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ORIGINAL PAPER

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Heavy metal tolerance by ectomycorrhizal fungi and metal amelioration by *Pisolithus tinctorius*

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Abstract Five ectomycorrhizal fungi, *Pisolithus tinctorius, Thelephora terrestris, Cenococcum geophilum, Hymenogaster* sp. and *Scleroderma* sp., which were demonstrated previously to be capable of forming ectomycorrhizas with some pine, eucalypt and fagaceous tree species were grown in vitro in liquid cultures for 3 weeks at six different concentrations of nine heavy metals, aluminium, iron, copper, zinc, nickel, cadmium, chromium, lead and mercury. Measurements of mean mycelial dry weight yields indicated that the local isolates of *Hymenogaster* sp. and *Scleroderma* sp., as well as the introduced fungal species *P. tinctorius,* were able to withstand high concentrations of Al, Fe, Cu and Zn and might, therefore, have potential for revegetation schemes in metal-contaminated soils. The metal amelioration mechanism in the metal-tolerant fungal species *P. tinctorius* was observed to involve extrahyphal slime and, as demonstrated by energy-dispersive X-ray spectrometry, was achieved by polyphosphate linkage of Cu and Zn.

Key words Metal tolerance · Ectomycorrhizal fungi
Pisolithus tinctorius · Metal amelioration
Energy-dispersive X-ray spectrometry

Introduction

In recent decades, great attention has been paid to the problem of heavy metal release from waste residues. It has been shown, for example, that severe contamination by pollutants such as heavy metals can result in widespread seedling mortality and in several decades of delay in revegetation schemes (Ritchie and Thingvold 1985). In contrast, many macrofungi exhibit tolerance to high concentrations of heavy metals that would normally cause severe toxicity symptoms in higher plants (Kuusi et al. 1981; Laaksovirta and Alakuijala 1978). Furthermore, some mycorrhizal fungi and other decomposer fungi have been shown to accumulate up to 63 times the concentration of mercury present in the soil from a mercury mining area (Bargagli and Baldi 1984).

Reclamation areas in Hong Kong originating from waste-disposal sites (landfills) may contain a large quantity of various heavy metals, and roadside areas are contaminated with metals emitted from automobiles etc., including a high proportion of Pb and other metals such as Cd, Cu, Ni and Zn (Seaward and Richardson 1990). Revegetation of such metal-contaminated areas may require ectomycorrhizal tree species tolerant to heavy metals. Previous studies in our laboratory indicated that several ectomycorrhizal fungi were capable of forming ectomycorrhizas with some pine, eucalypt and fagaceous tree species (Chan and Griffiths 1988, 1991; Tam and Griffiths 1993a) but their tolerance to heavy metals was not known.

The ion composition of polyphosphate granules of the ectomycorrhizal fungus *Pisolithus tinctorius* was determined by Orlovich et al. (1989) and Tam and Griffiths (1993b) by means of energy-dispersive X-ray analysis. Using the same technique, Vare (1990) demonstrated that aluminium polyphosphate granules were located in the ectomycorrhizal fungus *Suillus variegatus,* suggesting that this is the mechanism responsible for Al detoxification at high Al concentration in the growth medium.

The purpose of this present study was, firstly, to determine the tolerance of ectomycorrhizal fungi growing in vitro towards nine metals, Al, Fe, Cu, Zn, Ni, Cd, Cr, Pb and Hg, in order to assess their potential for the establishment of ectomycorrhizas at metal-contaminated sites. Secondly, by means of scanning electron microscopy coupled with energy-dispersive X-ray spectrometry (EDS), we investigated whether the metal amelioration mechanism in the metal-tolerant fungal species *P. tinctorius* involves polyphosphate linkage.

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Materials and method

Fungal cultures and media

Cultures of *Scleroderma* sp. and *Hymenogaster* sp. were isolated from sporophores growing at various sites and maintained on a modified Melin-Norkrans (MMN) agar medium (Marx 1969). Other fungal species obtained from the American Type Culture Collection (ATCC), *P. tinctorius* (Pers.) Coker and Couch (ATCC 38054), *Thelephora terrestris* Emhart ex Fries (ATCC 38058), and *Cenococcum geophilum* (Sow.) Fredinard et Winge (ATCC 38052), were also maintained on MMN agar medium.

MMN medium was prepared and dispensed in 25-ml portions to 100-ml screw-capped conical flasks and autoclaved. One, single, 5-mm-diameter disc cut from the margin of an actively growing colony of the test fungus was inoculated into the above medium and incubated stationary in the dark at 28° C for 3–7 days (depending on the growth rate of the fungal species) until visible hyphae emerged from the rims of the discs.

Stock solutions of sulphate, zinc sulphate, aluminium chloride, iron sulphate, cadmium sulphate, nickel sulphate, lead nitrate, potassium chromate and mercury choride were prepared at concentrations of 2500, 250 and 25 µg/ml and autoclaved for sterility. Each of the above metal stock solutions was pipetted aseptically to five conical flasks bearing the actively growing mycelium of the test fungus to give final concentrations of the metals in the medium of 400, 200, 100, 10, 1, and 0.1 µg/mL. A further five conical flasks containing the same test fungus growing in medium without added metals acted as the controls.

All cultures were incubated for a further 3 weeks under the same conditions as described above. The mycelium in each flask was harvested by filtration and washed with distilled water, and the mycelial dry weight yields of triplicate samples from each treatment were determined after oven-drying at 80° C. The inhibitory concentration of each metal for each ectomycorrhizal fungus was determined as the concentration at which the mean mycelial dry weight yield was reduced by 50% when compared with the controls.

Microscopic examination of hyphae

Small pieces of hyphae taken from the rim of the mycelial mat in each treatment were mounted in a drop of growth medium and observed under a light microscope. The external morphology of hyphae was photographically recorded.

Energy dispersive X-ray spectrometry

A small portion of hyphae, again taken from the rim of the mycelial mat in each treatment, was washed with distilled water and mounted on a transparent cellulose strip which had been coated with a drop of adhesive (0.05% egg albumen solution). The strips were placed on a hot-plate at 50° C until the hyphae in the adhesive were dry. The area of the strip containing the adhering hyphae was trimmed and dehydrated through a graded series of ethanol and coated with carbon in a vacuum evaporator. The specimens were examined in a Cambridge 150 scanning electron microscope equipped with an energy-dispersive X-ray analyser (eXL Link Analytical). The EDS analysis was carried out at an accelerating voltage of 20 KV. The electron beam was sharply focused on a hyphal portion giving a high peak for P (indicating the presence of polyphosphate granules at this particular spot). Triplicate spectra were collected for 100 s for each metal treatment and the controls.

Table 1 The 50% inhibition concentrations (µg/ml) of nine metals (metal tolerance) shown by the ectomycorrhizal fungi *Pisolithus tinctorius* (Pt), *Thelephora terrestris* (Tt), *Cenococcum geophilum* (Cg), *Hymenogaster* sp. (H), *Scleroderma* sp. (S). The values in each case are the means of triplicate samples

Fungus	Al	Fe	Cu	Zn	Ni	Cd	Cr	Pb	Hg
Pt	200	400	200	200	10	10	10	200	1
Tt	10	100	10	10	1	1	10	200	1
Cg	10	200	10	10	1	1	10	200	1
H	200	200	10	100	1	0.1	10	200	1
S	200	100	100	100	10	10	10	200	1

Results

The results obtained with the five ectomycorrhizal fungi and nine metals are summarized in Table 1. Differences were found in their abilities to withstand increasing concentrations of various metals. *P. tinctorius, Hymenogaster* sp. and *Scleroderma* sp. exhibited greater metal tolerance at higher concentrations of Al than *T. terrestris* and *C. geophilum*. All five fungi were tolerant of high concentrations of Fe, particularly *P. tinctorius, C. geophilum* and *Hymenogaster* sp. *P. tinctorius* and *Scleroderma* sp. exhibited greater tolerance of Cu at high concentrations than the other three fungi. *P. tinctorius, Hymenogaster* sp. and *Scleroderma* sp. were more tolerant of Zn than the other two fungi. All five fungi were sensitive to low concentrations of Ni, Cd, Cr

Table 2 Mean mycelium dry weight (mg) of *P. tinctorius* growing in vitro for 3 weeks in liquid media containing six concentrations of eight metals, and in a control medium without these metals. The values in each case are means of triplicate samples. The absence of data indicates negligible mycelial dry weight

Metal	Concentration (µg/ml)					
	0.1	1	10	100	200	400
Al	45.2	39.5	42.5	45.3	18.4	—
Cu	57.5	45.2	45.1	45.6	2.3	—
Zn	37.1	43.9	51.0	38.3	7.8	—
Fe	50.2	40.5	48.5	36.3	30.8	17.5
Ni	40.2	41.3	5.4	—	—	—
Cd	41.7	37.8	3.8	—	—	—
Cr	36.5	30.1	10.0	—	—	—
Hg	10.2	4.1	2.1	—	—	—

Control 39 7

Fig. 1 *Pisolithus tinctorius* hyphae growing in control medium ▶ and showing normal hyphal development and morphology. For Figs. 1–4, *bar*=10 µm

10 µg/L Cd)

Fig. 2 *P. tinctorius* hyphae growing in media at a [redacted] concentration resulting in inhibition of mycelial growth and distorted hyphal development

Fig. 3 A hypha of *P. tinctorius* with a swollen tip

Fig. 4 Mucilaginous substances usually seen associated with hyphae growing at high concentrations of metals inhibitory to mycelial growth

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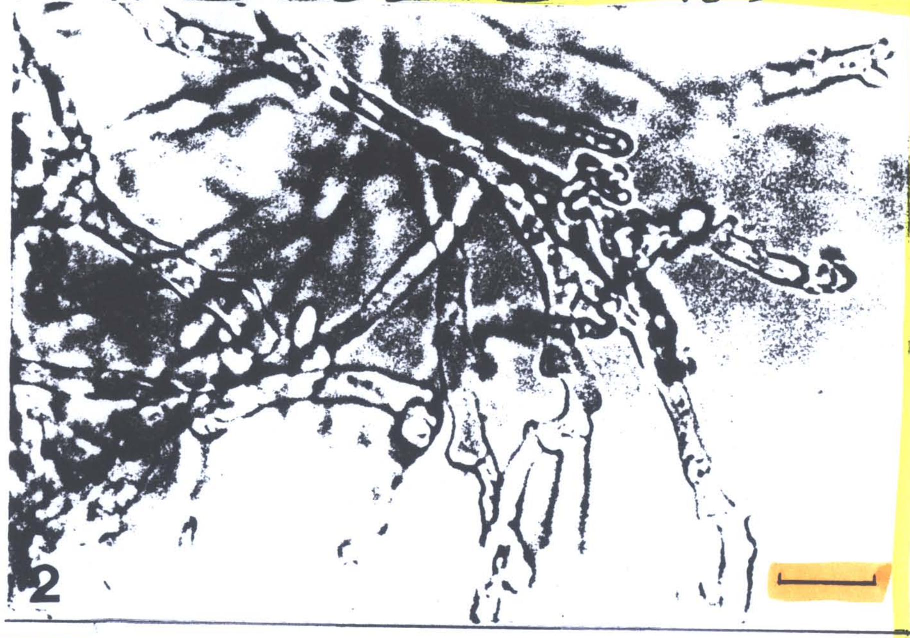
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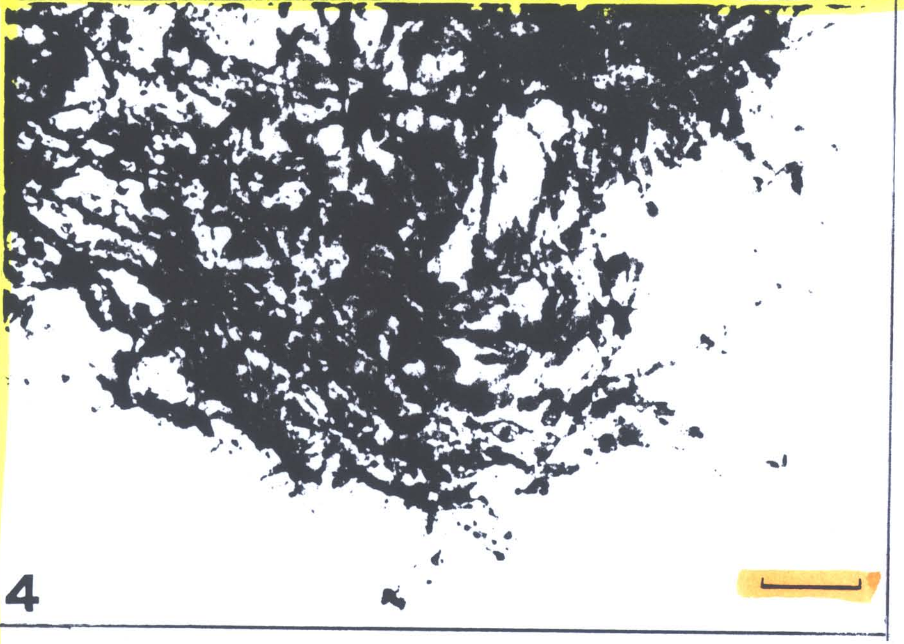
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and Hg, but were all tolerant of Pb at high concentrations.

The tolerance of *P. tinctorius* at six different concentrations of eight metals is summarized in Table 2. In media containing 0.1–100 μg/ml of Al, Cu, Zn and Fe, mycelial growth generally increased compared with the control. However, inhibition of mycelial growth occurred at concentrations above 200 μg/ml for Al, Cu, Zn and Fe, above 10 μg/ml for Ni, Cd, Cr; but only above 0.1 μg/ml for Hg.

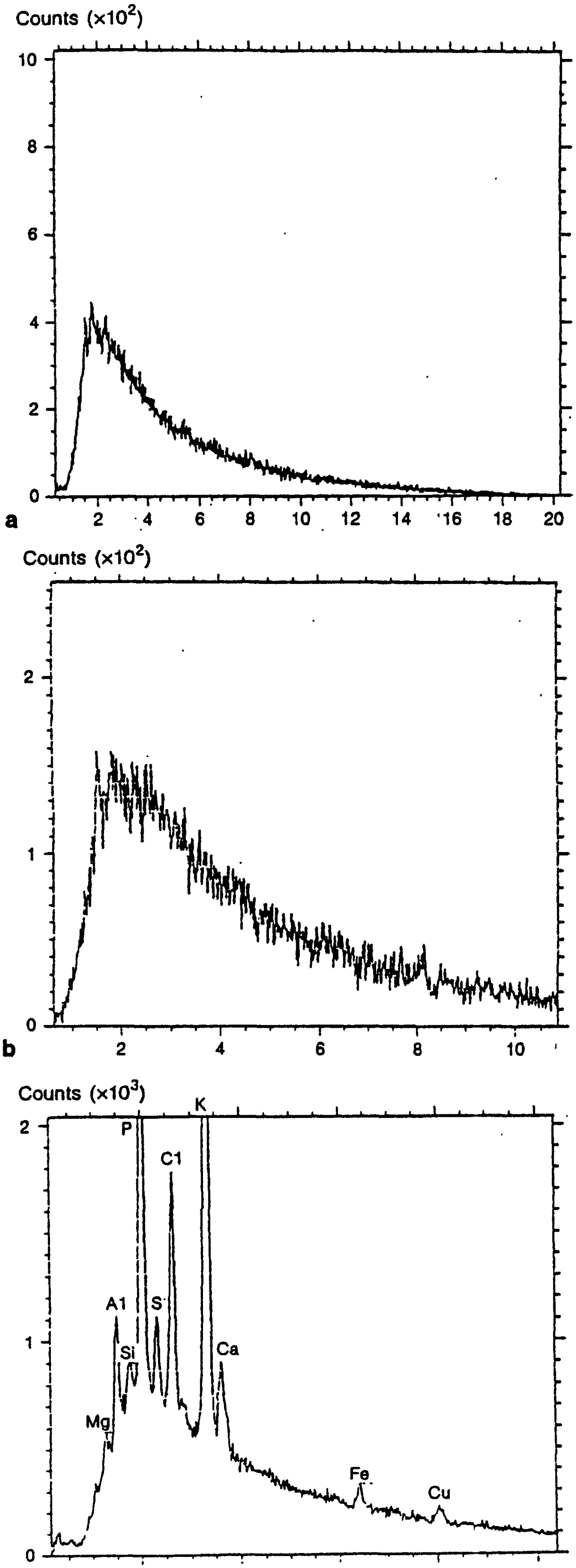
Photographs taken of various metal treatments indicated that hyphae growing at lower, nontoxic concentrations of metals usually exhibited normal hyphal development and the morphology of hyphae growing in the control medium (Fig. 1). At higher concentrations of metals resulting in more than 50% inhibition of mycelial growth, the hyphae exhibited morphological changes, e.g. distorted hyphal development and swollen hyphal tips (Figs. 2, 3); in these cultures, slimy substances were seen to accumulate on the hyphal surfaces (Fig. 4).

EDS spectra of the cellulose strip and albumen controls, of the *P. tinctorius* hyphae growing in the control medium, and of this fungus in media containing two different concentrations of Zn and Cu are presented in Fig. 5. Though variation was found in the peak heights of different ions, all spectra except the cellulose strip and albumen controls showed consistently high peaks for P; the other ions frequently occurring as peaks in these spectra were the cations Al, Si, K, Ca, Fe and Cu, and the anions S and Cl. The spectra from different concentrations of Cu and Zn (Fig. 5d–g) showed significantly higher peaks for Cu and Zn at the higher concentration (200 μg/ml) than at the lower concentration (10 μg/ml).

Discussion

It was observed during addition of Pb at high concentrations that the culture media became cloudy. It appeared that Pb was precipitated by some medium components such as phosphates. Precipitation may thus result in the apparent tolerance of Pb at high concentrations shown by the five ectomycorrhizal fungi.

The measurements of mycelial dry weight in general indicate that the local fungal species *Hymenogaster* sp. and *Scleroderma* sp. has a similar ability to withstand high concentrations of the metals Al, Fe, Cu and Zn as the known metal-tolerant *P. tinctorius*. Improved survival and growth of pine seedlings with abundant *P. tinctorius* ectomycorrhizas compared with naturally infected control seedlings (ectomycorrhizal with *T. terrestris*) has been widely reported to occur on acid coal spoils, kaolin spoils, severely eroded copper basin sites and borrow pits (Marx and Artman 1979). It might, therefore, be speculated that tree species ectomycorrhizal with the two local fungal isolates tested in the present study could be as successful as *P. tinctorius* ectomy-



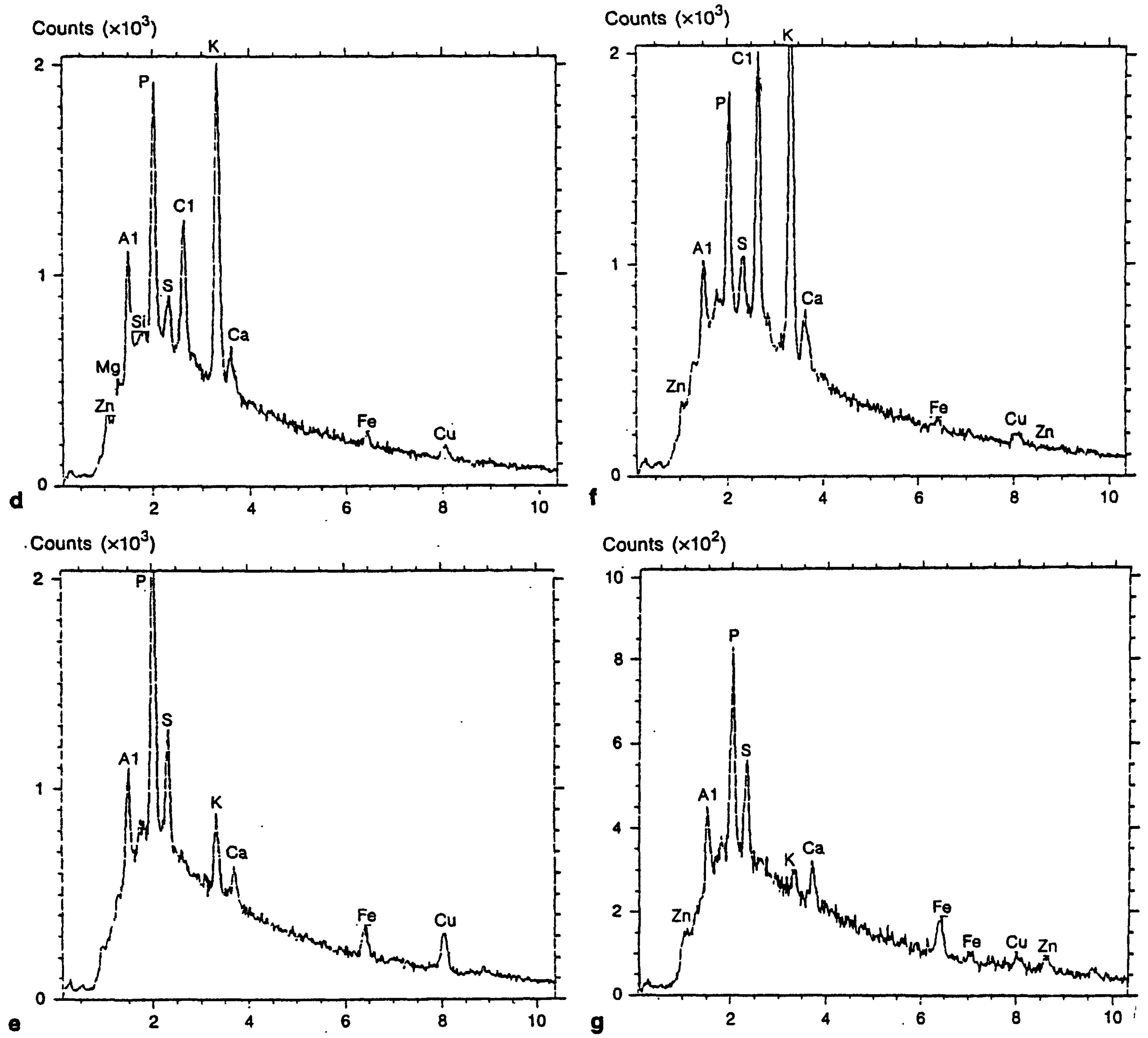


Fig. 5 Energy-dispersive X-ray spectra of cellulose strip control (**a**) albumen adhesive controls (**b**) and *P. tinctorius* hyphae growing in metal-free control medium (**c**) and in media containing Cu at 10 μg/ml (**d**) and 200 μg/ml (**e**) and Zn at 10 μg/ml (**f**) and 200 μg/ml (**g**)

corrhizas in conquering such adverse sites contaminated with metals.

The possible role of mycorrhizal fungi in the metal tolerance of higher plants is poorly understood. However, reports on ericaceous mycorrhizas (Bradley et al. 1982) and ectomycorrhizas (Brown and Wilkins 1985; Denny and Wilkins 1987b) indicated the importance of the mycobionts in improving metal tolerance of their host plants by primarily accumulating metals in the walls of extramatrical hyphae and extrahyphal slime; thus passage of metals to the shoots is restricted. It was further stressed by Denny and Wilkins (1987b) that the compatibility of fungal strain and plant host is more important to the success of the relationship in the presence of high heavy metal concentrations than fungal adaptation to the metal.

The detoxification of heavy metals within cells of several yeast species has been shown to be linked with polyphosphate granules located in the cytoplasm (Roomans 1980) and in vacuoles (Kunst and Roomans 1985). Metal-binding proteins, e.g. metal thiolate clusters and protein-bound sulphides have been detected in *P. tinctorius* but not in *C. geophilum* (Morselt et al. 1986). In order to better understand the metal amelioration mechanism in ectomycorrhizal fungi, the metal-tolerant strain *P. tinctorius* was selected for further study.

The higher mycelial dry weight yields of *P. tinctorius* at high concentrations of Al, Cu, Zn and Fe than the controls indicated the ability of this species to detoxify these metals. The enhancement of mycelial growth with Cu, Zn, and Fe recarded may be due to a requirement for these elements as micronutrients in their role of metal activators. However, Al is not known to be a micronutrient essential for fungal growth and, therefore, a detoxification mechanism ought to exist in this fungus.

Morphological changes often induced by high concentrations of heavy metals are not clearly understood, but may be either survival mechanisms or manifestations of toxicity (Gadd and Griffiths 1978). Extracellular mucilaginous substances were frequently excreted by fungal cultures with higher concentrations of added metals in this present study. Such materials may act to ameliorate heavy metals either in the form of hyphal slimes (Denny and Wilkins 1987[redacted]), or be chelates between heavy metals and organic acids excreted by the fungus (Bradley et al. 1982; Brown and Wilkins 1985).

The ion composition of polyphosphate granules in *P. tinctorius* was studied by Orlovich et al. (1989), who showed that Ca, K and Na composition may vary with the method of specimen preparation. This was further investigated by Tam and Griffiths (1993b) using wax-embedding and sectioning steps; they found that the major cation linked with the polyphosphate was consistently divalent Ca, whereas monovalent K occurred only as a small peak in some P treatments. However, the method of specimen preparation was modified in this investigation by employing intact hyphae in an attempt to minimize the loss of intracellular ions during histological processing. In the present study, a large peak of P was consistently found in all metal treatments as well as in the metal-free control, indicating a high P content of the polyphosphate granules of this fungus. The major cation was K, which was present in the control treatment as well as in the low-concentration metal treatments. This anomaly can be explained by the fact that intact hyphae were employed and the histological process omitted; thus the loss of intracellular monovalent ions was greatly reduced. In general, the peak heights of K and Cl increased with decreasing concentrations of added metals, demonstrating a dramatic displacement of K and Cl ions or a redistribution of ions in the polyphosphate granules in the presence of the added metals.

Since the cations Al, Si, Cu and Fe were also present in the control spectra of the albumen adhesive and the cellulose strip as well as in the spectrum of the control medium, they are either spurious background signals coming from the scanning electron microscope or artefacts. Thus, it is difficult to demonstrate convincingly a detoxification mechanism for these metals through phosphate-polymer linkage. However, it was observed in this study that the spectral peak for Cu in extracts from the high Cu treatment was significantly greater than the corresponding peaks at low Cu concentration and in the control treatment; this indicates metal amelioration of Cu via polyphosphate granules. Evidence for polyphosphate acting as a metal sequestering agent for Zn in this investigation was supplied by the presence of a spectral peak for Zn only at high Zn concentrations. Furthermore, Denny and Wilkins (1987a) demonstrated that the polyphosphate granules in zinc-treated roots of *Betula* consisted of similar ionic components. However, in this present study with *P. tinctorius*, metal-sequestering mechanisms via polyphosphate granules were not evident with Al, Fe, Ni, Cd, Cr or Hg.

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