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EXTRACELLULAR ENZYME ACTIVITY OF *TRICHODERMA* STRAINS ISOLATED FROM DIFFERENT SOIL TYPES

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Abstract
Enzymatic characterization of 16 *Trichoderma* strains, grown in potato dextrose broth was carried out semi-quantitatively by API-ZYM test. Strains that were used for test were isolated from different soil types and belonged to *T. harzianum* species complex, *T. koningiopsis, T. atroviride*, *T. brevicompactum, T. gamsii, T. citrinoviride* and *T. longibrachiatum*.
The activities of acid phosphatase and naphthol-AS-BI-phosphohydrolase were high in all examined strains and N-acetyl-β-glucosaminidase activity was detected in most of them. However, activities of β-glucuronidase, α-glucosidase, β-glucosidase were negative. Among all investigated *Trichoderma* isolates, *T.brevicompactum* showed moderate α-galactosidase and β-galactosidase enzymatic activity.
Obtained results are in accordance with previous results on very good antagonistic properties of examined *Trichoderma* strains against fungal pathogens, indicating that extracellular enzymes are important part of their antagonistic mechanism of biological control.
Key words: *Trichoderma*, N-acetyl-β-glucosaminidase, biocontrol, API-ZYM.

Introduction

Trichoderma spp. is cosmopolitan soil borne fungi with great abilities of colonizing their habitats either by efficient utilization of substrate or their secretion capacity for enzymes and antibiotics (Schuster and Schmoll, 2010). They play major role as biocontrol agents having capabilities of increasing crop yield acting as biopesticides, bioherbicides or plant growth promoters. Their antagonistic potential can be based on various mechanisms. *Trichoderma* strains exert biocontrol against fungal phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms with inducing plant systemic resistance to pathogens and antibiosis, or directly, by mechanisms such as mycoparasitism. The importance of these mechanisms is that they may act coordinately and their process of biocontrol depends on different factors. Activation of each mechanism follows the production of specific compounds and metabolites, such as plant growth factors, hydrolytic

enzymes, siderophores or antibiotics (Benítez *et al*., 2004). Strains belonging to the genus *Trichoderma* are well known to produce a lot of different extracellular enzymes. The amount and activity of these enzymes are important in the biofungicide efficacy of the strains, as they can determine the antagonistic properties of the strains (Sharma *et al.,* 2012).

One of the mechanisms of biocontrol which is based on production of extracellular enzymes which are responsible for degradation of fungal cell walls is mycoparasitism. The chitinase, laminarinase and protease systems play important roles in the mycoparasitism of *Trichoderma* spp. (Sivan and Chet, 1989). N-acetyl-β-glucosaminidase forms part of the chitinase complex: two isoenzymes were described in the most isolated species, *T. harzianum* (Haran *et al.* 1995). Chitinase encoding genes are also the most used to improve plant defence against fungal pathogens (Sharma *et al*., 2012). These enzymes are able of degrading the linear homopolymer of ß-1,4-Nacetyl- D-glucosamine, the major component of the cell wall of most phytopathogenic fungi, as well as are very strong inhibitors of their hyphal growth. (Harman *et al.,* 2004).

Enzyme β-1,3-glucanase is known to inhibit germination of spores or the growth of pathogens and often work in correlation with chitinase and antibiotics. These enzymes in fungi have functions in morphogenetic processes during their development and differentiation. In the fasting state these enzymes become autolytical, providing carbon and energy and finally they are involved in mycoparasitism (El-Katatny *et al*., 2001).

One of the methods used to detect extracellualar enzyme activity is the API ZYM system. This semi quantitative method is designed to detect 19 enzymatic reactions. It was used as one of the methods for the investigation of the taxonomy of different microorganisms including fungi (Bridge and Hawksworth, 1995). In this paper we have used API ZYM system to investigate extracellular enzyme activity of *Trichoderma* strains with very good antagonistic properties (Danilovic, *et al*.,2014) isolated from different soil types.

Material and methods

Trichoderma strains used in this study were isolated from different soil types in Serbia: Vertisol, Rendzic Leptosol, Chernozem, Regosol, Fluvisol, Calcic Gleysol, Eutric Cambisol and Dystric Leptosol. Sixteen strains that were used belonged to *T. harzianum* species complex (SZMC 20965; SZMC 20969; SZMC 20982; SZMC 20988; SZMC 20994)*, T. koningiopsis* (SZMC 20983 and SZMC 20984)*, T. atroviride* (SZMC 20968), *T. brevicompactum* (SZMC 22661 and SZMC 22663)*, T. gamsii* (SZMC 20985)*, T. citrinoviride* (SZMC 20987 and SZMC 22668) and *T. longibrachiatum* (SZMC 22664, SZMC 22669 and SZMC 22665). The strains were maintained on potato dextrose agar medium (PDA).

Detection of the enzymatic activity

Trichoderma strains were preincubated on PDA medium at 25^{o}C for 5 days. Mycelia discs of 5 mm diameter were cut from the edge of petri dish and inoculated into 200 ml of sterile potato dextrose broth (PDB) liquid medium. Flasks were shaken for 7 days on 25^{o}C in the dark on an orbital shaker BIOSAN (Lithuania). Liquid culture was filtrated through sterile gauze and filtrate was used for the detection of extracellular enzymatic activity by using

the semi quantitative API ZYM system (bioMerieux, France), according to the manufacturer protocol.

Sixty five micro liters of each inoculum was transferred into each of the twenty API ZYM strip microtubes prefilled with 5 ml of distilled water for maintaining humidity and incubated at 37^{o}C in thermostat for 4h. After incubation period a drop of ZYM A and ZYM B reagents was added to each of twenty wells.

The color reaction was read after 5 minutes according to the API ZYM reading color scale. According to this scale enzyme activities were scored as following: 0-no color, 1-low, 2-moderate and 3-high.

Results and discussion

Results of the enzymatic activity are presented in Table 1. The activities of acid phosphatase and naphthol-AS-BI-phosphohydrolase were high in the case of almost all examined isolates. Our results show that out of 16 examined strains only 2 are classified with low acid phosphatase activity. Altomare *et al*. (1999) have shown that *Trichoderma* is highly competitive for P-uptake with some phytopathogenic fungi, which are therefore suppressed. This might be based on high activities of acid phosphatase and naphthol-AS-BI-phosphohydrolase. In plants acid phosphatase increase phosphate availability, leading to improved growth of plants in the presence of *Trichoderma* strains in the rhizosphere (Kapri and Tewari, 2010). The activity of N-acetyl-β-glucosaminidase, was detected in most examined strains. This enzyme is included in chitinolitic system of *Trichoderma* species and responsible for breakdown of the pathogen cell walls, i.e. involved in antagonism mechanisms. Also it is used for the production of liquid, powder or solid antifungal compositions for plant protection and was firstly isolated from *T.harzianum* P1 strain (Harman *et al.,* 1995). Activities of β-glucuronidase, α-glucosidase, β-glucosidase were negative in case of all examined samples. Among all investigated *Trichoderma* isolates, *T.brevicompactum* showed moderate α-galactosidase and β-galactosidase enzyme activity. It is reported that α-galactosidase can be used in modifications of diverse agricultural products containing raffinose-family oligosaccharides to digestible sugars (Shabalin *et al,* 2002). Thus it could be presumed that *T.brevicompactum* strain is a good source of α-galactosidase enzyme and it could help in the hydrolysis of galactooligosaccharides.

Table 1. Extracellular enzyme activity as determined with API-ZYM system. Numbers in columns represent different *Trichoderma* strains, as explained at the bottom of the table.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Alkaline phosphatase	1	1	0	0	1	0	1	1	0	0	0	3	1	0	1	1
Esterase (C4)	3	3	0	0	1	1	0	2	1	0	2	3	0	0	0	2
Esterase Lipase (C8)	3	3	0	0	1	1	1	1	0	0	1	1	0	0	0	1
Lipase (C14)	2	2	0	0	1	1	1	1	0	0	0	1	1	0	0	1
Leucinearylamidase	0	0	0	0	0	0	0	1	1	0	0	1	0	0	1	0
Valinearylamidase	0	0	0	0	0	1	0	1	0	0	0	1	0	0	1	0
Cysteine arylamidase	0	0	0	0	0	0	0	1	1	0	0	0	0	0	1	0
Trypsin	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	1
α-chymotripsin	0	0	0	0	0	0	0	1	1	1	1	1	0	0	0	0
Acid phosphatase	3	3	2	3	3	3	2	3	2	3	2	3	1	1	3	3
Naphthol-AS-BI-phosphohydrolase	3	3	2	3	3	3	2	3	2	3	2	3	1	1	3	3
α-galactosidase	1	2	0	0	0	0	0	0	0	0	0	0	0	0	0	1
β-galactosidase	2	2	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β-glucuronidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-glucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
β- glucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
N-acetyl-β-glucosaminidase	1	1	1	3	2	3	0	0	2	3	3	3	0	1	2	0
α-mannosidase	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0
α-fucosidase	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0

1-*T.brevicompactum* SZMC 22661; 2- *T.brevicompactum* SZMC 22663; 3-*T.longibrachiatum* SZMC 22664; 4-*T.citrinoviride* SZMC 22668; 5-*T.longibrachiatum* SZMC 22669; 6-*T.longibrachiatum* SZMC 22665; 7-*T.harzianum* SZMC 20994; 8- *T.harzianum* SZMC20988; 9-*T.harzianum* SZMC 20969; 10- *T.citrinoviride* SZMC 20987; 11- *T.harzianum* SZMC 20965; 12-*T.koningiopsis* SZMC 20983; 13- *T.gamsii* SZMC 20985; 14- *T.harzianum* SZMC 20982; 15-*T.atroviride* SZMC 20968; 16- *T.koningiopsis* SZMC 20984

Previously communicated (Danilovic *et al*., 2014) very good antagonistic properties of examined strains are connected with high activities of acid phosphtase, naphthol-AS-BI-phosphohydrolase and N-acetyl-β-glucosaminidase. In case of *T.brevicompactum* strains, two more enzymes: α-galactosidase and β-galactosidase are contributing to their antagonistic potential.

Most of the examined Trichoderma straines are promising candidates for practical application within the frames of biological control. Our results indicate that extracellular enzymes are important part of their antagonistic mechanism against phytopathogens.

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