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Effect of Heavy Metals on Growth and Extracellular Enzyme Activities of Mycoparasitic *Trichoderma* Strains

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Trichoderma species are imperfect fungi, with teleomorphs belonging to the ascomycete order *Hypocreales*. Their mycoparasitic ability against plant pathogenic fungi, e.g. *Fusarium* and *Rhizoctonia* species, allows for the development of biocontrol strategies based on *Trichoderma* strains (Papavizas, 1985). Such strategies can be incorporated in a complex integrated plant protection. When planning the application of biocontrol strains, it is very important to consider the environmental stresses affecting microbial activities. The presence of heavy metals or pesticides in the soil, low temperature, and low water potential are among the most important stress factors. The study of the influence of such parameters on the growth of the fungi and on their extracellular enzyme activities is of great importance. Biocontrol strains of *Trichoderma* should grow fast in the soil and secrete active enzymes essential for mycoparasitism, such as chitinases, proteases or β-1,3-glucanases, which are involved in the degradation of fungal cell walls. Studies are available on the effect of low temperature (Antal et al. 2000), low water potential (Kredics et al. 2000), and pesticides (Beatty and Sohn, 1986; Stratton, 1983) on various activities of *Trichoderma* strains. Heavy metal sorption (Lokesha and Somashekar, 1989; Morley and Gadd, 1995), and accumulation (Ledin et al. 1996) by *Trichoderma*, and the effect of some heavy metals on the growth, sporulation (Babich et al. 1982; Somashekar et al. 1983), and differentiation (Frank et al. 1993) of these fungi were also examined. This study was designed to investigate the effect of heavy metals on growth and extracellular enzyme activities of mycoparasitic *Trichoderma* strains.

MATERIALS AND METHODS

Strains *T. aureoviride* T122, *T. harzianum* T66 and T334, and *T. viride* T124 and T228 were isolated by Manczinger et al. from the soil of the forest of Ásotthalom (southern Hungary). *T. viride* T114 was obtained from the strain collection of the Budapest Technical University. These are cold tolerant, mycoparasitic strains, antagonistic against *Fusarium* and *Rhizoctonia* species (Antal et al. 2000). The fungi were maintained on minimal agar medium (Manczinger and Ferenczy, 1985).

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4-mm-diameter plugs cut from the margin of actively growing colonies of the *Trichoderma* strains were inoculated centrally, onto Petri plates, 9 cm in diameter, containing yeast extract agar medium (1% glucose, 0.5% KH_2PO_4, 0.1% $NaNO_3$, 0.2% yeast extract, 0.1% $MgSO_4 \cdot 7H_2O$, and 1.5% agar) supplemented with heavy metal compounds ($AlCl_3 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, $NiSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $CdNO_3 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $PbNO_3$, $HgCl_2$, $FeSO_4 \cdot 7H_2O$) in different concentrations. After incubation at 25°C, the growth rates were determined by measuring the colony diameters daily along two perpendicular axes, in two replicates for each strain - heavy metal combination. Concentration values of the applied heavy metal compounds resulting in 50% inhibition of mycelial growth (IC_{50} values) were determined.

Depending on the enzyme activities to be examined, 1% skim milk powder, chitin or laminarin (Sigma) was incorporated as an inducer in 20 mL liquid media (0.5% KH_2PO_4, 0.1% $NaNO_3$, 0.1% $MgSO_4 \cdot 7H_2O$ in tap water). In 50 mL Erlenmeyer flasks, these solutions were inoculated with conidial suspensions to a final concentration of 10^5 conidia/mL, and incubated on a shaker at 200 rpm and 25°C. After 4 d of incubation, the mycelial pellets were removed by centrifugation, and enzyme activities were measured in the supernatants.

Trypsin-like, chymotrypsin-like, and β-1,4-*N*-acetylglucosaminidase activities were assayed using *N*-benzoyl-Phe-Val-Arg-p-nitroanilide, *N*-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, and p-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) substrate, respectively. In the case of the p-nitrophenol and p-nitroaniline derivatives, 100 μL of each supernatant was incubated with 50 μL substrate (2 mg/mL) and 50 μL heavy metal solution (4 m*M*) at 25°C for 1 hr. The enzymatic reactions were stopped with 50 μL 10% Na_2CO_3, and the optical density of the samples was determined with a Labsystems Uniskan II microtiter plate spectrophotometer at a wavelength of 405 nm.

β-1,3-Glucanase activities were assayed with dinitrosalicylic acid reagent (Miller, 1959): 100 μL of each culture filtrate was mixed with 100 μL laminarin solution (1 mg/mL) and incubated for 1 hr at room temperature. Thereafter, 400 μL dinitrosalicylic acid reagent was added to the samples, followed by incubation in a 100°C waterbath for 20 min. Finally, the optical density of the samples was determined at 570 nm.

All measurements were carried out in 3 replicates. For each substrate, the means of the σ values, determined for each measurement, were given as standard error values (S.E.) of the methods.

RESULTS AND DISCUSSION

Mycelial growth of the six *Trichoderma* strains in our experiments was significantly influenced by the heavy metals (Table 1). The smallest variation of IC_{50} values among the six strains was found for nickel and cobalt, while IC_{50} values for iron showed significant variation. The lowest IC_{50} values were found for copper, while

Table 1. The IC_{50} values of the applied heavy metal compounds in m*M*

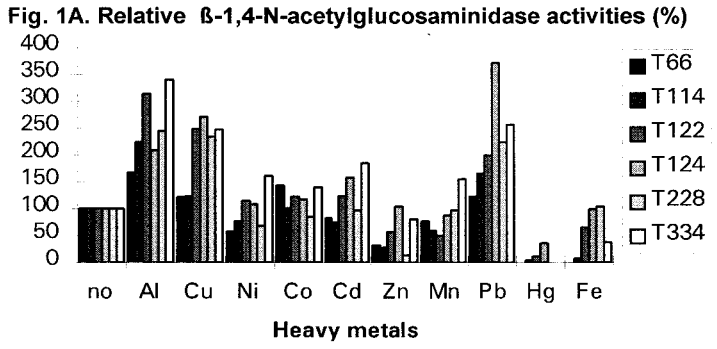
Strains	$AlCl_3 \cdot 6H_2O$	$CuSO_4 \cdot 5H_2O$	$NiSO_4 \cdot 7H_2O$	$CoCl_2 \cdot 6H_2O$	$Cd(NO_3)_2 \cdot 4\ H_2O$
T66	3.73	0.16	0.94	0.92	0.18
T114	5.00	0.20	0.78	0.80	0.17
T122	5.14	0.15	0.88	0.94	0.18
T124	4.14	0.19	0.82	0.82	0.17
T228	5.80	0.14	0.85	1.06	0.16
T334	4.97	0.14	0.80	0.88	0.23
	$ZnSO_4 \cdot 7\ H_2O$	$MnCl_2 \cdot 4\ H_2O$	$Pb(NO_3)_2$	$HgCl_2$	$FeSO_4 \cdot 7\ H_2O$
T66	0.72	5.66	1.70	0.27	1.14
T114	0.60	2.63	1.68	0.26	1.47
T122	0.72	3.03	1.62	0.31	1.28
T124	0.62	3.03	1.38	0.23	1.15
T228	0.70	2.83	1.22	0.32	1.47
T334	0.90	2.62	1.28	0.31	1.94

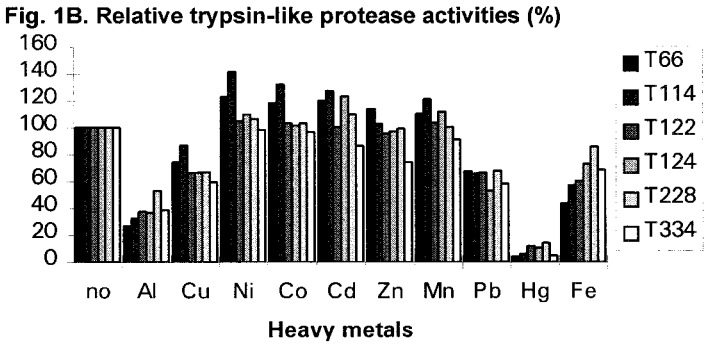
the highest were for aluminium. On the basis of the means of their IC_{50} values, the metal ions could be arranged in the following sequence of increasing toxicity:

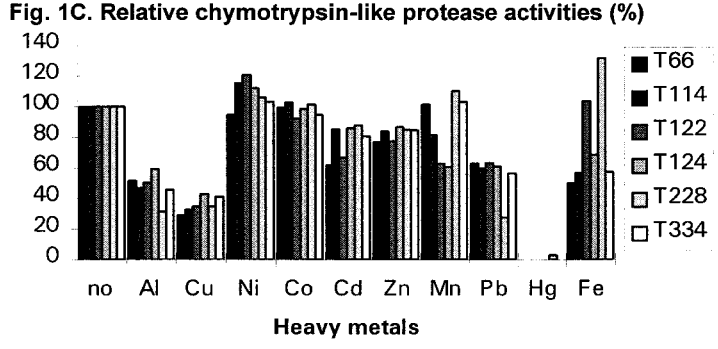
$$Cu^{2+} > Cd^{2+} > Hg^{2+} > Zn^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Pb^{2+} > Mn^{2+} > Al^{3+}$$

Trichoderma harzianum strain T66 showed the best tolerance levels for nickel and manganese. When all heavy metals were taken into consideration, *T. harzianum* T66 and *T. aureoviride* T122 were the most resistant, while *T. viride* strains T114 and T124 were the most sensitive.

The effect of a 1 m*M* concentration of the heavy metals on the in vitro activities of enzymes β-1,4-*N*-acetylglucosaminidase, trypsin-like, chymotrypsin-like proteases, and β-1,3-glucanase - all important factors in mycoparasitism - was also investigated. β-1,4-*N*-acetylglucosaminidase activities (Fig. 1A) were strongly inhibited by mercury. Iron and zinc inhibited the in vitro β-1,4-*N*-acetylglucosaminidase activities of some strains. Increased activities were found in the case of incubation with aluminium, copper and lead, while the other metal ions did not influence the in vitro activities of this enzyme significantly. According to the data published for a β-1,4-*N*-acetylglucosaminidase enzyme purified from a *T. harzianum* strain, copper increased the enzyme activities even at a concentration of 10 m*M* (Ulhoa and Peberdy, 1991). Both of the examined protease activities (Fig. 1B, 1C) were strongly inhibited by mercury and slightly by aluminium, copper and lead. Copper seems to inhibit chymotrypsin-like proteases to a larger extent than trypsin-like proteases. β-1,3-glucanase activities (Fig. 1D) were inhibited by mercury and activated by manganese. In an earlier study, manganese was found to be inhibitory to a laminarinase purified from *T. longibrachiatum*, but this inhibition was suggested to be unique to that particular enzyme (Tangarone et al. 1989).







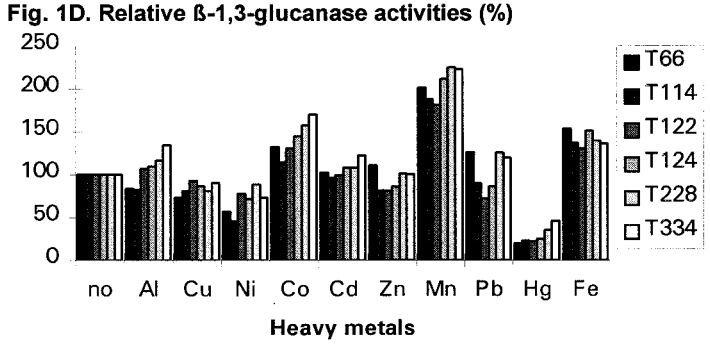


Figure 1. Effect of heavy metals on the extracellular enzyme activities of *Trichoderma* strains. no: control without heavy metals. A. β-1,4-*N*-acetylglucosaminidase (S.E.= ± 23.25%), B. trypsin-like protease (S.E.= ± 4.70%), C. chymotrypsin-like protease (S.E.= ± 5.71%), D. β-1,3-glucanase (S.E.= ± 9.37%)

A concentration of 1 m*M* mercury showed the strongest inhibition towards the in vitro activities of all examined enzymes; this concentration caused total inhibition in mycelial growth as well. The in vitro activities of the two proteases were inhibited by aluminium at 1 m*M*, which is significantly below its IC_{50} concentration. Lead in a concentration of 1 m*M* inhibited the two proteases to a similar extent to which it inhibited mycelial growth. The other examined heavy metals did not influence the in vitro enzyme activities of the mycoparasitic enzymes to the same extent as they inhibited mycelial growth. In some cases even increased enzyme activities were measured. These extracellular enzymes seem to be able to remain active even under heavy metal concentrations, where mycelial growth is already strongly inhibited. These results suggest, that breeding for heavy metal resistant *Trichoderma* strains could reveal biocontrol agents effective against plant pathogenic fungi even in soils with heavy metal contamination. Moreover, such mutants may be the preferred choice when applying biocontrol agents in combination with heavy metal-containing pesticides for complex integrated plant protection.

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