Review on Agriculture and Rural Development 2014. vol. 3 (1) ISSN 2063-4803

TRICHODERMA ISOLATES FROM VEGETABLE RHIZOSPHERE SAMPLES: POTENTIAL FOR THE BIOLOGICAL CONTROL OF *BOTRYTIS* SPECIES

PÉTER KÖRMÖCZI, TAMÁS MARIK, LÁSZLÓ MANCZINGER, ENIKŐ SAJBEN-NAGY, CSABA VÁGVÖLGYI, LÁSZLÓ KREDICS

University of Szeged, Faculty of Science and Informatics
Department of Microbiology
Közép fasor 52, H-6726 Szeged, Hungary
kormoczipeti@gmail.com

ABSTRACT
Members of the genus *Trichoderma* are wide-spread saprophytic fungi living in the soil and the rhizosphere of different plants. Due to their enzyme production abilities they are able to use complex biomolecules as carbon and nitrogen source. Many strains with very good biocontrol abilities against plant pathogenic fungi could be isolated from soils of agricultural areas. In this study we isolated *Trichoderma* strains from the rhizosphere of vegetables (pepper and lettuce) derived from gardens of different Hungarian cities (Szolnok, Kalocsa, Újszilvás, Kelebia). The isolates were identified by the sequence analysis of the ITS (internal transcribed spacer) region. The strains belonging to species that are not pathogenic to humans or cultivated mushrooms and possessing promising bicontrol potential were further investigated. The antagonistic abilities of the strains were studied against *Botrytis* species (*B. cinerea*, *B. pseudocinerea*) in *in vitro* confrontation tests and the extracellular enzyme systems of the strains were investigated in different liquid media. The knowledge of the correlation between the *in vitro* antagonistic abilities and enzyme production may contribute to our understanding of the biocontrol mechanism.

Keywords: *in vitro* antagonism, *Trichoderma*, *Botrytis*, extracellular enzymes

INTRODUCTION

The filamentous fungal genus *Trichoderma* (*Ascomycota*, *Hypocreales*, *Hypocreaceae*) was firstly divided into 9 species aggregates (*T. piluliferum*, *T. polysporum*, *T. hamatum*, *T. koningii*, *T. aureoviride*, *T. harzianum*, *T. longibrachiatum*, *T. pseudokoningii* and *T. viride*) which was later revised with the introduction of 5 sections: *Trichoderma*, *Longibrachiatum*, *Saturnisporum*, *Pachybasium* and *Hypocreanum* (DRUZHININA AND KUBICEK, 2005). Today, more than 200 species are known within the genus. They are mostly soil-borne, saprophytic fungi, living on the roots and in the rhizosphere of plants. *Trichoderma* species play key roles in biodegradation due to their abilities to produce extracellular enzymes: they can degrade different macromolecules including proteins, cellulose and chitin. Moreover, certain species of the genus include excellent biocontrol agents which are producing antifungal metabolites and having mycoparasitic abilities. Certain strains are also able to stimulate the plant growth, mainly in the root region, and they can induce drought tolerance and systemic resistance in plants. Because of these previously described phenomena, *Trichoderma* strains are applicable for biocontrol purposes against plant pathogenic fungi, e.g. species from the genus *Botrytis*.
Many previous studies focused on diversity of *Trichoderma* in natural soil ecosystems, e.g. in a mid-European, primeval floodplain-forest (WUCZKOWSKI, 2003), in the Danube floodplains (FRIEDL and DRUZHININA 2012), and habitats in Russia, Nepal, North-India (KULLNIG et al., 2000), Southeast Asia (KUBICEK et al., 2003), China (SUN et al., 2012), North Africa (SADFI-ZOUAOUI, 2009) and South-America (HOYOS-CARVAJAL, 2009). During these studies, many *Trichoderma* species and genotypes were identified and characterized, increasing our knowledge about the biodiversity of the genus. Unfortunately

only a few investigations were carried out in agricultural fields, monitoring the diversity of *Trichoderma* community in the plant rhizosphere (GHERBAWY, 2004; SADFI-ZOUAOUI et al., 2009; MULAW et al., 2010; NAEIMI et al., 2011). These community analyses could help us to find fungicide-resistant biocontrol strains with good antagonistic abilities, which could be applied for biocontrol purposes.

The genus *Botrytis* is belonging to the *Sclerotiniaceae* family (*Ascomycota, Helotiales*) counting more than 22 species including *B. cinerea, B. pseudocinerea, B. elliptica, B. tulipae* and *B. gladiolorum* which are able to cause serious infections in different plants, leading to significant production losses, e.g. the opportunistic pathogen *B. cinerea* can attack different parts of plants including roots, crops, bulbs or aging plant parts (ELAD et al., 2004). They are very widespread organisms; they could be found almost everywhere near their host plants. They can be identified easily, because of their numerous transparent conidia, grey, branching, tree-like conidiophores and sclerotia in the older cultures. *B. cinerea* is capable of attacking more than 230 different plants before and after harvesting as well. There are economically significant species among the host plants, like vegetables (tomato, pepper, lettuce and cucumber), fruits (strawberry, raspberry, grape and kiwi), onions or ornamentals. The most common disease caused by *B. cinerea* is grey mould. Infections are mostly developing through different lesions. The basis of plant protection is mostly the chemical control, however the emergence and spreading of fungicide resistant pathogenic strains is an increasing challenge. Moreover, in numerous countries there are strong limitations in the application of pesticides. These reasons highlight the significance of biological control as an environment-friendly alternative in plant protection.

Numerous studies are available in the literature about the opportunities of biocontrol application of *Trichoderma* species against *Botrytis* strains. The mycoparasitic process is well known in case of *T. harzianum* (BELANGER et al., 1995). It was concluded that the antibiotic agents produced are more important for biocontrol of *Botrytis* than the chitinase enzymes. The biocontrol agent (BCA) registered as TRICHODEX (strain T-39 of *T. harzianum*) could be applied in greenhouses and vineyards (ELAD, 1994; 2000ab), moreover TRONSMO and DENNIS (1977) reported that it could control grey mould of strawberry caused by *B. cinerea*. In this study we isolated and identified *Trichoderma* strains from the rhizosphere of different vegetables. We investigated the antagonistic abilities of the isolates against plant pathogenic *Botrytis* strains and the production of extracellular enzymes under different cultivation conditions.

MATERIAL AND METHOD

Isolation of *Trichoderma* strains from vegetable rhizosphere samples

Soil samples were collected at different locations in Hungary (Szolnok, Kalocsa, Újszilvás and Kelebia) from the root region of distinct vegetables (pepper and lettuce). Isolations were carried out on dichloran-Rose Bengal medium, which contained 5g peptone, 1g KH_2PO_4, 10g glucose, 0.5g $MgSO_4.7H_2O$, 0.5 ml dichloran-ethanol solution (0.2%), 0.25ml Rose Bengal from suspension (5%) and 20g agar per liter according to KING et al. (1979). After sterilization, 0.1g oxytetracyclin, 0.1g streptomycin and 0.1g chloramphenicol were added per liter to inhibit bacterial growth. The isolated strains were deposited at the Szeged Microbiological Collection (SZMC).

Molecular identification of the isolats

DNA extractions were carried with E.Z.N.A.® Fungal DNA Mini Kit-Omega Bio-Tek (VWR International, Radnor, USA) according to the instructions of the manufacturer. ITS

sequences (ITS1-5.8 rDNA-ITS2) were amplified and analysed as described previously by NAEIMI et al. (2011). *Trichoderma* isolates were identified by the barcoding program TrichOKEY 2.0 (DRUZHININA et al., 2005) available online at the home page of the International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy (www.isth.info).

In vitro confrontation tests in dual cultures of *Trichoderma* and *Botrytis* strains

In vitro antagonism was investigated in dual cultures tests in confrontation with 6 plant pathogenic *Botrytis* strains (*Table 1*.).

Table 1. Plant pathogenic *Botrytis* strains applied in the confrontation tests

Strain numbers	Species	Host plant
SZMC 21470	*Botrytis pseudocinerea* (*B. cinerea* group I)	Colza
SZMC 21471		Colza
SZMC 21472	*Botrytis cinerea* (*B. cinerea* group II)	Raspberry
SZMC 21473		Raspberry
SZMC 21474	*Botrytis pseudocinerea* (*B. pseudocinerea* group I)	Strawberry
SZMC 21475	*Botrytis cinerea* (*B. cinerea* group II)	Strawberry

Biocontrol Index Values (BCI) were determined by the image analysis-based method developed by SZEKERES et al. (2006).

Enzyme activity measurements

For enzyme production, 3 different liquid media were used: YEG (containing 5g glucose, 5g KH_2PO_4 and 1g yeast extract per liter), MIN (containing 5g mannitol, 5g KH_2PO_4, 2g $NaNO_3$ and $MgSO_4$ per liter) and BOT (containing 1g $NaNO_3$ and 2g dried mycelia of *B. cinerea* per liter). The strains were inoculated into these liquid media and after incubation for 5 days at 25°C in a rotary shaker (150 rpm), the cell free filtrates were used in the further experiments. We measured the activities of aminopeptidase, chymoelastase-like protease, chymotrypsin-like protease, β-1,4-*N*-acetyl-glucosaminidase, cellobiohydrolase and β-glucosidase with chromogenic substrates (*Table 2*.).

Table 2. Substrates used for the detection of extracellular enzyme activities

Extracellular enzyme activity	Substrates
Chymotrypsin-like protease	*N*-succinyl-Ala-Ala-Pro-Phe-pNA
Chymoelastase-like protease	*N*-succinyl-Ala-Ala-Pro-Pro-Leu-pNA
Aminopeptidase	*N*-benzoyl-L-Tyr-pNA
β-glucosidase	p-nitrophenyl-β-D-glucopyranoside
Cellobiohydrolase	p-nitrophenyl-β-D-cellobioside
β-1,4-*N*-acetyl-glucosaminidase	p-nitrophenyl-β-D-*N*-acetyl-glucosaminide

We measured the optical densities after one hour of incubation at 25°C with a Jupiter HD microtiter plate reader (ASYS Hitech GmbH, Austria) at a wavelength of 405 nm.

RESULTS

Isolation and identification

A total of 25 *Trichoderma* strains were isolated from the rhizospere of lettuce and pepper. The identification process revealed the presence of 4 different species: *Trichoderma atroviride, T. harzianum, T. longibrachiatum* and *T. koningiopsis* in the samples. The most frequently isolated species was *T. atroviride* with 16 isolates, followed by *T. koningiopsis* (4), *T. harzianum* (3) and *T. longibrachiatum* (2).

In vitro confrontation tests

For the antagonism assays, 5 *Trichoderma* strains: 3 *T. atroviride* (SZMC 22215, SZMC 22216, SZMC 22217), 1 *T. koningiopsis* (SZMC 22218) and 1 *T. harzianum* (SZMC 22219) were selected. *T. longibrachiatum* isolates were excluded from the experiments because of their potential to act as opportunistic human pathogens (HATVANI et al., 2013). In the case of *T. atroviride* (3 isolates) and *T. koningiopsis* (1 isolate), a BCI value of 100 could be measured against the 3 examined *B. cinerea* isolates (SZMC 21472, SZMC 21473, SZMC 21475) as well as against *B. pesudocinerea* SZMC 21474 (*Table. 3*).

Table 3. Biocontrol Index (BCI) values of the examined *Trichoderma* isolates against plant pathogenic *Botrytis* strains

Strain number	*TrichOkey* 2.0 diagnosis	Plant pathogenic *Botrytis* strains					
		SZMC 21470	SZMC 21471	SZMC 21472	SZMC 21473	SZMC 21474	SZMC 21475
SZMC 22215	*T. atroviride*	91.05	100	100	100	100	100
SZMC 22216	*T. atroviride*	89.46	84.2	100	100	100	100
SZMC 22217	*T. atroviride*	89.68	91.73	100	100	100	100
SZMC 22218	*T. koningiopsis*	100	100	100	100	100	100
SZMC 22219	*T. harzianum*	38.57	42.93	92.18	77.34	100	62.42

We also found the highest BCI values (100) in the case of the *T. koningiopsis* isolate SZMC 22218 (*Figure 1.*) against the *B. pseudocinerea* strains SZMC 21470 and SZMC 21471, while the *T. atroviride* isolates could not overgrow these two plant pathogenic fungi except from *T. atroviride* SZMC 22215 against *B. pseudocinerea* SZMC 21470.

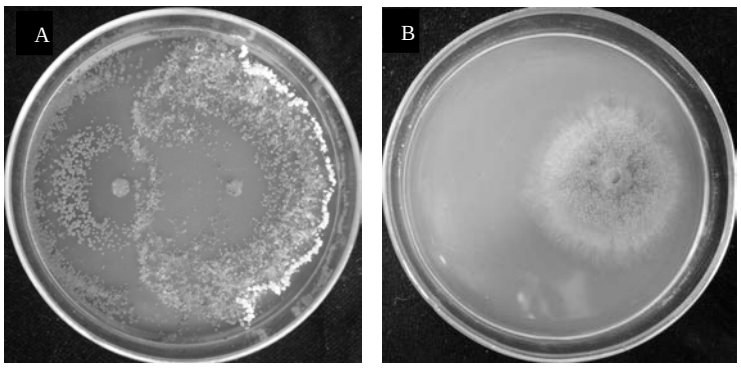

Figure 1. A: *In vitro* antagonism of *T. koningiopsis* SZMC 22218 (left) against *B. pseudocinerea* SZMC 21471 (right); B: *Botrytis pseudocinerea* colony of the same age without the presence of *Trichoderma*

We detected the lowest BCI values in the case of the *T. harzianum* isolate SZMC 22219. All of the examined *Trichoderma* strains could completely overgrow the colony of *B.*

pseudocinerea strain SZMC 21474, while *T. koningiopsis* strain SZMC 22218 could completely overgrow all of the examined *Botrytis* strains.

Enzyme activities

Measurement of cellobiohydrolase and β-glucosidase enzymes revealed low activities in liquid YEG and MIN media. In the case of the addition of powderized *Botrytis* mycelia (BOT) we could observe significant, 5× and 5.8 × increase in the cellobiohydrolase and β-glucosidase activities, respectively. For β-1,4-*N*-acetyl-glucosaminidase, higher activities could be detected in the case of almost all strains. We could detect 27× and 6× higher enzyme activities in BOT liquid media in the case of *T. atroviride* (SZMC 22216) and *T. harzianum* (SZMC 22219). Regarding the chymotrypsin-like protease and chymoelastase-like activities, low enzyme activities were measured, except from strains *T. atroviride* SZMC 22216 and *T. harzianum* SZMC 22219, where the production of these enzymes proved also higher in BOT liquid media. For aminopeptidase we also detected low enzymes activities, but in this case the liquid MIN and YEG media proved to be better than the liquid BOT medium.

CONCLUSIONS

In this study we isolated different *Trichoderma* strains from the rhizosphere of pepper and lettuce. We detected the presence of 4 *Trichoderma* species (*T. atroviride*, *T. harzianum*, *T. koningiopsis* and *T. longibrachiatum*). After the *in vitro* antagonism tests it can be concluded that *T. harzianum* has a lower capability to overgrow the tested plant pathogenic *Botrytis* strains. In contrast to that we detected high BCI values in the case of *T. atroviride* strains and we could observe complete overgrowth in the case of *T. koningiopsis*. We examined the production of extracellular enzymes in the case of these *Trichoderma* strains and the results showed that the β-glucosidase, cellobiohydrolase and β-1,4-*N*-acetyl-glucosaminidase enzyme activities were higher in liquid BOT media. Also in the case of *T. atroviride* SZMC 22216 and *T. harzianum* SZMC 22219 we measured higher enzymes activities in BOT liquid media. The results of this study (biodiversity data, antagonistic abilities and enzyme production data) could be useful for the selection of potential biocontrol agents, which could be used in the agriculture against plant pathogenic *Botrytis* strains.

ACKNOWLEDGEMENTS

The authors thank Dr. Erzsébet Karaffa (University of Debrecen) for kindly providing the *Botrytis* strains. Péter Körmöczi was supported by the European Union and the State of Hungary, co-financed by the European Social Fund in the framework of TÁMOP-4.2.4.A/ 2-11/1-2012-0001 ‘National Excellence Program’. The related research group was also supported by project TÁMOP-4.1.1.C-12/1/KONV-2012-0012 and the Hungary-Serbia IPA Cross-border Co-operation Programme (PHANETRI, HUSRB/1002/214/068).

REFERENCES

BELANGER, R.R., DUFOUR, N., CARON J., BENHAMOU N. (1995): Chronological events associated with the antagonistic properties of *Trichoderma harzianum* against *Botrytis cinerea*: indirect evidence for sequential role of antibiosis and parasitism. Biocontrol Science and Technology 5: pp. 41-53.

DRUZHININA, I.S., KUBICEK, C.P. (2005): Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters? Journal of Zhejiang University Science 6B: pp. 100-112.

ELAD, Y. (1994): Biological control of grape grey mould by *Trichoderma harzianum*. Crop Protection 13: pp. 35–38.

ELAD, Y. (2000A): *Trichodema harzianum* T39 preparation for biocontrol of plant diseases - control of *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Cladosporium fulvum*. Biocontrol Science and Technology 10: pp. 499-507.

ELAD, Y. (2000b): Biological control of foliar pathogens by means of *Trichoderma harzianum* and potential modes of action. Crop Protection 19: pp. 709–714.

ELAD, Y., WILLIAMSON, B., TUDZYNSKI, P., DELEN, N. (2004): *Botrytis*: Biology, Pathology and Controls. Kluwer Academic Publisher. The Netherlands.

FRIEDL, M.A., DRUZHININA, I.S. (2012): Taxon-specific metagenomics of *Trichoderma* reveals a narrow community of opportunistic species that regulate each other's development. Microbiology (UK) 158: pp. 69-83.

GHERBAWY, Y., DRUZHININA, I., SHABAN, G.M., WUCZKOWSKY, M., YASER, M., EL-NAGHY, M.A., PRILLINGER, H.J., KUBICEK, C.P. (2004): *Trichoderma* populations from alkaline agricultural soil in the Nile valley, Egypt, consist of only two species. Mycological Progress 3: pp. 211-218.

HATVANI, L., MANCZINGER, L., VÁGVÖLGYI, C., KREDICS, L. (2013) *Trichoderma* as a human pathogen. In: Mukherjee, P.K., Horwitz, B.A., Singh, U.S., Mukherjee, M., Schmoll, M. (eds.) *Trichoderma* - Biology and Applications. Wallingford: CABI, 2013. pp. 292-313.

HOYOS-CARVAJAL, L., ORDUZ, S., BISSETT, J. (2009): Genetic and metabolic biodiversity of *Trichoderma* from Colombia and adjacent neotropic regions. Fungal Genetics and Biology 46: pp. 615-631.

KING, A.D. JR., HOCKING, A.D., PITT, A.J. (1979): Dichloran-Rose Bengal medium for enumeration and isolation of molds from foods. Applied and Environmental Microbiology 37: pp. 959-964.

KUBICEK, C.P., BISSETT, J., DRUZHININA, I., KULLNIG-GRADINGER, C.M., SZAKACS, G. (2003): Genetic and metabolic diversity of *Trichoderma*: a case study on South East Asian isolates. Fungal Genetics and Biology 38: pp. 310-319.

KULLNIG, C.M., SZAKACS, G., KUBICEK, C.P. (2000): Molecular identification of *Trichoderma* species from Russia, Siberia and the Himalaya. Mycological Research 104: pp. 1117-1125.

MULAW, T.G., KUBICEK, C.P., DRUZHININA, I.S. (2010): The rhizosphere of *Coffea arabica* in its native highland forests of Ethiopia provides a niche for a distinguished diversity of *Trichoderma*. Diversity 2: pp. 527-549.

NAEIMI, S. KHODAPARAST, S.A., JAVAN-NIKKHAH, M. VÁGVÖLGYI, C., KREDICS, L. (2011): Species patterns and phylogenetic relationship of *Trichoderma* strains in rice fields of Southern Caspian Sea, Iran. Cereal Research Communications 39: pp. 560-568.

SADFI-ZOUAOUI, N., HANNACHI, I., ROUAISSI, M., HAJLAOUI, M.R., RUBIO, M.B., MONTE, E., BOUDABOUS, A., HERMOSA, M.R. (2009): Biodiversity of *Trichoderma* strains in Tunisia. Canadian Journal of Microbiology 55: pp. 154-162.

SUN, R.Y., LIU, Z.C., FU K., FAN, L. CHEN, J. (2012): *Trichoderma* biodiversity in China. Journal of Applied Genetics 53: pp. 343-354.

SZEKERES, A., LEITGEB, B., KREDICS, L., MANCZINGER, L., VÁGVÖLGYI, C. (2006): A novel, image analysis-based method for the evaluation of *in vitro* antagonism. Journal of Microbiological Methods 65, pp. 619-622.

TRONSMO, A. AND DENNIS, C. (1977): The use of *Trichoderma* species to control strawberry fruit rots. Netherlands Journal of Plant Pathology 83: pp. 449–455.

WUCZKOWSKI, M., DRUZHININA, I., GHERBAWY, Y., KLUG, B., PRILLINGER, H., KUBICEK, C.P. (2003): Species pattern and genetic diversity of *Trichoderma* in a mid-European, primeval foodplain-forest. Microbiological Research 185: pp. 125-133.