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# **Differentiation of polyphosphate metabolism between the extra- and intraradical hyphae of arbuscular mycorrhizal fungi.**

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## **SUMMARY**

Exopolyphosphatase activity and vacuolar acidity were assessed in order to have an overview of regulation of polyphosphate metabolism in arbuscular mycorrhizal symbiosis. Marigold (*Tagetes patula*) was inoculated with *Glomus coronatum* or *G. etunicatum* and grown in a mesh bag system. The extraradical hyphae were harvested from outside the mesh bag and the intraradical hyphae were collected from the mycorrhizal roots after enzymic digestion. Exopolyphosphatase activity was measured at pH 7.5 and 5.0. The extra- and intraradical hyphae of *G. coronatum* showed activity at pH 7.5 and 5.0, respectively. The extraradical hyphae of *G. etunicatum* showed activity both at pH 7.5 and 5.0, whereas intraradical hyphae of this fungus showed activity only at pH 5.0. The neutral (pH 7.5) activity showed lower  $K_m$  with long-chain polyphosphate, whereas the acidic (pH 5.0)

activity showed lower  $K_m$  with short-chain polyphosphate. The hyphae were stained with Neutral red, an acidic compartment probe, because a pH gradient across the tonoplast has been thought to be essential for polyphosphate accumulation in vacuoles. Both extra- and intraradical hyphae had acidic compartments which were identified as vacuoles. The inoculated plants were grown for 5 weeks with P-fertilizer (50  $\mu$ M  $\text{KH}_2\text{PO}_4$ ). P was then withheld from half of the plants, to provide low P and +P treatments. After 1 week, polyphosphate in the hyphae was stained by Toluidine blue. Polyphosphate was found in the hyphae from the +P treatment but not in the hyphae from the low P treatment. In contrast, exopolyphosphatase activity and acidity of vacuoles were relatively constant irrespective of polyphosphate status. Based upon these observations it is concluded that the fungi have at least two different exopolyphosphatase-type enzymes which are differently expressed between extra- and intraradical hyphae and that polyphosphate accumulation may be dynamically regulated by the balance between synthesis and hydrolysis.

## KEY WORDS

acid phosphatase, arbuscular mycorrhizal fungi, exopolyphosphatase, Neutral red, polyphosphate, acidic vacuole.

## INTRODUCTION

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with most land plants and promote the growth of the host through enhanced uptake of phosphorus (Smith & Read, 1997). Once the association is established, the fungi take up inorganic phosphate ( $\text{Pi}$ ) from the soil through extraradical hyphae, accumulate  $\text{Pi}$  into vacuoles as polyphosphate (polyP)

and translocate polyP to intraradical hyphae (Cox *et al.*, 1980). This is assumed to be followed by hydrolysis of polyP and subsequent release of Pi into the plant-fungus interfacial apoplast. PolyP is a linear polymer of Pi linked by high-energy phosphoanhydride bonds and has been found in nearly all classes of organisms (Kornberg *et al.*, 1999). Although the pathways involved in polyP metabolism in prokaryotes have been well documented, these have been yet to be elucidated in eukaryotes (Kornberg *et al.*, 1999). Several enzymes involved in polyP metabolism have been described. Polyphosphatekinase synthesizes polyP from ATP but also catalyzes the reverse reaction. Polyphosphateglucokinase transfers the terminal Pi of polyP to glucose, producing glucose-6-phosphate. These two enzymes, however, have only been found from prokaryotes (Kornberg, 1995; Kornberg *et al.*, 1999). There are two types of polyphosphate-hydrolyzing enzymes: endopolyphosphatase cleaves the internal linkages of polyP, shortening chain length; whereas exopolyphosphatase (PPX) hydrolyzes terminal residues and releases Pi. PPX is the only enzyme that has been well characterized in eukaryotic microorganisms. Yeast (*Saccharomyces cerevisiae*) has several PPXs in the cell envelope, cytoplasm, vacuoles, nuclei, and mitochondria, and these enzymes exhibit common features such as a pH optimum around 7.0 (Kulaev *et al.*, 1997). On the other hand, Capaccio & Callow (1982) found PPX-type activity in isolated intraradical hyphae of AM fungi with a pH optimum at 5.0. This suggests that the PPX found in AM fungi may be different from other PPX. In addition, it is of interest that the AM fungi have relatively shorter-chain polyP in the intraradical hyphae and longer-chain polyP in the extraradical hyphae (Solaiman *et al.*, 1999), suggesting that polyP metabolism between the two phases are different.

In eukaryotic microorganisms, almost all polyP is compartmentated in vacuoles,

e.g. in yeast (Kornberg *et al.*, 1999), filamentous fungi (Yang *et al.*, 1993) and algae (Pick & Weiss, 1991). Vacuolar pH may be one important regulatory factor of polyP metabolism, controlling both degradation and synthesis. Enzyme activity is, in general, highly pH-dependent and a vacuolar H<sup>+</sup>-ATPase defective mutant, which could not acidify the vacuolar lumen, could not accumulate polyP (Beauvoit *et al.*, 1991). Neutral red (NR), a pH-sensitive dye, has been used as an acidic compartment probe because of its properties as a weak base: the neutral form of NR is membrane-permeable but protonated NR is not, resulting in accumulation of the dye in acidic compartments. Accumulation of protonated NR can be observed with a fluorescent microscope (Chen *et al.*, 1999) based upon the shift in the optical absorption spectrum from pH 8.1 ( $\lambda_{\text{max}}$ =450 nm) to pH 5.3 ( $\lambda_{\text{max}}$ =535 nm) with constant emission wavelength at 637 nm (pK<sub>a</sub>=6.8) (Sousa *et al.*, 1996).

In this study, PPX-type activity in the extra- and intraradical hyphae of AM fungi, *Glomus coronatum* and *G. etunicatum*, was measured and compared. The enzyme properties and vacuolar status were examined using *G. coronatum*. Furthermore, the influence of P-nutrition on polyP status, PPX activity and vacuolar status of *G. coronatum* was investigated in order to have an overview of regulation of polyP metabolism in the AM symbiosis. *G. coronatum* was employed as it has relatively wide hyphae which facilitated microscopic observations. *G. etunicatum* was employed because it has been extensively used in previous studies on fungal enzymes (Ezawa *et al.*, 1995; Ezawa *et al.*, 1999).

## MATERIALS AND METHODS

### *Plant and fungal materials*

Dwarf marigold (*Tagetes patula* L. cv. Bonanza Spray) was inoculated with 100 spores of *G.*

*coronatum* Giovannetti (WUM 16) or 200 spores of *G. etunicatum* Becker & Gerd. (isolated by T. Wood, NPI, Utah) and grown in a compartmented system (mesh bag system) to facilitate harvesting of extraradical hyphae separate from colonized roots. The mesh bag (77  $\mu\text{m}$  mesh, 60 ml in volume) was buried centrally in a pot of sand of 270 ml volume (7 cm diameter, 10.5 cm high) and formed the root and hyphal compartment (R+H). The surrounding pot of sand formed the hyphal compartment (H). The R+H compartment was filled with 1:9 Mallala soil: washed river sand (pH 7.9, 6.28 mg P  $\text{kg}^{-1}$ , autoclaved), and the surrounding H compartment was filled with autoclaved washed river sand. The plants were grown in a greenhouse (20-25°C) and given liquid fertilizer (4 mM  $\text{NH}_4\text{NO}_3$ , 1 mM  $\text{K}_2\text{SO}_4$ , 0.75 mM  $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 0.5 mM Fe-EDTA, 50  $\mu\text{M}$   $\text{KH}_2\text{PO}_4$ , 46.25  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 9.14  $\mu\text{M}$   $\text{MnCl}_2$ , 0.81  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.32  $\mu\text{M}$   $\text{CuSO}_4$ , 0.10  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ ) 2-3 times per week until the solution flowed out from the drain holes. In experiments for 'influence of P-nutrition', P ( $\text{KH}_2\text{PO}_4$ ) was withheld from half of the plants a week before harvest to provide a low P treatment, whereas the other half of the plants received P as above (+P treatment).

Mycorrhizal roots were harvested from the R+H compartment at 6-7 weeks after sowing, cut into 5-10 mm segments, digested at 30°C for 60 min in a 10-fold volume (w/v) of 20 g  $\text{l}^{-1}$  Cellulase Onozuka RS (Yakult Pharmaceutical Ind., Tokyo), 20 g  $\text{l}^{-1}$  Driselase (Kyowa Hakko Kogyo, Tokyo or Sigma-Aldrich, Australia) and 10 g  $\text{l}^{-1}$  Pectolyase Y-23 (Seishin Corp., Tokyo) in 10 mM MES/NaOH (pH 5.5) and washed at 0°C with washing buffer (1 mM dithiothreitol, 0.3 M mannitol, 10 mM Tris/HCl, pH 7.6). The intraradical hyphae were collected from the digested roots as described by Ezawa *et al.* (1999) and stored in a 1.5 ml microtube at -75°C. The extraradical hyphae were collected from the H compartment by wet sieving, cleaned under a microscope and stored at -75°C. In order to

obtain sufficient material for assays 'batches' of extra- and intraradical hyphae were collected from groups of several plants grown at the same time in the greenhouse. Different batches of material were obtained from separate groups of plants, usually grown at different times.

#### *Enzyme preparation and assay conditions*

The fungal tissue (2-4 mg FW for intraradical hyphae and up to 100 mg FW for extraradical hyphae) was ground in a small mortar (6 cm diam) with 5 to 10-fold volume (w/v) of the extraction buffer (0.88 M sucrose, 100 mM Tris/HCl pH 7.5) at 0°C. The slurry was transferred to a 1.5 ml microtube. The mortar and pestle were washed with the same volume of extraction buffer. The solutions were combined and centrifuged at 10 000 g for 10 min at 4°C, and the pellet was resuspended in a 10 to 20-fold volume of the buffer and centrifuged. The supernatants were combined and designated as 'soluble fraction'. In the case of intraradical hyphae, the volume was adjusted to 100-fold volume (200-400 µl) with the buffer. The pellet was washed twice in the buffer, resuspended in 100-fold (in the case of intraradical hyphae) or 20 to 40-fold (in the case of extraradical hyphae) volume of the buffer and designated as 'insoluble fraction'.

PPX activity was assessed in the following two solutions: i) 20 mM Tris/HCl buffer pH 7.5, 50 mM ammonium acetate, 5 mM MgCl<sub>2</sub>, 1 mM (as Pi residue) polyP type 75+ (average chain-length 79, Sigma-Aldrich); ii) 100 mM sodium acetate buffer pH 5.0, 1 mM polyP type 75+. At the beginning of incubation, 10-15 µl enzyme extract was added, and the reaction mixture (total volume 300 µl) was incubated at 35°C for 60 min. The reaction was terminated by mixing with 150 µl of ice-cold 34% (w/v) trichloroacetate. The

solution was kept on ice for 10 min during which polyP was precipitated as well as protein. This step was essential to reduce background for colorimetric assay of Pi. After centrifugation at 10 000 g for 5 min at 4°C, 360 µl of supernatant was mixed with 240 µl of ammonium molybdate solution (Taussky & Shorr, 1953), and the amount of Pi released by the enzymic reaction was measured by absorbance at 710 nm. Control reaction mixtures consisted of: i) + enzyme, - substrate (polyP); ii) - enzyme, + substrate; iii) - enzyme, - substrate. Total protein in the soluble and insoluble fractions was precipitated and reextracted as described by Ezawa *et al.* (1999), and protein concentration was determined according to Lowry *et al.* (1951). One unit (U) of PPX activity was defined as the amount of enzyme which released 1 pmol of Pi per min under the specified conditions. Kinetic parameters were determined by the statistical method described by Wilkinson (1961) using either polyP type 75+ (long-chain polyP) or polyP type 5 (short-chain polyP, average chain-length 5, Sigma-Aldrich) as substrate.

#### *Staining of vacuoles with Neutral red*

The mycorrhizal roots were digested as above. Fifteen to 20 pieces of the colonized root were picked up under a microscope, and the epidermis was torn by needles to expose hyphae to the solution. Then the specimens were incubated at 25°C for 30 min with 0.5 mM NR in HEPES buffer (20 mM HEPES pH 7.2, 5 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 5 mM KNO<sub>3</sub>, 100 µM KH<sub>2</sub>PO<sub>4</sub>, 2 mM MgSO<sub>4</sub>, 2 mM glucose), blotted on filter paper briefly and washed with the HEPES buffer. Then the epidermis was removed, and the specimens were mounted on a glass slide. To manipulate vacuolar pH, the hyphae were treated with either a weak base [ammonium bicarbonate (NH<sub>4</sub>HCO<sub>3</sub>)] or protonophoric uncouplers [dinitrophenol (DNP) or

carbonylcyanide-*m*-chlorophenylhydrazine (CCCP)] as follows: i) the stained hyphae were incubated for 5 min at room temperature with 40 mM  $\text{NH}_4\text{HCO}_3$  in 20 mM HEPES pH 8.0 then mounted on a glass slide; ii) the specimens were preincubated at 25°C for 1 h with 5 mM DNP or 100  $\mu\text{M}$  CCCP in the HEPES buffer (without glucose), followed by incubation at 25°C for 30 min with the dye in the presence of uncoupler. The control specimens were preincubated and incubated without uncoupler.

The stained hyphae were observed using an Olympus IX70 inverted microscope equipped with a fluorescence observation attachment. The U-MWG (G-excitation) filter set (excitation filter: BP510-550, barrier filter: BA590) was employed. No autofluorescence in the hyphae was observed under these conditions. The number of hyphae which had fluorescent organelles (under dark field) and total number of hyphae (under bright field) within the camera field ( $430 \times 650 \mu\text{m}$ ) were counted in 10 different fields per batch, and the percentage of hyphae with fluorescent organelles was calculated.

#### *Visual assessment of polyP*

Extraradical hyphae were harvested from the H compartment and stained with 0.05% (w/v) Toluidine blue O for 30 min in 50 mM KCl/HCl buffer pH 1.0 (Orlovich & Ashford, 1993), rinsed in deionized water and spread on a glass slide. Percentage of hyphal length showing metachromasy was assessed in 10 different fields per batch under a microscope ( $\times 200$  magnification) based on the grid line intersect method (Giovannetti & Mosse, 1980).

#### *Statistical treatment*

All experiments except for 'Influence of P-nutrition' were repeated three times using

independent batches of material (see above). Each enzyme assay was replicated three times on the same batch of material. In the case of enzyme assays, one-way ANOVA or Student's *t* test (in the case of comparison between two means) was performed by StatView (Abacus Concept Inc., a statistical software for Macintosh) with the data of three assays of one batch of material, because absolute values ( $\text{U mg}^{-1}$  protein) were occasionally different between the batches although treatment effects were similar between the three replicate batches of material. In the case of assessment of vacuolar acidity, statistical analysis was performed using the means of ten assays (see above) of replicate batches of material. The experiments for 'Influence of P-nutrition' were carried out twice and similar results were obtained. In this case, Student's *t* test was performed between the treatments (low P and +P) with the data from the ten assays (assessment of polyP and vacuolar acidity) or the three assays (enzyme assay) on one batch of material.

## RESULTS

### *PPX activity in extra- and intraradical hyphae of G. coronatum and G. etunicatum*

In the extraradical hyphae of *G. coronatum*, only the insoluble fraction showed PPX activity (Fig. 1). The activity was much higher at pH 7.5 than at pH 5.0. In contrast, the intraradical hyphae showed about 3.5 times higher activity at pH 5.0 than at pH 7.5, and both the soluble and insoluble fractions showed similar activity.

In *G. etunicatum* both the soluble and insoluble fractions of the extraradical hyphae showed PPX activity (Fig. 2), with that in the soluble fraction slightly higher than in the insoluble fraction. The intraradical hyphae of *G. etunicatum* showed much higher activity at pH 5.0 than pH 7.5, similar to *G. coronatum*. The activity at pH 5.0 was observed

both in the soluble and insoluble fractions, but at pH 7.5 it was observed only in the soluble fraction.

#### *Characterization of PPX activity of G. coronatum*

Kinetic parameters were determined with respect to chain-length of substrate (Table 1).

Activity at pH 7.5 in the insoluble fraction of the extraradical hyphae showed much lower  $K_m$  and higher substrate specificity (expressed as  $V_{max}/K_m$ ) with long-chain polyP than with short-chain polyP. At pH 5.0 the situation was reversed with activity showing a lower  $K_m$  and higher specificity with short-chain polyP. In the intraradical hyphae, relatively higher specificity with short-chain polyP was observed at pH 7.5. In particular, the soluble enzyme showed very high  $V_{max}$  with short-chain polyP. It is noteworthy that the  $K_m$  of the insoluble enzyme with long-chain polyP was very low, resulting in relatively high specificity with long-chain polyP. At pH 5.0 both the soluble and insoluble enzymes showed lower  $K_m$  and higher specificity with short-chain polyP.

The three PPX-type activities, the neutral activity (pH 7.5) in the insoluble fraction of extraradical hyphae and the acidic activities (pH 5.0) in the soluble and insoluble fractions of intraradical hyphae, were chosen for investigation of the response to some inhibitors. As these enzymes showed different substrate specificities (see Table 1), long-chain polyP and short-chain polyP were used as substrates for the neutral and acidic activities, respectively. The neutral activity was markedly inhibited by beryllium and spermidine (Table 2). Heparin also inhibited the activity but a lesser extent. Only 10% inhibition by 10 mM fluoride was observed. EDTA increased the activity to about 170 % of the control. Response to inhibitors/activator was similar between the acidic activities in the

soluble and insoluble fractions (Table 3). Both 10 mM fluoride and 1 mM beryllium had a marked inhibitory effect (around 90%), but spermidine actually activated the enzymes.

#### *Vacuole as an acidic compartment*

After NR staining, the fluorescent organelles were observed in both the extra- and intraradical hyphae of *G. coronatum* (Figs. 3, 4). These fluorescent organelles were identified as vacuoles by the following observations: both bright and dark field photographs were taken from the same field, and diameters of vacuoles and that of corresponding fluorescent organelles were measured and found to be highly correlated ( $r = 0.974$ ).

The weak base,  $\text{NH}_4\text{HCO}_3$ , greatly reduced the percentage of hyphae with fluorescent vacuoles (Fig. 5a). The protonophoric uncouplers, DNP and CCCP, also reduced the percentage of hyphae with fluorescent vacuoles (Fig. 5b, c). Overnight incubation of the isolated intraradical hyphae in the HEPES buffer did not affect percentage of hyphae with fluorescent vacuoles but increased size of the vacuoles (Fig. 6a, b).

#### *Influence of P-nutrition on polyP, PPX activity and vacuolar status*

Forty-six percent of hyphal length of *G. coronatum* from the +P treatment (50  $\mu\text{M}$  P) showed metachromasy, but none of the hyphae from the low P treatment (0  $\mu\text{M}$  P) showed metachromasy (Fig. 7a). Significant but slight increase in PPX activities (except for the insoluble activity at pH 7.5) in the intraradical hyphae from the low P treatment was observed (Fig. 7b). No influence of P on percentage of intraradical hyphae with fluorescent vacuoles was observed (Fig. 7c).

## DISCUSSION

AM fungi clearly have at least two PPX-type activities indicated by different pH optima, and these enzymes were differently expressed between extra- and intraradical hyphae. High PPX activity at pH 5.0 in the intraradical hyphae of both *G. coronatum* and *G. etunicatum* observed in the present study is consistent with results obtained with *G. mosseae* by Capaccio & Callow (1982), although we found that considerable activity was associated with the insoluble fraction which Capaccio & Callow (1982) did not examine. These observations suggest that dominance of the acidic activity in the intraradical hyphae might be a common feature of the fungi. It must be noted that intraradical hyphae also showed neutral activity, which could be discriminated from the acidic activity not only by pH optimum but also by kinetic parameters. It is new and important information that extraradical hyphae, in which polyP synthesis may dominate, also had polyP-hydrolyzing activity. The extraradical hyphae of *G. etunicatum* showed extremely high activity of pyrophosphatase (soluble, optimum at neutral pH) (unpublished results). It is possible that the rather high activity in the soluble fraction of the extraradical hyphae of *G. etunicatum* might be due to high activity of pyrophosphatase, because yeast pyrophosphatase also shows polyphosphatase activity (Höhne & Heitmann, 1974; Lusby & McLaughlin, 1980). The difference in PPX activity in the extraradical hyphae between the two fungi may reflect functional diversity of AM fungi. Further investigation on PPX activity in extraradical hyphae of other fungi in relation to P-nutrition is required.

The activity found at neutral pH in the extraradical hyphae of *G. coronatum* showed similar characteristics to PPX (EC 3.6.1.11) found in yeast: optimum at neutral pH and inhibition by heparin (Kulaev *et al.*, 1997) and spermidine (Wurst & Kornberg, 1994).

Non-specific acid phosphatase (EC 3.1.3.2) may be responsible for the acidic activity in intraradical hyphae. Fluoride, a typical inhibitor for acid phosphatase (Arnold *et al.*, 1987), inhibited the acidic activity in intraradical hyphae, and spermidine, a PPX inhibitor, showed no inhibitory effect on the activity. Interestingly, beryllium, which is thought to be a specific inhibitor of alkaline phosphatase (Bell, 1972), inhibited both the acidic and neutral activities. It seems unlikely that the fungal alkaline phosphatase is responsible for the neutral PPX activity, because hydrolytic activity of glucose-6-phosphate, to which the fungal alkaline phosphatase showed highest specificity (Ezawa *et al.*, 1999), was much lower than polyP-hydrolyzing activity under the specified conditions. No inhibitory effect of beryllium on the fungal acid phosphatase was observed when *p*-nitrophenylphosphate or  $\alpha$ -naphthyl acid phosphate were used as substrates (Ezawa *et al.*, 1999; Ezawa *et al.*, 1995). Although the effect of beryllium on known PPXs has not yet been examined, specific interaction between beryllium and polyP which interferes with hydrolysis is suggested. Activation of the PPX of extraradical hyphae by EDTA also suggests that the reaction could be inhibited by divalent cations.

The results on substrate specificity of the acidic and neutral activity, which were expressed mainly in intraradical and extraradical hyphae respectively, agree with Solaiman *et al.* (1999), who found rather short-chain polyP in intraradical hyphae and longer-chain polyP in extraradical hyphae. Acid phosphatase of AM fungi seems to be localized in vacuoles (Ezawa *et al.*, 1995) in which polyP is accumulated. The results strongly suggest that the acidic PPX activity, possibly acid phosphatase, plays an important role in hydrolysis of (short-chain) polyP in vacuoles of intraradical hyphae prior to Pi release by the fungus into the plant-fungal interface.

Our study presents the first observation of acidic vacuoles in AM fungi.

Disappearance of fluorescence of vacuoles after ammonium bicarbonate (ammonia) treatment might be caused by alkalization of vacuolar lumen due to protonation of ammonia. Application of DNP or CCCP, protonophoric uncouplers, might reduce the proton gradient between the compartments and equilibrate vacuolar lumen with the cytosol, of which the pH might be around 7.5 (Ayling *et al.*, 1997). DNP and CCCP would also be expected to increase vacuolar pH and lower cytoplasmic pH, because these uncouplers inhibit ATP generation and thus inhibit vacuolar  $H^+$ -ATPase. These observations suggest that the vacuoles of the fungi would normally be acidified. The pH of fluorescent vacuoles is less than 6.8, which is the  $pK_a$  of NR, and possibly lower. It was expected that the overnight incubation would allow the isolated hyphae to recover from damage caused by enzymic digestion and that acidification would be greater. Vacuolar diameter in intact hyphae is probably around 1  $\mu m$  as seen in Fig. 3. Damage of hyphae, caused by cutting and digestion treatment in this investigation, stopped protoplasmic streaming (authors' observation), and in consequence the vacuoles may get stuck and fuse to each other, resulting in the formation of large vacuoles (see Fig. 6).

A metachromatic reaction of Toluidine blue was observed only in the fungus from the +P treatment, and this observation strongly suggests that the stained material is a polyanionic compound associated with P, such as polyP. We also observed metachromasy of intraradical hyphae in the sectioned roots of the plant from the +P treatment. Lack of metachromasy in the low P treatment does not necessarily mean that there is no polyP. The metachromatic reaction of Toluidine blue has been observed only with tetrapolyP and longer-chain polyP, tripolyP and pyrophosphate cannot be detected by this method (Lorenz

*et al.*, 1997). Furthermore, it is important to note that Toluidine blue is less sensitive to shorter-chain polyP (Lorenz *et al.*, 1997; Solaiman *et al.*, 1999). PolyP of AM fungi has been found as P-rich granules in vacuoles (Cox *et al.*, 1980; White & Brown, 1979). The granules were found even in fine-branched arbuscules (Peterson & Howarth, 1991) and size of the granules in arbuscules was not significantly different from that in the other stages of hyphae. On the other hand, Orlovich & Ashford (1993) employed a freeze-substitution technique and showed that granular polyP found in the ectomycorrhizal fungus, *Pisolithus tinctorius*, was an artefact of specimen preparation. After successive extraction of polyP from the extra- and intraradical hyphae of AM fungi, it was found that the fungi had both soluble and granular fractions of polyP (Solaiman *et al.*, 1999). ‘Soluble or granular (insoluble)’ is an important issue in terms of accessibility of the substrate for the hydrolyzing enzymes, especially in arbuscules in which the occurrence of polyP-degradation has been suggested. Analysis of polyP in freeze-substituted tissue would be a promising avenue for further research.

PolyP-hydrolyzing activity and vacuolar status (acidity) were relatively constant, irrespective of the presence or absence of polyP. Rapid hydrolysis of polyP and subsequent Pi release from cells by alkalization have been observed in yeast (Beauvoit *et al.*, 1991; Castrol *et al.*, 1999) and algae (Pick & Weiss, 1991). These observations suggest that hydrolyzing enzymes, such as acid phosphatase and PPX, may be expressed constitutively and thus polyP accumulation may be dynamically regulated by the balance between synthesis and degradation. PolyP synthesis seems to be energized and regulated by vacuolar H<sup>+</sup>-ATPase directly or indirectly, because the vacuolar H<sup>+</sup>-ATPase defective mutant of yeast could not accumulate polyP (Beauvoit *et al.*, 1991). There is some indirect evidence that

P-transfer (from the fungus to the plant) may occur at the arbuscular interface: a high-affinity plant Pi-transporter gene (*LePT1*), which is responsible for Pi uptake driven by a pH gradient across plasma membrane, was expressed in arbuscule-containing cells of tomato (Rosewarne *et al.*, 1999); ATP-hydrolyzing activity, possibly plasma membrane  $H^+$ -ATPase which generates a pH gradient across the plant plasma membrane, has been found on the periarbuscular membrane (Gianinazzi-Pearson *et al.*, 1991). Based upon these observations and our experimental results, we suggest the following model (Fig. 8): in intercellular hyphae polyP is maintained by the balance between synthesis and hydrolysis with vacuolar  $H^+$ -ATPase energizing polyP synthesis and acid phosphatase and/or PPX responsible for the hydrolysis. In contrast, in arbuscules, net hydrolysis of polyphosphate may be increased due to inactivation of vacuolar  $H^+$ -ATPase, and this may trigger release of Pi into the apoplast. Measurement of an absolute pH value of vacuoles at all stages of fungal development and investigation of the influence of vacuolar pH on hydrolysis of polyP are required to validate this hypothesis.

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**Table 1.** Kinetic parameters of exopolyphosphatase activity in the extra- and intraradical hyphae of *Glomus coronatum* with respect to chain-length of substrate.

| pH<br>/Parameter                         | Extraradical hyphae   |                      | Intraradical hyphae |                 |                  |                 |
|------------------------------------------|-----------------------|----------------------|---------------------|-----------------|------------------|-----------------|
|                                          | Insoluble             |                      | Soluble             |                 | Insoluble        |                 |
|                                          | PolyP 79 <sup>a</sup> | PolyP 5 <sup>a</sup> | PolyP 79            | PolyP 5         | PolyP 79         | PolyP 5         |
| pH 7.5                                   |                       |                      |                     |                 |                  |                 |
| $K_m$ (mM)                               | $0.36 \pm 0.06^b$     | $2.33 \pm 0.05$      | $0.30 \pm 0.13$     | $0.35 \pm 0.04$ | $0.077 \pm 0.03$ | $0.22 \pm 0.13$ |
| $V_{max}$ (U $\mu\text{g}^{-1}$ protein) | $60.9 \pm 2.7$        | $249.4 \pm 2.7$      | $9.14 \pm 1.4$      | $170.5 \pm 8.4$ | $13.5 \pm 0.8$   | $59.4 \pm 12.4$ |
| $V_{max}/K_m$                            | 169.2                 | 107.0                | 31.0                | 484.3           | 175.0            | 268.9           |
| pH 5.0                                   |                       |                      |                     |                 |                  |                 |
| $K_m$ (mM)                               | $10.8 \pm 2.8$        | $0.44 \pm 0.05$      | $0.58 \pm 0.06$     | $0.12 \pm 0.04$ | $0.60 \pm 0.11$  | $0.20 \pm 0.04$ |
| $V_{max}$ (U $\mu\text{g}^{-1}$ protein) | $25.0 \pm 6.1$        | $35.3 \pm 1.0$       | $24.0 \pm 0.8$      | $77.1 \pm 4.2$  | $23.9 \pm 1.5$   | $111.0 \pm 5.5$ |
| $V_{max}/K_m$                            | 2.3                   | 79.7                 | 41.4                | 642.5           | 39.8             | 555.0           |

<sup>a</sup> average chain-length of substrate.

<sup>b</sup> Kinetic parameter and standard error were calculated according to the statistical method described by Wilkinson (1961).

**Table 2.** *Inhibition and activation of exopolyphosphatase activity<sup>a</sup> in the insoluble fraction of the extraradical hyphae of Glomus coronatum at pH 7.5.*

| Inhibitors/activator               | Relative activity (%) |
|------------------------------------|-----------------------|
| Control                            | 100                   |
| 10 mM fluoride (F <sup>-</sup> )   | 91.8 <sup>b</sup>     |
| 1 mM beryllium (Be <sup>2+</sup> ) | 25.2                  |
| 1mM EDTA                           | 171.3                 |
| 1 µg/ml heparin                    | 59.9                  |
| 5 mM spermidine                    | 35.8                  |

<sup>a</sup> Long-chain polyphosphate (average chain-length = 79) was used as substrate at 1 mM.

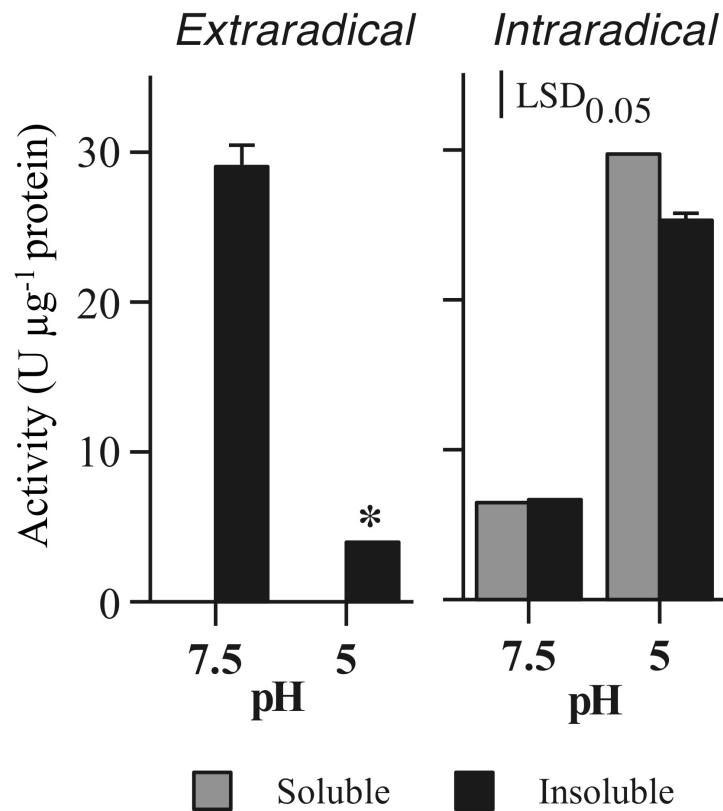
<sup>b</sup> Means of two replicate determinations on different batches of material (three assays per replication).

**Table 3.** *Inhibition and activation of exopolyphosphatase activity<sup>a</sup> in the intraradical hyphae of Glomus coronatum at pH 5.0 .*

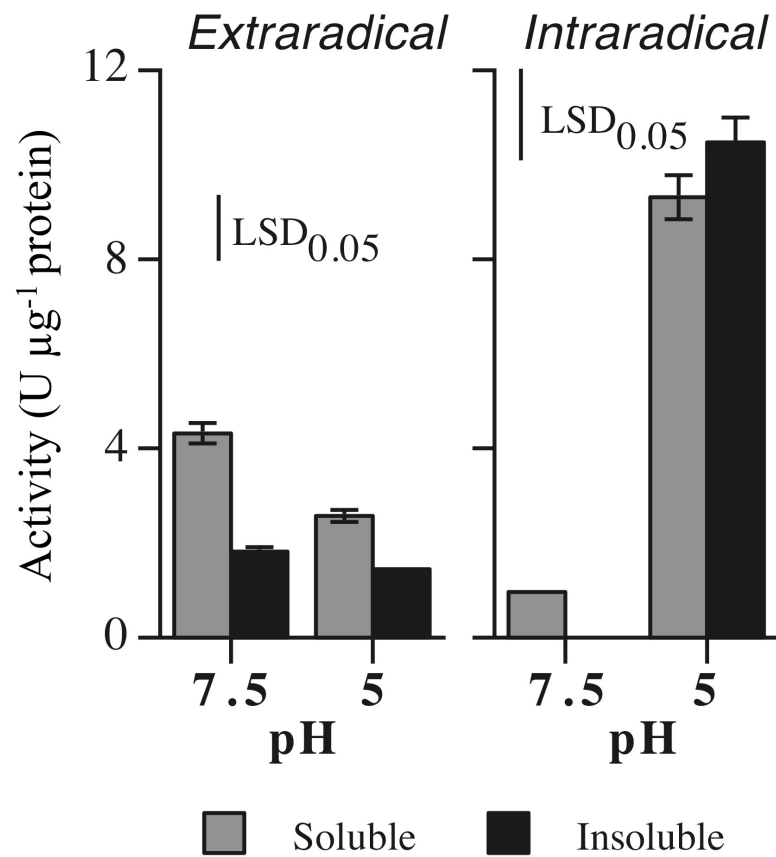
| Inhibitors                         | Relative activity (%) |                  |
|------------------------------------|-----------------------|------------------|
|                                    | Soluble enzyme        | Insoluble enzyme |
| Control                            | 100                   | 100              |
| 10 mM fluoride (F <sup>-</sup> )   | 16.5 <sup>b</sup>     | 11.2             |
| 1 mM beryllium (Be <sup>2+</sup> ) | 9.5                   | 4.9              |
| 5 mM spermidine                    | 132.6                 | 115.6            |

<sup>a</sup> Short-chain polyphosphate (average chain-length = 5) was used as substrate at 1 mM.

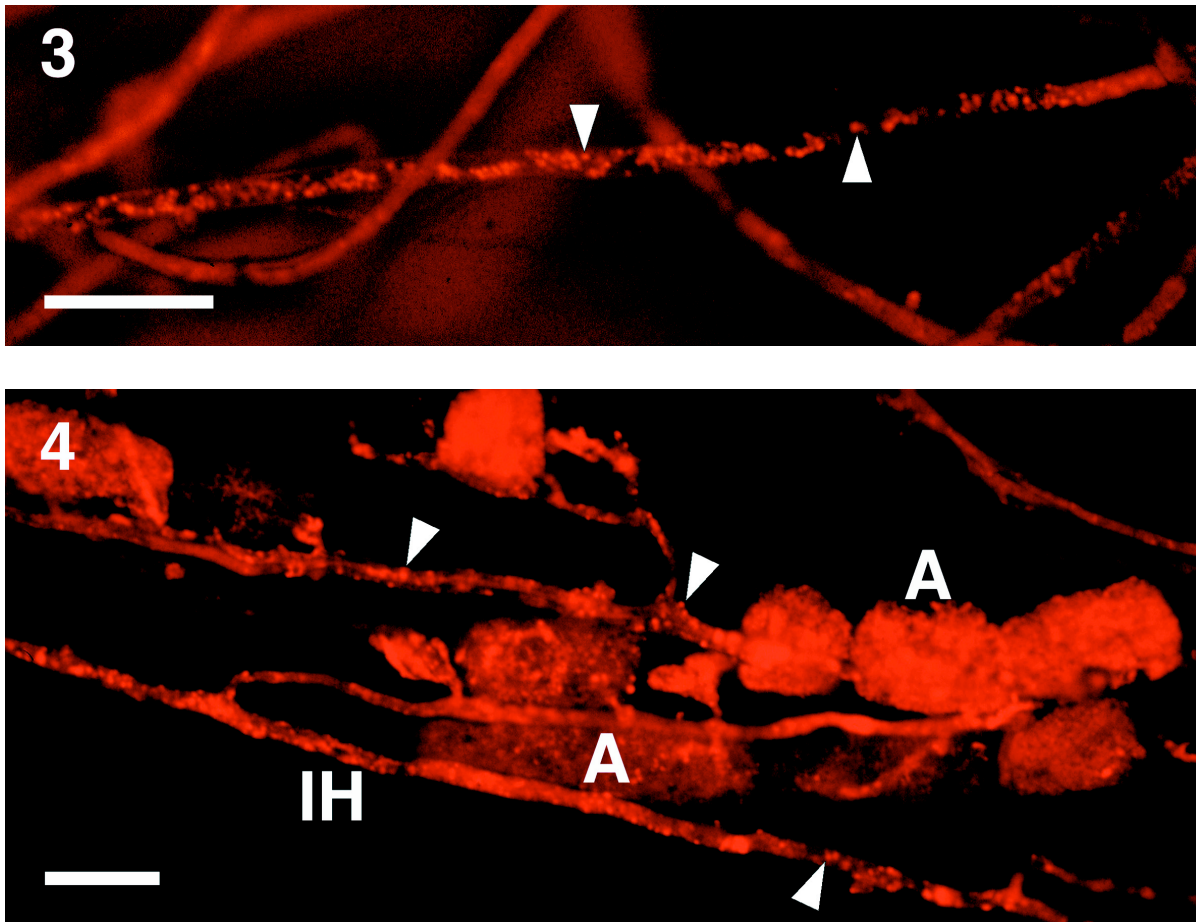
<sup>b</sup> Means of two replicate determinations on different batches of material (three assays per replication).



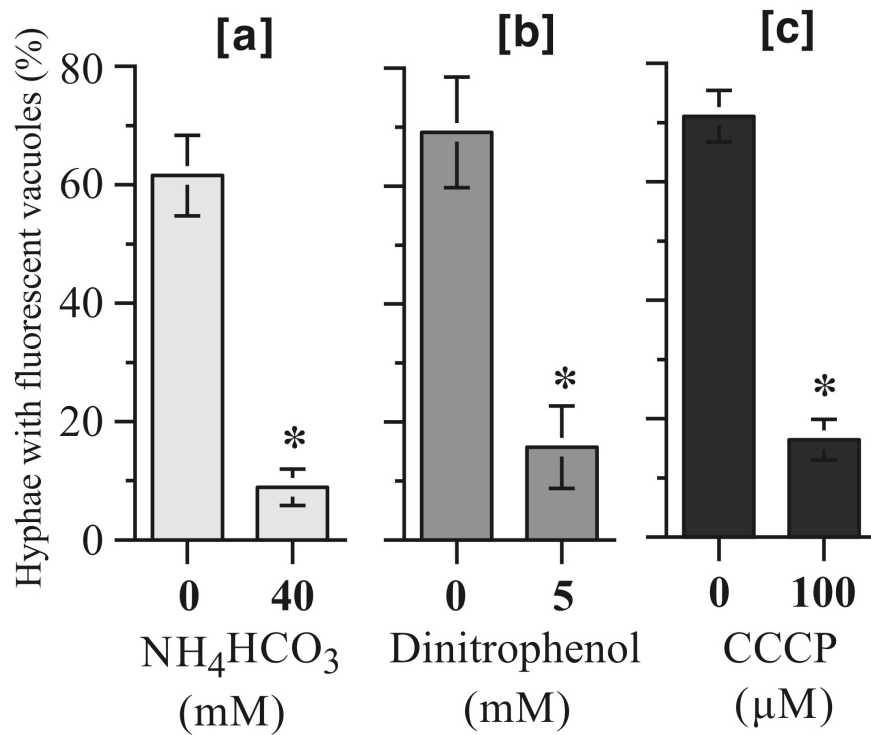
**Fig. 1.** Exopolyphosphatase activity in the soluble and insoluble fractions of the intra- and extraradical hyphae of *Glomus coronatum*. LSD ( $P < 0.05$ ) is presented to distinguish means (Intraradical). Asterisk indicates significant difference ( $P < 0.05$ , Student's *t* test) between the two means (Extraradical). Vertical bars indicate standard error ( $n=3$ ). The experiment was repeated three times using independent batches of material and statistical analyses were performed with the data from the three assays of one batch of material.



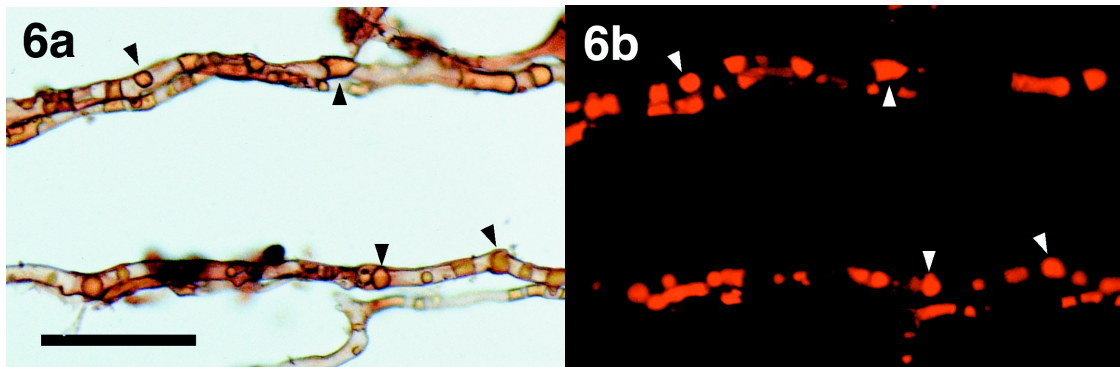
**Fig. 2.** Exopolyphosphatase activity in the intra- and extraradical hyphae of *Glomus etunicatum*. LSD ( $P < 0.05$ ) is presented to distinguish means. Vertical bars indicate standard error ( $n=3$ ). The experiment was repeated three times using independent batches of material and statistical analyses were performed with the data from the three assays of one batch of material.



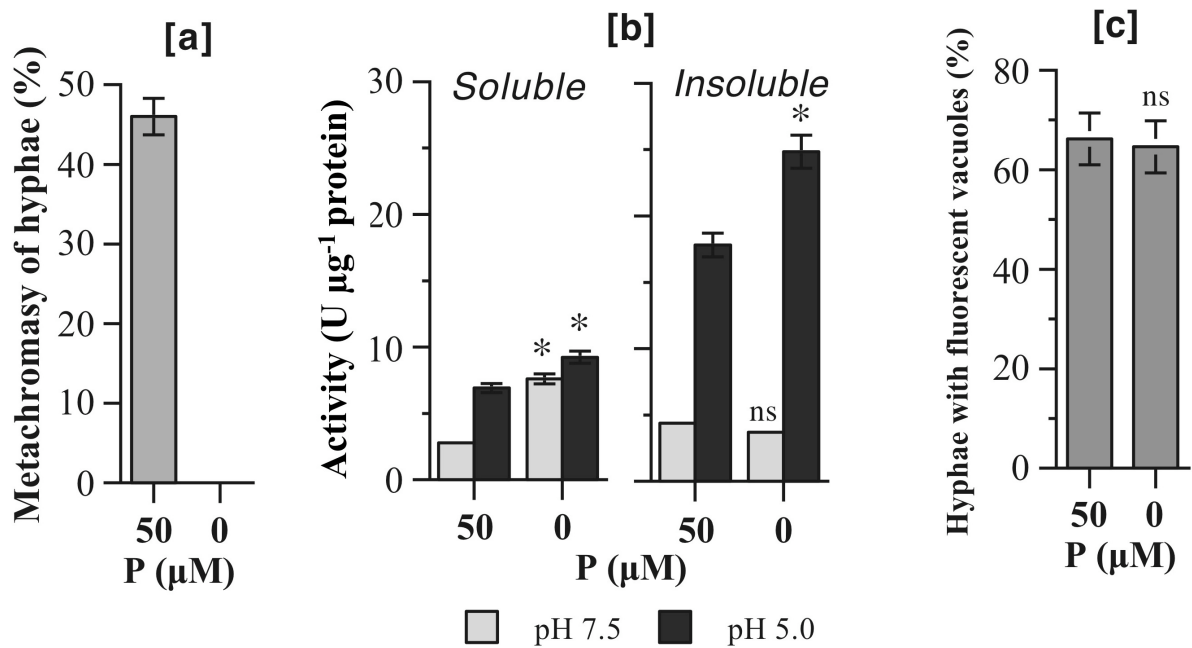
**Figs. 3-4.** Fluorescent vacuoles stained with 0.5 mM Neutral red. Fig. 3. The extraradical hyphae of *G. coronatum* were extracted by wet sieving from the hyphal compartment. Fig. 4. The intraradical hyphae of *Glomus coronatum* were isolated after enzymic digestion. Arrow heads indicate vacuoles. A, arbuscule; IH, intercellular hyphae. Bar=50  $\mu$ m.



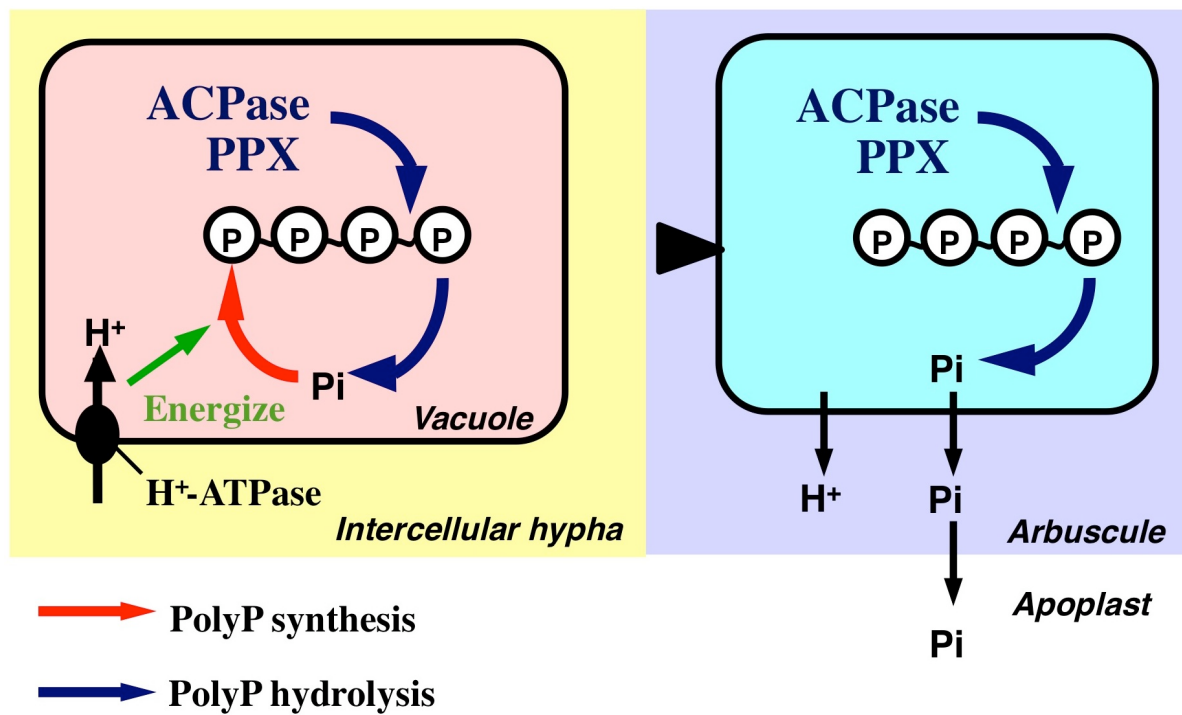
**Fig. 5.** Effect of ammonium bicarbonate (a), dinitrophenol (DNP) (b) and CCCP (c) on percentage of hyphae with fluorescent vacuoles in *Glomus coronatum*. Asterisks indicate significant difference ( $P < 0.05$ , Student's *t* test) between the two means. Vertical bars indicate standard error ( $n=3$ ). The experiment was repeated three times using independent batches of material and statistical analyses were performed using the means of replicate batches of material (three assays per replication).



**Fig. 6.** Large vacuoles in the intraradical hyphae of *Glomus coronatum* observed after overnight incubation: bright field (a) and dark field (b). Arrow head indicates corresponding vacuoles. Bar=50  $\mu\text{m}$ .



**Fig. 7.** Influence of P on metachromatic reaction in extraradical hyphae (a), exopolyphosphatase activity in intraradical hyphae (b) and percentage of intraradical hyphae with fluorescent vacuoles stained with Neutral red (c) in *Glomus coronatum*. The host plant (*Tagetes patula*) was grown with P-fertilizer (50  $\mu\text{M}$  P) for 5 weeks, then P was withheld from half of the plants (0  $\mu\text{M}$  P) and the other half received P as before (50  $\mu\text{M}$  P). After 1 week the hyphae were harvested (see MATERIALS AND METHODS). Asterisks indicate significant difference ( $P < 0.05$ , Student's *t* test) between the 50  $\mu\text{M}$  P and 0  $\mu\text{M}$  P treatments. ns, not significant. The experiment was repeated twice using independent batches of material and statistical analyses were performed using the data from the ten assays (assessment of polyP and vacuolar acidity) or three assays (exopolyphosphatase activity) of one batch of material. Vertical bars indicate standard error:  $n=10$ , polyP and vacuolar acidity;  $n=3$ , exopolyphosphatase activity.



**Fig. 8.** Model of polyphosphate metabolism in the intraradical hyphae of VA mycorrhizal fungi. ACPase, acid phosphatase; PPX, exopolyphosphatase; H<sup>+</sup>-ATPase, vacuolar H<sup>+</sup>-ATPase.