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Effects of copper, manganese and pH on the growth and several enzyme activities of mycorrhizal fungus *Cenococcum geophilum* Fr.*

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Abstract—— The effects of various concentrations of copper, manganese and pH on the growth, protein content and several enzyme activities of mycorrhizal fungus *Cenococcum geophilum* Fr. were investigated. The results showed that high concentration of copper (20 mg/L) and low pH (3.0—4.0) inhibited the mycelial growth (dry weight), however, the protein content increased or not significantly reduced in mycelia after copper or manganese was applied. The activities of acid phosphatase and α-mannosidase were induced by high concentration of Mn (200—400 mg/L); but the activities of G6PDH, MTLDH and trehalase were inhibited by high content of copper (10—20 mg/L), both of the responses of these enzymes and mycelial growth under the presence of copper were correlative each other. Measurement of these enzyme activities might therefore provide a useful criterion for the evaluation of the fungitoxicity of soil contaminated by copper.

Keywords: copper; manganese; pH; mycorrhizal fungus; enzyme activity.

1 Introduction

Acid precipitation and air pollution stresses may increase the availability of heavy metals in some soils and harm tree canopies directly (Aber, 1982). It is well known that plants infected with mycorrhizal fungi can increase the resistance to environmental stress (Brown, 1985), thus the kinds of plant may be suitable for metal-contaminated sites. However, this ability is not unlimited and not all fungi are equally effective. There are indications that the greater availability of heavy metal in acidic soils may interfere with the formation or function of ectomycorrhizae. So it is important to know the effects of toxic metal on the mycorrhizal fungi. In the past decade, the growth response and tolerance of different species of ectomycorrhizal fungi to Cu^{2+} and Ni^{2+} (Melanie, 1986), Zn^{2+} (Brown, 1985), Al^{3+} and Mn^{2-} (Gall, 1984), Hg^{2+}, Cd^{2+} and pH (Antonius, 1990) and acid precipitation (Dighton, 1987) have been reported. The suppression of protein synthesis in the fungi exposed to Al^{3+} was demonstrated by Oelbe-Farivar (Oelbe, 1985). But there is little further information on the physio-

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logical and biochemical response of mycorrhizal fungi to these toxic metals and acidification.

The purpose of this research is to investigate the effects of various concentrations of heavy metals Cu^{2+} and Mn^{2+}, and pH on the growth of mycorrhizal fungus *Cenococcum geophilum*, the variation of the activities of following enzymes in mycelia were measures: glucose-6-phosphate dehydrogenase, mannitol dehydrogenase, trehalase, α-mannosidase and acid phosphatase. Special attention will be paid to the relation between the enzymatic response and mycelial growth.

2 Materials and methods

2.1 Organism and culture

Cenococcum geophilum Fr. was obtained from Department of Botany, University of Tubingen (FRG), isolated from ectomycorrhizae of spruce in the field. Mycelia (homogenized with Ultra-Turrax, 9400 r/min for 30 sec) were cultured in a flask (250 ml) containing 60 ml liquid modified Melin-Norkran (MMN) medium (Guttenberger, 1992), the flasks were placed on a rotatory shaker at 20℃ in dark with continuous shaking (90 r/min).

2.2 pH treatments

The pH treatments were prepared by adjusting the pH of MMN solution with 1. 0 mol/L HCl to provide pH 3. 0, 4. 0, 4. 5, 5. 0, 6. 0 and 7. 0 before autoclaving, 10 g/L glucose was added as carbon source (Marx, 1965). Four replicate flasks containing 60 ml nutrient solution were made for each pH treatment. The flasks were autoclaved at 121℃ for 15 min. Each flask was then inoculated with 1 ml of fresh homogeneous mycelium and incubated on the shaker for 4 days. The mycelia contained in each flask were collected by filtration, frozen in liquid nitrogen, and dried and kept under vacuum for 14 days. Then, the mycelia were weighed and homogenized in liquid nitrogen using a Micro-dismembrator (Braun, Melsungen, FRG), the resulting powder was freeze-dried at temperature of below −30℃ and kept under vacuum until use.

2.3 Metal treatments

Cu^{2+} (as $CuSO_4$) and Mn^{2+} (as $MnSO_4$) were added into MMN solution. The concentration of Cu^{2+} and Mn^{2+} were 0. 0, 5. 0, 10. 0, 15. 0, 20. 0 mg/L and 0. 0, 100. 0, 200. 0, 300. 0, 400. 0 mg/L, respectively. The pH of all solution was adjusted to 4. 5 with1.0 mol/L HCl before autoclaving. Four replicates were made for each treatment. The cultures were incubated and the mycelia were collected and dried as above.

The dried mycelial powder (about 5 mg) was extracted with 1 ml Tris: Borate (0. 1 mol/L : 0. 3 mol/L) pH 7. 5, EDTA (5 mmol/L) and β-mercapto-ethanol (7 mmol/L) buffer on ice for 10 min, centrifuged at 10000g for 10 min. The extracts were stored at -80℃ until enzyme measurement.

2.4 Enzyme analysis

2.4.1 Glucose-6-phosphate dehydrogenase (G6PDH EC 1. 1. 1. 49)

The assay mixture contained 50 mmol/L Tris/Mes buffer, pH 8. 3, 5 mmol/L $MgCl_2$, 0. 5 mmol/L NADP, 20 μl sample in a total volume of 1. 0 ml, the reaction was started by

the addition of 2 mmol/L glucose-6-phosphate. The reduction of NADP was monitored at 30℃ by measuring absorbance at 340 nm (John, 1985).

2.4.2 Acid phosphatase and α-mannosidase

Acid phosphatase (EC 3.1.3.2) was assayed in the mixture contained 290 μl water, 100 μl acetic acid/acetate Na buffer, pH 5.6, and 100 μl sample. After the substrate *p*-nitrophenol-phosphate (PNP-P, 25 mmol/L, 100 μl) was added, the mixture was incubated at 37℃ for 60 min. The enzyme activity was assayed by measuring the liberation of PNP from the substrate per minute at 405 nm (Boller, 1979). For α-mannosidase (EC 3.2.1.24), the substrate was replaced by *p*-nitrophenol-α-mannopy-ranoside (PNP-α-MP, 10 mmol/L).

2.4.3 Trehalase

Trehalase (EC 3.2.1.28) activity was measured in the solution contained 100 μl citrate/phosphate (0.2 mol/L) buffer, pH 3.8, 30 μl trehalose (0.5 mol/L) and 20 μl sample, two contrasts were prepared by replacing the trehalose with bidistilled water and the sample with the extract buffer separately. The mixture was incubated at 30℃ for 80 min. After 10 μl NaOH (1.0 mol/L) was added, the mixture was placed at 105 ℃ for another 15 min in a dry bath. Glucose formed was quantified according to Jones and Outlaw (Jones, 1981).

2.4.4 Mannitol dehydrogenase (MTLDH, EC 1.1.1.138)

The assay mixture consisted of 2.2 ml of 0.5 mol/L mannitol in 50 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 9.0, 25 μl of 25 mmol/L NADP, 25 μl 25 mmol/L phenazine methosulfate (PMS) and 200 μl of 5 mmol/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Reaction was initiated with the addition of sample, and the reduction of MTT was monitored at 578 nm and calibrated using an extinction coefficient of 1.5×10^4mol/(L.cm) (Walter, 1982).

2.5 Protein measurement

Protein concentration in mycelium was determined by the method of Guttenberger and Hampp (Guttenberger, 1991) with the bovine serum albumin (BSA) as standard. The samples were applied to sheets of cellulose acetate membranes in aliquots of 2 μl and left to dry. For each sample, one dot area was formed. The membrane sheets were transferred to the staining solution [fluorescent dye benzoxanthene yellow, 2.5 mg in 5 ml of methanol/acetic acid (90/10), 10 min] on a laboratory shaker at ambient temperature in the dark. The membrane sheets were then washed in washing solution A [methanol/acetic acid (90/10), 5, 5 and 15 min] followed in washing solution B [Butanol/methanol/acetic acid (60/30/10), 3× 2 min] by gentle agitation. Prior to elution the membrane sheets were dry and the dots were cut from the membrane sheet. Elution [45 min, every dot in 2 ml of glycine-sulphuric acid buffer (0.25 mol/L, pH 3.6) containing 0.02% (w/v) SDS] was performed at ambient temperature in the dark. Readings were taken in a spectrofluorometer (SFM 25, Kontron) at 425 nm (excitation) and 475 nm (emission).

Enzyme activity was expressed in mU per mg soluble protein.

3 Results and discussion

3.1 Effect of Cu^{2+}, Mn^{2+} and pH on the growth

A distinct decrease in hyphal growth was induced by low pH and high level of Cu^{2+}. Especially at pH 3.0 and 20 mg/L Cu^{2+}, fungus showed the lowest mycelial biomass (Fig. 1a and 1c), the inverse relation was observed between Cu^{2+} content in medium and dry weight, the correlation coefficient was highly significant ($r = -0.94$). The dry weight of the mycelial was declined after Mn^{2+} was applied, as compared with the contrast, but the variation among the different concentration tested was smaller (100—400 mg/L, Fig. 1b). These results indicated that the high concentration of Cu^{2+} and stronger acidity appeared inhibitory effect on the growth of mycorrhizal fungus *C. geophilum*.

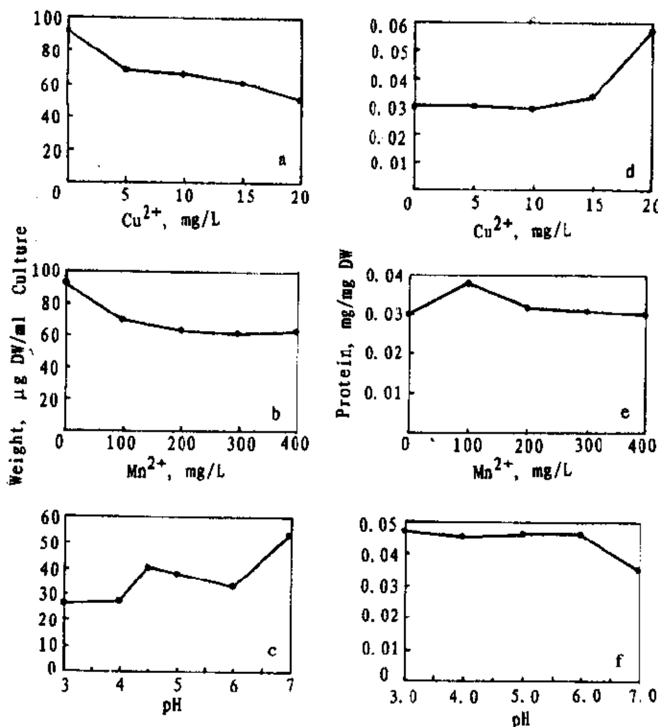

Fig. 1 Dry weight and protein in the mycelia of *C. geophilum* treated with copper (a,d), manganese (b,e) and varied pH (c,f) in the culture medium

The effects of Cu^{2+}, Mn^{2+} and pH on the growth of several ectomycorrhizal fungi had been investigated by Melanie (Melanie, 1986) and Gall (Gall, 1984). It was suggested that several fungi had less tolerance to Cu^{2+} (4 mg/L) and low pH (2.5) in liquid medium by measuring the dry weight of the final colony. But Mn^{2+} was less fungitoxic. At least 65% of the all tested strains could grow in the medium containing 500 mg/L of Mn^{2+}. The results of this paper were about the same as above.

3.2 Effect of Cu^{2+}, Mn^{2+} and pH on the protein content

The protein content (mg soluble protein per mg dry weight) was increased after the treatment with Cu^{2+}, the protein content of the fungus treated using 20 mg/L of Cu^{2+} was about two times as much as that in the contrast (Fig. 1d). For Mn^{2+} and pH treatment, the protein content in mycelium was not shown significant variation except for pH 7.0, where the content declined to the lowest level (Fig. 1f). The conclusions about the effect of metals on the protein synthesis were different from the previous results obtained by Oelbe-Farivar (Oelbe, 1985) who demonstrated that the protein synthesis was inhibited when the fungus was exposed to Al^{3+}, but the experiment data obtained by Assche *et al.* (Assche, 1988) showed that protein concentration (mg soluble protein per g fresh weight) was increased in roots and leaves of plant (*Phaseolus vulgaris*) after with toxic Cd^{2+} and Zn^{2+} treatment. In our experiment about the effect of Cu^{2+} and Mn^{2+} on *Amanita muscaria*, another ectomycorrhizal fungus, the protein content in mycelium showed similar tendency as that in *C. geophilum* (Kong, 1995).

3.3 The responses of enzyme activity to metals and pH

The responses of enzyme activity to metals and pH are shown in Fig. 2. The activities of G6PDH, MTLDH and trehalase were correlative with the copper content in culture medium, the high content of Cu^{2+} inhibited the activities of these enzymes (Fig. 2a). The activities of all enzymes measured did not show obvious decline after the strain was treated with Mn^{2+}. The activities of α-mannosidase and acid phosphatase were induced by high concentration of Mn^{2+} (200—400 mg/L; Fig. 2b). The effects of pH on the activities of enzymes are shown in Fig. 2c, the activity of G6PDH was reduced when the pH was lower than 4.0.

The induction of the activity of specific enzyme was demonstrated by Matthys (Matthys, 1975) and Lee *et al.* (Lee, 1976) as Cu^{2+}, Zn^{2+}, Cd^{2+} and Pb^{2+} were applied. Apparently, increases in activities of some enzymes seem to be a fairly general response on specified doses of several heavy metals. In this investigation, the high concentration of Mn^{2+} can stimulate the activities of some enzymes such as acid phosphatase and α-mannosidase. The specific concentration of Cu^{2+} can induce the activities of acid phosphatase (15 mg/L) and G6PDH (5 mg/L) as compared with the controls. Therefore, the effects of metal on the activity of enzyme might be depended on the species of metals and the doses applied.

The possible physiological mechanisms of induction or inhibition of activities of some enzymes in mycorrhizal fungi by soil metals and acidity have been studied. Some authors suggested that the response of fungi to stresses such as heavy metals might be related with detoxifying mechanisms that the heavy metals were sequestered, a process associated with a significant metabolic cost of carbon (Morselt, 1986). Among the studies of enzymes in this paper, acid phosphatase plays an important role in hydrolyse organic phosphate into free phosphate and contributes significantly the availability of phosphate in the soil. The trehalase, MTLDH and G6PDH are the enzymes concerning with the NADPH producing reactions and the metabolism of trehalose and mannitol, the fungus-specific storage carbohydrates. The proportion of these carbohydrates is various with the species of fungi and different environmental conditions. The different responses of these enzymes associated with the carbohy-

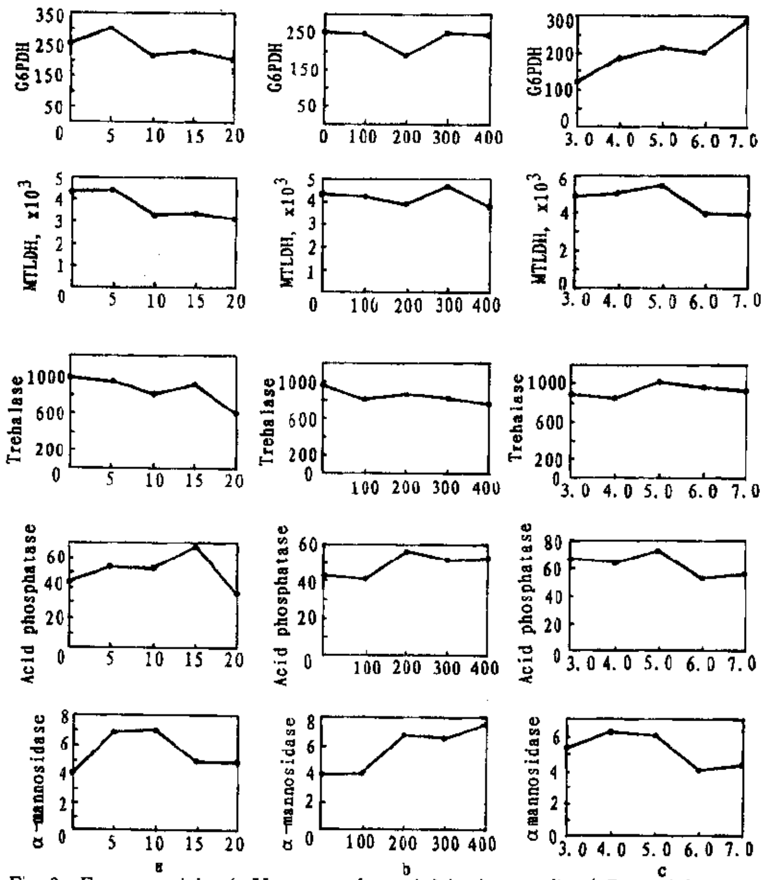

Fig. 2 Enzyme activity (mU per mg of protein) in the mycelia of *C. geophilum* treated with (a) copper, (b) manganese and (c) varied pH in the culture medium (Each point is the mean of the results from two independent experiments, $n=8$)

drate metabolism and phosphorus uptake in fungal mycelium were suggested that heavy metal might effect on the metabolism process or change the pathway of metabolism by varying distinctly enzyme activity or reducing ability in the cell. This hypothesis will be the basis for our future work.

3.4 The correlation between the growth and activities of enzymes

The results (Table 1) showed that the relationship of dry weight versus G6PDH, MTLDH and trehalase activity (positive relation) was particularly close after Cu^{2+} was applied. For Mn^{2+}, the correlation coefficients were generally smaller. Thus it is quite possible that the activities of the three enzymes might provide a useful biological criterion for the evaluation of the fungitoxicity of soil contaminated by copper.

Table 1 Correlation coefficients of dry weight versus enzyme activity and protein in *C. geophilum* treated with various concentrations of Cu and Mn in culture medium

	Dry weight mycelium treated with	
	Cu	Mn
G6PDH	−0.88[a]	−0.12
MTLDH	−0.86[a]	−0.32
Trehalase	0.82[a]	0.77[a]
Acid phosphatase	0.02	0.11
α-mannosidase	0.07	0.07
Protein	−0.75[a]	−0.76[a]

[a] Significantly correlated at 99% confidence level

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