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Effect of heavy metals on enzymes production by *Hebeloma crustuliniforme*

HANNA DAHM and EDMUND STRZELCZYK

Department of Microbiology, Institute of Biology and Environment Protection,
Nicolaus Copernicus University, Gagarina 9, 87-100 Toruń, Poland

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Studies were carried out in order to determine the effect of some heavy metals (Cu, Cd, Pb, Zn) on the production of enzymes (cellulases, pectinases, proteases) by ectomycorrhizal fungus *Hebeloma crustiliniforme* (Bull.: Fr.) Quél. All the heavy metals inhibited the general enzymatic activity regardless of the source of carbon used. The metals reduced the egzocellulolytic activity more in media with cellulose powder than with CMC (carboxymethylocellulose).

Among pectolytic enzymes heavy metals most strongly inhibited polygalacturonase (PG). The heavy metals did not harmful affect the activity of pectate lyase (PGL). Proteolytic activity of *Hebeloma crustuliniforme* was least affected by zinc (Zn). The degree of inhibition of enzymes by heavy metals can be presented in the following order: Pb < Zn < Cd < Cu.

INTRODUCTION

Plants gain many benefits from mycorrhizal fungi. They help plants aquire mineral nutrients from the soil, especially immobile elements such as P, Zn, Cu (T i n k e r, 1948) but also more mobile ions like S, Ca, Fe, Mg, N, and others. In soils where such elements may be defficient or less available, mycorrhizal fungi increase efficiency of mineral uptake, resulting in enhanced plant growth (S m i t h, 1987). They also have been shown to increase water uptake and/or otherwise alter the plant physiology to reduce stress response to soil drought (P a r k e, L i n d e r m a n, B l a c k, 1983). Furthermore mycorrhizal fungi can reduce plant response to other soil stresses such as high salt levels, heavy matals, toxicity due to minor elements or associated with mine spoils, reduce the disease respons to plant pathogens (L i n d e r m a n, 1988).

Heavy metals are imput to forest soils via both natural (soil and oceanic processes) and anthropogenics processes (fuel coal combustion for power generation, industrial processes, vehicle use and agricultural acivities) (S m i t h, 1983). It is suggested that heavy metals deposited from the atmosphere to forests are accumulated

in the upper soil horizons of forest floors (M i l l e r, M c F e e, 1983; F r i e d l a n d et al., 1984). Since these metals accumulate in the horizons with maximum root and soil microorganisms activity it was appropriate to study the effect of some heavy metals on mycorrhizal fungi. *Hebeloma crustuliniforme* (Bull.: Fr.) Quél. was chosen because apart from other genera it is one of the best growing mycorrhizal symbionts (M o l i n a, T r a p p e, 1982; P o k o j s k a et al., 1996).

MATERIALS AND METHODS

The ectomycorrhizal fungus (obtained from professor P a c h l e w s k i, Forest Res. Inst., Sękocin, 05-550 Raszyn, Poland) was isolated from a sporocarp in Nancy, France.

Culture conditions

The fungus was grown in a modified (without glucose) liquid Lamb's medium with cellulose powder (CF 11 Whatman), or carboxymethylocellulose (CMC) Koch--Light, U.K. in amound of 0.5 % and supplemented with the following filtered (Millipore 0.22 μm pore size filters) haevy metals: Pb $(CH_3COOH)_2 \cdot 3H_2O$; Cu $(CH_3COOH)_2 \cdot 3\ H_2O$; Cd $(CH_3COOH)_2 \cdot 3H_2O$; Zn $(CH_3COOH)_2 \cdot 3H_2O$ were used at the following concentrations 10, 100, and 500 ppm. Fifty cm^3 aliquots of sterile medium were inoculated with one agar disc (ø 1 cm) of Potato-Dextrose Agar (Difco) containing an eight, nine or ten-day old growth of the mycelium. The experiments were set up in triplicate. After 10 days of growth at 26°C the cultures were centrifuged (10.000 rpm) at 4°C for 10 min. and the cellulolytic activity was determined.

Enzyme assay

Studies on this activity were performed as indicated in previous paper of D a h m and S t r z e l c z y k (1995). The cellulolytic activity of the fungus studied was compared with the standard curve for glucose. The egzoglucanase activity unit was defined as the amound of enzyme which under the experimental condition relased 1 μg of glucose per hour.

Endoglucanase activity was studied according to W o o d (1980) as described by D a h m and S t r z e l c z y k (1995). Endoglucanase activity unit was assumed to represent the amount of enzyme of *Aspergillus niger* (Fluka, Swiss) which under the experiment conditions released 1 μmol of glucose per min. at 30°C and pH 4.8.

The studies on total cellulolytic activity in the presence of Cellulose Azur were carried out according to F e r n l e y (1963) as described D a h m and S t r z e l c z y k (1995). Cellulolytic activity unit was defined as the amound of enzyme which under the experimental conditions released 1 μmol of glucose per min. at 37°C and pH 5.0.

Pectolitic activity of these enzymes was estimated by the thiobarbituric acid – TBA test (S h e r w o o d, 1966). Methods of the studies on polygalacturonase (PG) polygalacturonate lyase (PGL) and pectin lyase (PL) were described in more detail

by D a h m and S t r z e l c z y k (1995). Solution of pectolyase (mixture of different pectolytic enzymes) of *Aspergillus japonicum* (Sigma, USA) served as a standard of activity of PG, PGL and PL which under the experimental conditions released 1 µmol of galacturonic acid per min. at 25°C and pH 5.5.

Studies on proteolytic activity were carried out using the H a z e n' s (1965) method (details: D a h m, S t r z e l c z y k, 1995). The fungi were grown in Lamb's medium supplemented with gelatine (Loba, Feinchemie, Austria) in amound of 2g/l. The same concentrations of heavy metals (as indicated above) were used. Solution of fungal protease, XIII (Sigma) in acetete buffer, pH 4.5 served as the standard of activity of acidic protease and Protease E (*Streptomyces griseus*, Sigma) in Tris buffer, pH 7.5 served as the standard of alcaline (neutral) protease. As the unit of activity of acid protease was assumed the amound of enzyme which under the experimental conditions hydrolyzed hemoglobin and released 1 µmol of tyrosine per min. at 37°C and pH 2.8. As the unit of activity of alkaline protease was assumed the amound of enzyme which by hydrolyzing casein released 1 µmol of tyrosine per min. at 37°C and pH 7.5.

The results of all estimations were evaluated using one factor ANOVA and Student's „t" test.

RESULTS

The results of our studies are presented in Tables 1-4 and Figures 1 a, b, c.

Table 1 (A) illustrates the effect of heavy metals on the activity of egzoglucanases in *H. crustuliniforme* in media with CMC or with cellulose powder. The activity of egzoglucanases was higher in media with CMC than with cellulose powder as a carbon source. All the heavy metals used reduced this activity in cellulose powder containing media. In the presence of copper and cadmium no activity of these enzymes was noted. Lead at concentration of 500 ppm and zinc at 10 ppm increased the activity of these enzymes in media with CMC. However no activity of these enzymes was recorded in the presence of copper (100 and 500 ppm) and cadmium at 500 ppm. In the case of zinc (500 ppm) the activity of egzoglucanases was also minimal (at the border of detectivy).

The effect of heavy metals on the activity on endoglucanases is shown in Table 1 (B) and Figure 1. All the heavy metals applied reduced the activity of these enzymes regardless of the source of carbon (CMC or cellulase powder). Copper had the strongest inhibitory effect whereas lead and zinc were least effective.

Table 2 shows the effect of heavy metals on total cellulolytic activity. Regardless of carbon, all the heavy metals used lowered the cellulolitic activity of the fungus studied.

Table 3 illustrates the influence of heavy metals on pectolytic activity of the fungus studied. The heavy metals at the concentrations used inhibited most strongly PG. No activity of this enzyme was detected in media containg Pb, Cd and Zn concentrations 100 and 500 ppm.

Table 1

Effect of heavy metals on egzoglucanases (A) and endoglucanases (B) activity of *H. crustuliniforme* in media with CMC or cellulose powder (units/h)

Metal and concentration (ppm)		Mean values (n = 6) ± standard deviation (SD)			
		A		B	
		CMC	cellulose powder	CMC	cellulose powder
Control (without metals)		134.713 b ± 64.231	103.540 c ± 15.765	140.7993 c ± 4.629	130.4493 g ± 3.273
Lead (Pb)	10	73.0424 ab ± 55.828	0	86.0306 c ± 18.760	93.7931 d ± 4.457
	100	83.997 ab ± 126.041	103.102 c ± 96.511	61.4493 b ± 36.799	101.1243 e ± 10.689
	500	285.172 c ± 95.974	0	120.0993 d ± 3.273	80.8556 c ± 3.809
Copper (Cu)	10	67.357 ab ± 45.836	0	76.97.43 c ± 3.896	0
	100	0	0	0	0
	500	0	0	0	0
Cadmium (Cd)	10	228.312 bc ± 145.755	0	79.9931 c ± 5.843	59.7243 b ± 4.818
	100	2.187 a ± 1.071	0	62.3118 b ± 5.824	32.1243 a ± 3.273
	500	0	0	0	0
Zinc (Zn)	10	429.945 d ± 87.612	44.312 b ± 38.774	109.7493 d ± 3.659	106.7306 f ± 5.766
	100	142.586 b ± 93.207	27.201 ab ± 27.742	112.7681 d ± 13.032	106.7306 f ± 1.948
	500	2.187 a ± 1.071	0	32.9868 a ± 2.113	32.1243 a ± 3.273

Explanations: mean values in a given column marked with the same letter do not differ significantly ($p \leq 0.05$)

Table 2

Effect of heavy metals on the total cellulolytic activity ("Cellulose Azure") of *H. crustuliniforme* in media with CMC or cellulose powder (units/ml)

Metal and concentration (ppm)		Mean values (n = 6) ± standard deviation (SD)	
		CMC	cellulose powder
Control (without metals)		0.002982 c ± 0.000173	0.004126 b ± 0.000031
Lead (Pb)	10	0.000568 a ± 0.000365	0.000838 a ± 0.000223
	100	0.000377 a ± 0.0004	0.001002 a ± 0.000121
	500	0.000807 a ± 0.000170	0.000707 a ± 0.000244
Copper (Cu)	10	0.000521 a ± 0.000228	0
	100	0	0
	500	0	0
Cadmium (Cd)	10	0.002355 b ± 0.000711	0.000823 a ± 0.000556
	100	0.000257 a ± 0.000211	0.000486 a ± 0.000296
	500	0	0
Zinc (Zn)	10	0.000929 a ± 0.0003	0.001006 a ± 0.000169
	100	0.001028 a ± 0.0007	0.000938 a ± 0.000366
	500	0	0

Explanations: see Table 1

Table 3

Effect of heavy metals on pectolytic activity of *H. crustuliniforme* (units/ml)

Metal and concentration (ppm)		Mean values (n = 6) ± standard deviation (SD)		
		polygalacturonase PG	pectate lyase PGL	pectin lyase PL
Control (without metals)		1.1942 b ± 1.5360	1.0658 a ± 0.4881	0.2239 b ± 0.1834
Lead (Pb)	10	0.0310 a ± 0.0068	1.1572 a ± 0.2340	0.0762 a ± 0.0669
	100	0	0.2337 a ± 0.0718	0.1237 a ± 0.1217
	500	0	0.8789 a ± 0.4443	0.0275 a ± 0.0186
Copper (Cu)	10	0	0.8983 a ± 0.6889	0.0689 a ± 0.0521
	100	0.0521 a ± 0.1701	0.2982 a ± 0.1279	0.1192 a ± 0.0114
	500	0	0.1675 a ± 0.0444	0.0135 a ± 0.0031
Cadmium (Cd)	10	0.0119 a ± 0.1327	0.9083 a ± 0.8127	0.0182 a ± 0.0051
	100	0	1.1069 a ± 0.9111	1.0214 a ± 0.0062
	500	0	0.1675 a ± 0.0444	0.0135 a ± 0.0031
Zinc (Zn)	10	0.0370 a ± 0.0962	1.7619 a ± 0.9228	0.0569 a ± 0.0216
	100	0	1.4155 a ± 0.2130	0.0891 a ± 0.0035
	500	0	0.2337 a ± 0.0718	0.0182 a ± 0.0051

Explanations: see Table 1

Table 4

Effect of heavy metals on proteolytic activity of *H. crustuliniforme* (units/ml)

Metal and concentration (ppm)		Mean values (n = 6) ± standard deviation (SD)	
		Acidic protease	Alkaline protease
Control (without metals)		0.0066607 b ± 0.001095	0.002618 c ± 0.001534
Lead (Pb)	10	0.003811 a ± 0.000841	0
	100	0	0
Cadmium (Cd)	10	0.004747 a ± 0.000747	0
	100	0	0
Zinc (Zn)	10	0.003658 a ± 0.001459	0.001950 c ± 0.000635
	100	0.001347 a ± 0.000116	0.001089 b ± 0.000495

Explanations: In media with metals at concentration of 500 ppm the fungus did not develop. The sp
did not grow at all in media with copper at the concentrations studied. For further explanations see Ta

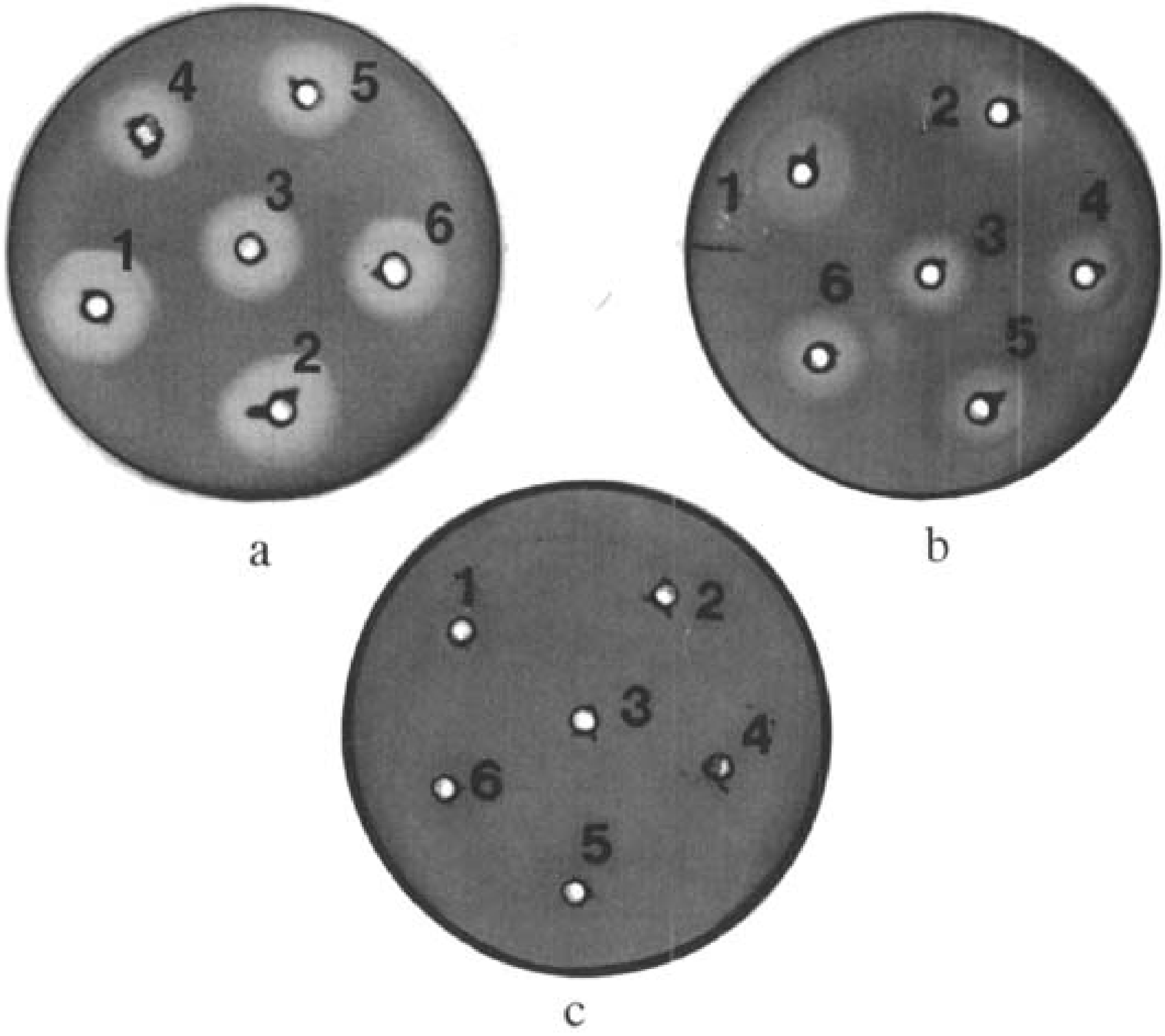

Fig. 1. Endoglucanases activity of *H. crustuliniforme* in media with CMC or cellulose powder
a — without heavy metals (control), b — with zinc – 100 ppm, c — with zinc – 500 ppm

In general the metals did not affect significantly the activity of PGL but all the concentrations applied inhibited the activity of PL.

The highest concentrations of the metals (500 ppm) and all the concentrations of copper completely inhibited the development of the fungus in gelatine containing media as the source of carbon (Table 4). The remaining concentrations of heavy metals (10 and 100 ppm) inhibited the growth of this fungus and significantly retarded the proteolytic activity. The inhibitory effect of the heavy metals on both types of enzymes (acidic and neutral proteases) was noted. An exception was zinc which at concentration of 10 ppm did not affect significantly the activity of neutral proteases. The highest toxicity for this fungus was exhibited by copper. The proteolytic activity was in general least affected by zinc.

In general were more sensitive to the action of heavy metals neutral than acidic proteases.

DISCUSSION AND CONCLUSION

Soil enzymes play an important role in the mineralization of organic substances and making nutrient ions available to plants. Due to reactions of specific enzymes like urease and phosphatase NH_4^+ and PO_4^- are made available to plants from organic substances in soil (R e d d y et al., 1987).

Accumulation of heavy metals in soils may decrease the rate of decomposition of organic matter – as a result of diminished soil microbial activity. However the degree of heavy metals on soil microorganisms often depend on many ecological factors like pH and organic matter content (B a d u r a et al., 1884), organic acids which bind metals, or fungal cell membranes which may cummulate these compounds (B å å t h, 1980). Differences in the sensivity of ectomycorrhizal fungi to lead, zinc, copper and nickel have ben found by P a c h l e w s k i and C h r u ś c i a k (1986) and J o n e s and H u t c h i n s o n (1988).

In our studies all the metals at the highest concentrations applied inhibited the enzymatic activity of the fungus studied. In media with gelatine and 500 ppm of Pb, Zn and Cd the growth of *H. crustuliniforme* was completely retarded. Pb and Cd used at 10 and 100 ppm inhibited the activity of alkaline proteases. Our results are in accordance with the observations of other authors who found that many heavy metals like Zn, Cu, Pb and Cd inhibit fungal proteases to different degree (W o r o w s k i, 1975).

Informations about the effect of heavy metals on enzymes of mycorrhizal fungi are rather scarce. Infection of plants by symbiotic fungi may occur either by their own enzymes or by those of associated microorganisms (G a r b a y e, 1994). At least some mycorrhizal fungi are capable of producing enzymes that degrade the main components of the cell wall of plants (D a h m, S t r z e l c z y k, M a j e w s k a, 1987; D a h m, S t r z e l c z y k, 1995). Data concerning the inhibitors of proteases occurring in nature are scarce and fragmentary.

In our experiments Zn was the least toxic to both the acidic and neutral proteases. Its toxicity was lower than that of Cu and Cd. Zinc as a component of many important enzymes is apparently used in greater amounds by the mycelium than other metals. Mycorrhizal roots of *Pinus radiata* absorb 1.4-4.5 times more zinc than non-mycorrhizal ones. The heavy metals used in our studies also affected negatively the productions of pectinases – most strongly the prolygalacturonase (PG) and least that of pectate lyase (PGL). The fungus produced more cellulases in media with CMC than with cellulose powder (in the presence of heavy metals).

The role of mycorrhizal fungi as protecting barrier of plant towards heavy metals is not unanimous and not yet clear cut. In our studies heavy metals were empoyed because of their importance in the natural environment and their possible involvement in the "acid rain" syndrome and because of their widespread occurrence as products in man-made environment. Solubility of most metals increases with decreasing pH, the natural acidic environments are likely to suffer from metal toxicity (R e a d, 1986).

In field studies the heavy metal pollution is never due to one single metal. This makes difficult to draw conclusions regarding toxicity of metals to organisms (B å å t h, 1990). However in order to understand the relationships between mycorrhiza infection and plant growth in metal enriched environments it is necessary first to obtain basic information concerning the resistance of the fungus to metals when growing in pure culture under standardized conditions (B u r t at al., 1986). T r a p pe in 1977 suggests several criteria which are important in selection of ectomycorrhizal fungi for inoculation of soils in tree nurseries. Among them the action of more than one single factor like pH tolerance should be considered. We have used heavy metals and studied their effect on some enzymes of one ectomycorrhizal fungus. Other mycorrhizal fungi certainly require similar studies.

According to B å å t h (1980) the relative toxicity of different metals is fairly constant. The following degree of toxicity appears to be most commonly found Cd > Cu > Zn > Pb. This was irrespective of the soils having high or low organic matter.

We have found the effects of heavy metals on enzymes of *H. crustuliniforme* to be: Pb < Zn < Cd < Cu. Thus differences in reaction to heavy metals between genera and perhaps between strains are possible.

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