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Suppressive Effect of *Trichoderma* spp. on toxigenic *Fusarium* species

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Abstract

The aim of the present study was to examine the abilities of twenty-four isolates belonging to ten different *Trichoderma* species (*i.e.*, *Trichoderma atroviride*, *Trichoderma citrinoviride*, *Trichoderma cremeum*, *Trichoderma hamatum*, *Trichoderma harzianum*, *Trichoderma koningiopsis*, *Trichoderma longibrachiatum*, *Trichoderma longipile*, *Trichoderma viride* and *Trichoderma viridescens*) to inhibit the mycelial growth and mycotoxin production by five *Fusarium* strains (*i.e.*, *Fusarium avenaceum*, *Fusarium cerealis*, *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium temperatum*). Dual-culture bioassay on potato dextrose agar (PDA) medium clearly documented that all of the *Trichoderma* strains used in the study were capable of influencing the mycelial growth of at least four of all five *Fusarium* species on the fourth day after co-inoculation, when there was the first apparent physical contact between antagonist and pathogen. The qualitative evaluation of the interaction between the colonies after 14 days of co-culturing on PDA medium showed that ten *Trichoderma* strains completely overgrew and sporulated on the colony at least one of the tested *Fusarium* species. Whereas, the microscopic assay provided evidence that only *T. atroviride* AN240 and *T. viride* AN255 formed dense coils around the hyphae of the pathogen from where penetration took place. Of all screened *Trichoderma* strains, *T. atroviride* AN240 was also found to be the most efficient (69–100% toxin reduction) suppressors of mycotoxins (deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, nivalenol, zearalenone, beauvericin, moniliformin) production by all five *Fusarium* species on solid substrates. This research suggests that *T. atroviride* AN240 can be a promising candidate for the biological control of toxigenic *Fusarium* species.

Key words: antagonism, dual culture bioassays, fungal interactions, microscopic observations, mycotoxins

Introduction

Fusarium species are considered to be some of the most important plant pathogens, causing head blight in small grain cereals and ear rot in maize. These pathogenic species produce a wide range of mycotoxins, such as trichothecenes (TCTs), fumonisins (FBs), zearalenone (ZEN), beauvericin (BEA) and moniliformin (MON), which have been determined to be common contaminants of cereal grains and derived products (Bottalico and Perrone, 2002; Jestoi *et al.*, 2008; Goetz *et al.*, 2010). Among the toxigenic *Fusarium* species that have been associated with infected grain are *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium avenaceum*, *Fusarium cerealis*, and *Fusarium temperatum* (Bottalico and Perrone, 2002; Logrieco *et al.*, 2002a; 2002b; Goetz *et al.*, 2010; Amarasinghe *et al.*, 2014; Czembor *et al.*, 2014). *F. graminearum* is capable of producing two major types of mycotoxins: estrogenic ZEN and type B trichothecenes, such as deoxynivalenol (DON) and, depending on the chemotype, its acetylated forms 3-acetyl-deoxynivalenol (3-AcDON) or 15-acetyl-deoxynivalenol (15-AcDON), as well as nivalenol (NIV) (Bily *et al.*, 2004; Glenn, 2007). The main mycotoxins biosynthesized by *F. culmorum* include DON, 3-AcDON, NIV, fusarenone X (FUS) and ZEN (Glenn, 2007; Wagacha and Muthomi, 2007). NIV, FUS and ZEN are also formed in cereals by *F. cerealis* strains (Logrieco *et al.*, 2003; Amarasinghe *et al.*, 2014). However, *F. avenaceum* and *F. temperatum* do not produce TCTs or ZEN, but they do produce other mycotoxins such as enniatins (ENs), BEA and MON (Logrieco *et al.*, 2002b; Jestoi *et al.*, 2008; Scauflaire *et al.*, 2012). *F. temperatum* additionally produces FB_1 (Scauflaire *et al.*, 2012).

As has been repeatedly demonstrated, *Fusarium* mycotoxins pose a significant health risk for humans and animals through food and feed prepared from contaminated cereal crops (Bennet and Klich, 2003; Glenn, 2007). Therefore, different strategies have been adopted

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to reduce *Fusarium* mycotoxin contamination in cereals (Palazzini *et al*., 2007). One of the more economically and environmentally attractive options is considered to be the use of biological control agents (BCAs) acting as natural antagonists to *Fusarium*.

Trichoderma spp. are among the most studied and promising microorganisms used in a biocontrol system (Benitez *et al*., 2004; Woo *et al*., 2014). Species of the ascomycete genus *Trichoderma* (teleomorph *Hypocrea*) are found in many ecosystems of all climatic zones, but the most common and natural habitat of these fungi is known to be soil and wood with symptoms of decay (Samuels, 2006; Kubicek *et al*., 2008; Druzhinina *et al*., 2011; Strakowska *et al*., 2014). These species succeed in various heterotrophic interactions, exhibiting saprotrophic and mycoparasitic (necrotrophic hyperparasitic, mycotrophic) lifestyles (Druzhinina *et al*., 2011). Their antagonistic abilities *i.e.* activities towards plant pathogens, such as *Botrytis cinerea*, *Fusarium* spp., *Pythium* spp., *Rhizoctonia solani*, *Verticillium dahilae*, and *Sclerotinia* spp. (Harman *et al*., 2004; Verma *et al*., 2007; Druzhinina *et al*., 2011) are a combination of several mechanisms, including nutrient and/or space competitions, antibiosis associated with the secretion of antibiotic metabolites and direct mycoparasitism, which involves the production of cell-wall-degrading enzymes (Vinale *et al*., 2008a; 2008b; Druzhinina *et al*., 2011). In addition, *Trichoderma* strains used as biocontrol agents are able to induce plant defense against pathogens and to promote plant growth (Hermosa, 2012; Nawrocka and Małolepsza, 2014).

Several studies indicate that *Trichoderma* species are effective biocontrol agents for phytopathogenic *Fusarium*. Although, particular attention has been paid to *Trichoderma harzianum* strains. It has been shown that seed treatment of maize with *T. harzianum* T22 and Th-8 strains reduces *Fusarium verticillioides* kernel colonization and FBs contamination under controlled and natural conditions as well as induces systemic resistance in maize against this pathogens (Nayaka *et al*., 2008; Ferriego *et al*., 2014a; 2014b). *T. harzianum* T16 and T23 strains have been found to be effective antagonists towards *F. verticillioides* and FBs production in maize kernels in liquid as well as agar medium (Altinok, 2009). Furthermore, *T. harzianum* isolate THF2/3 has been reported to reduce DON production by *F. graminearum* in agar medium bioassays (Cooney *et al*., 2001). Recently, it has been documented that *Trichoderma gamsii* 6085 and *Trichoderma atroviride* AN35 strains are able to suppress TCTs production by *F. graminearum* and *F. culmorum* species on natural substrates (Buśko *et al*., 2008; Matarese *et al*., 2012). In addition, *T. atroviride* AN35 grown in dual culture bioassays on rice (in a competition assay system) with *F. graminearum* and *F. culmorum* strains has also been found to substantially inhibit ZEN production (Gromadzka *et al*., 2009; Popiel *et al.*, 2008). However, very little is known on the impact of the various *Trichoderma* species on the phytopathogenic *Fusarium* and their capacity to inhibit mycotoxin production on solid substrates.

Therefore, the aim of this study was to examine the antagonistic ability of twenty-four isolates belonging to ten different *Trichoderma* species, including species that are not yet fully recognized as important biological control agents (BCA), against mycotoxigenic *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. graminearum* and *F. temperatum* species.

Experimental

Materials and Methods

Fungal isolates. The twenty-four *Trichoderma* isolates and five *Fusarium* strains investigated in this study were selected from the culture collection of the Institute of Plant Genetics, Polish Academy of Sciences, Poznań, Poland and are listed in Table I. Four selected *Trichoderma* strains (Table I) are deposited in the culture collection of the CBS-KNAW Fungal Biodiversity Centre, Utrecht, the Netherlands (CBS). All the studied *Trichoderma* strains sourced from different ecological niches in Poland had previously been identified to the species level by sequence analysis of the internal transcribed spacer regions ITS-1 and ITS-2 of the nuclear rDNA and a fragment of the translation-elongation factor 1-alpha (tef1) gene by Błaszczyk *et al.* (2011) and Jeleń *et al.* (2014). *Fusarium* strains of five species (*F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2870 and *F. temperatum* KF 506) were identified using species-specific markers validated during earlier studies (Stępień *et al*., 2011; Wiśniewska *et al*., 2014).

Dual culture bioassay. Antagonistic activities of all *Trichoderma* isolates towards *Fusarium* species were assessed in dual cultures on potato dextrose agar (PDA) medium. Pure cultures of *Trichoderma* and *Fusarium* were re-cultured aseptically from stock slants onto 8.5 cm diameter Petri dishes. They were grown on PDA for 7 days at 25 ± 2°C and were subsequently used as inoculum. Mycelial disks of 3 mm diameter from colony margins of each pathogen-antagonist combination were placed in Petri dishes containing 15 ml of PDA medium, 8.0 cm apart and diametrically opposed to each other. Each dual culture was set up in triplicate. The control consisted of individual cultures of the pathogen and antagonist. Plates were incubated at 25 ± 2°C, 12 h/12 h darkness/light. The radial growth of each fungus was measured daily with a ruler for

Table I
Trichoderma strains analysed for antagonistic activity against *Fusarium* species.

Culture code/ CBS[a] no.	Origin		NCBI GenBank Assession no.[b]	
	Locality	Source	ITS	*tef1*
T. atroviride				
AN152/ CBS136453	Central Poland	triticale kernel	HQ292792	HQ292957
AN182/CBS136454	Central Poland	forest wood	HQ292794	HQ292965
AN206/CBS136455	Central Poland	mushroom compost	HQ292804	HQ292960
AN240	Karkonosze Mts, SP	forest wood	JX184119	JX184096
AN497	Gorce Mts, SP	forest wood	JX184119	JX184096
T. citrinoviride				
AN262	Central Poland	forest wood	HQ292847	–
AN393	Gorce Mts, SP	forest wood	JX184109	JX184086
T. cremeum				
AN392	Gorce Mts, SP	forest wood	JX184117	JX184094
T. hamatum				
AN120	Central Poland	forest wood	HQ292855	–
AN277	Central Poland	forest wood	HQ292857	–
T. harzianum				
AN150	Central Poland	forest wood	HQ292878	–
AN278	Central Poland	forest wood	HQ292890	–
AN360	Tatra Mts, SP	forest wood	JX184113	JX184090
T. koningiopsis				
AN143	Central Poland	forest wood	HQ292929	HQ292992
AN251	Karkonosze Mts, SP	forest wood	HQ292939	HQ292993
T. longibrachiatum				
AN197	Eastern Poland	mashroom factory	HQ292780	–
AN213	Central Poland	mashroom compost	HQ292781	–
T. longipile				
AN359	Tatra Mts, SP	forest wood	JX184115	JX184091
T. viride				
AN255	Karkonosze Mts, SP	forest wood	JX184121	JX184098
AN401	Gorce Mts, SP	forest wood	JX184122	JX184099
AN430	Central Poland	forest wood	HQ292926	HQ293014
AN826	Karkonosze Mts, SP	forest wood	JX184122	JX184099
T. viridescens				
AN323	Tatra Mts, SP	forest wood	JX184127	JX184103
AN405/CBS136460	Gorce Mts, SP	forest wood	JX184127	JX184103

a – The culture collection of the CBS-KNAW Fungal Biodiversity Centre,Utrecht, the Netherlands
b – The same NCBI GenBank Accession number assigned to the isolates possessing identical alleles in the locus ITS or *tef1*

4 days (until contact). After 14 days of incubation, a qualitative evaluation of antagonism was carried out based on the modified scale of Mańka (1974): +8, antagonist completely overgrew the pathogen and entire medium surface; +6, antagonist occupies 85% of the medium surface; +4, antagonist occupies 75% of the medium surface, 0, antagonist occupies 50% of the medium surface (Popiel *et al*., 2008).

Microscopic observations. Co-cultures of *Trichoderma* spp. with *Fusarium* spp. were performed on PDA medium in Petri dishes. After solidification of the medium, a sterile strip (20 mm) of cellophane membrane (50-µm thick) was placed in the middle of each plate. Mycelia were collected from pure, actively growing colonies of each of *Trichoderma* and *Fusarium* species. Colonies were inoculated on opposite sides of Petri dishes at a distance of 5 mm from the edge. Incubation of co-cultures was performed at room temperature. After 7, 14 and 21 days of incubation, the cellophane membrane was cut with a razor blade in sterile

conditions and the mycelia were placed onto a drop of distilled water on a microscope slide and examined. Observations were carried out using a light microscope (Olympus CX-41-1 with UC-30 camera, Olympus, Japan). After the first 7 days, the samples were screened mainly for loops of the *Trichoderma* around *Fusarium* hyphae. After the following days of incubation the samples were screened for the anatomical damage of the *Fusarium* and other structures indicating potential mycoparasitism.

Solid substrate bioassay. In order to evaluate the ability of *Trichoderma* to inhibit mycotoxin production by *Fusarium* species, dual cultures were established on solid substrates (rice kernels) following the methods described by Buśko *et al.* (2008). The assay was performed using all of the *Fusarium* strains and five selected *Trichoderma* isolates characterized by the highest antagonistic potential against *Fusarium* species in dual cultures on PDA medium. Rice kernels (50 g) were added to 15 ml distilled water, left overnight and autoclaved at 121°C for 30 min in a 300 ml Erlenmeyer flask. The flasks were inoculated with four disks (4 mm in diameter) cut from the advancing edge of 7-day PDA culture of pathogen and/or antagonist. Three replicates for each experimental set (antagonist, pathogen and antagonist/pathogen variants) were performed. Uninoculated rice was used as negative controls. The incubation was carried out at 25 ± 2°C 12 h/12 h of darkness/light for 21 days. Flasks were shaken daily to prevent clumping and provide aeration.

Mycotoxin extraction and analysis

Chemicals and reagents. Mycotoxin standards (ZEN, DON and its derivatives 15-AcDON and 3-AcDON, NIV, MON and BEA) were supplied by Sigma-Aldrich (Steinheim, Germany). Acetonitrile and methanol (HPLC grade), disodium tetraborate, 2-mercaptoethanol, potassium hydroxide, sodium dihydrophosphate and *o*-phosphoric acid were also purchased from Sigma-Aldrich. Potassium chloride, dipotassium phosphate, *n*-hexane and *t*-butyl-ammonium hydroxide were purchased from POCh (Poland). Water of HPLC grade from Merck Millipore water purification system was used for analyses.

Sample preparation. Rice culture samples were powder-ground in a WŻ-1 laboratory mill (the Research Institute of Baking Industry Ltd., Bydgoszcz, Poland). ZEN, DON, NIV, 3-Ac-DON and 15-Ac-DON were extracted with acetonitrile:water (90:10, v/v) solution. MON and BEA were extracted with acetonitrile:methanol:water (16:3:1, v/v/v). A solvent mixture was used at a ratio of 2.5 ml of solvent per 1 g of ground samples. Extractions were performed by blending the samples with the solvents at high speed for 2 min in an H 500 blender jar (POL-EKO, Poland). The extract was filtered through filter paper (No. 5, Whatman), a glass microfiber filter (GF/B, Whatman) and a syringe filter (CHROMAFIL PET-45/15MS) and then the filtrate was collected. Where the toxin content in the sample was below the detection limit, the sample was purified by immunoaffinity columns (ZearalaTest, WB) according to the method of Visconti and Pascale (1998) for ZEN, and by using carbon columns for the determination of DON, MON, 3-Ac-DON and 15-Ac-DON by the method of Tomczak *et al.* (2002), Kostecki *et al.* (1999) and Yang *et al.* (2013) respectively. The filtrate was analyzed by HPLC chromatography according to the methodology described below.

HPLC analysis. The chromatographic system consisted of a Waters 2695 high-performance liquid chromatograph (Waters, Milford, USA), a Waters 2475 Multi λ Fluorescence Detector and a Waters 2996 Photodiode Array Detector. Millenium software was used for data processing. DON, NIV and MON analyses were performed according to the method described by Kostecki *et al.* (1999) and Tomczak *et al.* (2002) while 3-AcDON, 15-AcDON was detected by the method of Yang *et al.* (2013). Analysis of ZEN and BEA was performed according to Visconti and Pascale (1998) and Jestoi *et al.* (2008), respectively. The detection limit for DON, NIV, BEA and MON was 0.01 g kg $^{-1}$, for 3-AcDON, 15-AcDON was 1.0 g kg^{-1} and for ZEN was 0.3 g kg^{-1}. Recovery rates for NIV, DON, 3-AcDON, 15-AcDON, BEA and MON were 75, 87, 76, 75, 85, 90%, respectively. ZEN recovery was in a range from 97 to 99%. Relative standard deviation (R.S.D.) was below 1% for ZEN, below 5% for DON, 3-AcDON, 15-AcDON, NIV and BEA, and below 7% for MON. The recovery of mycotoxins were measured in triplicates by extracting toxins from solid substrates spiked with 1.0–100 g kg^{-1} of compounds. In order to confirm the presence of ZEN, a Photodiode Array Detector was used. Mycotoxin was identified by comparing retention times and UV spectra of purified extracted samples with pure standards. Quantification of mycotoxins was carried out by comparing peak areas of the analyzed samples to the calibration curve of peak areas obtained with authentic mycotoxin standards.

Statistical analysis. To analyse the inhibition (%) of pathogen growth on the fourth day after co-inoculation, inhibition coefficients and their standard errors were calculated according to the formula: (Rc-R) / Rc × 100, where Rc, R are the estimates of radial growth of a pathogen in control and dual culture, respectively (Edington *et al.* 1971). The same procedure was also applied to calculate relative toxin reduction (%), based on estimated toxin production level. The final assessment of inhibitory effectiveness of the antagonists was based on observations from visual assessment of colony state at day 14 of co-incubation. To identify the most

and the least efficient antagonists the values, expressed on a nominal scale (modified scale of Mańka, 1974), were averaged and compared.

To generally assess the influence of antagonists on the growth of pathogens during the initial days of the experiment the analysis of regression of pathogens' growth curves was performed. Linear regression was calculated for each pathogen in the presence/absence of antagonists (each combination in triplicate), based on measurements from days 0 to 4. Analysis of the variance of pathogen colony growth was performed for all pathogen-antagonist interactions on all days of observations at significance level $\alpha = 0.001$. To identify the effects of particular antagonists on pathogen colony size, Duncan's Multiple Range Test ($\alpha = 0.05$) was performed for (raw) growth observations at day 4 of co-incubation.

Statistical analysis of the results was performed using Genstat statistical software (regression analysis, ANOVA, modeling, correlation analysis) and R (Duncan test).

Results

Evaluation of the antagonistic activity of *Trichoderma* strains in dual cultures on PDA medium. Antagonistic tests showed that all *Trichoderma* strains significantly reduced *F. avenaceum* and *F. culmorum* growth during the initial days of co-incubation on PDA medium. Furthermore, five strains (AN240, AN262, AN359, AN826, AN323) significantly reduced the mycelial growth of *F. cerealis*, *F. graminearum* and *F. temperatum* (Supplemental online material 1 and 2). Among twenty-four *Trichoderma* strains screened for their antagonistic activity against five *Fusarium* species in dual cultures on PDA medium, thirteen strains (AN152, AN240, AN497, AN262, AN277, AN143, AN251, AN213, AN359, AN401, AN430, AN826, AN323) were able to inhibit mycelial growth of all investigated pathogens on the fourth day after co-inoculation (Table II). These strains provided from 1% to 80% inhibition (Supplemental online material 3). The remaining eleven *Trichoderma* strains (AN182, AN206, AN393, AN392, AN120, AN150, AN279, AN360, AN197, AN255, AN405) displayed a suppressive effect on mycelial growth of only *F. culmorum*, *F. avenaceum*, *F. graminearum* and *F. temperatum* (Table II, Supplemental online material 3). As shown in Table II and Supplemental online material 3, the range of action of *Trichoderma* strains against each of the *Fusarium* species was different. After 4 days of incubation, the estimated growth inhibition of *F. avenaceum* by *Trichoderma* ranged from 62% to 80%. The colony diameter of this species was significantly affected, compared to the control, by *T. harzianum* AN150. The growth inhibition of *F. cerealis* by *Trichoderma* ranged from 2% to 64% and the highest inhibitory effect on pathogen growth was achieved by *Trichoderma longibrachiatum* AN213. The *F. culmorum* growth inhibition rate caused by *Trichoderma* ranged from 27% to 80%. The maximum growth suppression of this species was recorded as a result of interaction with *Trichoderma koningiopsis* AN143. The growth inhibition of *F. gramineraum* ranged from 1% to 64% and *F. temperatum* ranged from 1% to 60%. The colony diameter of these pathogens was significantly retarded by the *T. atroviride* AN206 and *Trichoderma cremeum* AN392 strains, respectively.

The antagonistic profiles of all *Trichoderma* strains from qualitative evaluation of the interaction between the colonies after 14 days of co-culturing on PDA medium were present in Table III. Of the 24 *Trichoderma* strains, one strain, namely AN240, was efficiently antagonistic against the entire target *Fusarium* species in that it received the best evaluation (*i.e.*, 8) according to Mańka's classification (1974). Whereas, four *Trichoderma* strains (AN152, AN251, AN255, AN430) completely overgrew and sporulated on the colonies of all except one of the *Fusarium* species tested (Table III).

To investigate the mode of action of the antagonists, microscopic observation was conducted of the mycelium of confronting fungi in the interaction zone.

Supplemental online material 1
Analysis of variance of the growth rate of pathogens in the presence of antagonists/control across days; significance of all factors and their interactions.

Factor	*Df*	Sum Sq	Mean Sq	F value	Pr (> F)
Pathogen	5	14802	2960	2279.20	< 0.001 ***
Antagonist	24	6536	272	209.68	< 0.001 ***
Day	3	115885	38628	29739.56	< 0.001 ***
Pathogen × antagonist	120	15282	127	98.05	< 0.001 ***
Pathogen × day	15	7484	499	384.13	< 0.001 ***
Antagonist × day	72	5954	83	63.66	< 0.001 ***
Pathogen × antagonist × day	360	15481	43	33.11	< 0.001 ***

Supplemental online material 2

Linear regression analysis. Growth rate of *Fusarium* spp. facing *Trichoderma* spp. compared with the growth rate of *Fusarium* spp. in the control conditions (first row). R2 – determination coefficient; Δ slope – difference of growth rate (mm per day) and its significance. Δ int (intercept) – difference of elevation of the regression line. and its significance (determined only if Δ slope insignificant at < 0.001).

Pathogen	*F. avenaceum* KF 2818				*F. cerealis* KF 1157				*F. culmorum* KF 2795				*F. graminearum* KF 2870				*F. temperatum* KF 506			
	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope
T. atroviride																				
control	0.73	13.0		−7.4	0.95	8.0		−3.1	0.87	14.6		−7.5	0.96	11.6		−3.7	0.93	6.6		−2.9
AN152	0.97	−8.5	***	6.3	0.97	−2.8	***	2.0	0.97	−9.2	***	7.1	0.87	−3.5	***	0.6	0.98	−0.4		2.3
AN182	0.97	−7.1	***	6.1	0.92	0.0		1.9	0.88	−7.1	***	4.9	0.93	−4.5	***	1.5	0.91	−2.1	***	1.8
AN206	0.94	−8.0	***	6.8	0.93	1.6	*	−0.3	0.92	−9.0	***	7.9	0.97	−7.4	***	4.4	0.97	−1.4	**	1.2
AN240	0.97	−8.8	***	8.1	0.98	−2.9	***	1.5	0.97	−9.5	***	6.5	0.96	−3.5	***	2.7	0.97	−1.3	**	1.7
AN497	0.97	−8.5	***	6.3	0.92	−2.5	**	3.0	0.94	−5.9	***	4.8	0.94	−3.2	***	0.6	0.95	−1.6	**	0.9
T. citrinoviride																				
AN262	0.96	−8.3	***	7.0	0.96	−3.6	***	1.7	0.89	−4.5	***	4.1	0.90	−2.9	**	1.1	0.74	−1.7	***	1.5
AN393	0.97	−8.4	***	7.4	0.93	0.8		0.9	0.89	−6.0	***	4.7	0.95	−0.7		0.4	0.97	−1.3	*	1.3
T. cremeum																				
AN392	0.91	−8.2	***	5.7	0.90	3.9	***	−2.3	0.98	−4.1	***	5.5	0.96	−0.5		1.3	0.99	−3.8	***	3.1
T. hamatum																				
AN120	0.94	−8.0	***	6.1	0.90	0.5		0.3	0.92	−2.7	**	2.7	0.92	0.0		−1.7	0.94	−1.6	***	1.3
AN277	0.97	−8.8	***	6.3	0.92	−2.1	**	0.1	0.98	−7.5	***	5.9	0.96	−3.3	***	1.5	0.89	−1.0	*	0.5
T. harzianum																				
AN150	0.93	−9.5	***	6.5	0.92	3.9	***	−3.3	0.75	−6.2	***	4.6	0.82	−3.1	***	−2.3	0.97	−1.3	**	1.3
AN279	0.94	−7.0	***	6.0	0.95	0.6		1.0	0.97	−9.3	***	7.1	0.96	−4.2	***	1.8	0.96	−1.4	**	1.3
AN360	0.98	−7.8	***	6.2	0.94	0.5		0.1	0.93	−7.8	***	4.7	0.94	−1.7	*	−1.1	0.98	−0.3		0.5

Supplemental online material 2 – continued

Linear regression analysis. Growth rate of *Fusarium* spp. facing *Trichoderma* spp. compared with the growth rate of *Fusarium* spp. in the control conditions (first row). R2 – determination coefficient; Δ slope – difference of growth rate (mm per day) and its significance. Δ int (intercept) – difference of elevation of the regression line. and its significance (determined only if Δ slope insignificant at < 0.001).

Pathogen	*F. avenaceum* KF 2818				*F. cerealis* KF 1157				*F. culmorum* KF 2795				*F. graminearum* KF 2870				*F. temperatum* KF 506			
	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope	R^2	Δ int		Δ slope
T. koningiopsis																				
AN143	0.83	−9.6	***	5.8	0.92	−2.1	**	0.1	0.98	−11.4	***	6.9	0.96	−4.8	***	2.3	0.97	−1.6	***	1.5
AN251	0.98	−7.8	***	6.2	0.96	−0.8		0.6	0.96	−6.4	***	6.4	0.98	−6.4	***	2.9	0.76	−1.5	**	1.4
T. longibrachiatum																				
AN197	0.95	−7.8	***	5.2	0.95	0.7		1.1	0.97	−10.4	***	6.5	0.93	−6.3	***	4.1	0.98	−1.6	***	1.3
AN213	0.94	−9.5	***	8.4	0.95	−5.1	***	3.2	0.97	−7.6	***	5.3	0.96	−2.4	**	0.4	0.87	−0.4		−0.5
T. longipile																				
AN359	0.98	−7.8	***	6.1	0.92	−3.2	***	1.4	0.97	−4.0	***	4.5	0.94	−2.3	**	−0.5	0.97	−1.6	***	1.3
T. viride																				
AN255	0.97	−7.9	***	5.6	0.91	1.8	*	1.9	0.96	−8.3	***	7.2	0.97	−6.2	***	3.3	0.88	−1.1	*	1.0
AN401	0.97	−7.9	***	6.3	0.99	−2.7	***	2.1	0.91	−4.9	***	4.5	0.93	−1.4		−1.9	0.92	−2.8	***	2.3
AN430	0.98	−7.7	***	6.2	0.96	−0.3		1.0	0.98	−9.5	***	6.9	0.93	−4.8	***	1.9	0.97	−1.0	*	0.9
AN826	0.91	−6.8	***	5.2	0.99	−2.7	***	2.1	0.86	−4.8	***	2.3	0.98	−6.4	***	2.3	0.94	−1.4	**	1.6
T. viridescens																				
AN323	0.98	−7.8	***	5.8	0.98	−2.8	***	2.4	0.97	−10.3	***	8.3	0.94	−5.4	***	3.0	0.89	−1.7	***	1.7
AN405	0.93	−7.2	***	6.2	0.96	2.4	**	−1.1	0.91	−9.8	***	5.8	0.93	−2.0	*	0.3	0.89	−1.4	**	0.5

Significance codes: '***' for < 0.001; '**' for < 0.01 ; '*' for < 0.05

Table II
Duncan's Multiple Range Test (α = 0.05). Multiple comparisons procedure revealed pairwise differences between antagonists' influence on pathogen growth and assigned identity classes in terms of pathogen colony size.

Pathogen / Antagonist	*F. avenaceum* KF 2818			*F. cerealis* KF 1157			*F. culmorum* KF 2795			*F. graminearum* KF 2870			*F. temperatum* KF 506		
	Means[a]	Std[b]	M[c]	Means	Std	M	Means	Std	M	Means	Std	M	Means	Std	M
control[d]	60.67	1.15	a	30.00	0.00	d	61.00	1.73	a	45.67	0.58	a	26.67	5.77	a
T. atroviride															
AN152	17.67	1.15	efg	19.33	1.15	g	20.00	0.00	hi	35.00	0.00	d	22.67	2.31	cd
AN182	22.33	0.58	bc	30.00	0.00	d	26.67	2.89	efg	25.00	0.00	f	17.67	1.15	f
AN206	19.00	1.73	def	35.00	0.00	c	20.00	0.00	hi	16.33	1.15	i	19.67	0.58	ef
AN240	16.33	1.15	gh	19.67	0.58	g	19.33	0.58	hi	29.33	0.58	e	20.00	0.00	ef
AN497	17.67	1.15	efg	20.00	0.00	g	31.67	2.89	de	30.67	1.15	e	20.00	0.00	ef
T. citrinoviride															
AN262	17.33	0.58	fgh	16.67	0.58	h	35.00	0.00	cd	30.67	1.15	e	19.67	0.58	ef
AN393	17.33	0.58	fgh	30.00	0.00	d	30.67	1.15	def	39.67	0.58	b	20.33	0.58	def
T. cremeum															
AN392	20.33	0.58	cde	43.67	1.15	a	40.00	0.00	bc	40.00	0.00	b	10.67	1.15	h
T. hamatum															
AN120	20.67	1.15	cd	35.00	0.00	c	44.33	1.15	b	45.00	0.00	a	20.00	0.00	ef
AN277	16.33	0.58	gh	22.33	2.52	f	28.00	0.00	efg	30.00	0.00	e	23.00	2.65	bc
T. harzianum															
AN150	12.33	0.58	j	45.00	0.00	a	28.67	14.43	efg	35.00	0.00	d	20.00	0.00	ef
AN279	21.00	0.00	bcd	30.33	0.58	d	19.67	0.58	hi	30.00	0.00	e	20.00	0.00	ef
AN360	20.00	0.00	cdef	30.00	0.00	d	25.00	0.00	fgh	38.00	0.00	c	25.33	0.58	ab
T. koningiopsis															
AN143	15.00	0.00	hi	22.33	2.52	f	12.00	0.00	j	25.00	0.00	f	20.00	0.00	ef
AN251	20.00	0.00	cdef	28.33	0.58	e	30.00	0.00	def	20.00	0.00	h	19.67	0.58	ef
T. longibrachiatum															
AN197	19.33	0.58	def	30.67	0.58	d	16.33	0.58	ij	20.00	0.00	h	20.00	0.00	ef
AN213	13.67	1.15	ij	10.67	1.15	i	26.00	0.00	efg	35.33	0.58	d	26.33	0.58	a
T. longipile															
AN359	20.00	0.00	cdef	20.33	0.58	g	40.00	0.00	bc	35.00	0.00	d	20.00	0.00	ef
T. viride															
AN255	19.00	0.00	def	35.00	0.00	c	23.33	0.58	gh	20.00	0.00	h	23.00	0.00	bc
AN401	19.33	1.15	def	20.33	0.58	g	35.00	0.00	cd	39.00	0.00	bc	15.00	0.00	g
AN430	20.00	0.00	cdef	29.33	0.58	de	19.33	0.58	hi	24.33	2.31	f	22.00	0.00	cde
AN826	23.33	5.77	b	20.33	0.58	g	35.00	0.00	cd	20.00	0.00	h	20.00	0.00	ef
T. viridescens															
AN323	20.00	0.00	cdef	19.33	0.58	g	16.33	1.15	ij	22.67	2.31	g	20.00	0.00	ef
AN405	20.00	0.00	cdef	40.00	0.00	b	20.33	0.58	hi	35.00	0.00	d	20.33	0.58	def

a – Average colony size of the pathogen in the presence of the antagonist – a mean of three replicates
b – Standard error for three replicates
c – Values within columns followed by the same letter are not significantly different according to the test for α = 0.05.
d – The control consisted of individual cultures of the pathogen in the absence of the antagonist

After 7/14 days of incubation two of the 24 isolates, namely AN240 and AN255, showed coiling structures and branched hyphae with at least one of all five *Fusarium* species (Fig. 1A-B). For AN240, coiling was observed on all Petri dishes when growing in co-culture with *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. graminearum* and *F. temperatum* strain (Fig. 1). However, AN255 produce dense coils around the hyphae of *F. graminearum* in all Petri dishes. The analysis of the area of intermingling contact (pathogen-antagonist)

Supplemental online material 3

Estimated inhibition (%) of mycelial growth of *F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2878 and *F. temperatum* KF 506 by *Trichoderma* species after 4 days of co–incubation on PDA medium.

Pathogen / Antagonist	*F. avenaceum* KF 2818		*F. cerealis* KF 1157		*F. culmorum* KF 2795		*F. graminearum* KF 2870		*F. temperatum* KF 506	
	I[a]	se[b]	I	se	I	se	I	se	I	se
T. atroviride										
AN152	70.88	1.37	35.56	2.05	67.21	3.05	23.36	1.27	15.00	4.05
AN182	63.19	1.41	0.00	2.43	56.28	3.16	45.26	1.15	33.75	3.70
AN206	68.68	1.38	–16.67	2.65	67.21	3.05	64.23	1.07	26.25	3.83
AN240	73.08	1.37	34.44	2.06	68.31	3.04	35.77	1.20	25.00	3.85
AN497	70.88	1.37	33.33	2.07	48.09	3.26	32.85	1.22	25.00	3.85
T. citrinoviride										
AN262	71.43	1.37	44.44	1.97	42.62	3.34	32.85	1.22	26.25	3.83
AN393	71.43	1.37	0.00	2.43	49.73	3.24	13.14	1.34	23.75	3.88
T. cremeum										
AN392	66.48	1.39	–45.56	3.04	34.43	3.46	12.41	1.35	60.00	3.32
T. hamatum										
AN120	65.93	1.39	–16.67	2.65	27.32	3.58	1.46	1.42	25.00	3.85
AN277	73.08	1.37	25.56	2.15	54.10	3.19	34.31	1.21	13.75	4.07
T. harzianum										
AN150	79.67	1.35	–50.00	3.10	53.01	3.20	23.36	1.27	25.00	3.85
AN279	65.38	1.40	–1.11	2.45	67.76	3.04	34.31	1.21	25.00	3.85
AN360	67.03	1.39	0.00	2.43	59.02	3.13	16.79	1.32	5.00	4.25
T. koningiopsis										
AN143	75.27	1.36	25.56	2.15	80.33	2.95	45.26	1.15	25.00	3.85
AN251	67.03	1.39	5.56	2.37	50.82	3.23	56.20	1.10	26.25	3.83
T. longibrachiatum										
AN197	68.13	1.38	–2.22	2.46	73.22	3.00	56.20	1.10	25.00	3.85
AN213	77.47	1.35	64.44	1.83	57.38	3.15	22.63	1.28	1.25	4.33
T. longipile										
AN359	67.03	1.39	32.22	2.08	34.43	3.46	23.36	1.27	25.00	3.85
T. viride										
AN255	68.68	1.38	–16.67	2.65	61.75	3.10	56.20	1.10	13.75	4.07
AN401	68.13	1.38	32.22	2.08	42.62	3.34	14.60	1.33	43.75	3.54
AN430	67.03	1.39	2.22	2.41	68.31	3.04	46.72	1.15	17.50	4.00
AN826	61.54	1.41	32.22	2.08	42.62	3.34	56.20	1.10	25.00	3.85
T. viridescens										
AN323	67.03	1.39	35.56	2.05	73.22	3.00	50.36	1.13	25.00	3.85
AN405	67.03	1.39	–33.33	2.87	66.67	3.05	23.36	1.27	23.75	3.88

a – Inhibition (%)
b – Standard error for three replicates

after 14/21 days of incubation revealed differences in the hyphal morphology of the pathogen as vacuolization of hyphae and plasmolysis of mycelium (Fig. 1C). For the other *Trichoderma*/*Fusarium* combinations, mycoparasitic signs were either only observed occasionally on some of the replicates or were not observed at all (date not shown).

Evaluation of the antagonistic activity of *Trichoderma* strains in dual cultures on solid substrates. Five *Trichoderma* strains (AN152, AN240, AN251, AN255, AN430) selected on the basis of dual culture bioassay on PDA medium, and showing the highest antagonistic ability against at least four of all five *Fusarium* species (Table III), were further tested for

Table III
The final assessment of the interaction between the *Trichoderma*/*Fusarium* colonies after 14 days of co-culturing on PDA medium.

Pathogen Antagonist	*F. avenaceum* KF 2818	*F. cerealis* KF 1157	*F. culmorum* KF 2795	*F. graminearum* KF 2870	*F. temperatum* KF 506	Average[a]
T. atroviride						
AN152[b]	8	6	8	8	8	7.6
AN182	6	6	6	8	8	6.8
AN206	6	6	8	8	8	7.2
AN240	8	8	8	8	8	8
AN497	8	8	0	6	6	5.6
T. citrinoviride						
AN262	4	6	4	4	6	4.8
AN393	6	4	4	4	6	4.8
T. cremeum						
AN392	4	0	0	4	6	2.8
T. hamatum						
AN120	6	4	0	0	4	2.8
AN277	6	6	8	6	6	6.4
AN150	6	0	0	4	6	3.2
AN279	6	4	6	6	6	5.6
AN360	6	4	4	4	6	4.8
T. koningiopsis						
AN143	8	6	8	8	6	7.2
AN251	8	6	8	8	8	7.6
T. longibrachiatum						
AN197	6	4	6	6	6	5.6
AN213	6	6	4	4	6	5.2
T. longipile						
AN359	6	4	4	4	6	4.8
T. viride						
AN255	8	6	8	8	8	7.6
AN401	6	4	4	0	6	4
AN430	8	8	8	8	6	7.6
AN826	4	4	4	6	6	4.8
T. viridescens						
AN323	6	6	8	8	8	7.2
AN405	8	0	4	6	6	4.8

a – The values in modified Mańka (1974) scale (+8, antagonist completely overgrew the pathogen and entire medium surface; +6, antagonist occupies 85% of the medium surface; +4, antagonist occupies 75% of the medium surface, 0, antagonist occupies 50% of the medium surface) after visual assessment of the colony state (3 replicates, all observations equal in all cases) were averaged to identify the most efficient antagonist.
b – *Trichoderma* strains (grey) showing the highest antagonistic ability against at least four of all five *Fusarium* species

their ability to inhibit *Fusarium* mycotoxin production in dual cultures on rice kernels. *Fusarium* species used in the experiments represented five chemotypes (ZEN, DON and its derivatives 15-AcDON and 3-AcDON, NIV, MON, BEA). The estimated amount of mycotoxin production by these species after 21 days of incubation and co-incubation with *Trichoderma* on rice medium is shown in Table IV. The effect of *Trichoderma* strains on *Fusarium* mycotoxin synthesis in dual culture bioassay on rice after 21 days at 25 ± 2°C is presented in Table V. The experiments revealed that all *Trichoderma* strains assayed reduced mycotoxin production by *Fusarium* species from 21% to 100%, except for strain AN430 that showed no suppressive effect on ZEN production by *F. culmorum*. As shown in Table V, the presence of each of the four *Trichoderma*

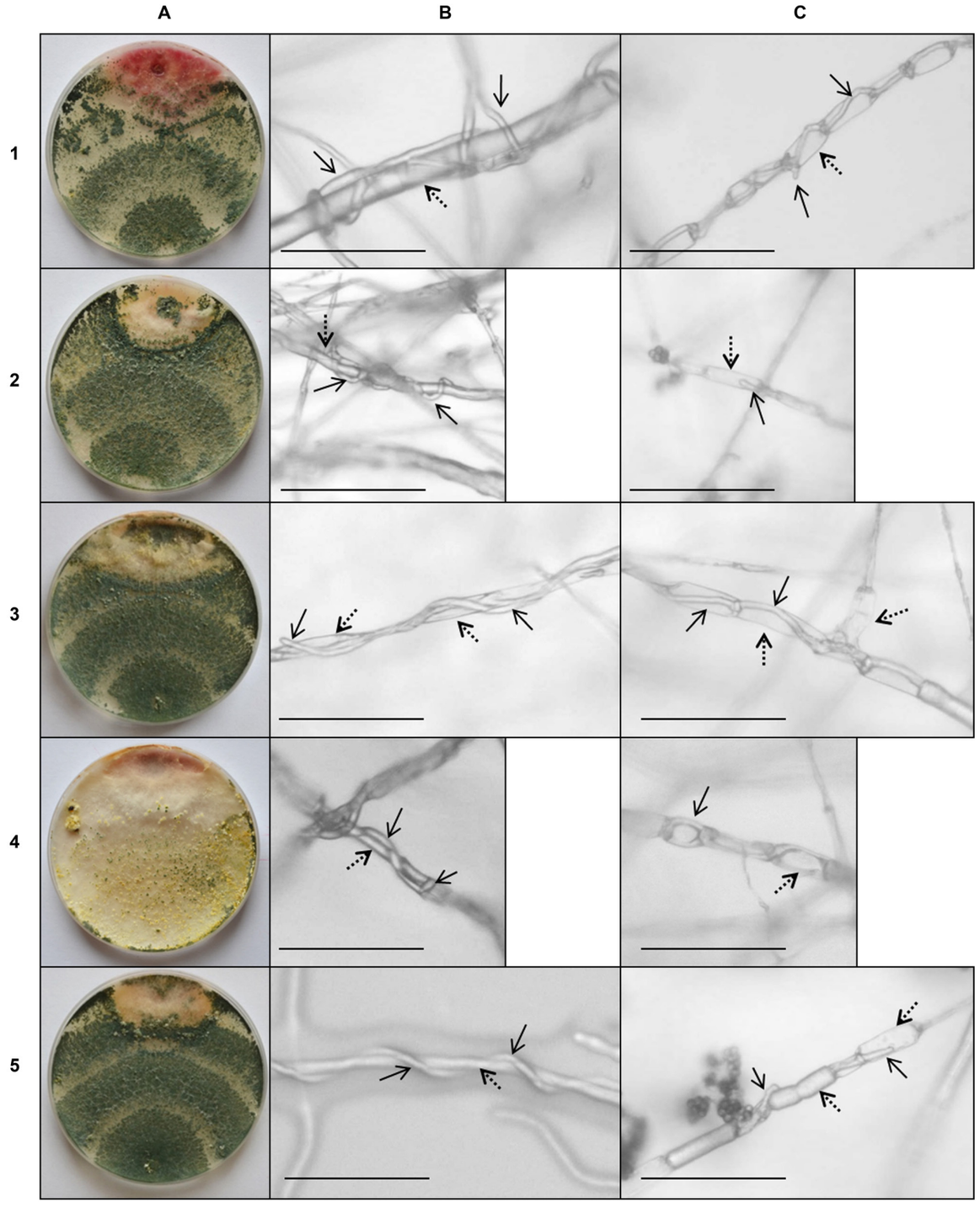

Fig. 1. Interactions between *T. atroviride* AN240 and *F. avenaceum* KF 2818 (1), *F. cerealis* KF 1157 (2), *F. culmorum* KF 2795(3), *F. temperatum* KF 506 (5) and between *T. viride* AN 255 and *F. graminearum* KF 2870 (4) in dual cultures on PDA medium: A – dense sporulation and overgrowth of antagonist on pathogen after 14 days of co-incubation; B – coiling of pathogen hyphae by antagonist after 7/14 days of co-incubation; C – antagonist hypha inside a partial degraded hypha of pathogen after 14/21 days of co-incubation. The black arrow indicate the hyphae of *Trichoderma* strain and the dotted arrow indicate the hyphae of *Fusarium* strains. Bar = 50 µm.

strains completely (100%) inhibited MON production by *F. avenaceum*. *Trichoderma atroviride* AN240 markedly (>99%) reduced the level of both BEA and MON synthesized by *F. temperatum*. In the presence of *Trichoderma viride* AN430, *F. cerealis* produced 92% less NIV than when alone. However, among the four

Table IV
Mycotoxin production (mg kg^{-1}) by *F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2870 and *F. temperatum* KF 506 on solid substrates (rice kernels) in the presence/absence of the *Trichoderma* strains (*T. atroviride* AN152, AN240, *T. koningiopsis* AN251, *T. viride* AN255, AN430).

Combination		Mycotoxin (mg kg^{-1})													
		DON		NIV		ZEN		3-AcDON		15-AcDON		BEA		MON	
		Means	se[a]	Means	se	Means	se	Means	se	Means	se	Means	se	Means	se
F. avenaceum KF 2818	control[b]	ND[c]		ND		ND		ND		ND		ND		1.08	0.18
	AN152													ND	
	AN240														
	AN255														
	AN430														
F. cerealis KF 1157	control	ND		19.24	0.85	ND		ND		ND		ND		ND	
	AN152			3.33	0.48										
	AN240			5.43	0.62										
	AN255			1.94	0.09										
	AN430			1.52	0.09										
F. culmorum KF 2795	control	11.06	4.71	43.81	2.5	45.76	16.96	5.77	1.16	ND		ND		ND	
	AN240	0.37	0.53	1.91	0.07	7.46	3.52	0.01	0.01						
	AN251	2.71	1.76	8.35	0.27	36.2	34.46	0.86	0.99						
	AN255	1.7	1.7	5.15	0.11	0.34	0.48	0.01	0.02						
	AN430	0.55	0.54	7.24	0.1	46.62	4.17	1.36	1.14						
F. graminearum KF 2870	control	39.79	1.19	49.72	0.43	34.27	0.81	ND		2.96	0.23	ND		ND	
	AN240	1.93	0.62	9.01	0.63	0.21	0.3			0.16	0.23				
	AN251	3.94	0.97	7.41	0.21	8.04	1.05			0.93	0.18				
	AN255	3.34	0.22	4.94	0.12	0.58	0.56			0.36	0.51				
	AN430	1.34	0.95	5.48	0.18	0.98	0.68			0.73	0.52				
F. temperatum KF 506	control	ND		ND		ND		ND		ND		19.59	1.66	63.34	2.09
	AN240											0.01	0.01	0.08	0.04
	AN251											7.56	1.28	0	0
	AN255											0.22	0.04	10.24	1.65
	AN430											0.54	0.28	1.47	0.68

a – Standard error for three replicates
b – The control consisted of individual cultures of the pathogen in the absence of the antagonist.
c – Not detected (*i.e.* < LOD)

Trichoderma strains, AN240 and AN255 showed the best capacity for reducing DON, 3-AcDON, ZEN, NIV concentrations during co-incubation with *F. culmorum* and DON, 15-AcDON, ZEN, NIV accumulation in dual cultures with *F. graminearum*.

Discussion

As is well known, antagonism by *Trichoderma* is a combination of several mechanisms, including nutrient and/or space competitions, antibiosis associated with the secretion of antibiotic metabolites and direct parasitism or mycoparasitism, which involves the production of cell-wall-degrading enzymes (Vinale *et al.*, 2008a; 2008b; Druzhinina *et al.*, 2011). The powerful tool for the study of the antagonistic activity of *Trichoderma* against some phytopathogenic fungi is considered to be the dual culture assays (Almeida *et al.*, 2007; Qualhato *et al.*, 2013; Schöneberg *et al.*, 2015). In the present study, this approach was used in the preliminary screening of 24 *Trichoderma* isolates, belonging to 10 species for *in vitro* antagonism towards five toxigenic *Fusarium*. The results of this work revealed a great variability in the level of biological activity between *Trichoderma* species and even among strains of the same species towards the same *Fusarium* strains. Moreover, the results obtained in the present study showed a different degree of biological activity of the same antagonistic agent against distinct *Fusarium* species (*i.e.* that the antagonism of the same *Trichoderma* strains varies when it is confronted with different *Fusarium* species).

Table V
Estimated reduction (%) of toxins production of five *Fusarium* species (*F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2878 and *F. temperatum* KF 506) by *T. atroviride* AN152, AN240, *T. koningiopsis* AN251 and *T. viride* AN255, AN430 strains in dual culture on solid substrates (rice kernels).

Combination		DON		NIV		ZEN		3- AcDON		15-AcDON		BEA		MON	
		R[a]	se[b]	R	se	R	se	R	se	R	se	R	se	R	se
F. avenaceum KF 2818	AN152	–[c]		–		–		–		–		–		100.00	5.29*
	AN240													100.00	5.29*
	AN255													100.00	5.29*
	AN430													100.00	5.29*
F. cerealis KF 1157	AN152	–		82.69	1.94	–		–		–		–		–	
	AN240			71.78	1.99										
	AN255			89.92	1.92										
	AN430			92.10	1.92										
F. culmorum KF 2795	AN240	96.63	15.34	95.63	1.82	83.70	27.17	99.83	10.47	–		–		–	
	AN251	75.53	15.78	80.95	1.85	20.88	34.20	85.16	10.58						
	AN255	84.63	15.51	88.24	1.83	99.26	26.81	99.77	10.47						
	AN430	95.00	15.35	83.47	1.84	–1.88	38.29	76.50	10.75						
F. graminearum KF 2870	AN240	95.16	1.53	81.87	0.53	99.39	1.49	–		94.48	8.73	–		–	
	AN251	90.11	1.53	85.09	0.52	76.55	1.53			68.47	9.14				
	AN255	91.61	1.53	90.07	0.52	98.32	1.49			87.84	8.78				
	AN430	96.63	1.53	88.99	0.52	97.14	1.49			75.23	8.98				
F. temperatum KF 506	AN240	–		–		–		–		–		99.95	3.42	99.87	1.37
	AN251											61.41	3.66	100.00	1.37
	AN255											98.88	3.42	83.83	1.39
	AN430											97.26	3.42	97.68	1.37

a – Reduction (%)
b – Standard error for three replicates
c – Not applicable
* The standard error of the estimate for MON toxin comes only from variation in the control conditions. All antagonists reduced MON completely (toxin was not found in the measurements).

These findings are consistent with several earlier studies on the antagonistic potential of *Trichoderma* species against *F. graminearum* and *F. cerealis*, *Fusarium solani*, *R. solani*, *Sclerotinia rolfsii*, *Sclerotinia sclerotiorum* (Dubey *et al.*, 2007; Inch and Gilbert, 2007; Shaigan *et al.*, 2008; Amin *et al.*, 2010; Anees *et al.*, 2010; Qualhato *et al.*, 2013; Schöneberg *et al.*, 2015) and could suggest that the *in vitro* antagonistic potential of *Trichoderma* is determined by types of antagonist/pathogen interactions unique and specific for each strain. It is worth noting that this type of interaction might also depend on the experimental conditions, as was recently observed by Schöneberg *et al.* (2015).

Dual-culture assay on PDA medium clearly documented that all of the *Trichoderma* strains used in the present study were capable of influencing the mycelial growth of at least four of all five *Fusarium* species on the fourth day after co-inoculation, when there was the first apparent physical contact between antagonist and pathogen. Ten of *Trichoderma* strains – AN152, AN182, AN206, AN240, AN497, AN277, AN143, AN255, AN430, AN323, AN405 – completely overgrew and sporulated on the colony at least one of the tested *Fusarium* species. Whereas, the microscopic assay provided evidence that only *T. atroviride* AN240 and *T. viride* AN255 formed dense coils around the hyphae of the pathogen from where penetration took place. Coiling has been considered to be an indicator for mycoparasitic potential, which could play an important role in making contact with the pathogen (Benítez *et al.*, 2004; Schöneberg *et al.*, 2015). In the present study, not all highly antagonistic *Trichoderma* strains were able to coil around the hyphae of the pathogen. This observation is in accordance with previous reports where no correlation between coiling and other features of antagonistic potential of *Trichoderma* was found (Almeida *et al.*, 2007; Anees *et al.*, 2010; Schöneberg *et al.*, 2015) and supports the statement that coiling could also depend on various biotic and abiotic factors and may be part of a more general response to a filamentous substrate (Inbar and Chet, 1992; Almeida *et al.*, 2007).

As observed here, those *Trichoderma* strains which displayed the highest antagonistic activity against at least four *Fusarium* species in co-culturing on PDA medium, namely AN152, AN240, AN255, AN430 and AN251, were also found to be effective suppressors of mycotoxin production by these pathogens on solid substrates. A similar study performed by Cooney *et al.* (2001) showed that *T. harzianum* isolate THF2/3 grown in a competition assay system with *F. graminearum* displayed an inhibitory effect on mycelial growth and trichothecene mycotoxin (DON) production by *Fusarium*. The capacity of highly antagonistic *T. gamsii* and *T. atroviride* strains to inhibit trichothecene and ZEN production by *F. graminearum* and *F. culmorum* on rice kernels was also described by Buśko *et al.* (2008), Popiel *et al.* (2008) and Matarese *et al.* (2012). Furthermore, Matarese *et al.* (2012) clearly demonstrated that the reduction of DON production by *Fusarium* species is correlated with the reduction of the pathogen biomass by *Trichoderma*. This finding could suggest that the ability all of the *Trichoderma* strains tested in the present study to suppress mycotoxin synthesis by *Fusarium* species on solid substrates was also the result of their high activity related to pathogen growth inhibition. The only exception is *T. viride* AN430. As was shown here, this strain was not able to reduce ZEN production by *F. culmorum* KF 2795, whereas the level of the other mycotoxins synthesized by KF 2795 was markedly reduced by AN430. Thus, the cause could not be as that described by Matarese *et al.* (2012). It is supposed that the presence of AN430 could stimulate the pathogen to selectively overproduce ZEN (Elmholt, 2008). However, the explanation of this aspect requires further studies.

In the present work, the most efficient strain overall was *T. atroviride* AN240 which was able to reduce mycelial growth and mycotoxins production of *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. graminearum* and *F. temperatum* species. To the best of our knowledge, this is the first report of the suppressive effect of *T. atroviride* on five toxigenic *Fusarium* species. The only reports of an efficient antagonistic strain of *T. atroviride* towards *F. avenaceum*, *F. culmorum* and *F. graminearum* were available from Buśko *et al.* (2008) and Popiel *et al.* (2008). The results of present study indicate that *T. atroviride* AN240 strain was able both to inhibit the pathogen's growth before the contact in confrontation plates and to overgrow and sporulate on the pathogen's colony. Moreover, the microscopic observation revealed a direct development of AN240 on the mycelium of *Fusarium* species. This research has suggested a possible antibiosis and mycoparasitism of AN240 and the production of antifungal components and extracellular metabolites by this antagonist (Druzhinina *et al.*, 2011; Verma *et al.*, 2007). Many studies of the *in vitro* biological activity of *Trichoderma* against fungal plant pathogens demonstrated the involvement of cell-wall degrading enzymes (CWDEs), which are also capable of acting synergistically with diffusible, volatile and non-volatile secondary metabolites and a complex system for fungal prey detection (Almeida *et al.*, 2007; Qualhato *et al.*, 2013). It is significant to note that our associated studies (unpublished data) on the examination of the 186 *Trichoderma* strains originating from wood with decay symptoms and analyses of their ability to produce hydrolytic enzymes have shown *T. atroviride* AN240 as the best producer of β-1,3-glucanase – an enzyme which have been found to be directly involved in the hydrolysis of the fungal pathogen's cell walls (Druzhinina *et al.*, 2011). Furthermore, in our previous study on the formation of the 6-n-pentyl-2H-pyran-2-one and other volatile compounds by different *Trichoderma* species grown on PDA medium, *T. atroviride* was found to be the most efficient species in this respect (Jeleń *et al.*, 2014). In the light of this and our previous findings, it can be assumed that *T. atroviride* AN240 represents a good candidate for the biological control of toxigenic *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. graminearum* and *F. temperatum*. However, it will be necessary to verified the reproducibility of the suppressive effect of AN240 also on *Fusarium* species (*i.e.*, *F. avenaceum*, *F. culmorum*, *F. cerealis*, *F. graminearum* and *F. temperatum*) originating from other fungal collections and to undertake the field trials in order to determine the ability of these strains to control of mycotoxin production in grain as well as to reduce toxigenic *Fusarium* inoculum levels in cereal debris under natural conditions.

Conclusion

The present survey has led to the emergence of the most efficient antagonist – *T. atroviride* AN240, able to inhibit the mycelial growth, completely overgrown and sporulated on the pathogen's colony, as well as to reduce the level of mycotoxins produced by *F. avenaceum* KF 2818, *F. cerealis* KF 1157, *F. culmorum* KF 2795, *F. graminearum* KF 2870 and *F. temperatum* KF 506. Considering the biological activity of this strain, being the synergistic effect of the several factors, it can be concluded that AN240 is a candidate fungus for the biological control of toxigenic *Fusarium* species by reducing their inoculum, as well as preventing mycotoxin accumulation in plant tissues.

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