had formed at the mesh interface. The density of the root mat was proportional to the root length density measured in RC (results not shown). For non-mycorrhizal plants the depletion zone in HC extended only 1.5 mm before it reached the initial content of P_i

Table 1. Plant dry weight (DW), P concentration, total and colonized root length in root compartments (RC), and hyphal length in hyphal compartments (HC). Different letters in the same column indicates significant ($P < 0.05$) differance between means

			Root length in RC		
	Plant DW (g)	Plant P (mg g^{-1} DW)	Total (cm cm^{-3})	Colonized (cm cm^{-3})	Hyphal length in HC* (m cm^{-3})
Control	12.05a	1.26b	79.6a	0c	0
G. invermaium	7.77b	1.29b	34.9b	25.9a	22.4a
G. caledonium	6.26b	1.39a	21.4c	14.1b	22.9a

*Hyphal background measured in BSC has been subtracted.

(8.5 mg P kg^{-1}). In mycorrhizal treatments the soil was depleted beyond this zone of root influence. Up to 10 mm from the mesh interface the concentration of P_i was reduced to 5.6 mg kg^{-1} by *G. caledonium* and to 4.4 mg kg^{-1} by *G. invermaium*. P depletion by mycorrhizal hyphae also extended 0.5 mm beyond the membrane interface of the BSC. The concentration $NaHCO_3$-extractable P_o was not influenced by roots or hyphae in any of the compartments [Fig. 2(b)].

Both acid and alkaline phosphatase activity was greatly stimulated by roots [Fig. 3(a, b)]. The highest rates of phosphatase activity at both pH values were found in the non-mycorrhizal treatment. Increased enzymatic activity extended 0.75–1.0 mm into HC before it reached a background level. Neither acid nor alkaline phosphatase activity in HC was affected by the presence of mycorrhizal hyphae [Fig. 3(a, b)]. Consequently, there were no differences in phosphatase activity between treatments in BSC. The sampling procedure of soil containing hyphae (microtome cutting or scooping with a spatula) did not alter the measured phosphatase activity, as shown in samples taken 10 mm from the roots [Fig. 3(a, b)].

DISCUSSION

The use of a three-compartment system allowed the comparison of soil phosphatase activity and P depletion influenced by mycorrhizal and non-mycorrhizal roots at four different levels: (1) in a true rhizosphere within the RC where samples were taken between roots at high root length densities; (2) in a two-dimensional rhizosphere created by a root mat and dominated by planar nutrient and exudate diffusion (Jungk, 1987); (3) in a true hyphosphere influenced by AM hyphae but not roots; and (4) in a two-dimensional hyphosphere where the influence of AM fungal hyphae excluded by a membrane could be monitored. Slicing the soil in a microtome facilitated a high resolution for the measured values regarding the distance from the two interfaces. Extra P was added to soil in RC of non-mycorrhizal plants to compensate for an expected increase in P uptake and growth due to mycorrhizal colonization. The added P and the resulting differences in root length complicates the comparisons of P depletion and phosphatase activity in soil influenced by roots.

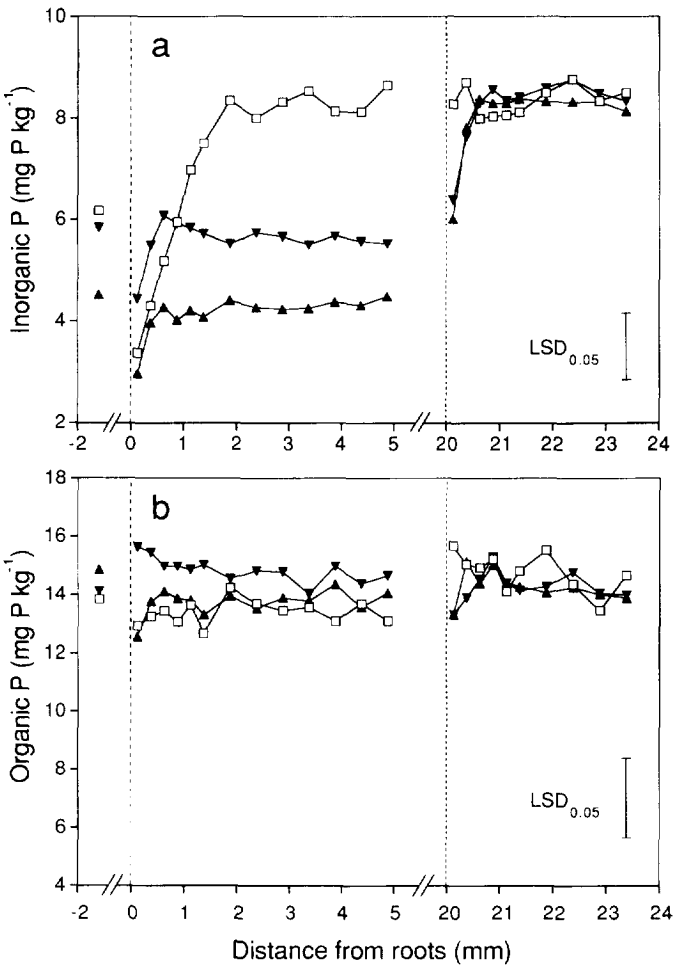

Fig. 2. Soil inorganic (a) and organic (b) phosphorus. Vertical dashed lines indicate the positions of 37 μm mesh (left) and 0.45 μm membrane (right). □ control, ▲ *G. invermaium*, ▼ *G. caledonium*.

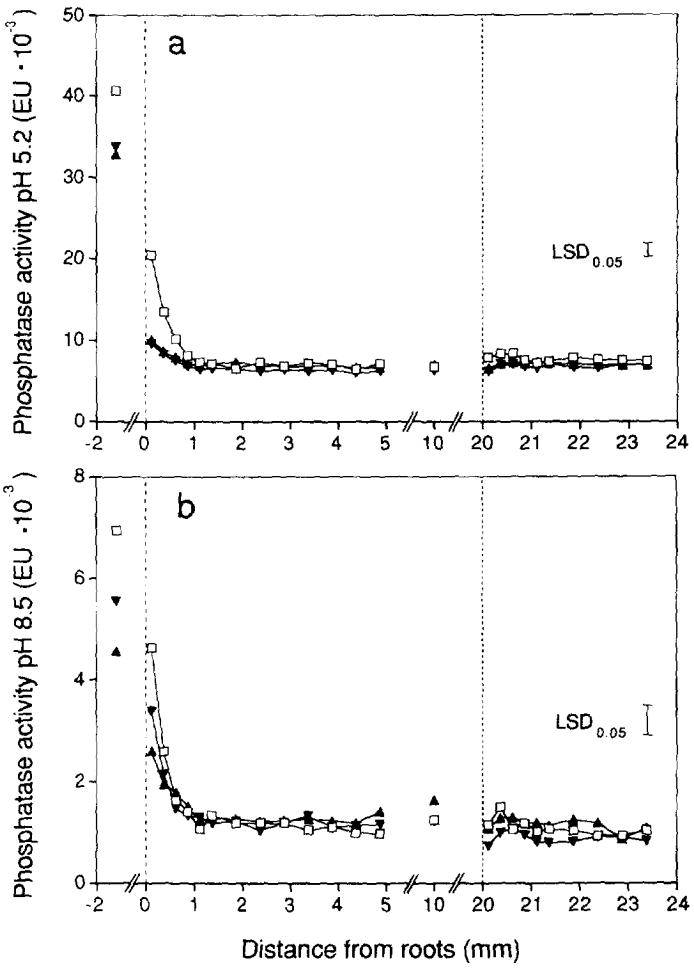

Fig. 3. Extracellular acid (a) and alkaline (b) phosphatase activity in soil. Symbols as in Fig. 2.

Plants colonized by *G. invermaium* had depleted the soil in RC of more P_i than plants colonized by *G. caledonium*. This difference could have been caused by higher root length density in the former treatment. The soil in RC of the non-mycorrhizal treatment received additional P and thus eludes comparisons with respect to P_i depletion. For all treatments, the formation of a root mat at the mesh interface resulted in an additional P depletion in the two-dimensional rhizosphere as compared to RC. Though the root mats were less dense in the mycorrhizal treatments, the non-mycorrhizal and *G. invermaium* treatments had depleted P_i to the same concentration in the two-dimensional rhizosphere. In comparison, *G. caledonium* showed less depletion of P_i in this zone. This indicates that hyphae of *G. invermaium* had contributed to P depletion in the rhizosphere, which is usually not the case when root length densities are as high as in our experiment (Pearson and Jakobsen, 1993; Joner and Jakobsen, 1994). In spite of similar hyphal length densities there were differences in P depletion in the hyphosphere of the two fungi suggesting that *G. invermaium* had the most efficient P uptake of the two fungi. Apparently this is in contrast to the results of Pearson and Jakobsen (1993) who found a 4-fold increase in hyphal P uptake from a 70 mm long HC by *G. caledonium* as compared to *G. invermaium*. However, the distance between the roots and the site of P uptake may explain this divergence, as AM fungi differ in their P transport capacity over short and long distances (Jakobsen *et al.*, 1992).

Depletion of P_i by AM fungal hyphae across a membrane retaining the hyphae was described by Li *et al.* (1991), who partly attributed their results to acidification mediated by supplying N exclusively as NH_4^+. We found a comparable depletion of P_i with NH_4NO_3 as N source for roots and no N added to HC or BSC (soil originally contained 3.3 mg kg^{-1} NH_4^+-N and 7.1 mg kg^{-1} NO_3^--N, determined by extraction with 2 M KCl). pH measurements were not carried out in our experiment, but we assume that acidification was also responsible for P mobilization in the two-dimensional hyphosphere in our experiment, since rhizosphere acidification is known to occur if NH_4^+-N constitutes more than 6% of the supplied N (Gahoonia and Nielsen, 1992a).

Previous studies of extracellular phosphatase in the rhizosphere of AM plants have shown increased activity (Dodd *et al.*, 1987; Tarafdar and Marschner, 1994), no difference (Azcon *et al.*, 1982; Dodd *et al.*, 1987) as well as reduced activity (Azcon *et al.*, 1982; Sainz *et al.*, 1987) due to mycorrhizal colonization. When more than one host–fungus combination have been tested, the outcome has varied with the choice of host plant and fungal endophyte (Azcon *et al.*, 1982; Dodd *et al.*, 1987). In our experiment, differences in root length density between mycorrhizal and non-mycorrhizal treatments have probably masked the possible effect of mycorrhiza for our host–endophyte combinations. This is assumed since the quantity of root derived phosphatases increases with increasing root length density (Tarafdar and Jungk, 1987; Rubio *et al.*, 1990).

P deficiency often increases the production of extracellular phosphatases. This has been demonstrated for roots (Goldstein *et al.*, 1988; Helal and Sauerbeck, 1991) and for pure cultures of several microorganisms (Torriani, 1960; Nyc, 1967; Calleja and d'Auzac, 1983). Plant P and soil P concentrations both suggest that P deficiency was severe in our experiment. Nevertheless, no differences in phosphatase activity was found between soil with or without a dense AM fungal mycelium. In our experiment AM hyphae therefore seem to release none or very small amounts of extracellular phosphatases. This supports the assumption of Dodd *et al.* (1987) that increased phosphatase activity in the rhizosphere of mycorrhizal plants was due to phosphatase derived from the roots. Tarafdar and Marschner (1994) found that mycorrhizas increased acid and alkaline phosphatase activity in both rhizosphere and hyphosphere soil in an experimental setup similar to ours. Conclusions concerning the causal components must be drawn with care in that work as plant growth responded to inoculation with an AM fungus, as hyphal length densities were rather low, as no data on hyphal lengths were presented for the controls, and as no corrections for phosphatases leaking from fungal cytoplasm after microtome cutting were made. However, the conflicting results could be due to the absence (Tarafdar and Marschner, 1994) or presence (our experiment) of microorganisms other than AM that could produce or inactivate extracellular phosphatases, or differences inherent to given host–endophyte combinations.

The high phosphatase activities measured in RC and in the two-dimensional rhizosphere did not mediate any measurable depletion of $NaHCO_3$-extractable P_o. One reason for this could be that P_o was stabilized and not accessible for breakdown by phosphatases (Stewart and Tiessen, 1987). This can be assumed since the soil had not been cultivated for 2 years. Nevertheless, P_o in $NaHCO_3$-extracts is considered to be rather labile (Bowman and Cole, 1978), and depletion of P_o caused by rape plants has been found in comparable soils with similar physical and chemical properties (Gahoonia and Nielsen, 1992b).

Some authors have found that depletion of P_o is correlated with soil phosphatase activity (Tarafdar and Jungk, 1987; Helal and Sauerbeck, 1991). Others have found that phosphatases in soil are unable to hydrolyze P_o (Hedley *et al.*, 1982; Helal and Sauerbeck, 1984). The inconsistency of these findings may again be related to the forms of P_o present in soil. Barrett-Lennard *et al.* (1993) suggested that since simple forms of P_o only exist in minute amounts in soil, phosphatases would be of minor importance in improving plant utilization of stabile soil P_o. Rather, the function of root derived phosphatases may be cleavage of P-esters leaked from roots before re-absorption.

The absence of detectable phosphatase activity due to the presence of AM fungal hyphae under P deficient conditions indicates that AM hyphae have no or only a minor role in mineralization of soil P_o. A possible response to additions of less stable P_o (e.g. manure or plant residues) still merits investigation.

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