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Excessive Cu and Zn affecting on distribution of the metals and activities of glycolytic and nitrogen incorporating key enzymes in mycelia of ectomycorrhizal fungi *Suillus bovinus*

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Abstract: Concentration of copper and zinc in isolated *Suillus bovinus* mycelia, used nutrient solution and 0.5 mol/L EDTA mycelia washing solution were measured to investigate the distribution of heavy metals in mycelia growth in excess copper or zinc nutrient solution. Treated with zinc, most of added zinc maintained in used solution, and 9.8%/14.6% was in/on mycelia in treatment, and in treatment 2 was 3.9%/8.0% in/on mycelia. In the copper applications, copper stimulated in more than on mycelia, i.e., 25.9%/4.5% in/on mycelia in treatment, and 7%/18.8% in/on mycelia while most of copper retained in used nutrient solution. Certain amount of copper or zinc uptake by mycelia led to pronounced influence on glycolysis and nitrogen incorporating process of *Suillus bovinus*, while the tested enzymes kept constant in treatment. In crude extracts of copper treatment 2 mycelia, activities of HK, PFK and GS were inhibited and decrease to 63%, 48% and 38% and GlDH increased by 68% of the control, respectively. The behaviors of these tested enzymes toward zinc corresponded in general with that towards copper. The potential protection of *Suillus bovinus* for its host plant under excess copper or zinc threaten was discussed.

Key words: *Suillus bovinus*; ectomycorrhizal fungous; copper; zinc; glycolysis; nitrogen incorporate; enzymes

Introduction

Ecto-mycorrhizal fungi are known to ameliorate metal toxicity to the associated higher plants (Bradley, 1981; 1982; Brown, 1985; Bucking, 1995). The improvement of heavy metal tolerance by mycorrhizal infection is related to a reduction of shoot tissue concentration, couple with an increased accumulation in mycorrhizal root system (Brown, 1985). They suggested that extramatrical mycelia kept heavy metal ions off plant roots and the interface with fungal cell walls might be the principal site of metal accumulation in ericaceou mycorrhiza. However, because of impossible split mycelia away roots, it is unclear that the heavy metals accumulated in roots system is in extrahyphal glycoprotein (Bradley, 1982) or transferred to roots by mycelia (Jones, 1988). Therefore, for better understanding heavy metal behaves in myucorrhizal plant, it is necessary to study on heavy metals distribution as well as it influence on metabolism in isolated fungi.

For investigation of metabolism affected by heavy metals, concerning the heterotrophic fungus, carbohydrate-degrading processes as the energy producing reaction for growth were of primary interest. There are already information in literature that in some ectomycorrhizal fungi this proceeds aerobicall via glycolysis and tricarboxylic acid cycle (McDonald, 1978; Martin, 1987; Criffin, 1994). Meanwhile, fungous need reduced nitrogen for protein biosynthesis, the basis for cell amplification and growth. Ammonia, in pre-experiments, was found to be the most suitable exogenous nitrogen source. NADH-/NADPH-dependent glutamate dehydrogenase (GlDH), Glutamine synthetase (GS) and NADH-/NADPH-dependent glutamine-2-oxoglutarate aminotransferase (GOGAT), as key enzymes involved in the assimilation of ammonia (Chalot, 1991; Martin, 1994), were of considered.

The objective of this study was to investigate metabolic response of isolated *Suillus bovinus* growing under excess copper or zinc nutrient solution by measuring key enzymes in glycolytic pathway and ammonia assimilating pathway, so as to understand the influence of heavy metals on metabolism of ectomycorrhizal fungi and the possibility of fungi to protect toxicity from its host plant.

1 Materials and methods

1.1 Growth condition

Mycelium was suspended in nutrition solution after Kottke *et al*. (Kottke, 1987). 300 ml samples of such suspensions were transferred into culture tubes (length 45 cm, ϕ 4cm, previously sterilized for 4h at 160℃) with gas inlet at the bottom. These were placed in a thermostat at 25℃ in the dark. For an ample supply with oxygen for heterotrophic growth and to prevent the hyphae from settling down, the suspensions were continuously aerated with compressed air, purified by a passage through cotton filters.

1.2 Treatments

Besides the copper and zinc in the base nutrition solution, additional copper or zinc was added as $CuSO_4$ or $ZnSO_4$ to the

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solution at pH 4.5 before autoclaving. The treatments were (1) 25 mg $CuSO_4 \cdot 5H_2O$/L (Cu treat 1); (2) 100 mg $CuSO_4 \cdot 5H_2O$/L (Cu treat 2); (3) 250 mg $ZnSO_4 \cdot 7H_2O$/L (Zn treat 1) and (4) 1000 mg $ZnSO_4 \cdot 7H_2O$/L (Zn treat 2). These concentrations were chosen mainly from results of preliminary experiments as harmful but not lethal for fungi mycelia. It was estimated using a geochem-program (Parker, 1987) that, at pH 4.5, 97.0% and 95.1% of copper was available as free ions respectively in Cu treat 1 and 2, and 93.9% and 87.3% of zinc was available respectively in Zn treats 1 and 2. It resulted that the free copper ions were 6.5 ppm and 24.5 ppm in Cu treats 1 and 2, and free zinc ions of 55 ppm and 200 ppm Zn treats 1 and 2, respectively.

Exposed to the heavy metal excessive nutrient solution for one week, fungus mycelia were harvested for content of heavy metals and enzymes activity measurement.

1.3 Determinations

Mycelia from liquid cultures were harvested by filtration and washed with 0.5 mol/L EDTA. These materials were dried at 100℃ for 10h, then weighted out for measurement of biomass. Or dried mycelia, used nutrient solution and washing solution were transferred separately to teflon vessels containing concentrated HNO_3, and digested at 170℃ for 7h. After cooling and appropriate dilutions the concentrations of Cu and Zn were determined by atomic absorption spectrometry with Perkin-Elmer 380 and 5100 (Carnrick, 1991; Slavin, 1989).

Of the enzymes activity measurement, 1.0g of filtered and washed mycelia was frozen in liquid N_2 and ground with 50 ml ice-cold 0.1 mol/L phosphate buffer pH 7.0 in an ice-cooled mortar and pestle. The resulting homogenate was centrifuged at 20000g for 20 min at 4℃ in refrigerated centrifuge. The supernatant (crude extract) was used for all enzyme assays performed.

In vitro activities of mycelia hexokinase (HK, EC 2.7.1.1), phosphofructokinase (PFK, EC 2.7.1.11) and pyruvate kinase (PK, EC 2.7.1.40) were determined in control, copper treated and zinc treated fungus. Assays of activity of HK, PFK and PK were determined using the methods described by Bergmeyer (Bergmeyer, 1983) and modified by Thiemann (Thiemann, 1996). And assays of GlDH and GOGAT was after Kowallik and Neuert (Kowallik, 1984), GS was modified after Meya and Kowallik (Meya, 1994).

Soluble protein of crude cell extracts was determined after Lowry *et al*. (Lowry, 1951).

2 Results

2.1 Biomass and contents of heavy metals

For study of the cell metabolism influenced by copper and zinc, mycelia were grown in normal or heavy metals supplemented nutrient solution in culture tube at continuous aeration as stated before. 7 days under this condition, the biomass increase of mycelia was marked inhibited in Cu treat 1 (low concentration) and very pronounced inhibited in treat 2 (high concentration). Under zinc treatments, the fresh weight of mycelia was proved inhibited as well (Table 1).

Table 1 Biomass influenced by excessive Cu and Zn in liquid cultures of *Suillus bovinus* ($n = 6$)

Treatment	Control	Cu treat 1	Cu treat 2	Zn treat 1	Zn treat 2
Fresh weight, g/culture tube	2340 ± 120	1140 ± 460	570 ± 10	1720 ± 50	1260 ± 80

Copper and zinc as essential elements were found accumulated in large amount in or tightly bound in the cells. The rather low amount of the both elements in intracellular in normal supply were increased by factor 7 and 10 under copper treat 1 and treat 2 respectively. The zinc treatments led to 5 and 9 times higher than control. The growth of mycelia was inhibited seriously as consequence of up taking of heavy metals into cells. Biomass were only about 50% and 24% of control under copper treatments, and 74% and 54% of control under zinc treatments, respectively, after growth in continuously aerated liquid cultures for 7 days (Fig.1a).

Fig.1b shows, in addition, that there were only minor amounts of copper attached to the cell walls, only 2%—4% of the total could be removed by washing with 0.5 mol/L EDTA. 70% or 90% of initially applied amount, respectively, could still be determined in the nutrition solution. In zinc applications about 76% in the treat 1 and about 90% in the treat 2 were left when zinc content of the normal nutrition solution was down to about 20% after this period of growth. That indicated that the cells of mycelia could not take up heavy metals unlimitedly.

For the ability of mycelia retained heavy metals, if heavy metal ions washed away by aqueous EDTA were supposed to be the part of heavy metals bound on the surface of hypha and extra-phal polysaccaride slime, copper was located more in than on mycelia under copper applications. And zinc shows a reverse result. These results corroborate and extend those previous published by.

2.2 The activity of glycolytic regulatory enzymes

In crude extracts of mycelia grown under copper treat 1 for 5 days, there were no measurable alterations in the specific

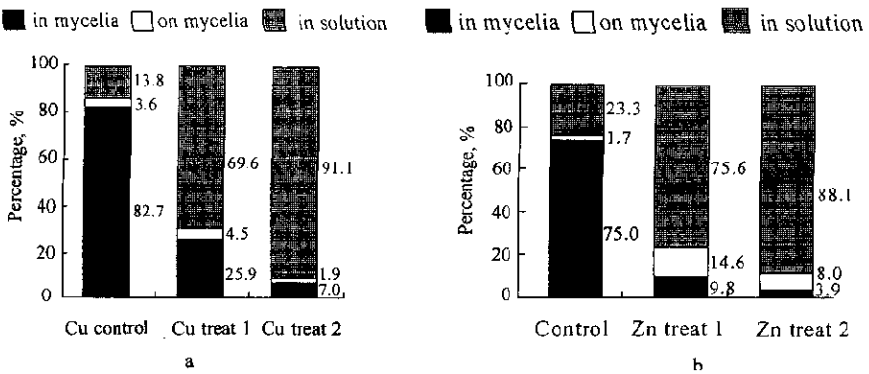

Fig. 1 Cu and Zn distribution in liquid cultures of *Suillus bovinus* ($n = 6$)
a. Cu distribution; b. Zn distribution

activity of all three glycolytic kinase (Table 2). There were, however, pronounced effects at exposure to treat 2 for the same period. Hexokinase and phosphofructokinase exhibited markedly lower specific activities, reaching only 63% and 48% of their untreated control respectively. The pyruvate kinase was still almost identical as no significant difference of activity between control and treat 2.

Table 2 Influence of Cu^{2+} and Zn^{2+} on the activities of some glycolytic enzymes of *Suillus bovinus*

(nmol/min × mg protein; $n = 6$—8)

Enzymes	Cu control	Cu treat 1	Cu treat 2	Zn control	Zn treat 1	Zn treat 2
HK	355.6 ± 10.77	361.1 ± 18.40	222.6 ± 9.8*	212.7 ± 10.6	178.0 ± 8.9	129.7 ± 5.2*
PFK	11 5 ± 0.50	11.7 ± 0.55	5.5 ± 0.29*	13.1 ± 0.29	9.5 ± 0.25	2.5 ± 0.18*
PK	512.1 ± 26.1	517.8 ± 29.7	548.1 ± 19.8	444.1 ± 8.87	505.0 ± 10.2	571.7 ± 34.3

Note: HK: hexokinase; PFK: phosphofructokinase; PK: pyruvate kinase; * significantly different from control ($P < 0.05$) using ANOVA

The behavour of hexokinase and phosphofructokinase towards zinc corresponded in general with that towards copper. Pyruvate kinase, in contrast, was increase by about 30% at higher zinc concentration.

2.3 Activity of nitrogen incorporating enzymes

The specific activities of 3 important enzymes in nitrogen metabolism, NADH-/NADPH-GIDH, GS and NADH-/NADPH-GOGAT were measured in unpolluted mycelia. NADPH-GIDH and NADPH-GOGAT were undetectable in crude cell extract of the fungal mycelia. Since these are usually found in green plant tissues, they might not be existent in the hyphase of *Suillus bovinus*. Therefore, only NADH-dependent GIDH and GOGAT were measured under copper or zinc treatments.

The specific activities of GIDH, GS and GOGAT were unaltered in crude extracts of copper treat 1 exposed mycelia, but different from those in the copper treat 2 treated cells. GIDH and GOGAT activities were higher by 68% and 40% respectively, and GS activity was lower by 62% comparing with control (Table 3).

Table 3 Influence of Cu^{2+} and Zn^{2+} on the activity of some nitrogen incorporating enzymes in crude extracts of *Suillus bovinus*

(nmol/min × mg protein; $n = 6$—9)

Enzymes	Cu control	Cu treat 1	Cu treat 2	Zn control	Zn treat 1	Zn treat 2
GIDH	109.0 ± 2.85	110.9 ± 4.28	182.8 ± 100.5*	155.3 ± 4.7	207.7 ± 5.20	230.8 ± 6.0*
GS	205.7 ± 1.88	224.0 ± 13.44	77.7 ± 1.65*	179.6 ± 4.0	177.2 ± 7.63	109.7 ± 6.6*
GOGAT	8.8 ± 0.45	9.9 ± 0.30	12.3 ± 1.62*	9.3 ± 0.28	16.9 ± 0.50	9.3 ± 0.56

Note: GIDH: glutamate dehydrogenase; GS: glutamine synthetase; GOGAT: glutamine-2-oxoglutarate aminotransferase (NADH-dependent); * significantly different from control ($P < 0.05$) using ANOVA.

As shown in Table 3, none of the amino acid producing enzymes investigated exhibited significant influences on their activities after exposure of the mycelia to zinc treat 1 for 5 days. At mycelia growth under zinc treat 2, however, only GOGAT activity was found unaltered. Activity of GIDH was higher by about 50%, while that of GS was lower by about 40% than in the control.

3 Discussion

Results of biomass of mycelia growth under zinc concentration 174 times of its control (zinc treat 2) was 2.2 time higher than that growth under copper concentration 80 time of its control (copper treat 2), indicate that ectomycorrhizal gung *Suillus bovinus* has higher tolerance to zinc and sensitive to copper. That is consistent with the work of Jone and Hutchinson (Jone,

1986). Tolerance of mycorrhizal fungi to heavy metals is related to the ability of fungal mycelia to limit uptake for them (Denny, 1987; 1992; 1995). In the present study, the amount of zinc took up by mycelia was located on hyphae more than accumulated in cell. Therefore the high zinc tolerance may be interpreted as that zinc ions are bound and detoxified externally by adsorption on hyphal cell wall or slime layer.

Under the excess heavy metals stress, as measured enzymes shown, the glycolysis of mycelia was pronounced influenced. The activities of HK and PFK in crude extract of mycelia growth in copper or zinc application were extremely inhibited. The two enzymes carry out ATP-dependent phosphorylations. Magnesium ion is required in these reactions, because the reactive form of ATP is their chelated complex with Mg^{2+} (Mathews, 1990). The fact of HK and PFK was decreased by copper or zinc may be interpreted as Cu^{2+} or Zn^{2+} competed with Mg^{2+} so that effected ATP activity and led to the both phosphorylation inhibited. However, in the present study, in contrast with HK and PFK, another regulatory enzyme PK in crude extract of mycelia was increased slightly in copper and significantly in zinc application. As Mathews and Van Holde (Mathews, 1990), aldolase from bacteria and lower eukaryotes is a dimeric protein that requires Zn^{2+} for activity. Therefore, if Zn^{2+} stimulated activity of aldolase leading to increase activity of PK will be need to further investigate.

There are reports of employing different pathway to NH_4^+ incorporated into carbon skeletons to form amino acids in both fungi and higher plants: glutamate dehydrogenase (GlDH) is the primary NH_4^+ assimilating enzyme in the former (Patman, 1976), and the primary route of NH_4^+ assimilation in roots and photosynthetic tissues of the latter is via the glutamine synthetase-glutamate synthase (GS/GOGAT) pathway (Miflin, 1980; Oaks, 1985). Results from this study indicate that the two pathways were employed by *Suillus bovinus* to assimilate ammonia, and GS rather acting de-amidating than amidating *in vivo*. The extremely low activity of GOGAT may be interpreted as that the activity of GOGAT was only from part of this enzyme because ferredoxi-dependent GOGAT was not checked in the study.

In the copper or zinc applications, the fact of activity of GlDH increased and GS decreased suggest that Cu^{2+} or Zn^{2+} inhibited the NH_4^+ incorporation via GS/GOGAT route in mycelia cell (Griffin, 1994).

The change of specific activity of measured enzymes of treated mycelia here indicated that the excess copper or zinc in nutrient solution was not only stimulated on mycelia surface and in slime, but also transferred into mycelia cells. The constant of activities in relative low copper and zinc treated mycelia (treat 1) suggested that *Suillus bovinus* have great potential for protection host plant from copper or zinc toxicity. The high amount of zinc stimulated in fungi slime illustrated the ability of the fungous to retain excess zinc ion and reconfirmed its potential protection.

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