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Protease over-production in the presence of copper by a *Trichoderma harzianum* strain with biocontrol potential

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Abstract: The effect of copper on the production of extracellular trypsin-like proteases of *Trichoderma harzianum* T66 was examined. Trypsin-like protease enzyme activities were significantly enhanced in the presence of copper ion. It was demonstrated that instead of raising the activity of previously produced enzymes, copper ions are able to induce the secretion of extracellular trypsin-like proteases. Supernatants of control and $CuSO_4$-containing cultures with the highest enzyme activities were separated by gel filtration chromatography and enzyme activities were measured in the fractions collected. It turned out that in the presence of copper certain extracellular trypsin-like protease izoenzymes are produced in a larger degree both under inductive and non-inductive conditions. Our results suggest that the antagonistic abilities of *Trichoderma* strains could be enhanced by adding certain sublethal amounts of $CuSO_4$. Consequently, an appropriate level of crop protection could be ensured by the application of reduced amounts of copper-containing fungicides in combination with biocontrol *Trichoderma* strains within the frames of integrated pest management.

Key words: biocontrol, copper, fungicide, plant pathogenic fungi, proteases, *Trichoderma*

Introduction

Copper ion is a fungicide used widely in agriculture, which is due to the fact that it is a strong inhibitor of fungal growth. On the other hand, copper is necessary for normal growth in a certain small amount, as it is an important trace element.

Trichoderma species are imperfect filamentous fungi with teleomorphs belonging to the *Hypocreales* order of the Ascomycota division. Their effective antagonistic abilities against plant pathogenic filamentous fungi are based on different mechanisms including competition, antibiosis and mycoparasitism (Manczinger et al., 2002). Several *Trichoderma* species are effective agents to control plant pathogenic fungi, e.g. *Fusarium*, *Pythium* and *Rhizoctonia* species, which allows for the development of biocontrol strategies.

Integrated pest management is based on the combined application of physical, chemical and biological means of control. As copper is an effective chemical pesticide and *Trichoderma* strains are potential biofungicides, integrated plant protection strategies can be worked out based on their combined application. Croatian field-trials for the control of grey mould (*Botrytis cinerea*) on grapevines revealed that *T. harzianum* can be mixed with pesticides based on copper sulphate and copper hydroxide (Topolovec-Pintaric et al., 1999). Mahanty et al. (2000) examined the efficacy of promising fungicides in combination with *Trichoderma* for the management of foot rot of betelvine (*Piper betle*) and have found that *T. harzianum* along with Bordeaux mixture (1% copper sulphate) at monthly soil drenching was the best solution for controlling *Phytophthora parasitica.* For the successful development of

such integrated control strategies it is very important to study the effects of copper on *Trichoderma* extracellular enzymes important for mycoparasitism. Data are available for six *Trichoderma* strains about the effects of copper-sulphate and other metal compounds on the *in vitro* activities of extracellular enzymes involved in mycoparasitism: copper slightly inhibited trypsin- and chymotrypsin-like protease and enhanced β-1,4-*N*-acetylglucosaminidase activities at a concentration of 1 mmol (Kredics et al., 2001a).

The aim of this study was to examine the effect of different concentrations of copper on the secretion and *in vitro* activities of trypsin-like proteases, which are among the extracellular enzymes important for mycoparasitism of *Trichoderma* strains.

Material and methods

Microorganisms and culture conditions

Strain *Trichoderma harzianum* T66 derived from the Microbiological Collection of the University of Szeged. For the induction of extracellular proteases, cultures were grown in liquid minimal medium containing skim-milk powder (1 g/l mannitol, 1 g/l $NaNO_3$, 1 g/l KH_2PO_4, 0,1 g/l $MgSO_4$ and 3 g/l skim-milk powder). Non-inductive conditions were ensured by growing cultures in liquid minimal medium (5 g/l mannitol, 1 g/l $NaNO_3$, 1 g/l KH_2PO_4 and 0,1 g/l $MgSO_4$). Media contained distinct sublethal concentrations of $CuSO_4$, the applied concentrations were 0, 10, 20, 30, 40, 50 and 80 µg/ml under inductive and 0, 2, 4, 6, 8, 10, 15, 20, 30 and 50 µg/ml under non-inductive conditions. Cultures were shaken on a shaker at 25°C with 100 rpm for 3 and 6 days in the case of induced and non-induced cultures, respectively. Enzyme activities were measured in the culture supernatants.

Measurement of extracellular trypsin-like protease activities

To provide the appropriate pH for the function of the enzymes, 50 µl phosphate buffer (pH 6.6) was added to 50 µl of the culture supernatants. After adding 50 µl of *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide (1 mg µg/ml, dissolved in dimethyl-sulphoxide) as a substrate for trypsin-like proteases, incubation followed at 25°C for 1 h and enzyme activities were measured with a Labsystems Uniskan microtiter plate spectrophotometer at a wavelength of 405 nm. Based on our experience about the susceptibility of measurements using *p*-nitroaniline substrates, the enzyme activities were considered significant, if the OD_{405} values were above 0.050.

Gel filtration chromatography

The gel filtration chromatography was performed on a 0.9 x 60 cm K-90 column (Pharmacia) containing Sephadex G-100 gel swollen in distilled water with 1 g/l NaCl. Two ml of the supernatant was loaded to the column and 80 fractions of 0.7 ml were collected, the eluent contained 1 g/l NaCl. Activities of trypsin-like proteases were measured in the fractions after 3 h of incubation using the method described above.

Results

Significant trypsin-like protease activities could be measured after 3 and 6 days of culturing in the case of induced (Figure 1a) and non-induced (Figure 1b) cultures, respectively. It is apparent that the activity of extracellular trypsin-like proteases rises in the presence of certain sublethal concentrations of $CuSO_4$. Activity rising appears both under inductive and non-inductive conditions, but as compared with the control which contained no $CuSO_4$, the rate of activity rising is much higher in the case of cultures that were grown under non-inductive conditions. As copper has no significant inhibitory effect on mycelial growth at the applied concentrations, the elevated levels can not be related to differences in growth. To determine whether the presence of $CuSO_4$ enhances the degree of enzyme production or just raises the

activity of previously secreted enzymes, $CuSO_4$ in a concentration of 20 and 40 μg μg/ml was added to the supernatant of the control cultures grown under inductive conditions and the activities were measured. No activity rising appeared suggesting that the production and not the activity of extracellular trypsin-like proteases is influenced positively by $CuSO_4$.

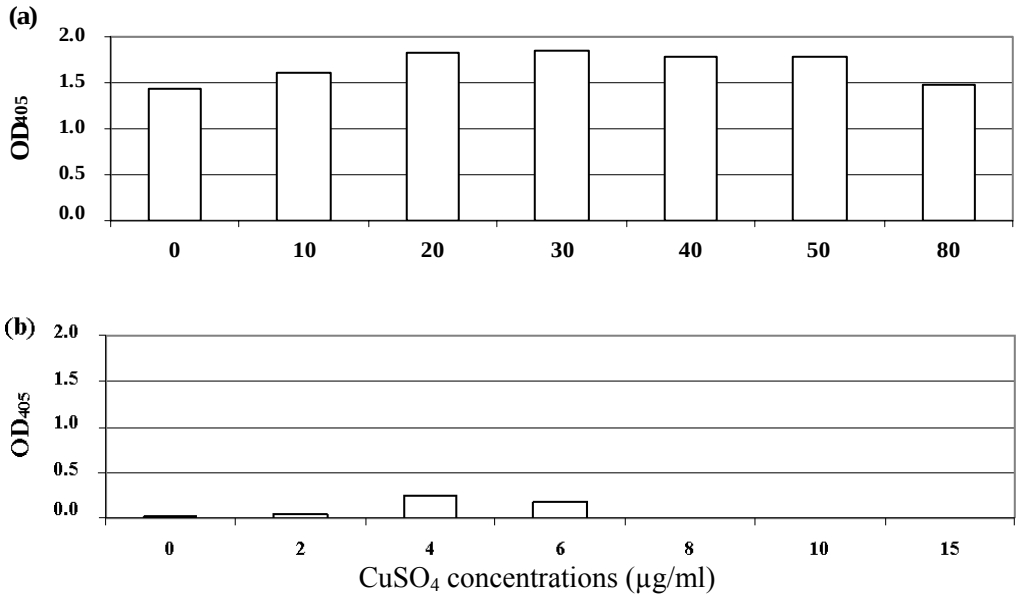

Figure 1. Trypsin-like protease activities measured in supernatants derived from (a) induced and (b) non-induced $CuSO_4$-containing cultures of strain *T. harzianum* T66

Gel filtration chromatography profiles of trypsin-like proteases of strain *T. harzianum* T66 are shown in Figure 2. Control cultures grown under non-inductive conditions showed very low trypsin-like protease activities, which are not measurable after gel filtration. Two peaks of activity appeared in the supernatant of the induced control culture, suggesting the presence of at least two extracellular trypsin-like proteases. The production of both isoenzymes seem to rise in the presence of $CuSO_4$ both under inductive and non-inductive conditions.

Discussion

Copper in certain sublethal concentrations appeared to enhance the production of extracellular trypsin-like proteases both under inductive and non-inductive conditions. These results suggest that the combination of biocontrol *Trichoderma* strains with copper-containing fungicides may result in more effective integrated plant protection strategies than the application of *Trichoderma* or copper alone. Besides the addition of the inhibitory effects of the biocontrol agent and the chemical pesticide, the enhancement of *Trichoderma* protease production by copper may also contribute to the efficiency of integrated control. As copper ion has an inhibitory effect on mycelial growth with an IC_{50} concentration of 0.16 mmol for strain T66 (Kredics et al., 2001a), the isolation of copper-tolerant strains seems to be important for the purposes of integration with copper-containing fungicides. Direct UV-mutagenesis with the selection at 1.6 mmol copper sulphate was ideal for the improvement of copper tolerance in *Trichoderma* strains and the resulting mutants proved to be effective *in vitro* antagonists of *Fusarium*, *Pythium* and *Rhizoctonia* strains even under copper stress

(Kredics et al., 2001b). The results of the present study indicate that the antagonistic potential of *Trichoderma* strains can be raised by enhancing the secretion of extracellular proteases by copper, suggesting that an elevated level of crop protection could be ensured by using reduced amounts of copper-containing fungicides in combination with copper tolerant *Trichoderma* strains within the frames of complex integrated plant protection.

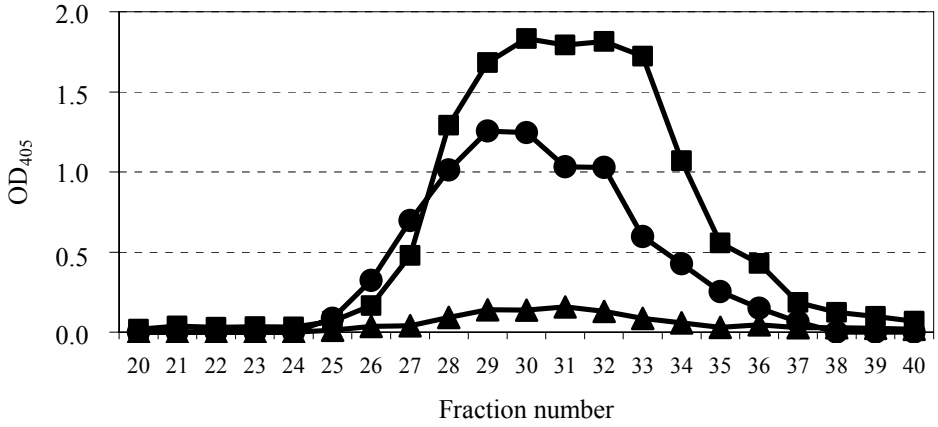

Figure 2. Gel filtration chromatography profiles of trypsin-like proteases of strain *T. harzianum* T66 : induced control (●); induced+30 µg/ml $CuSO_4$ (■); non-induced+4 µg/ml $CuSO_4$ (▲)

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