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COPPER TOLERANCE OF *TRICHODERMA* SPECIES

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Abstract - Some *Trichoderma* strains can persist in ecosystems with high concentrations of heavy metals. The aim of this research was to examine the variability of *Trichoderma* strains isolated from different ecosystems, based on their morphological properties and restriction analysis of ITS fragments. The fungal growth was tested on potato dextrose agar, amended with Cu(II) concentrations ranging from 0.25 to 10 mmol/l, in order to identify copper-resistant strains. The results indicate that some isolated strains of *Trichoderma* sp. show tolerance to higher copper concentrations. Further research to examine the ability of copper bioaccumulation by tolerant *Trichoderma* strains is needed.

Key words: *Trichoderma* sp., copper, tolerance, ITS fragments

INTRODUCTION

Intensive agriculture, industrialization and technological innovations have led to the problem of environmental pollution. The introduction of metal pollutants in various forms in the environment can pose a severe threat to the ecological system due to their negative impact on most life forms. Soil contamination from the improper handling and disposal of hazardous materials and waste is faced by all countries (Caliman et al., 2011). Although some heavy metals are required for physiological processes (as components of metalloenzymes), their excessive accumulation in living organisms is always detrimental. Generally, toxic metals cause enzyme inactivation, damage cells by acting as antimetabolites or form precipitates or chelates with essential metabolites (Sobolev and Begonia, 2008). Bioremediation of heavy metals, in particular copper, is a new technology gaining increased attention since it is an eco-friendly and innovative method. The first step in the development of bioremediation techniques is screening for potential agents tolerant to increased concentrations of pollutants.

The genus *Trichoderma* comprises cosmopolitan soil-borne fungi found in different ecosystems. They are highly successful colonizers of their habitats, which is the result of both efficient utilization of the substrate as well as the capacity for secretion capacity of antibiotic metabolites and enzymes. They are capable of dealing with different environments such as compost, agricultural soils, rhizosphere and waste material. Therefore, different strains of *Trichoderma* have been applied in agriculture, bioremediation, waste management and biotechnology (Schuster and Schmoll, 2010). It is comprised of a great number of fungal strains that act as biological control agents and plant growth promoters, of which *T. virens, T. viride* and, above all, *T. harzianum*, are the best known, showing mycoparasitic ability against phytopathogenic fungi such as *Pythium*, *Phytophthora*,

Sclerotinia, Rhizoctonia, Fusarium and *Botrytis* (Benitez et al., 2004).

Trichoderma harzianum was identified as an effective agent for solid waste bioconversion, as it produces extracellular enzymes (Rahman et al., 2011). It was shown that Cu (II) can bind to the cell wall surface of *T. viride*, a mechanism of metal tolerance making it less available in the medium (Anand et al., 2006). Thus, it could be considered a possible bioremediation agent.

When planning the application of *Trichoderma* strains, it is important to consider the effect of biotic and abiotic stresses on the microbial communities present in the soil. Among the most important environmental stresses in soil are the presence of heavy metals and different chemicals, variations of temperature or water potential (Kredics et al., 2001). The copper tolerance of *Trichoderma* sp. has been rarely explored (Kredics et al., 2001, Anand et al., 2006, Ting and Choong, 2009). Therefore, the present research was undertaken to study the tolerance of *Trichoderma* strains isolated from different types of soil, compost and waste material to increased concentrations of copper.

MATERIALS AND METHODS

Fungi were isolated from different types of soil (vitisol, cambisol, regosol, pseudogley and ranker), compost made of the sop from plum brandy production and sewage sludge from the Kolubara coalmine (KEK).

For isolation of *Trichoderma* strains, a serial dilution technique was applied. Half a milliliter of 10^{-3} dilution was used to inoculate Rose Bengal Agar (RBA) (Peper et al., 1995), which was incubated for one week on 25°C. Culture plates were examined and colonies supposed to belong to *Trichoderma* sp. were picked and transferred on fresh potato dextrose agar (PDA) plates (Merck, Germany). Culture purification was done by standard procedures. In order to confirm affiliation to the order *Trichoderma*, morphological properties were observed on Petri dishes by using microscopic slide techniques. Pure cultures were stored at + 4°C.

The copper tolerance test was performed by measuring the diameter of fungal colonies on plates containing Cu(II) amended PDA medium and comparing the diameter with colonies on control PDA plates.

PDA medium amended with 0.25, 0.5, 0.75, 1, 2, 4 and 10 mmol Cu(II)/l, in the form of $CuSO_4x5H_2O$, was prepared and autoclaved. For concentrations of 4 and 10 mmolCu(II)/l, the pH of the medium was set with 1 mol/l NaOH solution prior to autoclaving. PDA without additional Cu (II) was used for the control plates.

Fungal isolates were preincubated on PDA medium at 25°C for 5 days.

Mycelia discs of 5 mm diameter were cut and placed on the center of the control and Cu (II)-enriched plates. Three replicate plates were used per treatment. Fungi were incubated in the dark at 25°C for 48 h. Fungal colony diameter was measured and the percentage of inhibition calculated according to the formula:

$$PI = \frac{(C-T)}{C} x100$$

where PI is the percentage inhibition, C is the colony diameter (mm) on the PDA plate and T is the colony diameter (mm) on the Cu-amended PDA plate.

For molecular analysis, mycelia were harvested after 3 days of growth on PDA at 25°C. Genomic DNA was isolated using a ZymoResearch Soil Microbe DNA MiniPrep Kit following the manufacturer's protocol.

PCR reaction was carried out in a total volume of 50 ml containing 2.5 mmol/l $MgCl_2$, 1 x Taq Buffer, 0.5 μmol/l of each primer, 0.2 mmol/l of each dNTP and 2.5 units of Taq Polymerase (Fermentas). The amplification program consisted of 5 min initial denaturation at 94°C, 30 cycles of amplification

for 1 min at 94°C, 1 min at 48°C and 1 min at 72°C, and a final extension period of 10 min at 72°C. A specific fragment of the internal transcribed spacer region (ITS1 – 5.8S rDNA – ITS2) was amplified by the primers ITS1 (5'-TCCGTAGGTGAACCT-GCGG-3') and ITS4 (5'-TCCTCCGCTTATTGA-TATGC-3').

PCR products obtained after amplification with primers ITS1-ITS4 were incubated for 2 h at 37°C with restriction enzymes AluI, HhaI, Hae III and RsaI (Fermentas). Ten microliters of each PCR-amplified product was digested with restriction endonucleases as recommended by the manufacturer. Amplified DNA (before restriction) and restriction fragments (after restriction) were detected by electrophoresis on a 3% agarose gel in 1xTBE (Tris-Borate-EDTA) buffer. The gels were stained with ethidium bromide (0.6 µg/ml). Gel images were visualized with the Bio-Print system (Vilber Laurmat, France) and fragment size was determined in comparison with the standard 100 bp Plus DNA Ladder (Fermentas).

RESULTS

In this study, thirteen characteristic white-green isolates were obtained. Morphological examination confirmed that eight of these isolates belong to *Trichoderma* sp., and their pure cultures were used for further analysis (Table 1).

The concentrations of ≥2 mmol Cu(II)/l completely inhibited the growth of all tested strains. The growth of most isolated *Trichoderma* strains was inhibited from 20 to 80% by copper concentrations ranging from 0.5 to 1 mmol Cu(II)/l, respectively.

Table 1. The origin of isolated *Trichoderma* strains

Isolate	Sample
Tv	Vitisol (0-30cm)
Tk	KEK
10/5	compost made of sop from plum brandy production
9	Pseudogley (0-30cm)
10	Pseudogley (30-60cm)
11	Regosol (0-30cm)
15b	Ranker (10-20cm)
17	Cambisol (5-30cm)

Concentrations of 0.25, 0.5 mmol and 0.75 mmol Cu(II)/l had a stimulative effect on the growth of isolate 10. The colony diameter of isolate 10 on the PDA plate amended with 0.5 mmol Cu(II)/l was greater by 24% in comparison to the control (Fig. 1). The lowest concentration that inhibited the growth of isolate 10 was 1 mmol Cu(II)/l, and percentage inhibition was only 8%. The growth of isolates 15b and 9 was significantly stimulated on medium with 0.25 mmol Cu(II)/l (Table 2).

After amplification with primers ITS1-ITS4, only one fragment was amplified in all of the examined samples (Fig. 2). The PCR product in all examined samples was 680 bp.

After digestion with AluI, two fragments (≈480 bp and ≈200 bp) were detected in samples 9 and 10, while other analyzed samples do not have a restriction site for this enzyme (Fig. 2).

Similarly, after digestion with the restriction enzyme HhaI, samples 9 and 10 had a different digestion pattern compared to the other examined samples (results not shown). The amplified fragment was not digested by restriction enzymes RsaI and HaeIII.

DISCUSSION

Several heavy metal ions are trace elements required for fungal growth, but these elements can be toxic at high concentrations. Fungal environment can be contaminated with copper in different ways, for example by the application of some pesticides. The resistance of some *Trichoderma* sp to several heavy metals was examined previously by Kredics et al. (2001). According to their results, the IC_{50} values for copper were in the range of 0.14 to 0.20 mmol $CuSO_4x5H_2O$/l. In this paper, for the six examined isolates (Tv, Tk, 10/5, 11, 15b and 17), the IC_{50} values were higher. They varied between 0.5 and 0.75 mmol Cu/l for most of the isolates. How-

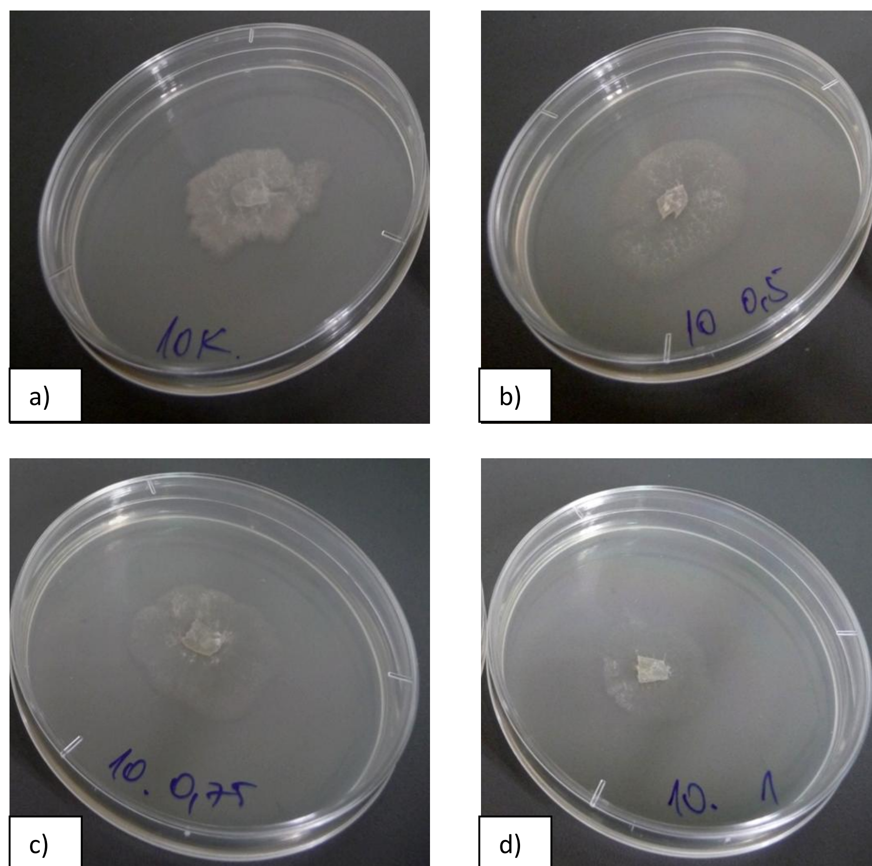

Fig. 1. The growth of *Trichoderma* isolate 10 in 0 (a), 0.5 (b), 0.75 (c) and 1 mmolCu(II)/l

ever, isolates 9 and 10 had IC_{50} values between 1 and 2 mmol Cu/l. Similar results were obtained by Ting and Choong (2009) and Anand et al. (2006) with *Trichoderma* strains isolated from polluted areas.

Results of PCR based on the size of amplified ITS fragments indicate the differences in ITS sequence among the examined isolates. Furthermore, restriction enzyme analysis with AluI and HhaI showed that there are at least two different species.

Table 2. Growth inhibition percentage of isolated strains by different Cu concentrations

Isolate	Percentage inhibition			
	0.25 mmolCu(II)/l	0.5 mmol Cu(II)/l	0.75 mmolCu(II)/l	1 mmolCu(II)/l
Tv	-3.6*	29	68.1	79.0
Tk	-4.6*	32	72.7	81.0
10/5	-2.2*	34.2	61.6	74.0
9	-20.0*	7.1	16.2	21.2
10	-10.9*	-24.1*	-2.3*	8.0
11	1.3	25.1	71.3	79.7
15b	-16.9*	23.3	60.3	69.8
17	-1.7*	38.9	75.8	77.6

*- the presence of Cu stimulated mycelial growth of some isolates

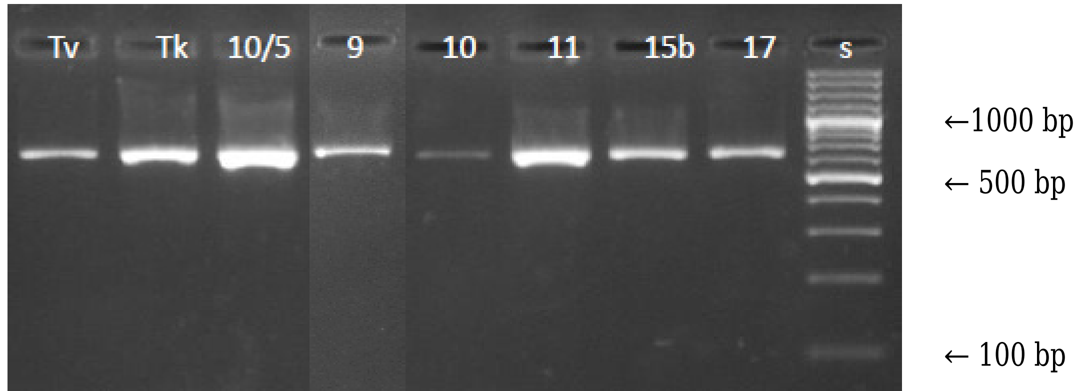

Fig. 2. Amplification patterns with primers ITS1-ITS4. Lane S=100bp Plus DNA Ladder Fragments of 1000bp, 500bp and 100bp are marked with arrow.

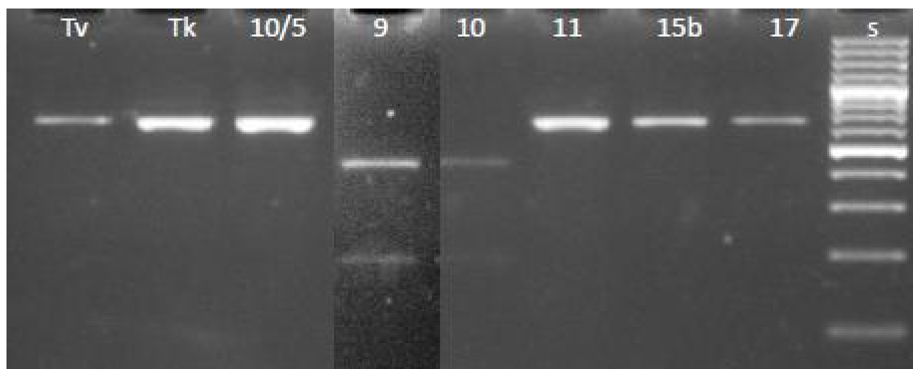

Fig. 3. Digestion patterns of ITS1-ITS4 PCR amplification products with restriction enzyme AluI

Isolate 9 and 10 are different in comparison to the other isolates in showing a higher tolerance to elevated copper concentrations. These isolates have different morphological properties as well as a different sequence of ITS fragments.

The results suggest that copper-tolerant *Trichoderma* strains might be the preferred choice for bioremediation agents. For approving their use in bioremediation it is necessary to examine bioaccumulation of copper by isolated *Trichoderma* strains, especially isolates 9 and 10.

This study resulted in a collection of *Trichoderma* sp. isolated from different environments. The next step in the investigation of genetic polymorphism between pure cultures of *Trichoderma* will be to identify isolates based on their complete ITS sequences and further characterization of isolated strains.

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