Mycorrhiza (1995) 5:205–211

ORIGINAL PAPER

K. Turnau · J. Dexheimer

Acid phosphatase activity in *Pisolithus arrhizus* mycelium treated with cadmium dust

Abstract The influence of cadmium dust (containing lead, cadmium, copper, zinc, silicium and other elements) on acid phosphatase activity of *Pisolithus arrhizus* was observed by means of electron microscopy. Dust-treated mycelium showed increased activity of the enzyme, especially on the surface of the cell wall. There was an increase in abundance of autophagic vacuoles marked by a strong phosphatase reaction. An increase in the number of hyphae with diffuse enzyme activity within the cytoplasm coincided with a decrease of lifespan of the fungus, rapid changes in the mictoplasm stage, earlier closing of the dolipori and presumably the earlier autolysis of cell cytoplasm. Hyphae showing strong autolytic activity were separated from other hyphae by the material deposited within the doliporus and this whole area was devoid at that stage of acid phosphatase activity. The role of the enzyme in the mechanism of resistance to toxic elements is discussed.

Key words *Pisolithus arrhizus* · Acid phosphatase Toxic element resistance · Cytochemistry

Introduction

Acid phosphatase consists of a broad group of enzymes that share the ability to hydrolyse various phosphate esters with the release of phosphate ions (Essner 1973; Hall and Hawes 1991). Acid phosphatase activity, with a pH optima of 5.0–6.5, has been shown to be typically associated with cellular membranes and organelles and absent from the cytosol in healthy cells. A great deal of information on cytochemical localization has been obtained for *Pisolithus arrhizus*, which is also of interest because of its resistance to toxic elements. Acid phosphatase activity in *P. arrhizus* mycelium cultivated in agar media was reported on the outer surface of the cell wall, and in vacuoles, nuclei and the endoplasmic reticulum (Lacaze 1983). In mycorrhizal association with *Pinus pinaster* (Dexheimer et al. 1986), the enzyme in this fungus has been shown to be localized along the plasma membranes of the Hartig net but was not present on cell walls. Strong activity was found in the matrix of the mantle, but only weak activity in the cement material of the Hartig net. An increase in diffuse phosphatase activity was found in the cytoplasm of senescent hyphae (Dexheimer et al. 1986). The above observations were later confirmed by immunolocalization of the enzyme in the same species (Gourp and Pargney 1991).

As already reported (Turnau et al. 1994), *P. arrhizus* belongs to a group of mycorrhizal fungi highly resistant to toxic elements. The mycelium grew well in liquid and agar media containing high doses of cadmium dust collected from chimney electrofilters, but marked changes in glycogen and calcium accumulation were observed. Though toxic elements were found in some of the polyphosphate granules, in most cases they were either absent or below the level of detection. As acid phosphatase has already been reported to be involved in heavy metal resistance in *Citrobacter* sp. (Macaskie and Dean 1990), the activity of this enzyme in *P. arrhizus* mycelia treated with cadmium dust was investigated. The present paper complements the studies of plant and fungal ecology and physiology in relation to industrial dusts carried out since 1980 (Greszta et al. 1987; Turnau 1991; Turnau et al. 1993, 1994).

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Materials and methods

Mycelium of *P. arrhizus* (obtained from INRA, Champenoux, France) was cultivated on 2% agar medium containing 0.25 g di-

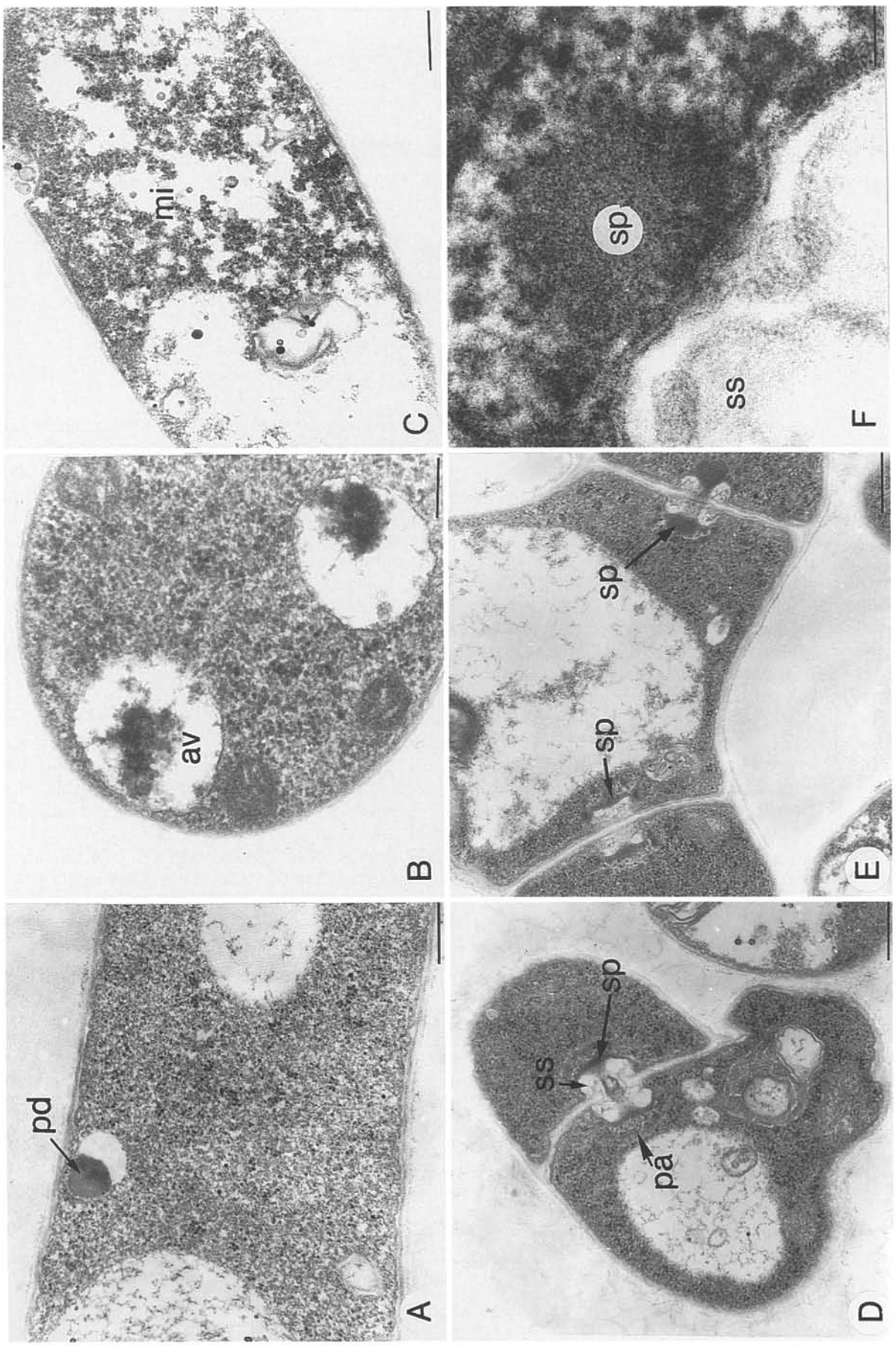

Fig. 1A–F

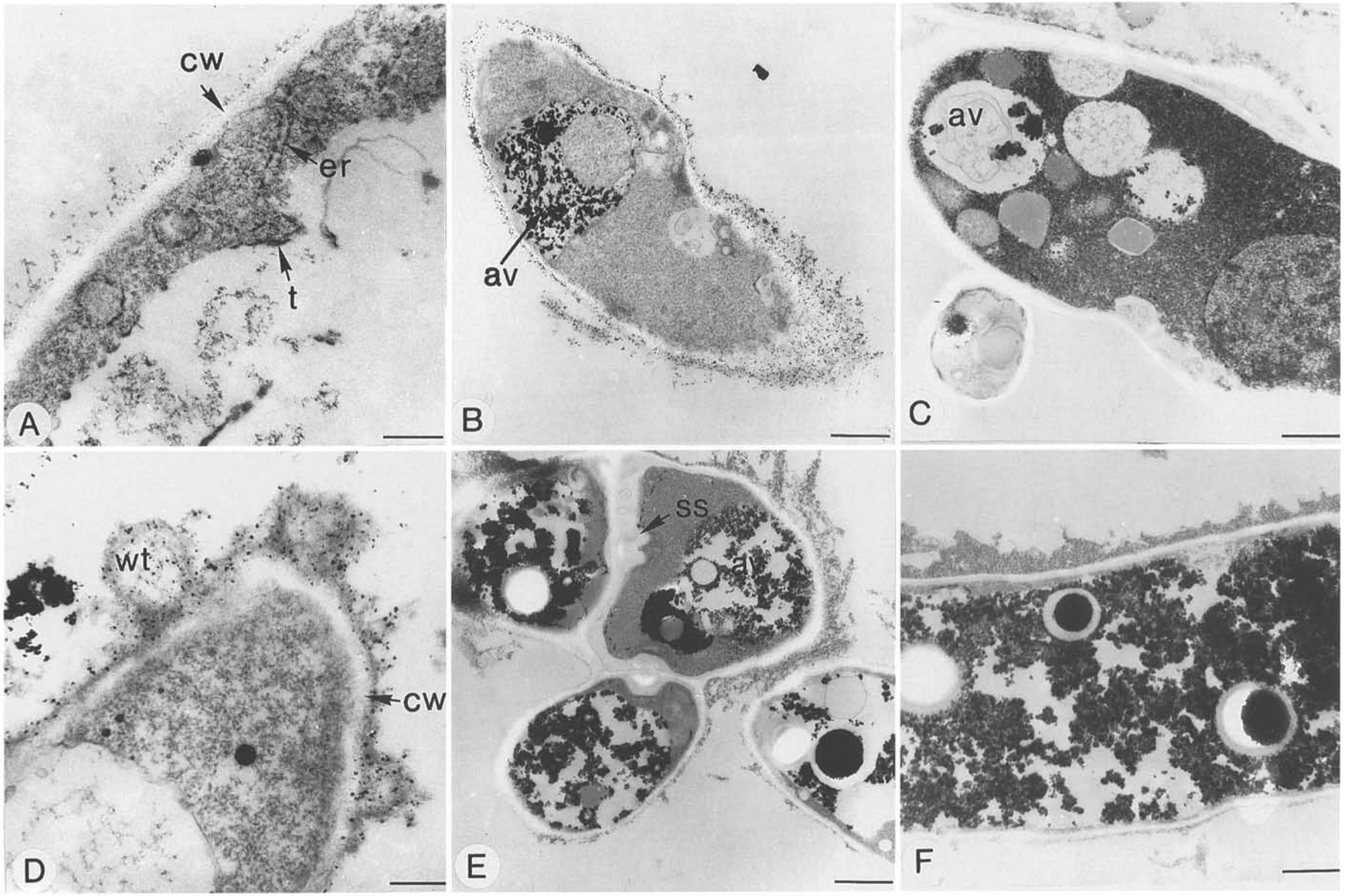

Fig. 2A–F

ammonium tartrate, 0.5 g KH_2PO_2, 0.25 g $MgSO_4$, thiamine, microelements, 20 g glucose and 5 g maltose. To study the influence of toxic elements on the mycelium, sterilized cadmium dust (collected from industrial electrofilters) was added to partly solidified medium (2 g/l). Small pieces of 4-week-old cultures were collected from the outer part of the mycelium for analysis.

Collected mycelium was prefixed in 2% glutaraldehyde in a cacodylate buffer at pH 7.2 and 0° C (1 h). This was followed by a 5% saccharose solution in the cacodylate buffer for 2 h (changed every 0.5 h). Samples were then washed in a 5% saccharose solution in distilled water for 1 h (changed every 20 min) and a 5% saccharose solution in 0.02 M sodium acetate (1h). The samples were then incubated in a standard medium containing 10 mM sodium glycerophosphate, 50 mM acetate buffer and 3.6 mM $Pb(NO_3)_2$ for 12 h at 0° C followed by incubation in a freshly prepared staining medium at 37° C. To verify that the precipitate observed was enzymatically produced, two procedures were used: (1) the staining medium was devoid of sodium glycerophosphate; (2) 20 mM sodium fluoride was added to the complete staining medium as an inhibitor of phosphatase activity. The specimens were then postfixed in 2% osmium tetroxide (1 h), dehydrated in acetone and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate for 15 min.

For routine electron microscopy, thin layers of agar with the mycelium were fixed in a 2% glutaraldehyde/0.2 M Hepes buffer (1:1), postfixed in osmium, dehydrated and embedded in Epon 812 or in Spurr resin. For conventional transmission electron microscopy, ultrathin sections of 80–120 nm were stained with uranyl acetate and lead citrate.

Results

Early developmental stages of *P. arrhizus* mycelium are characterized by the presence of two types of vacuoles, those in which phosphorus-rich material is deposited (Fig. 1A), and those in which autolysis is proceeding (Fig. 1B). As the cytoplasm ages, empty areas appear, vacuoles disappear and "mictoplasm" is formed (Fig. 1C). Though *P. arrhizus* is a fungus extremely resistant to cadmium dust, the life-span of the hyphal cells seems to be decreased in the presence of the dust. The formation of mictoplasm appears much earlier, when the cytoplasm is still very dense. In a double-stained section it is often indicated by a patch-like organisation of the cytoplasm (Fig. 1C), which is presumably the first hint of cytoplasm disorganization. In untreated cells, this stage usually follows the formation of autophagic vacuoles. In cadmium dust-containing media, the mycelium often shows no formation of vacuoles but only spaces between patches of cytoplasm. As a result of mictoplasm formation, the organelles, cytoplasm and electron-opaque granules are not separated by vacuolar membranes. The area close to the septal porus formed between cells of the hyphae seems almost always to be surrounded by much better preserved cytoplasm. The plug within the porus appears in untreated mycelium while the cells are already strongly vacuolized and mictoplasm formation is beginning. At this stage, all the organelles are still clearly visible. In cadmium-treated mycelium, the plug is formed much earlier, sometimes when the cytoplasm appears to be still in good condition (Fig. 1D–F). In the area between the porus and the parenthesome, small aggregations of electron-opaque material appear and form the plug (Fig. 1F). On the inner surface of the parenthesome, material of similar appearance is often visible. The comparison of the dust-treated and untreated mycelium using an energy-filtering microscope shows the presence of phosphorus-rich granules in both cultures. No difference in the abundance of these granules were produced by application of the dust. In both cases, the granules were found either within the vacuole or in spaces between patches of degenerating cytoplasm.

In untreated young mycelium the activity of acid phosphatase shows the typical distribution already described for *P. arrhizus* by Lacaze (1983), Dexheimer et al. (1986) and Gourp and Pargney (1991). A positive reaction was usually observed on the surface of the cell wall (Fig. 2A), in all cell membranes (Fig. 2A) and within autophagic vacuoles (Fig. 2B). In degenerating hyphae, the acid phosphatase activity was diffused in the cytoplasm (Fig. 2C). Strong staining was occasionally observed in some of the polyphosphate rich granules and in the tonoplast close to the granules (Fig. 3A). Mycelium treated with cadmium dust showed much stronger acid phosphatase activity both extracellularly

◀ **Fig. 1A–F** Transmission electron micrographs of *Pisolithus arrhizus* hyphae treated or not with cadmium dust. **A**, **B** Mycelium with vacuoles containing phosphorus-rich depositions and autophagic vacuoles present in the dust-treated or untreated mycelium respectively; **C** patch-like organization of dust-treated mycelium; **D** beginning of plug formation in quite young mycelium treated with cadmium dust; **E** septal pores closed by plugs; **F** formation of a plug with visible electron-opaque particles appearing between plug and parenthesome and similar material close to the inner surface of the parenthesome. *Bars:* **A**, **D**, **E** 0.4 μm, **B** 0.15 μm, **C** 0.25 μm, **F** 0.03 μm. *Abbreviations used in all figures: av* autophagic vacuole, *cw* cell wall, *er* endoplasmic reticulum, *mi* mictoplasm, *pa* parenthesome, *pd* phosphorus-rich deposition, *pl* plasmalemma, *sp* septal plug, *ss* septal pore swelling, *t* tonoplast, *wt* wall thickening

Fig. 2 Acid phosphatase activity in hyphae of *P. arrhizae* treated (**D–F**) or not (**A–C**) with cadmium dust. **A** Positive reaction on the surface of the cell wall, in the endoplasmic reticulum and tonoplast in young mycelium; **B** positive reaction within autophagic vacuoles; **C** diffuse activity in cytoplasm of untreated degenerating hyphae; **D** wall thickenings of mycelium growing over the medium showing phosphatase activity; **E** abundant autophagic vacuoles in cadmium-treated hyphae; **F** strong activity of degenerating cytoplasm. *Bars:* **A** 0.15 μm, **B**, **C**, **E**, **F** 0.6 μm, **D** 0.25 μm

Fig. 3A–F Acid phosphatase in the region of the septal pore apparatus in *P. arrhizus* mycelium of different ages. **A** Positive reaction of septal pore swelling, parenthesome and plasmalemma adjoining pore swelling in young hyphae; **B** negative reaction of septal pore swelling in older hyphae; **C** presence of electron-opaque material on the inner surface of the parenthesome in control mycelium (treated with NaF), probably connected with plug formation but more weakly stained than the real phosphatase activity; **D** parenthesome appearance in degenerating cell adjoining cell with a much weaker reaction; **E** more advanced stage of cell degeneration (note the lack of activity in the septal apparatus); **F** two degenerating cells strongly marked with the septal pore, plug and parenthesome showing no activity. *Bars:* **A** 0.25 μm, **B** 0.09 μm, **C** 0.05 μm, **D** 0.15 μm, **E** 0.6 μm, **F** 0.4 μm ▶

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and within the cytoplasm. This was partly related to the shorter life-span of the cells but was also observed in younger cells. The outer layer of the cell wall forms an irregular, presumably mucilaginous, surface when the mycelium is in contact with the agar surface (Fig. 2E–F), or highly ornamented thickenings (Fig. 2D) when the hyphae grows over the surface of the medium. Acid phosphatase activity was detected in both cases in the outer layer of the cell wall (Fig. 2D–F). Autophagic vacuoles were much more abundant than in mycelium not treated with the dust. Degenerating hyphae showed a very strong reaction (Fig. 2F), sometimes preventing sectioning.

Enzyme activity of the septal pore apparatus was of particular interest whenever it was found in a section. In open dolipori, the activity was visible in the septal pore swelling (dolipore) surrounding the pore (Fig. 3A), in the parenthesome (Fig. 3A), especially on the inner surface, and on the plasma membrane adjoining the surface of the annulus. The acid phosphatase activity disappeared first from the septal pore swelling when the doliporus was still open (Fig. 3B). At this stage, the activity of the parenthesome and plasma membrane adjoining the pore swelling was slightly stronger than that observed in the endoplasmatic reticulum and in the plasmalemma. Septal pores in control samples treated with sodium fluoride showed the presence of electron opaque material deposited on the inner surface of the parenthesome in some hyphae (Fig. 3C). Though it was sometimes hard to decide whether the staining was due to enzyme activity or to a deposition of unknown nature (especially in older hyphae), the large number of dolipori observed leaves no doubt as to the presence of phosphatase activity in the young parenthesome. The activity of the porus area seemed to decrease with age and once the plug formation had started, it was no longer observed to be connected with membranes. The activity of the parenthosome likewise was hardly distinguishable later, as degeneration of the cytoplasm resulted in increase of phosphatase activity spread over the whole cytoplasm, including the region between the porus and the parenthesome (Fig. 3D). In degenerating cells, where the cytoplasm shows diffuse phosphatase activity, the only place devoid of activity was the cell wall and the septal pore swelling, including the plug and the parenthesome (Fig. 3E, F).

Discussion

Pisolithus arrhizus belongs to the group of mycorrhizal fungi that can grow at heavily polluted sites. Its importance lies in the fact that phosphorus is one of the nutrients often deficient in such places, and association with this species leads to an increase in phosphatase activity by a factor of two to three (Fortin 1969). As our results show, the presence of cadmium dust increases the activity of this enzyme in fungal mycelium. Especially in young cells, extracellular activity was increased, which is a measure of the resistance of the enzyme to toxic metals. This is similar to the phenomenon described by Macaskie and Dean (1984, 1990) in *Citrobacter* sp., where heavy metal accumulation was mediated by an atypical acid-type phosphatase produced during pregrowth. This continued to function in resting and immobilized cells and liberated HPO_4^{2-} that precipitated stoichiometrically with metal ions M^{2+} to form $MHPO_4$ tightly bound to the cell surface. Additional evidence supporting this hypothesis comes from the precipitation of toxic elements on the surface of the mycelium of *P. arrhizus* shown by electron energy loss spectroscopy (Turnau et al. 1994). The increase of phosphatase activity in the cytoplasm of degenerating cells also suggests that a similar detoxification mechanism is also present intracellularly in degenerating or nonliving cells. If so, the fungal biomass might serve as a kind of biological filter. The mechanism of biosorption has been described already by several authors, e.g. for metal recovering processes used in industry, but it may also be applicable to ectomycorrhizal fungi, where it could be an extremely important process in toxic metal detoxification within the mantle. Although it has often been shown that this part of the mycorrhizae is nonliving, this does not imply an absence of function. Unfortunately, ageing mycorrhizae are not a common subject of research; one reason is that this sort of material is extremely difficult to prepare for electron microscopy, due to the removal of precipitates during sectioning and element redistribution during preparation.

As has already been described by Dexheimer et al. (1986) for mycorrhizal fungi and by Coulomb and Coulomb (1972) and Mesquita (1972) for plant cells, the autolytic processes we observed in the vacuoles of young cells occur naturally and may be important for the turnover of nutrients. Lysis of the cytoplasm may be more prominent under unfavourable conditions e.g. starvation or prolonged dormancy (Villiers 1972), and the data for cadmium-treated *Pisolithus* described above are in accordance with these findings. In addition, the cutting off of dead cells of the hyphae by the early closing of the dolipori is an interesting feature that may help to keep out detoxified elements from healthy cells.

In conclusion, the phosphatase activity reported above for *P. arrhizus* seems to be an important mechanism in heavy metal detoxification as well as that associated with phosphate-rich material deposited in the cell wall and within vacuoles (Turnau et al. 1994).

Acknowledgements We are very grateful to Joel Gerard (University of Nancy, France) for the preparation of the plates. This research was supported financially by the Ministre de la Recherche et de la Technologie (France) and Polish KBN (grant P205037 05).

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