Mycorrhiza (1993) 2:133–139

Mycorrhiza

Mycorrhizal associations in Hong Kong Fagaceae

IV. The mobilization of organic and poorly soluble phosphates by the ectomycorrhizal fungus *Pisolithus tinctorius*

Paul C. F. Tam, D. A. Griffiths

Department of Botany, University of Hong Kong, Pokfulam Road, Hong Kong

Abstract. *Pisolithus tinctorius* (Pers.) Coker and Couch is capable of mobilizing non-mobile phosphates. Organic phosphates are broken down enzymatically by the fungal surface acid phosphatases whereas poorly soluble phosphates are solubilized by the ability of the fungus to excrete protons and organic acids. The accumulation of phosphate in the form of insoluble polyphosphate stored in the vacuoles is positively correlated with the external available phosphate pool. The major cation linked to the stored phosphate polymers in this fungus is calcium.

Key words: *Pisolithus tinctorius* – Phosphates – Energy dispersive X-ray analysis – Ectomycorrhiza

Introduction

Pisolithus tinctorius has a broad host range and is distributed throughout many areas of the world (Marx 1977). It has been demonstrated that pine seedlings inoculated with *P. tinctorius* ectomycorrhizas survive and grow faster than those bearing other ectomycorrhizas on adverse sites (Marx and Artman 1979).

In Hong Kong, both naturally occurring and introduced isolates of *P. tinctorius* form successful ectomycorrhizas with members of the Pinaceae (Chan and Griffiths 1988) and with various exotic species of *Eucalyptus* (Chan and Griffiths 1991). We have shown that the introduced isolate *P. tinctorius* (Pers.) Coker and Couch is able to form ectomycorrhizal associations with three members of the local Fagaceae: *Quercus*, *Castanopsis* and *Lithocarpus* (Tam and Griffiths 1993a); more recently we have reported an increase in phosphorus uptake by *Quercus* seedlings grown in artificial soil inoculated with this fungus. The higher level of P and moderate level of Ca found in such mycorrhizal root samples led us to propose a linkage between these two ions in the composition of polyphosphate granules observed as metachromatically stained bodies in the ectomycorrhizal hyphae (Tam and Griffiths 1993b).

Soluble phosphate is present in limiting amounts in many soils and consequently may be a factor limiting growth (Bielski 1973). Phosphates may be present in the humus layers of soil in organic forms such as inositol phosphate or phytic acid salts (Harley and Smith 1983); in the deeper soils, a large part of the phosphates are in poorly soluble forms (Lapeyrie et al. 1990). Hong Kong soils are mainly derived from decomposed granite and are generally deficient in available phosphate (Grant 1962).

Harley (1978a, b) showed that the ectomycorrhizal sheath probably acts as an important storage organ for inorganic nutrients and estimated that about 40% of the dry weight of a typical ectomycorrhiza is made up of fungal components. Thus, we can speculate that a study of phosphate mobilization by the mycorrhizal fungus in vitro would reflect the physiological characteristics of the mycobiont in situ.

The purpose of the present study was to evaluate the efficiency of *P. tinctorius* (Pers.) Coker and Couch in exploiting various sources of non-mobile phosphate in vitro and to examine the ionic composition of polyphosphate granules found in this fungus by means of energy dispersive X-ray spectrometry.

Materials and methods

Fungal culture

An isolate of *P. tinctorius* (Pers.) Coker and Couch (ATCC 38054) obtained from the American Type Culture Collection was maintained on a modified Melin-Norkans agar medium (Marx 1969). It was inoculated on P-free medium (Lapeyrie et al. 1984) and grown for 2 weeks at 25° C in the dark to produce P-starved mycelium.

Media

Two 5-mm plugs removed from the margins of actively growing colonies of P-starved mycelia were each transferred to a screw-capped Erlenmeyer flask containing 25 ml of P-free liquid media

Correspondence to: P. C. F. Tam

(Lapeyrie et al. 1984) supplemented with one of the following P sources:

(1) Phytic acid 5 mM
(2) β-Glycerophosphate 5 mM
(3) $CaHPO_4$ < 100 mg in 25 ml medium
(4) $Ca_3(PO_4)_2OH$ < 100 mg in 25 ml medium
(5) $FePO_4$ < 100 mg in 25 ml medium
(6) $NaHPO_4$ 5 mM
(7) Controls consisted of the original P-free medium

Phytic acid (Sigma P3168) and β-glycerophosphate (Sigma G6251) were dissolved in autoclaved P-free medium which was then filter-sterilized through a 0.45 µm Millipore filter.

Insoluble calcium and ferric phosphates were autoclaved in P-free medium and the supernatant discarded after sedimentation. The undissolved particles were resuspended in sterile P-free medium after being washed several times in sterile water.

Seven replicates of each treatment were prepared, of which six were inoculated and one was retained as an uninoculated control. These still, liquid cultures were incubated at 25° C in the dark for 30 days.

Determination of pH and the soluble P content of the culture medium

After 30 days, three replicates of each treatment were harvested, the mycelial mats removed by filtration through Whatman no. 54 filter paper and the volume of the filtrate measured. The pH of the filtrate was measured on an Orion Digital pH meter model 611, and the soluble P determined colorimetrically by the molybdenum blue method (Allen 1989).

Acid phosphatase activity

Mycelial mats from each treatment were washed repeatedly in distilled water and then in several changes of a modified universal buffer (MUB) (Tabatabai and Bremner 1969) at pH 6, and transferred to 25-ml flat-bottomed universal bottles containing 4 ml of MUB at pH 6.

Three replicates were set up for each experimental treatment. Each sample was allowed to equilibrate for 1 h at the experimental pH and temperature before 1 ml of 25 mM disodium *p*-nitrophenyl phosphate (pNPP, Sigma 104) was added; a further universal bottle containing 4 ml MUB plus 1 ml pNPP acted as a control. After incubation for 1 h at 21° C, 1 ml aliquots of each reaction mixture were removed and added to test tubes containing 4 ml of 0.5 M NaOH and also to test tubes containing 4 ml distilled water (acting as blanks against fungal pigments). Absorbance was read at 410 nm using a Hitachi 150-20 spectrophotometer and the amount of *p*-nitrophenol (pNP) released was determined from standard curves obtained with alkaline solutions of pNP.

The mycelial mats were dried overnight at 60° C and then weighed. Enzyme activity was measured as µg of pNP released per milligram of fungal dry weight per hour.

Filtrate samples of 3 ml were buffered with 1 ml of stock MUB at pH 6 (at a concentration 5 times greater than the working buffer) in 25-ml flat-bottom universal bottles, mixed with 1 ml of 25 mM pNPP and incubated for 1 h at 21° C. The procedure was repeated as described above and the enzyme activity was determined as µg pNP released per mg fungal dry weight.

Microscopic examination of hyphae

Small pieces of hyphae taken from the rim of the mycelial mats in each treatment were mounted in 50% glycerol, observed under a light microscope and photographed. Hyphae with particulate material in the $FePO_4$ treatment were further examined in a laser scan confocal microscope (Bio-Rad MRC600) using an Argon ion laser as a light source at 488 nm with a blue high-sensitivity filter.

Polyphosphate granules

Small portions of fungal mycelia from each treatment were stained and photographed by the technique of Chilvers and Harley (1980).

Energy dispersive X-ray spectrometry

Portions of hyphae taken from the rim of the mycelial mats of each treatment were fixed in 95% ethanol and dehydrated in a graded series of ethanol-butyl alcohol mixtures (Berlyn and Miksche 1976), infiltrated with paraffin and sectioned with a rotary microtome. Serial sections were cut and mounted on microscope slides, the paraffin was removed in xylene and the sections were stained with toluidine blue at pH 4.4 for the presence of metachromatic granules. Other dewaxed sections were mounted on cellulose strips, air-dried and coated with carbon in a carbon

Table 1. Comparative phosphate mobilization in vitro by the ectomycorrhizal fungus *Pisolithus tinctorius* 30 days after inoculation in still, liquid cultures

Treatment	Medium pH		ΔpH	Soluble P mg/l in medium		Yield of mycelium (mg dry wt.)
	Uninoculated	Inoculated		Uninoculated	Inoculated	
Organic P						
(1) Phytic acid	5.6	2.8	2.8	18	4	14.7
(2) β-Glycerophosphate	6.4	5.8	0.6	1	40	7.3
Insoluble P						
(3) $CaHPO_4$	7.0	5.7	1.3	92	84	15.7
(4) $Ca_3(PO_4)_2OH$	7.0	5.8	1.2	8	11	8.6
(5) $FePO_4$	5.0	2.8	2.2	8	9	18.5
Soluble P						
(6) $NaHPO_4$	5.2	2.8	2.4	230	180	27.8
No P						
(7) P-free	6.1	4.3	1.8	0	0	1.2

vacuum evaporator. The specimens were examined in a Cambridge 150 scanning electron microscope equipped with an energy dispersive X-ray (EDX) analyser (eXL Link Analytical). EDX analysis was carried out at an accelerating voltage of 20 kV. Spectra were collected for 100 s for each sample, and six spectra were obtained from random areas of the sections in each treatment.

Results

From Table 1 it can be seen that there was a drop in the pH of the various media after 30 days of fungal growth. Mycelial dry weight was greatest in media containing soluble phosphate and varied considerably with other treatments. The amount of soluble phosphate in the media at the end of the growth period was also very variable, with the highest, as would be expected, in media containing soluble phosphate and the lowest in phytic-acid-supplemented medium. The greatest overall change in soluble phosphate occurred in treatments containing β-glycerophosphate.

The results in Table 2 demonstrate that the surface-associated acid phosphatase enzyme was active in all treatments while the activity of the excreted enzyme was generally inversely proportional to the phosphate concentration of the media; the media supplemented with tricalcium phosphate and ferric phosphate were exceptions.

Microscopic examination of hyphae taken from the various media showed that hyphae were generally smooth but that those growing in ferric and tricalcium phosphate frequently bore fine particulate material adsorbed onto the mycelial surface (Figs. 1a, b, 2b). Mycelium growing in tricalcium phosphate medium also bore surface crystalline material (Fig. 2a, b).

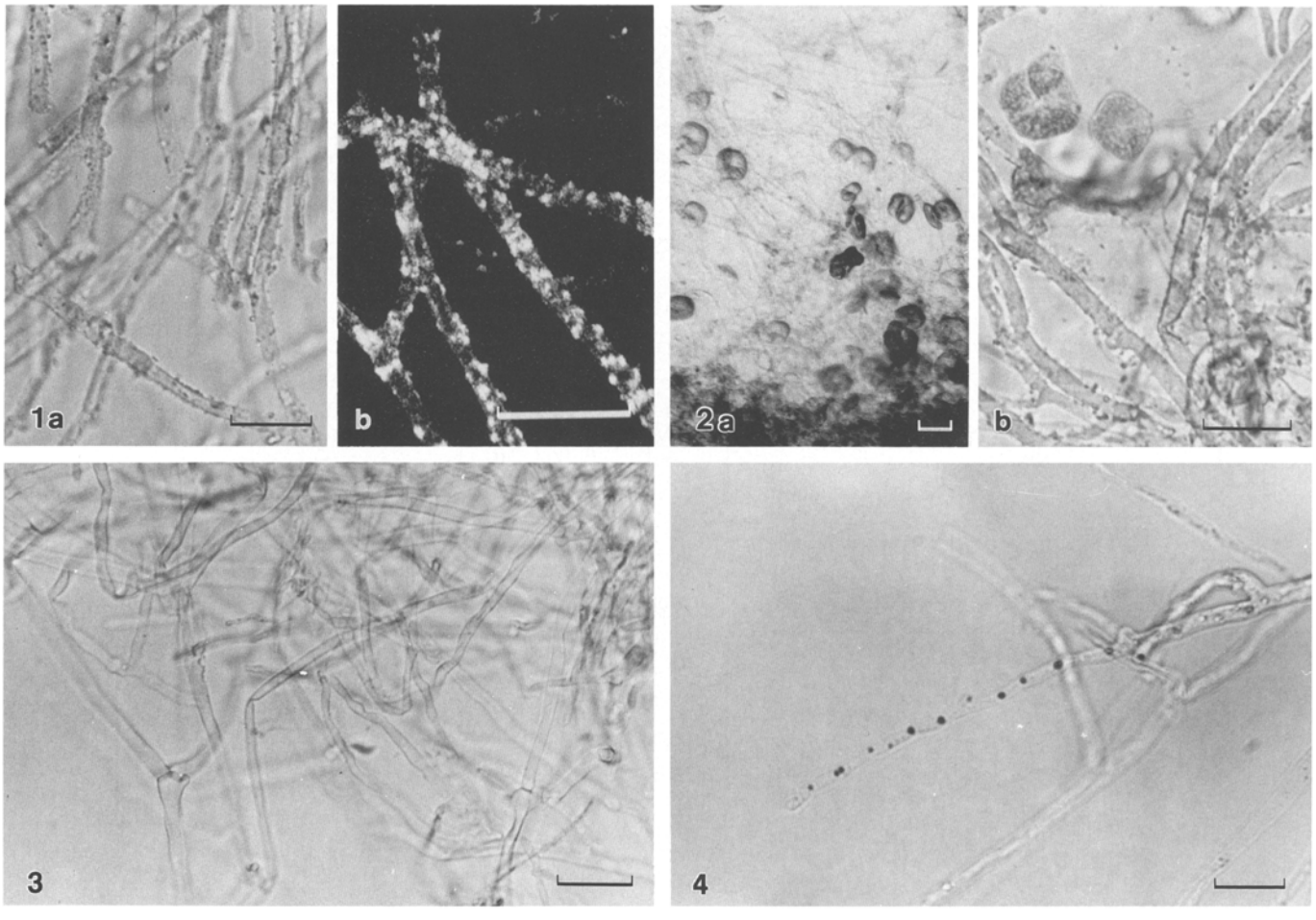
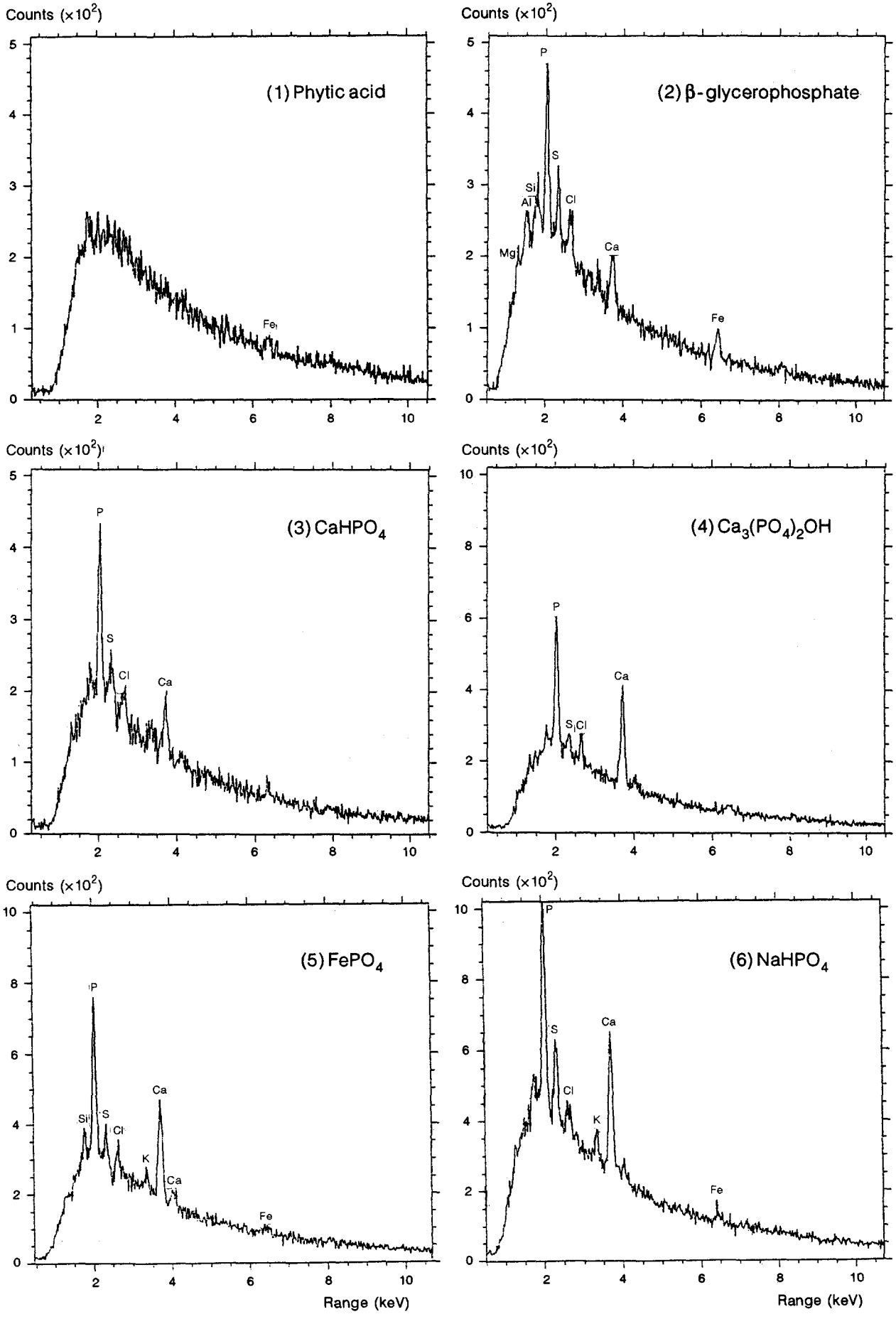


Fig. 1a, b. Solubilizing and crystallizing activity of *Pisolithus tinctorius*. **a** Photomicrograph of the fine particulate material adsorbed onto the hyphal surface in the ferric phosphate medium. **b** The same material seen under confocal microscopy. *Scale bar*, 10 μm

Fig. 2a, b. Solubilizing and crystallizing activity of *P. tinctorius*. **a** Abundant crystalline material adhering to the hyphae in the tricalcium phosphate medium. **b** Crystalline material (enlarged) found near hyphae together with fine particulate material. *Scale-bar*, 10 μm

Fig. 3. Hyphae of *P. tinctorius* stained for metachromatic granules. Hyphae grown in phosphate-free medium. Note the empty hyphae and absence of granules. *Scale bar*, 10 μm

Fig. 4. Hyphae of *P. tinctorius* stained for metachromatic granules. Hyphae grown in the β-glycerolphosphate medium. Note the dark, discrete and variable sized metachromatic granules in the hypha. *Scale bar*, 10 μm


Counts (×10²)
(1) Phytic acid
Fe
(2) β-glycerophosphate
P
S
Si
Al
Cl
Mg
Ca
Fe
(3) CaHPO4
P
S
Cl
Ca
(4) Ca3(PO4)2OH
P
Ca
S,Cl
(5) FePO4
P
Si
S
Cl
K
Ca
Ca
Fe
(6) NaHPO4
P
S
Ca
Cl
K
Fe
Range (keV)

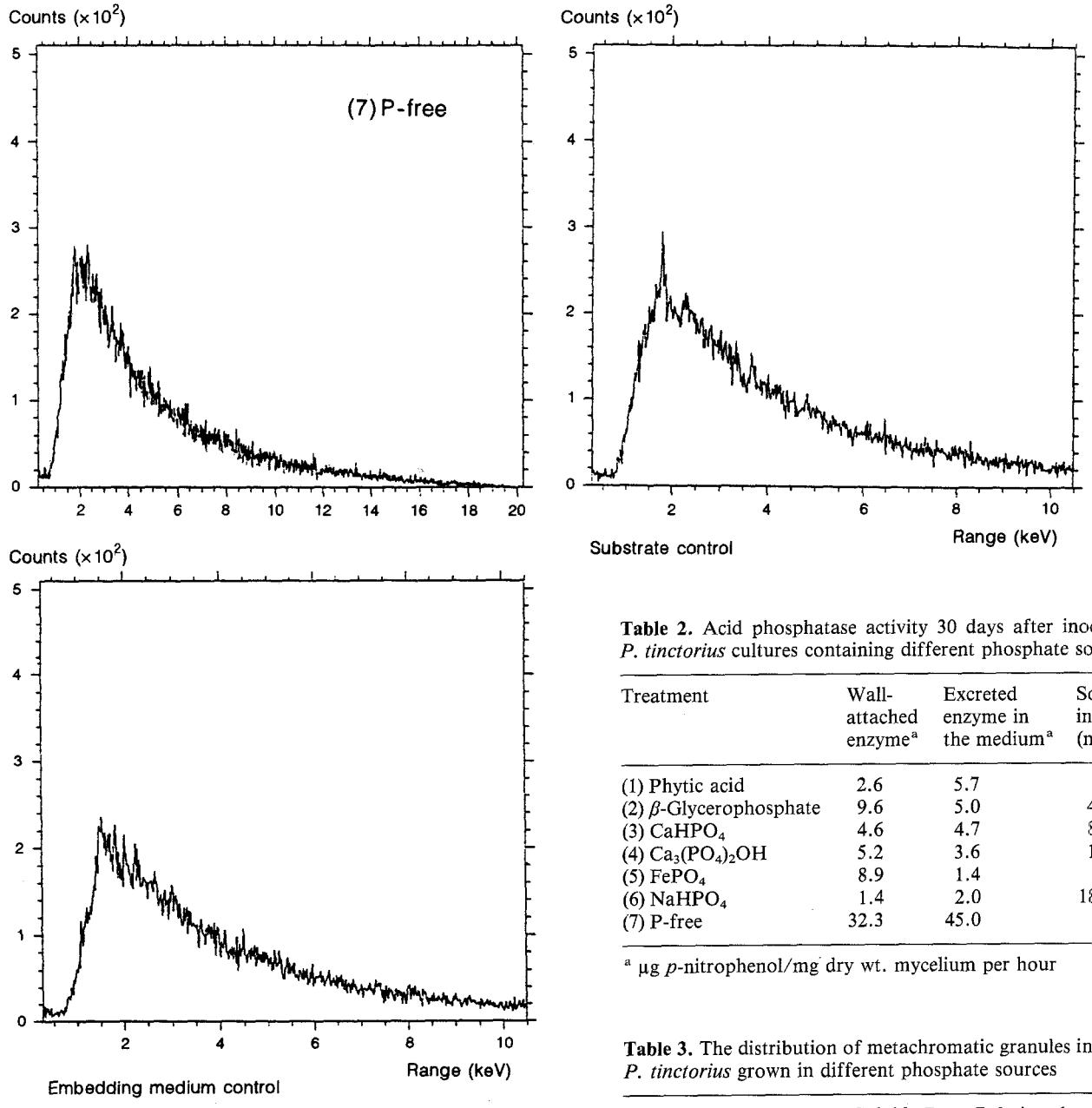


Fig. 5. Energy dispersive X-ray spectrometry spectra of *P. tinctorius* hyphae growing in seven phosphate treatments and of two controls

Table 2. Acid phosphatase activity 30 days after inoculation in *P. tinctorius* cultures containing different phosphate sources

Treatment	Wall-attached enzyme[a]	Excreted enzyme in the medium[a]	Soluble P in medium (mg/l)
(1) Phytic acid	2.6	5.7	4
(2) β-Glycerophosphate	9.6	5.0	40
(3) $CaHPO_4$	4.6	4.7	84
(4) $Ca_3(PO_4)_2OH$	5.2	3.6	11
(5) $FePO_4$	8.9	1.4	9
(6) $NaHPO_4$	1.4	2.0	180
(7) P-free	32.3	45.0	0

[a] µg *p*-nitrophenol/mg dry wt. mycelium per hour

Table 3. The distribution of metachromatic granules in mycelia of *P. tinctorius* grown in different phosphate sources

Treatment	Soluble P in medium (mg/l)	Relative abundance of metachromatic granules
(1) Phytic acid	4	Very scarce
(2) β-Glycerophosphate	40	Moderate
(3) $CaHPO_4$	84	Abundant
(4) $Ca_3(PO_4)_2OH$	11	Scarce
(5) $FePO_4$	9	Moderate
(6) $NaHPO_4$	180	Very abundant
(7) P-free	0	None

In P-free medium, P-starved hyphae contained no granules (Fig. 3) but hyphae in all media supplemented with phosphates bore metachromatic granules (Fig. 4); the relative abundance of these is summarized in Table 3. The relative abundance of these granules was generally proportional to the concentration of soluble phosphate in the medium, the most abundant being found in soluble sodium phosphate and the least abundant in phytic acid. The granules also varied considerably in both size and number dependent upon the phosphate source.

EDX analysis of the metachromatic granules showed large peaks of P and Ca, and their presence in randomized samples corresponded exactly to the hyphae examined microscopically, i.e. those which exhibited granules microscopically always produced significant peaks while those on which metachromatic granules were rarely visible (e.g. in the phytic acid treatment) showed very weak peaks (Fig. 5, spectra 1–7).

In addition to the major peaks for Ca and P, smaller peaks for Al, K, Fe, Mg, Si, S and Cl were also present

in some samples (Fig. 5, spectra 2–6). A peak for Si was also found in the substrate control (cellulose strip) and in the wax-embedding medium and was, therefore, considered an artefact. In general, peaks for P were higher than those for Ca.

Discussion

The results presented here indicate that *P. tinctorius*, as well as utilizing soluble phosphate, can also mobilize sources of both organic and poorly soluble phosphate. It would appear that organic phosphates can be enzymically degraded both by cell wall-attached as well as extracellular excreted acid phosphatases. These results confirm those of Calleja et al. (1980) with the same fungus, who showed that the increase in total phosphatase activity under conditions of phosphate deficiency could be explained by an increase in cell wall-attached enzyme.

We found, in general, that the excreted acid phosphatase activity in the medium was inversely proportional to the orthophosphate concentration. This inverse relationship was explained by Juma and Tabatabai (1977) as competitive inhibition between orthophosphate and acid phosphatases.

Insoluble phosphates was also solubilized in acid media, particularly where nitrogen was supplied as ammonium salts (Raven and Smith 1976). Another mechanism for the mobilization of insoluble phosphates is the extracellular excretion of organic acids. In our study, for example, the presence of extrahyphal crystals in tricalcium phosphate treatment may indicate the precipitation of organic acids by this calcium salt.

Fe and Ca ions are needed in trace amounts by mycorrhizal fungi (Marx 1969). However, despite their release during mobilization of insoluble phosphate sources, they never reach toxic levels in the media. It is thus reasonable to assume that they are converted into some form which is non-toxic to the fungi. The formation of Ca and Fe chelates in the presence of excreted organic acids is one possible explanation, and we did observe particulate material on hyphal surfaces.

The positive correlation between the relative abundance of polyphosphate granules and the concentration of soluble phosphate in the medium indicates that the uptake of soluble phosphate by hyphae relies on an available external pool of phosphate; the hyphae store the excess in the form of granules and thus reduce the damaging effects of osmotic changes and hyphal buffering systems, as suggested by Harley and Smith (1983).

In order to analyse the composition of the polyphosphate granules, we adopted a fixation and sectioning method in which no water was used. Hence we are confident that our results are an accurate analysis and do not suffer from the problems associated with cryofixation (Orlovich et al. 1989) and aqueous phosphate buffer systems, as well as with the wet-sectioning step used in conventional transmission electron microscopy methods. Our specimens were fixed in 95% ethanol, which is the reagent of choice for the precipitation of polyphosphate in biochemical and histochemical studies (Bennett and Scott 1971; Callow et al. 1978; Chilvers and Harley 1980).

As shown in our spectral data (Fig. 5), the peaks for P differed with the various phosphate sources employed. The ratio between the peak heights of P and Ca also varied but Ca was consistently the major cation associated with phosphate in the granules. The other cations Al, K, Mg and Fe present in some samples could also play a role in stabilizing the negative charge of polyanion phosphate, while the anions of S and Cl seen in most samples might also be involved in maintaining the ionic neutrality of the phosphate polymers.

Following the mobilization of poorly soluble phosphate, a high concentration of both calcium and ferric ions would be expected in the media. However, high peaks were recorded for Ca but only a small peak for Fe, indicating a strong linkage between the P polymers and Ca but not Fe cations. This shows the intricacy of the intracellular homeostatic regulation mechanism of these ions by the fungus.

Acknowledgement. The authors wish to thank Mr. T. K. Cheung of the Electron Microscope Unit, University of Hong Kong for his advice and assistance in processing the EDX spectra.

References

Allen SE (1989) Chemical analysis of ecological materials, 2nd edn. Blackwell, London

Bennett J, Scott J (1971) Inorganic polyphosphates in wheat stem rust fungus and in rust-infected wheat leaves. Physiol Plant Pathol 1:185–198

Bielski RL (1973) Phosphate pools, phosphate transport, and phosphate availability. Annu Rev Plant Physiol Plant Mol Biol 24:225–252

Berlyn GP, Miksche JP (1976) Botanical microtechnique and cytochemistry. Iowa State University Press, Ames, Iowa

Calleja M, Monsain D, Lecouvreur B, D'Auzae J (1980) Influence de la carence phosphatée sur les activités phosphatases acides de trios champignons mycorrhiziens: *Hebeloma edurum* Metrod, *Suillus granulatus* (L. and Fr.) O. Kuntze et *Pisolithus tinctorius* (Pers.) Coker and Couch. Physiol Veg 18:489–504

Callow JA, Capaccio LCM, Parish G, Tinker PB (1978) Detection and estimation of polyphosphate in vesicular-arbuscular mycorrhizas. New Phytol 80:125–134

Chan WK, Griffiths DA (1988) The mycorrhizae of *Pinus elliottii* Engel. and *P. massoniana* Lamb. in Hong Kong. Mem Hong Kong Nat Hist Soc 18:11–17

Chan WK, Griffiths DA (1991) The induction of mycorrhiza in *Eucalyptus microcorys* and *E. torelliana* grown in Hong Kong. For Ecol Manage 43:15–24

Chilvers GA, Harley JL (1980) Visualization of phosphate accumulation in beech mycorrhizas. New Phytol 84:319–326

Grant CJ (1962) The soils and agriculture of Hong Kong. Government Printer, Hong Kong

Harley JL (1978a) Ectomycorrhizas as nutrient absorbing organs. Proc R Soc London B 203:1–21

Harley JL (1978b) Nutrient absorption by ectomycorrhizas. New Phytol 87:325–332

Harley JL, Smith SE (1983) Mycorrhizal symbiosis. Academic Press, London New York

Juma NG, Tabatabai MA (1977) Effects of trace elements on phosphatase activity in soils. Soil Sci Soc Am J 41:343–346

Lapeyrie FF, Chilvers GA, Douglass PA (1984) Formation of me-

tachromatic granules following phosphate uptake by mycelial hyphae of an ectomycorrhizal fungus. New Phytol 98:345–360

Lapeyrie F, Ranger J, Vairelles D (1990) Phosphate-solutilizing activity of ectomycorrhizal fungi in vitro. Can J Bot 69:342–346

Marx DH (1969) The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. I. Antagonism off mycorrhizal fungi to root pathogenic fungi and soil bacteria. Phytopathology 59:153–163

Marx DH (1977) Tree host range and world distribution of the ectomycorrhizal fungus *Pisolithus tinctorius*. Can J Microbiol 23:217–223

Marx DH, Artman JD (1979) *Pisolithus tinctorius* ectomycorrhizae improve survival and growth of pine seedlings on acid coal spoils in Kentucky and Virginia. Reclam Rev 2:23–31

Orlovich DA, Ashford AE, Cox GC (1989) A reassessment of polyphosphate granule composition in the ectomycorrhizal fungus *Pisolithus tinctorius*. Aust J Plant Physiol 16:107–115

Raven JA, Smith FA (1976) Nitrogen assimilation and transport in vascular land plants in relation to intracellular pH regulation. New Phytol 76:415–431

Tabatabai MA, Bremner JM (1969) Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol Biochem 1:301–307

Tam PCF, Griffiths DA (1993a) Mycorrhizal associations in Hong Kong Fagaceae. I. Techniques for the rapid detection and observation of ectomycorrhizas in local genera. Mycorrhiza 2:111–115

Tam PCF, Griffiths DA (1993b) Mycorrhizal associations in Hong Kong Fagaceae. III. The ontogeny of mycorrhizal development, growth and nutrient uptake by *Quercus myrsinaefolia* seedlings inoculated with *Pisolithus tinctorius*. Mycorrhiza 2:125–131