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Identification of new *Trichoderma* strains with antagonistic activity against *Botrytis cinerea*

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ABSTRACT

The antagonistic activity of 52 isolates of *Trichoderma* spp. against *Botrytis cinerea* was tested in *in vitro* conditions using the dual culture technique. The results revealed that all of the *Trichoderma* isolates had the ability to inhibit the mycelial growth of grey mould. The percentage reduction in the growth of *Botrytis cinerea* after six days of incubation at 25ºC varied between 45-78%. The isolates Tr43 and Tr52 showed the highest antagonistic activity (Tr43 – 76%; Tr52 – 78%). Biochemical and molecular identification indicated that both isolates were *T. atroviride*. The isolates showed differences in the utilisation of 11 to 96 different carbon sources. Additional biochemical tests revealed the ability of Tr43 and Tr52 to produce siderophores, indole-3-acetic acid and chitinases. Neither of the isolates gave positive results regarding phosphate solubilisation on Pikovskaya's medium.

Key words: antagonistic potential, grey mould, identification, *Trichoderma* spp.

INTRODUCTION

Grey mould caused by the fungus *Botrytis cinerea* Pers. ex Fr. is one of the most common crop diseases that is responsible for serious crop losses in more than 200 plant species worldwide (Williamson et al. 2007). This fungus can negatively affect all of the aboveground organs of plants, especially the buds, flowers and fruits (Elad et al. 2007). It normally enters through a wound or infects plants that are under stress, although it can also infect healthy plants, especially under humid conditions. There are a large number of fungicides with a high level of activity against grey mould (De Kock and Holz 1994, Markoglou and Ziogas 2002). Unfortunately, chemical protection negatively affects fruit and plant crops, the environment and human health. The use of fungicides may also lead to the occurrence of new resistant strains of plant pathogens. Recently, a worldwide tendency has been to use eco-friendly methods in plant protection (Hajieghrari et al. 2008). Biological control includes, for example, antagonistic microorganisms that naturally occur in the soil (Karkachi et al. 2010, Abano and Sam-Amoah 2012). *Trichoderma* is a group of filamentous fungi that are well known for their antagonism against several soil phytopathogens, involving fungi such as: *Fusarium oxysporum*, *Rhizoctonia solani, Sclerotium rolfsii* and *Verticillium dahliae* (Spiegel and Chet 1998, Jabnoun-Khiareddine et al. 2009). The antagonistic activity shown by *Trichoderma* species is connected with mycoparasitism, competition for nutrients

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and niche, production of antibiotics and enzymes (Howell 2003, Benitez et al. 2004, Verma et al. 2007). The antagonism of *Trichoderma* spp. has been observed both in *in vitro* conditions (Mishra et al. 2011) as well as in greenhouse and field trials (Kexiang et al. 2002). Some strains of *Trichoderma* also promote plant growth and yielding through enhanced production of plant hormones and vitamins, improved nutrient uptake and acquisition, etc. (Shanmugaiah et al. 2009, Joshi et al. 2010). Consequently, the antagonistic potential of *Trichoderma* spp. against pathogens is considered to be successfully used in biological control instead of the application of chemical plant protection products against phytopathogens.

The objectives of this study were to evaluate the antagonistic activity of *Trichoderma* isolates originating from Polish soils against *Botrytis cinerea* in *in vitro* conditions and to identify isolates with the highest capacity for pathogen inhibition.

MATERIAL AND METHODS

Pure culture of Botrytis cinerea

A pure culture of *B. cinerea* (isolate FFBC001) was isolated from the fruit of the 'Regent' grapevine cultivar and was stored for further use in the collection of microorganisms called SymbioBank, established in the Rhizosphere Laboratory of the Institute of Horticulture in Skierniewice (Poland).

Pure cultures of Trichoderma spp.

Fifty-two isolates of *Trichoderma* spp. were obtained from field soils and old orchard soils in central Poland (Tab. 1). Pure cultures were established with the use of soil-plate technique on Rose-Bengal Chloramphenicol Agar medium and incubated at 25ºC for 5-7 days. The cultures were maintained in a deep freezer at -80º C in Eppendorf tubes with 99.5% glycerol as a cryoprotectant. The *Trichoderma* isolates were identified to the genus level with the use of a morphological key (Watanabe 2010).

Testing of the antagonistic activity of Trichoderma isolates

In vitro tests were performed using the dual culture technique (Morton and Stroube 1955) on a PDA (potato dextrose agar) medium. Petri dishes with the medium were inoculated with discs six millimetres in diameter of the tested *Trichoderma* isolates and the *B. cinerea* isolate (six-day-old culture of each fungus). The discs of *Trichoderma* and *Botrytis* were placed on the opposite sides of each dish. The dishes were incubated at 26ºC for six days. Three replicates (dishes) were used in each test and for each *Trichoderma* isolate. After six days of radial growth of *B. cinerea* colonies, the extent of the infection was measured and compared with the control (pure culture of *B. cinerea*). The reduction in the growth of *B. cinerea* colonies caused by the *Trichoderma* isolates was determined as follows (El-Naggar et al. 2008):

$$R = (A\text{-}B)/A \times 100,$$

where: R – percentage reduction in the growth of pathogen, A – radius (cm) of pathogen colony in control culture, B – radius (cm) of pathogen colony in test dish.

The degree of antagonistic activity was estimated as follows (Sookchaoy et al. 2009): 4 – very high antagonistic activity (R > 75), 3 – high antagonistic activity (R = 61-75), 2 – moderate antagonistic activity (R = 51-60), 1 – low antagonistic activity (R < 51).

Data were analysed using ANOVA. Tukey's multiple range test at p = 0.05 was used for specific comparisons of the means. All calculations were done by means of the STATISTICA v.10 package (StatSoft, Inc. 2011).

Identification of Trichoderma isolates

The isolates of *Trichoderma* spp. that showed the best efficacy in inhibiting mycelial growth of *B. cinerea* were identified to the species level with the use of molecular and biochemical methods.

Molecular identification of Trichoderma isolates

Fungal genomic DNA of *Trichoderma* spp. was extracted using a commercial DNeasy Plant Mini Kit (Qiagen). PCR (polymerase chain reactions) were performed in a total volume of 20 µl, containing 1× reaction buffer, 0.2 mM dNTPs, 0.2 µM of each primer, 0.5 U of Taq DNA polymerase (DreamTaq™ Green, ThermoScientific) and 10 ng of template DNA. PCR reactions were carried out in an S 1000 Thermal Cycler (BioRad) under the conditions involving an initial denaturation step at 95°C for 2 min., followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, extension at 72°C for 1 min., and the final extension step at 72°C for 10 mins. ITS regions 1 and 2 and the 5.8S rDNA gene was amplified using the universal primers ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') and ITS6 (5'-GAA GGT GAA GTC GTA ACA AGG-3') (White et al. 1990). The PCR products were sequenced using sequencing system 3730xl DNA Analyzer and BigDye®Terminator

v.3.1 kit (Applied Biosystems). Related sequences were searched using the BLAST program from the NCBI (National Center for Biotechnology Information) database (http://www.ncbi.nlm.nih.gov/blast).

Biochemical identification of Trichoderma isolates

The biochemical characteristics of *Trichoderma* isolates were determined with the use of the Biolog Identification System (Biolog Inc., USA). Fresh cultures of *Trichoderma* spp. were streaked on a 2% MEA (malt extract agar) medium and incubated at 26ºC for seven days. The fungal suspension prepared in the IF-F inoculant's solution (quantification of 65%) was inoculated into FF microplate and incubated at 26ºC for seven days. The results were read off daily by inserting the microplate with a *Trichoderma* isolate into the Biolog's reader apparatus operated by the software of the Biolog Identification System (Microlog 3 v. 5.2.01). The fungi were identified down to the species level.

Biochemical characterisation of Trichoderma isolates

The *Trichoderma* isolates that showed the best antagonistic activity against *B. cinerea* on the Petri dishes were additionally tested to determine their ability to produce siderophores on the (CAS chrome azurol S) agar medium (Alexander and Zuberer 1991), indole-3-acetic acid (Gordon and Weber 1951), chitinase (Hsu and Lockwood 1975) and whether they were able to solubilise phosphate (Pikovskaya 1948).

Preparation of the CAS agar medium

The CAS agar medium was prepared from four solutions. The Fe-CAS indicator solution was prepared by mixing 10 ml of 1 mM $FeCl_3 \cdot 6H_2O$ (in 10 mM/l HCl) with 50 ml of an aqueous solution of CAS (1.21 g/l) and adding it to 40 ml of an aqueous solution of hexadecyltrimethylammonium bromide (1.821 g/l). The buffer solution (solution 1) was prepared by dissolving 30.24 g of piperazine-N,N-bis (2-ethanesulfonic acid) in 800 ml of a salt solution (solution 2) containing 0.3 g K_2HPO_4, 0.5 g NaCl, 1.0 g NH_4Cl. The pH was adjusted to 6.8 with 50% KOH. Before autoclaving 15 g of agar was added. Solution 3 contained (in 70 ml water): 2 g glucose, 2 g mannitol, 493 mg $MgSO_4 \cdot 7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4 \cdot H_2O$, 1.4 mg H_3BO_3, 0.04 mg $CuSO_4 \cdot 5H_2O$, 1.2 mg $ZnSO_4 \cdot 7H_2O$, 1.0 mg $NaMoO_4 \cdot 2H_2O$. Solution 4 contained 30 ml of 10% casamino acids. All of the solutions were sterilised separately before mixing. Each of the *Trichoderma* isolates was inoculated into a Petri dish with CAS agar medium. A yellow halo surrounding the *Trichoderma* isolates indicated a positive reaction.

Testing for indole-3-acetic acid production

The production of indole-3-acetic acid was estimated using the Salkowski reagent (1 ml 0.5 mol/l $FeCl_3$ and 49 ml 35% $HClO_4$). The *Trichoderma* isolates were cultured in a sterilised Czapek broth (30 g sucrose, 3 g $NaNO_3$, 1 g K_2HPO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4$, 1000 ml distilled water) with L-tryptophan (1 g/l) on a rotary shaker. After 96 h of incubation at room temperature, 500 µl of each *Trichoderma* culture was transferred to microtubes and centrifuged at 14,000 rpm for two minutes. Afterwards 500 µl of the Salkowski reagent was added. The microtubes were left for 30 minutes to allow colour development. A pink colour of the samples indicated the production of indole-3-acetic acid.

Preparation of chitin agar medium

The ability to produce chitinases was investigated using a chitin agar medium. Colloidal chitin was prepared by dissolving 15 g of powdered chitin in 200 ml of concentrated HCl. Chitin was dialysed by distilled water until the suspension adjusted a pH value of 5.5-6.0. Afterwards, 4 g of colloidal chitin was mixed with mineral salts: 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$, 20 g agar and 1000 ml distilled water. The agar medium was adjusted to pH 8.0 with 50% KOH and autoclaved. The *Trichoderma* isolates were inoculated onto the Petri dishes. The production of chitinases was observed as a discoloration of the agar medium.

Preparation of Pikovskaya's agar medium

The phosphate-solubilising ability was evaluated on Pikovskaya's agar medium consisting of 0.5 g yeast extract, 0.5 g $(NH_4)_2SO_4$, 5 g $Ca_3(PO_4)_2$, 0.2 g KCl, 0.1 g $MgSO_4$, 0.0001 g $MnSO_4$, 0.0001 g $FeSO_4$, 10 g glucose, 15 g agar and 1000 ml distilled water. Each of the *Trichoderma* isolates was inoculated onto a Petri dish with Pikovskaya's agar medium. A clear dissolution zone around the isolates indicated a positive reaction.

RESULTS

In the present study, 52 isolates of *Trichoderma* were screened for antagonistic activity against

Table 1. Inhibition of the growth of *Botrytis cinerea* by 52 *Trichoderma* isolates and their antagonistic activity against this pathogen in dual culture tests

Trichoderma isolates	Location of sampling	Species of fruit trees	Average degree of growth inhibition after 6 days of incubation (%)	Antagonistic activity (on 1-4 scale*)	*Trichoderma* isolates	Location of sampling	Species of fruit trees	Average degree of growth inhibition after 6 days of incubation (%)	Antagonistic activity (on 1-4 scale*)
Tr3	Willanów	cherry	63 c-j**	3	Tr29	Dębowa Góra	cherry	62 c-j	3
Tr4	Willanów	cherry	58 f-j	2	Tr30	Stryczowice	pear	67 b-g	3
Tr5	Willanów	cherry	67 b-h	3	Tr31	Stryczowice	pear	68 b-g	3
Tr6	Nowy Dwór	cherry	64 c-j	3	Tr32	Stryczowice	pear	67 b-g	3
Tr7	Nowy Dwór	cherry	63 c-j	3	Tr33	Stryczowice	pear	66 b-i	3
Tr8	Nowy Dwór	cherry	62 c-j	3	Tr34	Stryczowice	pear	67 b-g	3
Tr9	Nowe Berezowo	apple	65 c-i	3	Tr35	Stryczowice	pear	64 c-j	3
Tr10	Nowe Berezowo	apple	54 jk	2	Tr36	Stryczowice	pear	72 a-c	3
Tr11	Nowe Berezowo	apple	64 c-j	3	Tr37	Stryczowice	plum	68 b-g	3
Tr12	Nowe Berezowo	apple	65 b-i	3	Tr38	Stryczowice	plum	71 a-d	3
Tr13	Nowe Berezowo	apple	58 f-j	2	Tr39	Stryczowice	plum	70 a-e	3
Tr14	Nowe Berezowo	apple	55 ij	2	Tr40	Stryczowice	plum	70 a-e	3
Tr15	Nowe Berezowo	apple	56 ij	2	Tr41	Stryczowice	plum	56 ij	2
Tr16	Nowe Berezowo	apple	60 e-j	3	Tr42	Stryczowice	plum	64 c-j	3
Tr17	Nowe Berezowo	apple	56 ij	2	Tr43	Przeworsk	apple	76 ab	4
Tr18	Nowe Berezowo	apple	61 d-j	3	Tr44	Przeworsk	apple	61 d-j	3
Tr19	Nowe Berezowo	apple	61 d-j	3	Tr45	Przeworsk	apple	58 g-j	2
Tr20	Nowe Berezowo	apple	59 f-j	2	Tr46	Przeworsk	apple	61 d-j	3
Tr21	Dębowa Góra	cherry	60 e-j	3	Tr47	Przeworsk	apple	61 d-j	3
Tr22	Dębowa Góra	cherry	63 c-j	3	Tr48	Przeworsk	apple	58 f-j	2
Tr23	Dębowa Góra	cherry	57 h-j	2	Tr49	Przeworsk	apple	67 b-g	3
Tr24	Dębowa Góra	cherry	67 b-h	3	Tr50	Przeworsk	apple	56 h-j	2
Tr25	Dębowa Góra	cherry	65 c-i	3	Tr51	Przeworsk	apple	62 c-j	3
Tr26	Dębowa Góra	cherry	63 c-j	3	Tr52	Przeworsk	apple	78 a	4
Tr27	Dębowa Góra	cherry	59 f-j	2	Tr53	Przeworsk	apple	45 k	1
Tr28	Dębowa Góra	cherry	61 d-j	3	Tr54	Przeworsk	apple	68 b-f	3

*1 = low antagonistic activity (R < 51), 2 = moderate antagonistic activity (R = 51-60), 3 = high antagonistic activity (R = 61-75), 4 = very high antagonistic activity (R > 75)
**Values marked with the same letter do not differ significantly at p = 0.05

B. cinerea. All of the tested isolates restricted the growth area and intensity of grey mould colonies (Tab. 1). The average level of this growth inhibition varied between 45-78%. Over 60% of the isolates showed a high level of antagonistic activity, ranging from 61% to 75%. Among the tested *Trichoderma* isolates, six isolates showed the best efficacy in inhibiting mycelial growth of *B. cinerea* at a level of 70% for Tr39 and Tr40, 71% for Tr38, 72% for Tr36, 76% for Tr43 and 78% for Tr52. In comparison with the other *Trichoderma* isolates, the differences were statistically significant. However, according to the scale used by Sookchaoy et al. (2009), very high antagonistic activity (4 points on a 1-4 scale) was shown by two strains: Tr43 and Tr52 (Fig. 1).

Results of Trichoderma identification

A comparison of sequences (the sequence of 601 nucleotides for the isolate Tr43 and the sequence of 600 nucleotides for the isolate Tr52) with the NCBI sequences database allowed the identification of both isolates as the *Trichoderma atroviride* P. Karst. The identities of the results were as follows: 99% for isolate Tr43 and 100% for isolate Tr52.

Table 2. Results for the utilisation of different carbon sources after 72 h of incubation at 26ºC obtained with the Biolog Identification System

Utilisation of different carbon sources	Isolate		Utilisation of different carbon sources	Isolate		Utilisation of different carbon sources	Isolate	
	Tr43	Tr52		Tr43	Tr52		Tr43	Tr52
Water (control)	-	-	D-Ribose	+	+	Lactulose	-	-
Tween 80	+	+	Salicin	+	+	Maltitol	-	-
N-Acetyl-D-Galactosamine	-	-	Sedoheptulosan	-	-	Maltose	+	-
N-Acetyl-D-Glucosamine	+	+	D-Sorbitol	+	+	Maltotriose	+	+
N-Acetyl-D-Mannosamine	-	-	L-Sorbose	+	+	D-Mannitol	+	+
Adonitol	-	-	Stachyose	+	+	D-Mannose	+	+
Amygdalin	+	+	Sucrose	+	+	D-Melezitose	-	-
D-Arabinose	-	+	D-Tagatose	+	-	D-Melibiose	+	+
L-Arabinose	+	+	D-Trehalose	+	+	α-Methyl-D-Galactoside	+	+
D-Arabitol	+	+	Turanose	+	+	β-Methyl-D-Galactoside	-	-
Arbutin	+	+	Xylitol	+	+	α-Methyl-D-Glucoside	-	-
D-Cellobiose	+	+	D-Xylose	+	+	β-Methyl-D-Glucoside	+	+
α-Cyclodextrin	-	-	γ-Amino-butyric Acid	+	+	Palatinose	-	-
β-Cyclodextrin	-	-	Bromosuccinic Acid	+	+	D-Psicose	-	-
Dextrin	+	+	Fumaric Acid	-	+	D-Raffinose	+	+
i-Erythritol	+	+	β-Hydroxy-butyric Acid	+	+	L-Rhamnose	-	-
D-Fructose	+	+	γ-Hydroxy-butyric Acid	+	+	L-Alanyl-Glycine	+	+
L-Fucose	-	-	p-Hydroxyphenyl-acetic Acid	-	-	L-Asparagine	+	+
D-Galactose	+	+	α-Keto-glutaric Acid	-	+	L-Aspartic Acid	+	+
D-Galacturonic Acid	-	-	D-Lactic Acid Methyl Ester	-	+	L-Glutamic Acid	+	+
Gentiobiose	+	+	L-Lactic Acid	-	+	Glycyl-L-Glutamic Acid	-	+
D-Gluconic Acid	-	-	D-Malic Acid	+	+	L-Ornithine	+	+
D-Glucosamine	-	-	L-Malic Acid	-	-	L-Phenylalanine	+	+
α-D-Glucose	+	+	Quinic Acid	+	+	L-Proline	+	+
Glucose-1-Phosphate	+	+	D-Saccharic Acid	-	+	L-Pyroglutamic Acid	+	+
Glucuronamide	-	-	Sebacic Acid	-	-	L-Serine	+	+
D-Glucuronic Acid	+	+	Succinamic Acid	-	-	L-Threonine	+	+
Glycerol	+	+	Succinic Acid	-	+	2-Amino Ethanol	+	+
Glycogen	+	+	Succinic Acid Mono-Methyl Ester	-	-	Putrescine	-	+
m-Inositol	-	-	N-Acetyl-L-Glutamic Acid	-	-	Adenosine	+	+
2-Keto-D-Gluconic Acid	+	+	Alaninamide	+	+	Uridine	-	+
α-D-Lactose	-	+	L-Alanine	+	+	Adenosine-5'-Monophosphate	-	+

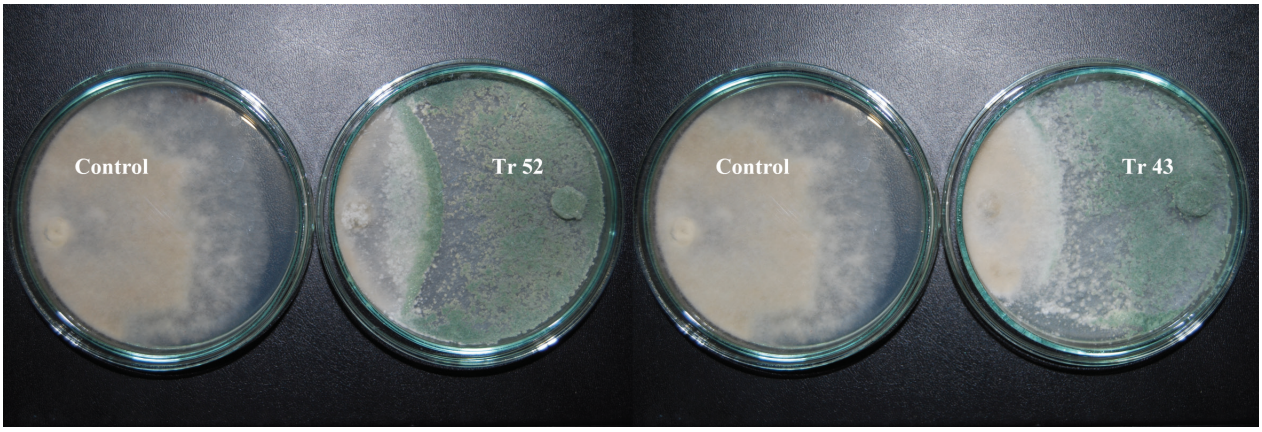

Figure 1. Antagonistic activity of isolates Tr43 and Tr52 against *Botrytis cinerea* (on the left: pure culture of *B. cinerea*, on the right: the dual culture of *B. cinerea* and *Trichoderma isolate*)

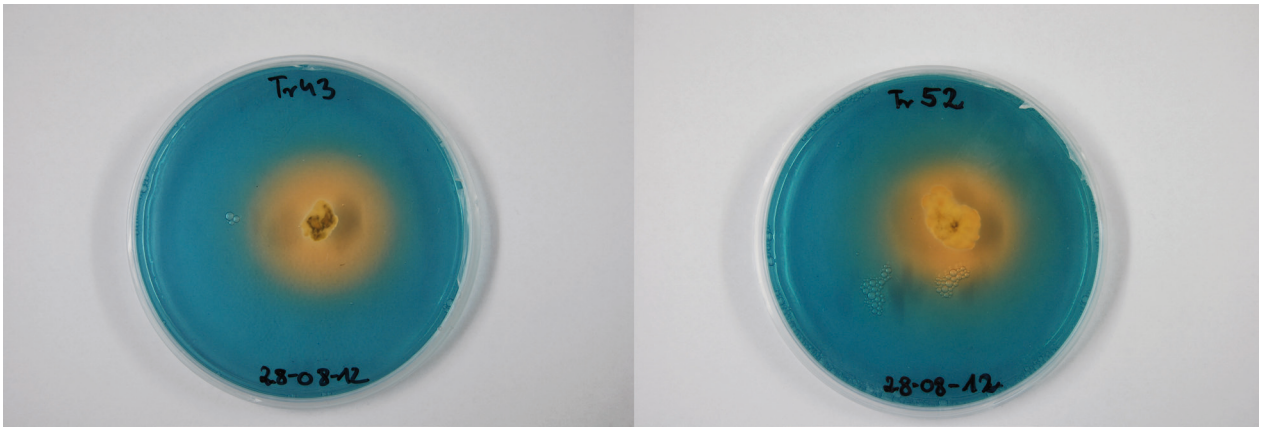

Figure 2. Siderophore production by isolates Tr43 and Tr52 on CAS agar medium

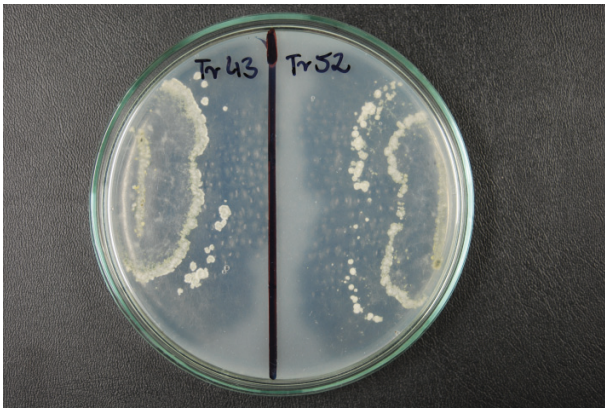

Figure 3. Visualisation of chitynolytic activity by *Trichoderma* isolates Tr43 and Tr52. The clear zone around the isolates indicates chitinase production

Biochemical identification using the Biolog Identification System was performed during seven days of incubation at 26ºC. Using this method, isolate Tr52 was identified after 96 h as *T. atroviride*. The probability of correct identification was 94% and the similarity to standard *T. atroviride* was 0.604. Isolate Tr43 was not positively identified, but the results indicate that it is the most similar to *T. atroviride* (similarity was 0.512). The isolates Tr43 and Tr52 showed differences in the utilisation of 11 to 96 different carbon sources. In contrast to Tr52, *Trichoderma* Tr43 utilised maltose and D-tagatose, whereas isolate Tr52 utilised in wells D-arabinose, α-D-lactose, fumaric acid, α-keto-glutaric acid, D-lactic acid methyl ester, L-lactic acid, D-saccharic acid, succinic acid and glycyl-L-glutaric acid (Tab. 2).

Results of additional biochemical tests on Tr43 and Tr52 Trichoderma isolates

Both of the *Trichoderma* isolates produced siderophores, which was visualised on the CAS agar medium as an orange halo developed around the isolates (Fig. 2). The halo was caused by siderophores chelating Fe from the Fe-CAS dye complex. Production of indole-3-acetic acid from L-tryptophan was observed as a change in the colour of the medium from colourless to a pink colour (addition of the Salkowski reagent). Chitynolytic activity was also exhibited by both *Trichoderma*

Table 3. Results of additional tests for the biochemical characterisation of *Trichoderma* isolates Tr43 and Tr52

Trichoderma isolates	Siderophores production	Indole-3-acetic acid production	Phosphate solubilisation	Chitynolytic activity
Tr43	+	+	-	+
Tr52	+	+	-	+

isolates, which was observed as a discoloration of the agar medium (Fig. 3). Neither of the isolates gave positive results regarding phosphate solubilisation on Pikovskaya's medium since the clear zone did not appear nor was visible in this medium (Tab. 3).

DISCUSSION

Trichoderma spp. are widespread in the soil as saprophytic fungi highly competitive to plant pathogens. Among *Trichoderma* isolates, the most studied are *T. harzianum* (Chaur-Tsuen and Chien-Yih 2002), *T. reesei* (El-Naggar et al. 2008), *T. atroviride* (Brunner et al. 2005) and *T. viride* (Mishra et al. 2011). The biological control activity of the *Trichoderma* strains against fungal phytopathogens has been tested and described in several research papers (Meszka and Bielenin 2009, Joshi et al. 2010, Lone et al. 2012). *Trichoderma* isolates have been shown to be successful in controlling soil-borne diseases in the greenhouse and under field conditions. Some of the *Trichoderma* strains are currently available as components of commercial bioproducts: KRL-AG2 (*T. harzianum*) controls a wide range of soil-borne diseases (Spiegel and Chet 1998), Trichodex (*T. harzianum*) is used against *B. cinerea, Sclerotinia sclerotiorum, Cladosporium fulvum* diseases in greenhouse-grown tomato and cucumber, and in vineyards (Freeman et al. 2004), Binab T (*T. harzianum* and *T. polysporum*) controls wound decay and wood rot (Mehrotra and Aggarwal 2003), Supresivit (*T. harzianum*) inhibits the growth of *Phytophthora* spp. and *Pythium ultimum* and might stimulate the growth of plants (Brožová 2004).

In this study, the results of the dual culture tests revealed antagonistic activity of all 52 *Trichoderma* isolates against *B. cinerea*. The *Trichoderma* isolates grew rapidly and intensively covered the entire surface of the Petri dishes after 10 days. The most effective strains revealed more than 70% of the growth inhibition of *B. cinerea*. An isolate of *T. reesei* studied by El-Naggar et al. (2008) showed only a 30% reduction in the growth of *B. cinerea*, 40.2% in the growth of *B. fabae* and only 4% in the growth of *B. allii* after five days of incubation. Fiume and Fiume (2006) observed the antagonistic activity of *T. harzianum* against grey mould at a range from 4.7% after three days of incubation and up to 75.76% after seven days of incubation. They also reported no inhibition halo between *B. cinerea* and *T. harzianum* colonies, which suggests that the antagonistic effect of *T. harzianum* isolates is based on the competition for niche and nutrients and not on a chemical aggressiveness or classic antibiosis. In the present study all the *Trichoderma* isolates achieved an average percentage of growth reduction above 45% after six days. A clear zone between all of the *Trichoderma* isolates and *B. cinerea* was also not observed. However, additional biochemical tests revealed the ability of the isolates Tr43 and Tr52 identified as *T. atroviride* to produce the chitinases. An isolate of *T. atroviride* studied by Matroudi et al. (2009) showed chitinase and β-1.3 glucanase activity. Both of these extracellular enzymes are connected with mycoparasitism that is initiated against phytopathogenic fungi. Chitinases are able to lyse the hard chitin cell wall of mature hyphae, conidia, chlamydospores and sclerotia (Harighi et al. 2007).

T. atroviride is well-known as a biological control agent for a wide range of economically important aerial and soil-borne plant pathogens (Brunner et al. 2005). McLean et al. (2012) observed antagonistic activity of *T. atroviride* against *Sclerotium cepivorum*, whereas Anita and Ponmurugan (2011) reported that *T. atroviride* were highly effective in controlling Phomopsis canker diseases in tea plants. The tests performed by Matroudi et al. (2009) also revealed the high antagonistic activity of *T. atroviride.* That isolate produced 85% inhibition in the growth of *S. sclerotiorum* after three days and 93% after four days of incubation. The dual culture tests against other fungal phytopathogens (for example *Verticillium dahlia* or *Fusarium oxysporum*) are essential to perform. The published literature data clearly indicate that the antagonistic activity of *Trichoderma* species is based on mycoparasitism, the production of antibiotics and enzymes, and is usually directed against the development of a few pathogens. Hajieghrari et al. (2008) observed an inhibitory effect of

Trichoderma isolates on the growth of *Rhizoctonia solani, Macrophomina phaseoli, Phytophthora cactorum* and *Fusarium graminearum*. In a study by Joshi et al. (2010), the antagonistic activity of *Trichoderma* was shown against *Sclerotium rolfsii*, *R. solani* and *S. sclerotiorum,* whereas Siameto et al. (2010) described antifungal properties of *T. harzianum* against *F. oxysporum* f. sp *lycopersici*, *F. oxysporum* f. sp *phaseoli* and *F. graminearum*.

Additional biochemical tests for siderophore production and indole-3-acetic acid production suggest that the isolates Tr43 and Tr52 might also stimulate plant growth. Indole-3-acetic acid is an auxin that stimulates plant growth and development. Siderophores reduce Fe^{3+} ions to Fe^{2+} ions that can be taken up by plants and efficiently transported from the roots to the shoots. Iron is an important microelement that participates in a variety of redox reactions associated with many important metabolic processes, such as respiration, photosynthesis and the metabolism of nitrogen compounds. Microorganisms that produce siderophores competitively inhibit the growth of plant pathogens with a less efficient iron uptake system. Hoyos-Carvajal et al. (2009) evaluated the production of potential growth-promoting metabolites by 101 isolates of *Trichoderma*. More than 50% of the assessed strains showed an ability to produce siderophores on a CAS agar medium. The production of indole-3-acetic acid was observed in 60% of the isolates. Some of the *Trichoderma* strains that revealed plant growth promotion mechanisms in laboratory tests also showed an ability to enhance the growth of bean seedlings in the early stages of development. Both of the *Trichoderma* isolates gave negative results on Pikovskaya's medium. Microorganisms dissolve phosphates by producing inorganic and organic acids. The tricalcium phosphate solubilsing ability depends on various factors like carbon sources, salinity, pH of medium, etc. Yadav et al. (2011) observed the maximum significant tricalcium phosphate solubilisation of *Aspergillus niger* strain at 1% $CaCl_2$ in saline conditions and with glucose used as a carbon source. Mahamuni et al. (2012) used dextrose and 1% NaCl to isolate phosphate solubilising fungi from the rhizosphere soil of sugarcane and sugar beet. In our studies, we used Pikovskaya's medium containing 1% KCl and glucose as a carbon source to estimate the phosphate solubilising activity. According to the literature, this standard medium is considered to be a good selective medium for the isolation of phosphate solubilising microorganisms.

CONCLUSIONS

1. In *in vitro* conditions, *Trichoderma* isolates Tr43 and Tr52 exhibited the highest antagonistic activity against *B. cinerea*.
2. Additional biochemical tests (siderophore production, indole-3-acetic acid production) revealed the production of potential growth promoting metabolites by isolates Tr43 and Tr52.

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IDENTYFIKACJA NOWYCH SZCZEPÓW *TRICHODERMA* O AKTYWNOŚCI ANTAGONISTYCZNEJ PRZECIWKO *BOTRYTIS CINEREA*

Streszczenie: 52 izolaty grzybów z rodzaju *Trichoderma* zostały przebadane z użyciem techniki podwójnych kultur w celu oceny ich antagonistycznego oddziaływania przeciwko *Botrytis cinerea*. Wszystkie spośród badanych izolatów hamowały wzrost szarej pleśni. Wartość inhibicji wzrostu *B. cinerea* po 6 dniach inkubacji w temperaturze 25ºC wynosiła 45-78%. Największą aktywność antagonistyczną wykazały izolaty Tr43 i Tr52 (Tr43 – 76%, Tr52 – 78%). Izolaty te zostały zidentyfikowane jako *Trichoderma atroviride*. Na podstawie identyfikacji biochemicznej izolatów Tr43 i Tr52 z użyciem systemu do identyfikacji mikroorganizmów BIOLOG stwierdzono różnice w utylizacji 11, spośród 96 źródeł węgla. Dodatkowe testy biochemiczne wykazały zdolność izolatów Tr43 i Tr52 do syntezy sideroforów, kwasu indoilo-3-octowego i chitynaz. Nie stwierdzono zdolności do rozpuszczania związków fosforu na podłożu wg Pikowskiej.

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