BioControl (2016) 61:755–768
DOI 10.1007/s10526-016-9743-2

Optimal *Trichoderma* strains for control of stem canker of brassicas: molecular basis of biocontrol properties and azole resistance

Adam Dawidziuk · Delfina Popiel · Joanna Kaczmarek · Judyta Strakowska · Malgorzata Jedryczka

Received: 21 May 2016 / Accepted: 23 May 2016 / Published online: 3 June 2016

Abstract Genus *Trichoderma* contains the most powerful agents used in biocontrol. Our study demonstrated that *T. harzianum*, *T. hamatum* and *T. longibrachiatum* can effectively control phytopathogenic fungi *Leptosphaeria maculans* and *L. biglobosa*. The effects of *Trichoderma* on pathogens' growth (in dual cultures on agar media) and on disease severity (on seedlings in controlled conditions) were also confirmed by field experiments. Additionally, spraying with conidiospores in the autumn was helpful in reducing the incidence and severity of phoma leaf spots. It has also accelerated the degradation of plant stubble and the decomposition of pathogens' fruiting bodies. Furthermore, all *Trichoderma* isolates showed higher cellulolytic activity and enhanced resistance to flusilazole treatments as compared to *Leptosphaeria* spp., which coincided with upregulation of 14α-sterol demethylases and an AbcG5 transporter. The effects we observed justify the use of *Trichoderma* to enhance the resistance of oilseed rape against pathogens, which in turn may lead to a decrease in the use of pesticides.

Authors Adam Dawidziuk and Delfina Popiel are to be regarded as two first authors with equal contribution.

Handling Editor: Fouad Daayf

A. Dawidziuk · D. Popiel · J. Kaczmarek · J. Strakowska · M. Jedryczka (✉)
Institute of Plant Genetics, Polish Academy of Sciences, 34 Strzeszynska, 60-479 Poznan, Poland
e-mail: mjed@igr.poznan.pl

Keywords 14α-sterol demethylase · Azole transporter · Cellulolytic activity · Fungicide resistance · Oilseed rape

Introduction

Oilseed rape (*Brassica napus* L.) is one of the fastest expanding crops worldwide, with the European Union currently producing the highest amount of oilseed rape seeds and oil (http://faostat.fao.org). The great increase of oilseed rape production and its common use in crop rotations necessitates the introduction of new agronomic systems and of solving numerous problems related to crop protection against various pests, including weeds, insects and pathogens. The most damaging pathogens of oilseed rape are fungi. The incidence and severity of fungal species depend on field geographic location.

Leptosphaeria maculans (Desm.) Ces. et de Not is one of the most damaging pathogens to oilseed rape production worldwide. It is responsible for phoma leaf spotting and stem canker, also termed blackleg. The fungus is highly polymorphic (Kaczmarek et al. 2014a) and is responsible for yield losses of economic significance in all areas of intensive rapeseed cultivation, including Europe, Australia and Canada (Khangura and Barbetti 2001; West et al. 2001). In some areas, especially in Central and Eastern Europe, this species is accompanied by *L. biglobosa* (Kaczmarek

and Jedryczka 2011; Piliponyte-Dzikiene et al. 2014). The latter pathogen is often associated with less damaging upper stem lesions (Fitt et al. 2006). However severe plant infections with *L. biglobosa* can also lead to substantial yield losses (Jedryczka 2007). Most of these diseases can be controlled using chemical treatments. However, the current EU policy of integrated pest management mandates a search for biological methods to combat these pathogens.

Antagonistic and hyperparasitic fungi have increasingly been used in integrated pest management technologies to reduce the amount of pesticides introduced into the environment (Chet 1987; Wachowska et al. 2013). Antagonists are naturally occurring organisms with traits enabling them to interfere with pathogen growth, survival, infection or plant colonization (Chernin and Chet 2002). Members of the genus *Trichoderma* are the most potent biocontrol agents (Druzhinina et al. 2011; Blaszczyk et al. 2014). The mechanisms underpinning the capacity of *Trichoderma* species to restrict plant pathogens include hyperparasitism. It is a complex process which can involve several events such as host recognition, direct attack as well as competition for nutrients and space (Roco and Perez 2001). This process also involves antibiosis, that is the production of secondary metabolites inhibiting the growth of other microorganisms without physical contact (Hjeljord and Tronsmo 1998; Bailey et al. 2008). Recent experiments have provided information to elucidating the mechanisms of antibiosis and disease suppressive activities of some *Trichoderma* species (Zhang et al. 2015). The fungi belonging to the genus *Trichoderma* produce over 180 secondary metabolites, including various toxins and antibiotics (Reino et al. 2008). Furthermore, *Trichoderma* produce extracellular cell wall degrading enzymes, including chitinases and β-1,3-glucanases or peptaibols (Degenkolb et al. 2003). The mycolytic activity of these enzymes is considered to be a key factor in the hyperparasitic mechanism (Elad et al. 1982).

Many pathogens of crop plants can be successfully controlled by fungicides. Resistance of fungal strains to toxic compounds depends on the development of various mechanisms against these synthetic antifungal agents. These mechanisms relate to qualitative factors such as the absence or presence of a sensitive target site, or to quantitative factors such as uptake, transport, storage and metabolism (Deising et al. 2008). The cytochrome P450 sterol 14α-demethylase (CYP51) is an essential enzyme in the biosynthesis of sterols which are the components of cell membranes in eukaryotic organisms required for the regulation of membrane stability. Azole fungicides form the main class of antifungals used in agriculture and are known to target fungal sterol synthesis (Price et al. 2015). CYP51 mediated sterol biosynthesis is a target for the control of many fungal pathogens (Sheehan et al. 1999). The study of Becher et al. (2011) has shown that CYP51 is not a single gene, but there are two or more paralogues. Species with multiple copies of CYP51 can be resistant to some azoles. In fungi, drug transporters modulate the baseline sensitivity to fungicides, contribute to multidrug resistance (MDR) and virulence on host plants (de Waard et al. 2006). Two major groups of drug transporters that mediate membrane transport of foreign metabolites are the superfamilies of ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporter. The role of ABC and MFS transporters in the efflux of fungicides is well known among some filamentous fungi (Sorbo et al. 2000; de Waard et al. 2006; Kretschmer et al. 2009). However no studies have been conducted with respect to *Trichoderma* spp.

Kowalska and Remlein-Starosta (2011) demonstrated the potential of a commercial product containing *T. asperellum* to control some diseases of oilseed rape, including phoma stem canker. In our study the hypothesis that morphological and molecular properties of *Trichoderma* spp. can help in protection of oilseed rape against stem canker of brassicas was tested. The studies included laboratory experiments to evaluate growth rate under different conditions, bioassays in dual cultures and analysis of cellulolytic properties as well as three-year field experiments on decomposition of oilseed rape stubble, and the potential of sprays with the spores of *Trichoderma* spp. to decrease phoma leaf spotting. We have also examined the reaction of *Trichoderma* spp. to flusilazole, a representative azole fungicide commonly used against stem canker of oilseed rape. The analysis included the main molecular mechanisms of biosynthesis of sterols (CYP51). We compared the characteristics of several species of *Trichoderma*, including those obtained

from oilseed rape, as well as ones isolated from soil and plants other than oilseed rape.

Materials and methods

Fungal isolates

Leptosphaeria maculans and *L. biglobosa* isolates (Table 1) used in the study were collected at three sites located in the region of Wielkopolska (central-west Poland) over three seasons (2004–2006). *Trichoderma* spp. (hereafter *Trichoderma*) strains were obtained from oilseed rape plants (*Brassica napus*), soil from a greenhouse experiment with oilseed rape plants grown on peat compost and from yellow lupine (*Lupinus luteus*). The identification of species was based on fungal morphology and in the case of *Trichoderma* confirmed by DNA sequencing of ITS and tef-1α (Blaszczyk et al. 2014).

Expression of CYP51A, CYP51B and AbcG5 genes

Primer design

Primers allowing the detection of homologs of genes coding for 14α-sterol demethylases were designed for the purpose of this study (Table 2) on the basis of back translated codon alignments created from protein sequence alignments of homologous genes from NCBI/RefSeq and Ensembl/Fungi. To normalise gene expression calculations two housekeeping genes were used: β-tubulin and actin (Table 2). The propensity of primers for homodimer and heterodimer formation was assessed on the basis of nearest neighbour energy/

Table 1 The origin of fungi used in the experiment

No.	Fungal species	Isolate symbol	Plant organ/habitat	Location (country, site)	Year	Pathogen collection/NCBI accession no.
Leptosphaeria species						
1	*L. biglobosa*	LBC101	Leaf	PL, Cerekwica	2004	Collection of the molecular plant pathology team, IPG PAS, Poznan
2		LBC102	Mature stem	PL, Pawlowice		
3		LBC103				
4		LBC104	Stem debris	PL, Cerekwica	2005	
5		LBC105		PL, Kroscina Mala	2006	
6	*L. maculans*	LMC101	Leaf	PL, Cerekwica	2004	
7		LMC102				
8		LMC103	Mature stem			
9		LMC104	Leaf	PL, Pawlowice	2005	
10		LMC105	Stem debris		2006	
Trichoderma species						
11	*T. atroviride*	TA1	Soil	PL, Poznan	2008	FJ710062
12		TA2		PL, Poznan	2008	FJ710065
13		TA3		PL, Poznan	2008	FJ710066
14	*T. hamatum*	TH7	*Lupinus luteus*	UA, Bucza	2005	FJ710058
15	*T. harzianum*	T1S	*Brassica napus*	PL, Zielecin	2006	FJ710061
16		T3B		PL, Zielecin	2006	FJ710060
17		T4B		PL, Zielecin	2006	FJ710059
18		T13		PL, Zielecin	2006	FJ710063
19	*T. koningii*	TE2		PL, Lublin	2006	FJ710057
20	*T. longibrachiatum*	TLA1		PL, Poznan	2008	FJ710064

PL Poland, *UA* Ukraine

Table 2 The sequences of the primers used for gene expression analyses

Gene targeted	Primer name	Sequences (5′–3′)	Efficiency (%)
Azole transporter (*AbcG5*)	rt_ABCG5_fB2	TAC TGT GAA CAA CTC GAT GT	101.8
	rt_ABCG5_rB2	GAT CTC TTG GAA CTT CAC	
Cytochrome P450 51A	rt_CYP51A_fA2	TTC ACC CCC ATC AAC TTC AT	100.9
	rt_CYP51A_rA2	GCT CCT TGA TAG TGT CCA T	
Cytochrome P450 51B	rt_CYP51B_am_fA2	CCC TAC CTC ACA ACC GAA AA	98.9
	rt_CYP51B_am_rA2	CAG CCC ATC AAG TTG GCT AT	
β-tubulin (reference)	rtBtubTRICHOR2	AGC GAA TCC GAC CAT GAA GA	100.7
	rtBtubTRICHOF2	CAC CGT CGT TGA GCC CTA	
Actin (reference)	rt_act_All_am_fA1	ATC GGT ATG GGT CAG AAG GA	99.2
	rt_Btub_ALL_am_rA1	GTC ATC TTC TCA CGG TTG GA	

The measurement of the efficiency was calculated according to the following equation: Efficiency $= -1 + 10^{(-1/\text{slope})}$

melting temperature calculations with IDT OligoAnalyzer.

Efficiency of the PCR reaction

In order to obtain accurate and reproducible results, PCR reactions should have efficiency close to 100 %. The efficiency of the PCR between 90 and 110 % means doubling of the amplicon at each cycle. This corresponds to a slope of −3.1 to −3.6 in the C_t vs. log-template amount standard curve. The measurement of the efficiency was calculated according to the following equation:

$$\text{Efficiency} = -1 + 10^{(-1/\text{slope})}$$

RNA isolation and gene expression analysis

The protocols of PCR, sequencing and thermal cycling conditions followed the methodologies described by Dawidziuk et al. (2014) and Popiel et al. (2014). For the analysis of expression of *CYP51A*, *CYP51B* and *AbcG5* genes, the isolates were incubated four days before the addition of fungicides (3 mg l^{-1} of flusilazole). Two days after chemical treatment mycelia were collected from the Czapek-Dox medium, excess medium was separated from mycelium using a vacuum pump and then each sample was weighed on laboratory scales (Sartorius AG, Göttingen, Germany). Total RNA was purified from frozen mycelium, using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturers' protocol with an additional DNase digestion step. The quality of total RNA was estimated by Nanodrop (Thermo Scientific, Wilmington, DE, USA) and via Bioanalyzer (Bio-Rad, Hercules CA, USA). The RT-PCR reaction was conducted using the SYBR® Green Quantitative RT-qPCR Kit (Sigma-Aldrich, UK). The total reaction volume was 25 µl: 12.5 µl SYBR Green Taq Ready Mix, 1 µl RNA (<35 ng), 0.5 µl of each primer (10 µM), 0.125 µl reverse transcriptase and 5.125 µl nuclease free water. Gene expression profiles were determined through quantitative real-time PCR using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). The reaction was carried out using the following protocol: initial denaturation 94 °C for 2 min, followed by 40 cycles at 94 °C for 15 s, 61 °C for 1 min. The test was performed on the delta Ct values from the second day after fungicide exposure. The melting curve analysis (from 70 °C to 95 °C) confirmed the specificity of primer pairs. In the experiment, we used three biological and two technical replicates together with a template-free negative control in each analysis of target and control genes. As a control, we used mycelium samples cultivated on medium without the addition of a fungicide. Relative quantification of gene expression was done using the 2-ΔΔCt method (Bio-Rad, Hercules, CA). All data were normalised to actin and β-tubulin (Table 2) genes as internal

controls (Real-Time PCR Application Guide, Bio-Rad, Hercules CA).

Measurement of cellulolytic activity

Fungal isolates were cultured in induction medium. Mycelial pellets were removed by centrifugation (15 min at 12.000*g*) and enzyme activities were measured in the obtained supernatant. Total cellulase activity was determined using the Filter Paper Activity (FPA) assay (Ghose, 1987). Whatman No. 1 filter paper (50 mg, 1 × 6 cm strip; Whatman International, UK) was incubated for 60 min in 1 ml of 0.05 M Na-citrate buffer solution (pH 4.8) at 50 °C with an addition of 0.5 ml enzyme solution (supernatant). The reaction was stopped by adding 1 ml of DNS (3,5-dinitrosalicylic acid), according to Eveleigh et al. (2009). The released sugars were monitored spectrophotometrically (UV-1800 Spectrophotometer, Shimadzu, Japan). A calibration curve was established with glucose (POCH, Poland). Final cellulase activity was expressed in Filter Paper Units (FPU, [μmol × ml^{-1} × min^{-1}]), defined as the amount of enzyme which forms 1 μmol of glucose per minute under the assay conditions. All samples were analysed in triplicate. A control strain, *T. reesei* QM 9414, originating from the Czech Collection of Microorganisms at Masaryk University in Brno was used as a reference. This mutant strain, with high cellulolytic activity (0.30 μmol × ml^{-1} × min^{-1}) is used for industrial production of cellulases.

Bioassays in laboratory conditions

Dual cultures

The bioassay analysis was performed using 9 cm diameter Petri dishes containing 20 ml of PDA medium. For each pathogen-*Trichoderma* pairing, 5 mm diameter disks of pathogen and *Trichoderma* colonies were placed opposite each other at a distance of 1 cm from the edge of the plate. To allow the initial development of *Leptosphaeria* spp., the disks with the mycelium of *L. maculans* were placed at first (due to the slowest growth of this pathogen), the disks of *L. biglobosa* were placed on the medium four days later (this pathogen grows *ca.* 2.0–2.6 times faster than *L. maculans*), and the disks with *Trichoderma* were placed on the opposite site three more days later. As a result, the co-cultivation of the fungus pair *L. biglobosa-Trichoderma* spp. was shifted by three days and for the pair of *L. maculans* and *Trichoderma* spp. this time difference was seven days. Each variant of this 'shifted co-cultivation' (pathogen × antagonist) was replicated three times. Control plates contained single cultures of all tested fungi. Samples were incubated at 25 °C in the dark. Antagonistic effects on pathogen isolates was evaluated using a modified scale ranging from 0 to +8, where 0 means no inhibition and +8 indicates total inhibition of a pathogenic fungus by an antagonist (Popiel et al. 2008). Growth rate of each control culture was measured at daily intervals starting three days after inoculation.

Growth rate of fungal isolates treated with flusilazole

Fungal response to the flusilazole was analysed by measurement of surface area of the mycelium (*Leptosphaeria biglobosa*, *L. maculans*, *T. atroviride*, *T. hamatum*, *T. harzianum*, *T. longibrachiatum* and *T. koningii*). Morphological changes of the isolates were studied on PDA and Czapek-Dox media with additional flusilazole (3 mg l^{-1}) in simulated day/night conditions at 25 °C. In the final test, each assay was performed with ten replicates. The surface area of the mycelium was calculated as the area of an ellipse, based on measuring of the length and the width of the mycelium.

Decomposition of plant residues

Ten fragments of stems (10 cm long, 10 mm thick) of oilseed rape (cv. Californium) were soaked with water for 24 h, then drained and placed in plastic containers. Subsequently they were sprayed with 1 × 10^6 or 1 × 10^7 spores ml^{-1} of the particular isolate of *Trichoderma*, covered with the lid and kept at room temperature (±20 °C). The decomposition of stubble was described at one week time intervals, using a 0–9 scale, where 0 was no difference as compared to control and 9 was a full decomposition of stubble. The effect of *Trichoderma* was assessed by subtracting the decomposition values of control plants from corresponding values obtained from the plants treated with spore suspension. At each time-point of the

experiment the number of fungal fruiting bodies on the oilseed rape stubble was calculated.

Seedling bioassay in controlled environment conditions

Fungal spores were produced on V8-agar on 90 mm Petri plates incubated at 25 °C. Spore concentrations were adjusted to 1×10^7 spores ml^{-1} for inoculation. Oilseed rape plants were grown in four rows of 12 seedlings in 28 × 35 cm plastic trays on peat compost manufactured by Klassmann (pH 5.5–6.5). Inoculations were made on 12-day old plants, as described by Stachowiak et al. (2006). To perform the inoculation, each half-cotyledon was punctured with a needle. Spore suspensions of plant pathogens (10 µl droplets) were deposited directly onto each plant wound. After the inoculation, trays were covered with plastic lids and put in the darkness for 48 h and then transferred into a growth chamber with alternating 12 h periods at 24 °C (light)/16 °C (dark) at 70 % RH. Disease symptoms were scored 14 and 21 days after inoculation using a 0–6 rating scale. Every variant was studied in three replicates. Plant material consisted of an open pollinated cultivar of winter oilseed rape cv. Californium (Monsanto, USA), cv. Brise (Deutsche Saatveredelung AG) and cv. Bosman (Plant Breeding Strzelce Ltd).

Field experiments

Weather conditions during field experiments

Basic meteorological data in the five month periods, from July (harvest of the previous crop) to November (start of the winter pause) showed considerable differences between the summer-autumn seasons over the three years of the study. The weather in 2010 was the wettest, yet warm. The rainfall in the period lasting from mid July until the end of September, which is the usual time of pseudothecia formation, reached 263.2 mm, whereas in the other years it did not exceed 100 mm. 2009 was the hottest and driest year, the rainfall in the designated period was only 68.2 mm. The 2008 season was intermediate, the rainfall was sufficient and not excessive (98.8 mm) and (with the exception of October) the mean monthly temperatures did not exceed respective means of the other experimental years.

Decomposition of plant residues

Field studies were conducted over three seasons (2008/2009–2010/2011) at the Field Station of IPG PAS, located in Cerekwica, Poland (N 52°31′10.0″ E 16°41′33.0″) on plots of oilseed rape cv. Californium (Monsanto). The standard size of an individual plot was 1.5 × 2 m (3 m^2). The experiments were performed during the autumn seasons of 2008–2010. Standardised fragments of stubble (10 cm, 10 mm thick) of oilseed rape were kept in the field after harvest, with a density of 60 fragments per plot of 3 m^2. The stubble was sprayed with spore suspensions of five species of *Trichoderma* (1×10^6 spores or 1×10^7 spores per ml) and then kept in natural conditions and under plastic tents (1 m diameter). The number of viable fruiting bodies (pycnidia and pseudothecia) was evaluated at weekly intervals (0, 7, 14 and 21 days post inoculation). The assessment of pseudothecia was done based on the A–D developmental scheme, as proposed by CETIOM and used by Dawidziuk et al. (2012).

Leaf treatment with conidiospores of Trichoderma spp.

Seeds of the oilseed rape cultivar Californium (Monsanto) were sown on individual plots of 1.5 × 2 m size, with a sowing density of 50 plants per m^2, using 25 cm rows. Plants were subjected to natural infection with the ascospores of *L. maculans* and *L. biglobosa* released from infected stubble originating from the previous season. When the plants reached BBCH stage 16 they were sprayed with spore suspensions of the studied *Trichoderma* species (1×10^6 or 1×10^7 spores per ml). Half of the plots were then covered with transparent foil tunnels for seven days to examine the effect of a raised temperature. Each variant (year of experiment × *Trichoderma* species × spore density × temperature) was carried out with three replicates, measuring 30 leaf spots per replicate (width × length).

Statistical analysis

Statistical analyses comprised analyses of variance (ANOVA) and post-hoc means comparisons (Tukey–Kramer honestly significant difference [HSD]; $p \leq 0.05$) performed with the Statistica 9.0 software package (Stat Soft, USA). The differences in gene

expression between non-treated and treated samples were analysed with Wilcoxon signed-ranks non-parametric tests ($p \leq 0.05$) with the use of a one-tailed hypothesis. In RT-PCR reactions, the test was performed on the delta Ct values from the second day after fungicide exposure.

Results

Molecular identification of *Trichoderma* species

Based on the sequences of ITS1-5.8S rDNA-ITS2 region, ten *Trichoderma* isolates were ascribed to five species: *T. harzianum* Rifai (4 isolates), *T. atroviride* P. Karst (3), *T. hamatum* (Bonord.) Bainier (1), *T. koningii* Oudem (1) and *T. longibrachiatum* Rifai (1). All strains originating from plants of oilseed rape belonged to one of the three species: *T. harzianum*, *T. koningii* and *T. longibrachiatum.* The strains isolated from soil were identified as *T. atroviride* and the isolate obtained from yellow lupin belonged to *T. hamatum*. The sequences of the isolates were deposited in the NCBI Database (Table 1).

Growth rate of isolates

All *Trichoderma* species formed fast expanding colonies. The highest growth rate (29 mm day^{-1}) was observed for *T. atroviride* and *T. longibrachiatum*. The growth rate of *T. hamatum* and *T. harzianum* (27 mm day^{-1}) was followed by *T. koningii* (23 mm day^{-1}). The plant pathogens tested were among the slowest growing strains with growth rates of 3 mm day^{-1} for *L. biglobosa* and 1 mm day^{-1} for *L. maculans* (Table 3).

Dual culture bioassays

The growth of the pathogenic isolates in dual cultures with competitive *Trichoderma* species was significantly reduced in all tested combinations except *L. maculans* co-inoculated with *T. longibrachiatum*. The isolates of *T. atroviride* caused growth inhibition of all tested fungal strains whereas *T. hamatum*, *T. harzianum* and *T. koningii* were more efficient in the assays with the isolates of *L. biglobosa*. In the case of dual culture of *T. harzianum* and *T. longibrachiatum* with *L. maculans* an inhibition zone between the pathogen and the antagonist was observed, which suggests the existence of antibiosis (Table 3).

Seedling bioassay in controlled environment conditions

Protective abilities of antagonistic fungi against pathogenic *Leptosphaeria* isolates were examined with cotyledon tests. In the first experiment, young oilseed rape plants were co-inoculated simultaneously with the antagonistic fungi alongside the phytopathogens. The co-inoculation of *L. maculans* with *T. harzianum* and with *T. atroviride* resulted in decreased disease severity on cv. Brise (Fig. 1a). A similar effect was observed on cv. Californium co-inoculated with *L. maculans* and *T. longibrachiatum*. The effect of decreased symptom severity was also found for co-inoculation of *L. biglobosa* with *T. atroviride* and with *T. hamatum* on both oilseed rape varieties.

Three weeks after the treatment of oilseed rape seedlings with *Trichoderma* isolates, followed by inoculation with the pathogens *L. maculans* and *L. biglobosa*, the protective abilities of all tested *Trichoderma* species were observed, with the exception of *T. hamatum* (Fig. 1b). A decrease of disease symptoms occurred in seven out of 24 co-inoculation combinations. In most cases it was one score point less in the evaluation scale, compared to inoculation with conidia of the pathogen only. The highest inhibition of pathogen activity was observed in 'shifted' co-cultivation with *T. atroviride* pre-treatment. The use of this species resulted in a decrease of the disease symptoms observed on cultivar Brise inoculated with *L. maculans* and on cultivar Californium inoculated with *L. biglobosa*. No protective effect of *Trichoderma* was found on cv. Californium treated with *L. maculans*, but we have observed the activity of *T. longibrachiatum*, *T. harzianum* and *T. atroviride* against *L. biglobosa* (Fig. 1b).

Growth rate of fungal strains treated with flusilazole

Three species of *Trichoderma* were resistant to flusilazole treatment. The most resistant isolates of *T. harzianum* and *T. longibrachiatum* had their growth reduced by 10 %. In contrast the growth of *T. hamatum* was reduced by 40 %. Both the isolates of *Leptosphaeria* spp. and *Trichoderma* spp. were

Table 3 The properties of *Trichoderma* isolates used in this study

Trichoderma species	Isolate symbol	Growth rate (mm ± SE)[a]	Cellulolytic activities (FPU per min ± SE)[a]	Dual cultures[b] *Trichoderma-Leptosphaeria*	
				L. biglobosa	*L. maculans*
T. atroviride	TA1	29.01 ± 0.02 d	0.20 ± 0.017 abcd	+6	+6
	TA2	28.95 ± 0.13 d	0.17 ± 0.006 ab	+6	+6
	TA3	29.04 ± 0.18 d	0.22 ± 0.017 abcd	+6	+6
T. hamatum	TH7	27.07 ± 0.06 bc	0.18 ± 0.017 abc	+6	+5
T. harzianum	T13	26.83 ± 0.19 b	0.32 ± 0.012 ef	+5	+5[c]
	T4B	26.75 ± 0.19 b	0.28 ± 0.057 cdef	+6	+5[c]
	T3B	26.81 ± 0.17 b	0.37 ± 0.029 f	+6	+5[c]
	T1S	26.91 ± 0.07 b	0.21 ± 0.050 abcd	+6	+5[c]
T. koningii	TE2	22.90 ± 0.31 a	0.10 ± 0.000 a	+6	+5
T. longibrachiatum	TLA1	29.00 ± 0.16 d	0.21 ± 0.017 abcd	+5	+3[c]
T. reesei	QM 9414	–	0.30 ± 0.046 def	–	–

[a] Shared letters denote no statistical differences between *Trichoderma* isolates, with $p \leq 0.05$

[b] Scale range from 0 to +8, where 0 means no inhibition and +8 indicates total inhibition of a pathogenic fungus

[c] 1 mm inhibition zone between the pathogen and the antagonist

– not studied

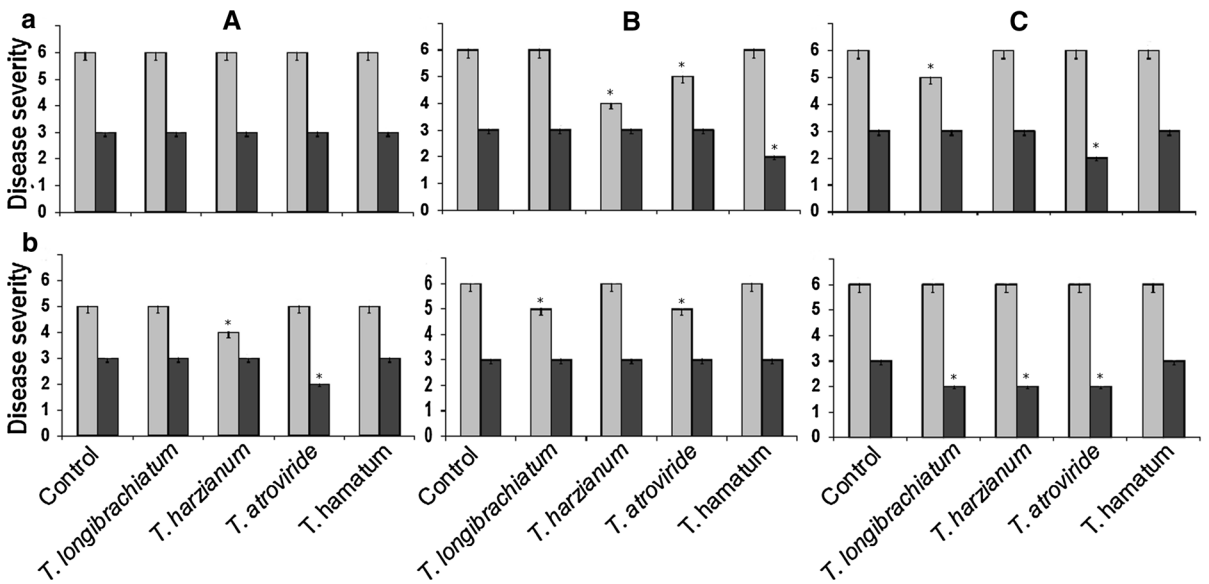

Fig. 1 Disease severity estimated: 14 days (**a**) and 21 days (**b**) after co-inoculation of *Leptosphaeria maculans* (*grey bars*) or *L. biglobosa* (*black bars*) with different *Trichoderma* species. Disease severity tested on three cultivars of oilseed rape: Bosman (*A*), Brise (*B*), and Californium (*C*). *Bars* indicate SE. *Asterisks* (*) mark statistical differences at $p \leq 0.05$ as compared to control

susceptible to flusilazole. However, the isolates of *T. hamatum*, *T. harzianum* and *T. longibrachiatum* were significantly more resistant to flusilazole than *T. atroviride*, *T. koningii* and both *Leptosphaeria* species.

Expression of sterol 14α-demethylase (CYP51A and CYP51B) in *Trichoderma* cultures treated with fungicides

As the growth rate of *T. harzianum*, *T. hamatum* and *T. longibrachiatum* was not reduced significantly in the presence of the flusilazole, the expression of sterol 14α-demethylases was examined. All tested genes exhibited increased activity in the presence of flusilazole. The strongest reaction was observed in *T. longibrachiatum* in which the relative normalised expression of both sterol 14α-demethylases was raised over two times (CYP51A—2.8 fold; CYP51B—2.5 fold) (Fig. 2a, b). The isolates of *T. harzianum* and *T. hamatum* have also exhibited up-regulated expression of both sterol demethylases, but the normalised expression did not exceed 2.5-fold increase. Increases in the expression of CYP51A and CYP51B genes were statistically significant at $p \leq 0.05$ in all tested samples except in the case of CYP51B gene in *T. harzianum*. The expression of each gene was normalised using two housekeeping genes: β-tubulin and actin (Table 2). For each primer set, efficiency values ranged between 97 and 102 % (Table 2), which corresponds to highly reliable and reproducible results.

Expression of the azole transporter AbcG5 in fungal cultures treated with fungicides

Degenerate, pan-species specific primers allowed amplification of parts of the AbcG5 gene and its possible orthologues in *T. harzianum*, *T. hamatum* and *T. longibrachiatum.* The orthologues of the AbcG5 in *T. harzianum*, *T. hamatum* and *T. longibrachiatum* were significantly upregulated in the presence of flusilazole ($p \leq 0.05$). The highest relative expression was found in *T. longibrachiatum* where the increase was 9.57-fold (Fig. 2c).

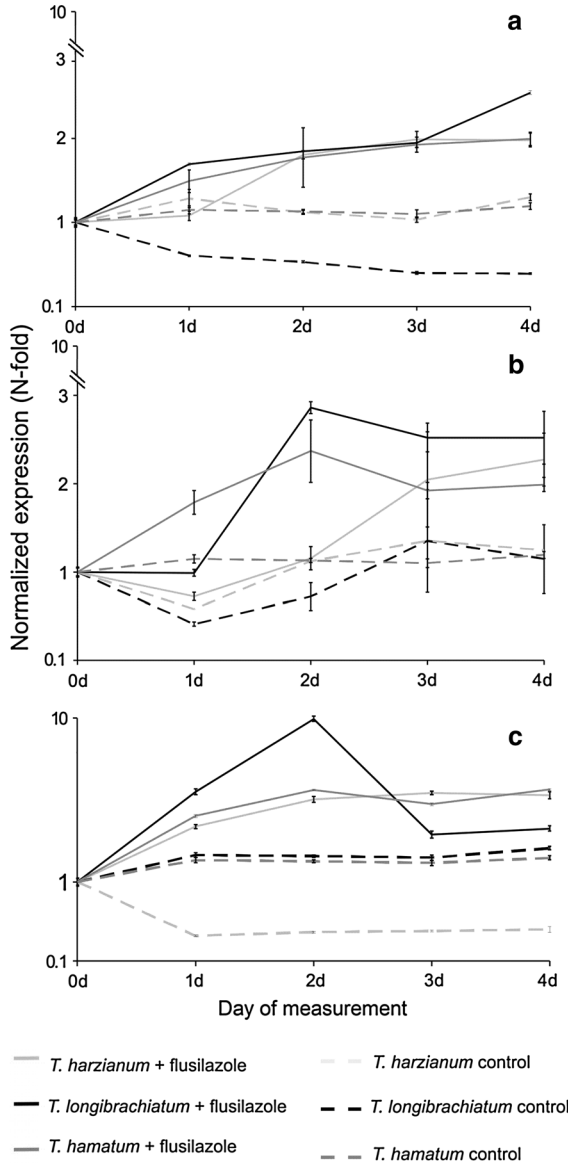

Fig. 2 Expression of CYP51A (**a**) CYP51B (**b**) and ABCG5 (**c**) genes in T. *harzianum*, *T. longibrachiatum* and *T. hamatum* isolates after flusilazole treatment. *Bars* indicate SE. Differences in gene expression were verified with Wilcoxon signed-ranks tests. One-tailed test performed on the second day after fungicide treatment (CYP51A-*T. harzianum* ($p = 0.0128$), *T. longibrachiatum* ($p = 0.0139$), *T. hamatum* ($p = 0.0178$); CYP51B-*T. harzianum* ($p = 0.1356$), *T. longibrachiatum* ($p = 0.0029$), *T. hamatum* ($p = 0.0027$); ABCG5-*T. harzianum* ($p = 0.0019$), *T. longibrachiatum* ($p = 0.0011$), *T. hamatum* ($p = 0.0012$))

Cellulolytic activity of *Trichoderma* strains

All tested isolates were able to degrade cellulose. However, the levels of decomposition efficiency measured by cellulase activity differed between isolates belonging to the same *Trichoderma* species and between the species. Among tested antagonistic strains, the highest specific cellulase activity (FPA) was observed for *T. harzianum* isolates (T3B—0.37 µmol × ml^{-1} × min^{-1} and T13—0.32 µmol × ml^{-1} × min^{-1}) and it was higher than in the control isolate *T. reesei* QM 9414. The lowest value of cellulolytic activity was noted in *T. koningii* isolate TE2 (0.1 µmol × ml^{-1} × min^{-1}). The rest of examined isolates belonging to *T. atroviride*, *T. hamatum* and *T. longibrachiatum* demonstrated medium levels of cellulolytic activity (Table 3).

Decomposition of plant residues in laboratory conditions

The fragments of stems were gradually decomposed by the species of *Trichoderma* and the speed of stubble decomposition was related to the growth rate of the isolates: the slowest being *T. koningii* while the other four tested species were faster. There was a great reduction in the number of fruiting bodies of *L. maculans–L. biglobosa* species complex from 60 to 38 on average (reduction of 36.7 %) at one week after inoculation. Two weeks after inoculation, the average number of fruiting bodies still visible on stems was further reduced by half. At the same time the number of fruiting bodies on stubble treated with the Czapek-Dox liquid medium was also slightly reduced. This reduction was only 13.4 % as compared to the start of the experiment. One week later, the reduction of the fruiting bodies of untreated stubble was 26.8 % but at the same time new pycnidia and the first pseudothecia (classes A–C) were formed on stems. The stubble of oilseed rape treated with different species of *Trichoderma* was nearly totally decomposed and only few pycnidia were still visible (3.3 pycnidia on average per stem fragment). The strongest effect was observed for *T. harzianum*, and the weakest for *T. koningii*.

Leaf treatment with conidiospores of *Trichoderma* spp.

The year of the experiment (weather conditions) and the density of spores in the suspension had a significant effect on the incidence and size of phoma leaf spots caused by *L. maculans–L. biglobosa* species complex (Fig. 3a, b). The most efficient were the sprays with *T. harzianum* and *T. atroviride*. Treatment of leaves by conidial suspension spray with any of *Trichoderma* species was ineffective in variants when the use of plastic covers raised temperatures (Fig. 3c).

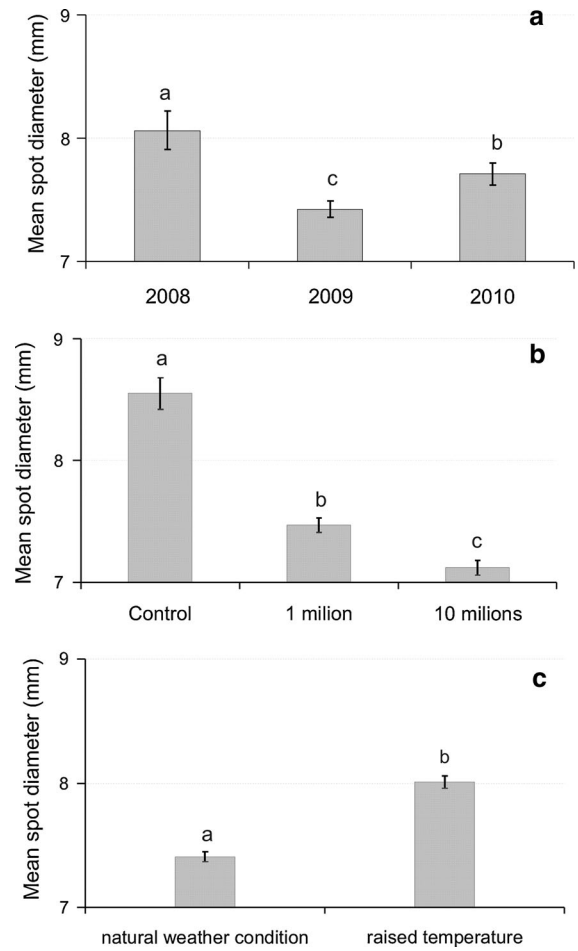

Fig. 3 Effect of leaf treatments with conidiospores of *Trichoderma* spp. in relation to: year (**a**), spore density (**b**), temperature (presence of the plant cover) (**c**). *Shared letters* in each panel denote no statistical differences at $p \leq 0.05$ (*a*: $F_{2,\ 6477} = 21.9$, $p < 0.001$; *b*: $F_{2,\ 6477} = 141.95$, $p < 0.001$; *c*: $F_{1,\ 6477} = 65.36$, $p < 0.001$). *Bars* indicate SE

Discussion

Our studies demonstrated that fungal species of the *Trichoderma* genus potentially can help to control stem canker of oilseed rape. The most effective was *T.*

harzianum, which not only reduced the growth of oilseed rape pathogens in dual cultures, but also exhibited the inhibition zone, which is a typical symptom of antibiosis. This species was also most resistant to flusilazole—an active substance of azole fungicides. We have observed an increased expression of sterol 14α-demethylases and the AbcG5 azole transporter. In the seedling bioassays we observed that *T. harzianum* most effectively reduced symptoms of the disease, similarly to the results of dual culture tests. In some species desired traits were less consistently observed. For example, the results of the in vitro assays using *T. longibrachiatum* were discouraging, but the isolate of this species was the most resistant to an azole fungicide. In the presence of flusilazole the orthologues of the AbcG5 transporter in *T. longibrachiatum* were upregulated with the highest relative expression (nearly ten-fold). The isolates of *T. atroviride* were fast growing and caused growth inhibition of all tested fungal strains. However they were susceptible to the fungicide.

An additional advantage of *Trichoderma* species as a biocontrol measure is their resistance to azole fungicides (Khan and Shahzad 2007). This enables the simultaneous use of biological and chemical protection. Reduced amount of fungicides can weaken the pathogen and increase their susceptibility to attack by the resistant antagonist (Hjeljord and Tronsmo 1998). Degenerate, pan-species specific primers, designed by us for the purpose of this study, facilitated the amplification of parts of the *AbcG5* gene and its possible orthologues. Under our experimental conditions *L. maculans* and *L. biglobosa* were susceptible to flusilazole, which is one of the most common and efficient components of chemical treatments (Alert®, Capitan®) against phoma stem canker (Kaczmarek et al. 2014b). In our study the isolates of *T. harzianum*, *T. hamatum* and *T. longibrachiatum* were resistant towards this compound. Due to the common recognition of the main molecular mechanism of fungal resistance to the fungicide, the experiments we performed offered an explanation of this phenomenon at the molecular level. The reasons were sequence differences in sterol demethylase gene or upregulation of this gene. It is very likely that this additional quantitative resistance is a multidrug resistance (MDR). Literature data concluded that azole resistance was conditioned by changes in gene *CYP51A*, whereas *CYP51B* gene was associated primarily with the production of ascospores, and *CYP51C*, found only in fungi of the *Fusarium* genus, was associated with the virulence of strains (Fan et al. 2013). In our experiments we observed significant increases in the expression of *CYP51A* and *CYP51B* genes. Sequence differences at the CYP51A loci may explain differences in flusilazole resistance between the *Trichoderma* isolates. Additionally, the exposure to flusilazole treatment led to increased expression of *AbcG5* gene in all resistant *Trichoderma* isolates. Based on the Ensembl Fungi programme (Kersey et al. 2010), the orthologue of this gene is a potential azole ABC transporter (Dubey et al. 2014).

The reduction of the disease symptoms varied greatly between different cultivars of oilseed rape tested. Despite the fact there were no considerable differences of the resistance towards the pathogens, the most favourable results of *Trichoderma* biocontrol were obtained for the cultivar Brise and the smallest effect was observed in cv. Bosman. The solution of this problem might be the use of a mixture of antagonistic isolates, compensating for differences associated with cultivars, connected with the rate of their development in the autumn, plant morphology (especially the number, size and shape of leaves), genetic resistance to stem canker and other characteristics. The number of fruiting bodies on plant stubble treated with *Trichoderma* rapidly decreased, as the stubble decomposed. Natural decomposition of the stubble and fruiting bodies was also observed when the stubble was moistened with the Czapek-Dox liquid medium. The rate of this process was much slower than with *Trichoderma*. It was connected with the activity of numerous fungi present in the air, such as *Cladosporium* spp., *Alternaria* spp., *Penicillium* spp. and others.

Our observations are in accordance with the results of previous studies, showing that chemical treatments are not the only way to combat plant pathogens (Chet 1987). Another approach is to apply antagonistic organisms as biological control agents. In case of the diseases of apples, carrots and peas, caused by *Rhizoctonia*, *Sclerotinia* and *Fusarium* species, effective control agents belonged to the genus *Trichoderma* as well as *Gliocladium* (Hjeljord and Tronsmo 1998). Among them *T. harzianum* is considered to be one of the most important species in biocontrol (Blaszczyk et al. 2014). This organism is commonly used to prevent the development of several soil pathogenic

fungi (Roco and Perez 2001). The first commercialised biocontrol product contained an isolate of *T. harzianum*. It was registered for greenhouse crops and vineyards to control grey mould caused by *Botrytis cinerea* (Elad 2000).

At present, a considerable number of biocontrol products are used against *S. sclerotiorum* (Hjeljord and Tronsmo 1998). Antagonists, such as *Coniothyrium minitans* are effective mycoparasitic fungi controlling white mould (Li et al. 2006). The process of antibiosis caused by *Pseudomonas* spp. inhibited the germination of *S. sclerotiorum* ascospores (Savchuk and Fernando 2004). Although much is known about biocontrol of other potential oilseed rape pathogens, there is no sufficient information about the influence of antagonistic fungi on *Leptosphaeria* spp. The only published studies related to *Bacillus amyloliquefaciens* (Danielsson et al. 2007) and *T. asperellum* (Kowalska and Remlein-Starosta 2011).

The most important element of biological protection against plant pathogens is the efficiency in field conditions. In our experiments, the spray with *Trichoderma* conidiospores caused a statistically significant decrease of the disease incidence and severity. Moreover, the treatment with *Trichoderma* accelerated the degradation of the stems of oilseed rape, which was explained by high cellulolytic activity of these strains. In some cases it was higher to a standard isolate of *T. reesei*, which is commercially used to produce cellulases (Blaszczyk et al. 2014). There was also a significant reduction of the number of fruiting bodies of *L. maculans–L. biglobosa* species complex, both pycnidia and pseudothecia. Therefore, although the treatment had a relatively weak impact on the disease symptoms, it caused significant reduction of the primary inoculum. The results are partially in agreement with the effect of *T. asperellum* introduced in a commercial product, Trifender WP, to control *Botrytis cinerea*, *Alternaria* spp. and *Phoma lingam* in oilseed rape (Kowalska and Remlein-Starosta 2011). In the study performed by these authors, the treatment was done in stages BBCH61 and BBCH67 and the protection effectively limited symptoms of oilseed rape diseases and prevented the reduction of seed yield.

The proper use of biocontrol, such as the application of an adequate mixture of antagonistic isolates, resistant to chemical treatments, combined with agrotechnical methods and supplemented with decision support systems, can eliminate the most harmful pesticides and, as such, it can help to reduce the pollution of the environment. Future research should concentrate on biocontrol agents which not only directly influence the development of the pathogen in plant tissues but are also capable of reducing primary inoculum.

Acknowledgments Research partially funded under the project: "Molecular mechanisms of multidrug resistance to synthetic fungicides in fungi of the *Fusarium* genus" UMO-2011/03/D/NZ9/02061. The authors thank to the Czech Collection of Microorganisms at the Masaryk University in Brno for the mutant strain *T. reesei* QM 9414 with increased production of cellulases. The revision of the manuscript was done with great help of Dr. Magdalena Sadys from Rothamsted Research, UK and Dr. William Truman from the Institute of Plant Genetics of the Polish Academy of Sciences, Poland.

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Adam Dawidziuk received a PhD at the Institute of Plant Genetics of the Polish Academy of Sciences (IPG PAS) in Poznan, on modelling of the life cycle of oilseed rape (OSR) pathogens. Currently he is working in the Department of Pathogen Genetics and Plant Resistance (DPG&PR) on functional evolution of transmembrane transporters.

Delfina Popiel has completed her PhD at the Institute of Plant Genetics of the Polish Academy of Sciences. Her PhD dissertation concerned the biological role, biocontrol potential and genetic diversity of *Trichoderma* strains obtained from decaying wood. Her work as a post-doc at DPG&PR concerns research on molecular mechanisms of fungicide resistance.

Joanna Kaczmarek received a PhD at IPG PAS on plant-pathogen interactions between oilseed rape and fungi causing stem canker. Currently her work at DPG&PR concentrates on quantitative detection of pathogens in air, soil and plant tissues.

Judyta Strakowska has completed her PhD at IPG PAS on biochemical characters and genetic diversity of *Trichoderma* strains. Part of her work was studies on enzymatic activities of fungi. She is currently employed as a post-doc at the Division of Biology and Protection of Agriculture and Forestry Systems, Department of Agriculture, the University of Naples, Italy.

Malgorzata Jedryczka is a full professor in agronomy (since 2013), the head of the DPG&PR at IPG PAS. Her work concerns the identification and characterization of fungi and plant resistance to pathogens using classical and molecular tools. The main models in her work are fungal pathogens of OSR. She is involved in creating decision support systems in crop plants.