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In vitro interactions between *Trichoderma harzianum* and pathogenic fungi damaging horse-chestnut (*Aesculus hippocastanum*) leaves and fruits

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Abstract: Interactions between 3 pathogenic fungi damaging horse-chestnut (*Aesculus hippocastanum*) leaves and fruits – *Phyllosticta sphaeropsoidea*, *Phomopsis carposchiza*, and *Diaporthe padi* – and the antagonistic fungus *Trichoderma harzianum* were studied to determine their mutual influence *in vitro*. Antibiosis of colonies developing on 5 nutrient media was tested. The 3 studied *T. harzianum* isolates differed in their antagonistic potential. Although *T. harzianum* isolates significantly inhibited the growth of *Phomopsis carposchiza*, the mycelium growth of some of the re-isolates on fresh medium indicates an inadequate antagonistic effect of *T. harzianum* on this species. The tested *Trichoderma* isolates showed stronger antagonism towards the other pathogens, reflected in overgrowing of *Phyllosticta sphaeropsoidea* and *Diaporthe padi* and reducing their growth. Granulation of the cytoplasm and lysis of hyphae of the fungal pathogens were the most frequently observed effects of the interaction.

Keywords: *Aesculus hippocastanum*, fungal pathogens, antagonist, dual cultures, hyphal interaction, *Trichoderma harzianum*, *Phyllosticta sphaeropsoidea*, *Phomopsis carposchiza*, *Diaporthe padi*

INTRODUCTION

Horse-chestnut (*Aesculus hippocastanum* L.) is very widely planted as an amenity tree across Europe. Extracts from its leaves, flowers, bark, and seeds have been used in many herbal medicines for centuries and more recently also in cosmetics (Wilkinson & Brown 1999; Dudek-Makuch & Studzińska-Sroka 2015). The species is affected by the fungal pathogens *Guignardia aesculi* (Peck) V.B. Stewart (asexual stage *Phyllosticta sphaeropsoidea* Ellis & Everh.) and *Diaporthe padi* G.H. Otth (asexual stage *Phomopsis carposchiza* Fairm.), causing leaf blotch and fruit rot disease, respectively. The application of fungicides to large horse-chestnut trees

growing in urban green areas is difficult and may cause serious ecological problems along with environmental and public health hazards, but biocontrol offers alternatives or supplements to the use of conventional methods for plant disease management. Many studies have proved the potential of antagonistic fungi as biocontrol agents of numerous plant pathogens (Elad et al. 1983; Zimand et al. 1996; Calistru et al. 1997; Padmaja et al. 2013). The fungus *Trichoderma harzianum* Rifai, producing antifungal metabolites, is an antagonist or competitor to plant pathogenic fungi that cause not only root rot but also fruit and foliage diseases (Harman et al. 1996; Küçük et al. 2007). *Trichoderma* spores are the basis of commercial products used in biological protection against plant pathogenic fungi (Woo et al. 2014). According to Lo et al. (1997), *Trichoderma* has the ability to survive on the phylloplane, and this is a desirable trait for potential antagonists used as biocontrol agents against foliar diseases. There have been no studies on microorganisms antagonistic to fungal pathogens of horse-chestnut so far. For this reason, the aim of this study was to verify the effectiveness of *T. harzianum* as a potential antagonist against important horse-chestnut pathogens: *G. aesculi* with its asexual stage *Phyllosticta sphaeropsoidea*, and *D. padi* with its asexual stage *Phomopsis carposchiza*. The antagonistic potential or tolerance between *T. harzianum* and horse-chestnut pathogens was investigated *in vitro*.

MATERIAL AND METHODS

Fungal isolates

The fungal pathogens used in this study – *Phyllosticta sphaeropsoidea*, *Phomopsis carposchiza*, and *Diaporthe padi* – were isolated from infected leaf and fruit tissue of *Aesculus hippocastanum* and maintained on potato dextrose agar (PDA). *In vitro* isolation of *Guignardia aesculi* (the sexual stage of *Phyllosticta sphaeropsoidea*) was unsuccessful. The potential antagonistic fungus used was *Trichoderma harzianum*: 2 isolates (Th01, Th02) from roots of horse-chestnut seedlings, and isolate Th03 from soil, provided by Michal Ondřej (Agritec Ltd., Šumperk, Czech Republic). The fungal isolates were identified on the basis of their morphological characteristics, using taxonomic manuals (Rifai 1969; van der Aa 1973; Ellis & Ellis 1985; Uecker 1988).

Media and dual culture tests

Initially, radial growth rates of 3 fungal pathogens and *T. harzianum* were determined by inoculating centrally 3 replicate Petri dishes of 5 nutrient media: PDA, carrot agar (CA), 2% water agar (WA), Czapek-Dox agar (CDA), and malt extract agar (MEA) (Biomark Lab., Sigma), with a 5 mm diameter disc of each fungus taken from the growing margin of cultures on PDA plates. The growth (in mm) of the fungal isolates was recorded every day.

The growth and interactions between horse-chestnut fungi and *T. harzianum* isolates were examined on each of the 5 tested media in 2 variants of dual cultures (Fig. 1). In variant I, a mycelial disc of the fungal pathogens and a mycelial disc of *T. harzianum* were seeded opposite each other near the periphery of the plate. In variant II, 4 mycelial discs of *T. harzianum* isolate were seeded at 4 equidistant points

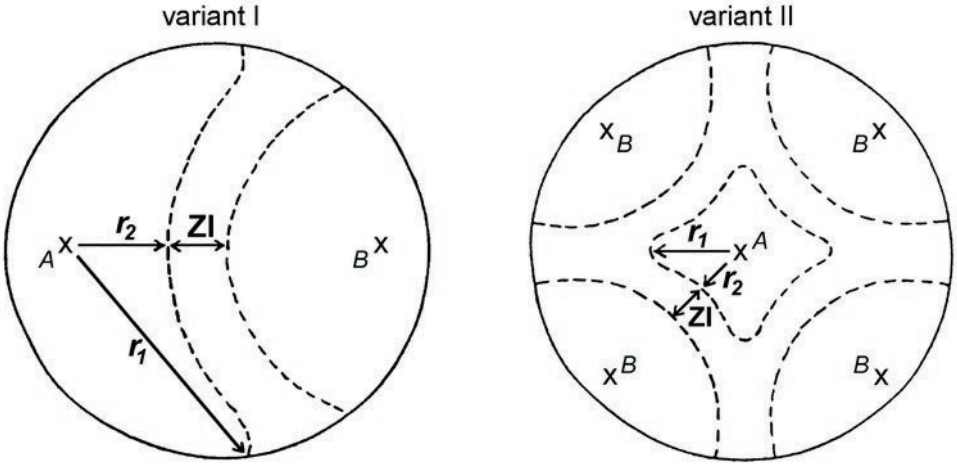

Fig. 1. Models of inoculation of a fungal pathogen (*A*) and potential antagonist (*B*) on agar plates, explaining the parameters of inhibition: width of the zone of inhibition (ZI) and percentage inhibition of radial growth of the fungal pathogen, IRG (%) = $[(r_1 - r_2)/r_1] \times 100$, according to Royse & Ries (1978) and Dhingra & Sinclair (1995)

near the periphery of the plate and a mycelial disc of a fungal pathogen was seeded in the centre of the culture plate (according to Dhingra & Sinclair 1995). Due to the different growth rate of the fungal pathogens, *P. sphaeropsoidea* (slow-growing species) was seeded 5 days prior to seeding with *T. harzianum* isolates, whereas *P. carposchiza* and *D. padi* (fast-growing), only 1 day prior to seeding with the potential antagonist. Each combination was replicated 5 times. The plates were incubated in a growth chamber at 22°C.

Antagonistic activity

Inhibition of radial growth of the fungal pathogens in dual cultures was evaluated by 2 parameters: (i) percentage inhibition of radial growth (IRG), calculated using the formula: IRG (%) = $[(r_1 - r_2)/r_1] \times 100$, where r_1 is the control value, i.e. the furthest radial distance grown by the fungal pathogen, and r_2 is the inhibition value, i.e. the distance grown on the line between the inoculation position of the fungal pathogen and the antagonist (Fig. 1); and (ii) width of the zone of inhibition ZI (mm), according to Royse & Ries (1978). The above parameters were evaluated after 14 days of cultivation.

The type of colony interactions of the examined fungi was assessed visually based on modified methods described by Skidmore & Dickinson (1976), Chand & Logan (1984), and Whipps (1987). The interacting colony growth was classified as mutually intermingling growth (type 1/2 and 2/1), overgrowth by antagonist or fungal pathogens (type 1 and 2, respectively), mutual slight inhibition (type 3), or mutual inhibition at a distance (type 4).

Hyphal interference was observed under a standard light microscope (BX51, Olympus, Tokyo, Japan). On the day of colony contact, a 20 mm × 20 mm square of agar from the interaction zone or overlap region was removed with a scalpel and put on a slide. Mycelium on the surface of the agar was stained with lactophenol blue solution (Merck, Darmstadt, Germany) and a cover glass was placed on top. The following signs of antibiosis were searched for: coiling of the antagonist on the surface of the fungal pathogen, penetration of the hyphae of the fungal pathogen by the antagonist, granulation and vacuolation of hyphae of the fungal pathogen, abnormal branching of its hyphae, and lysis of its hyphae.

The effect of antagonistic activity of *T. harzianum* was verified by re-isolation of fungal pathogens on fresh medium (PDA). Re-isolates were evaluated after 10 days of cultivation.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) and the Tukey HSD (honestly significant difference) test at $p = 0.05$, using STATISTICA version 10 software (StatSoft, Inc., 2011).

RESULTS

Growth rates

The growth rates of the mycelia of the fungal pathogens and 3 isolates of the potential antagonist on 5 nutrient media (PDA, CA, WA, CDA, MEA) varied significantly ($p = 0.05$) according to the Tukey test (Table 1). *Phyllosticta sphaeropsoidea*, *Phomopsis carposchiza* and *Diaporthe padi* had maximum growth rates on CA, while all isolates of *Trichoderma harzianum* had maximum growth rates on PDA. The minimum daily growth of mycelium of all fungi was observed on WA. Fungal pathogens grew generally slower on any given medium than isolates of *T. harzianum* did (Fig. 2).

Table 1. Mean radial growth rates of pathogenic and antagonistic fungi

Fungus	Mean radial growth rate (mm/day)				
	PDA	CA	WA	CDA	MEA
Phyllosticta sphaeropsoidea	1.8 a	2.6 a	1.2 b	1.6 c	1.3 c
Phomopsis carposchiza	10.1 b	16.1 b	9.2 a	9.3 a	10.7 b
Diaporthe padi	14.5 c	18.9 c	6.9 c	11.6 a	12.5 b
Trichoderma harzianum Th01	28.8 d	28.0 d	9.0 a	27.4 b	22.3 a
T. harzianum Th02	41.0 f	40.5 f	12.3 d	25.7 b	22.9 a
T. harzianum Th03	38.8 e	37.5 e	14.9 e	30.5 d	23.7 a

Media: PDA = potato dextrose agar; CA = carrot agar; WA = water agar; CDA = Czapek-Dox agar; MEA = malt extract agar. Values are means of 3 replicates. Means followed by the same letter within columns do not differ significantly at $p < 0.05$ (Tukey test).

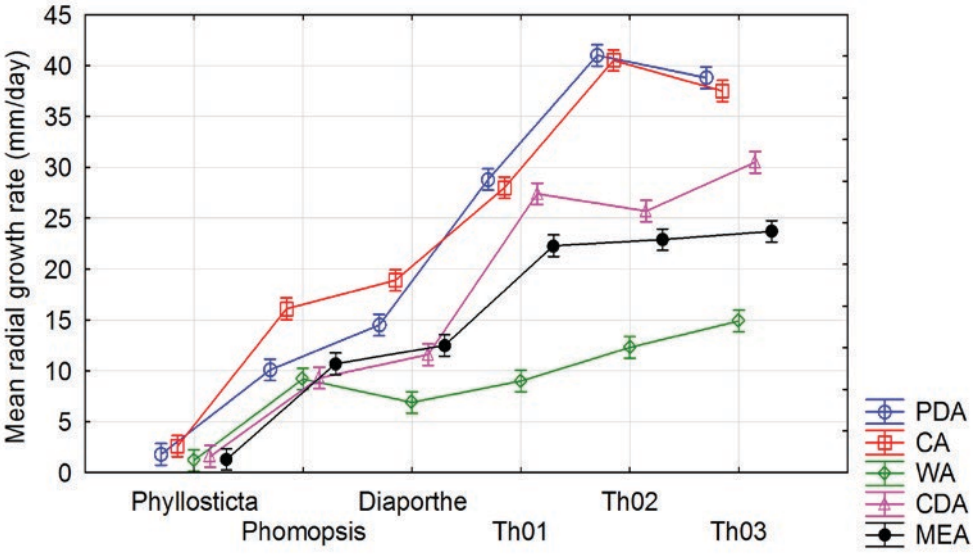

Fig. 2. Radial growth rates of fungal pathogens and *Trichoderma harzianum* isolates (Th01–Th03) in dual cultures on 5 nutrient media (PDA = potato dextrose agar; CA = carrot agar; WA = 2% water agar; CDA = Czapek-Dox agar; MEA = malt extract agar). Values are means of 3 replicates; vertical bars represent 95% confidence intervals

Colony interactions in dual cultures

Mean percentage inhibition of radial growth of horse-chestnut fungi in dual cultures (Tables 2 and 3, Fig. 3) varied significantly between *Trichoderma* isolates ($F_{4,180} = 24.289$, $p < 0.001$) due to markedly higher inhibition by isolate Th03. Medium also had a significant effect on the inhibition of radial growth of pathogens ($F_{8,180} = 63.266$, $p < 0.001$, Fig. 4). On PDA and CA, the greatest inhibition of *Diaporthe* growth was found. A maximum mean inhibition of 36% was reached with isolates Th01 and Th03. On PDA, *Phomopsis* was consistently inhibited by all *T. harzianum* isolates. On WA, all *Trichoderma* isolates were able to inhibit *Phyllosticta*, as the antagonist grew superficially over its colony and inhibited its growth by up to 20%. Sporadically, an inhibition zone was created around the colony of *P. sphaeropsoidea* (4.5 mm wide on CDA, variant II). Mutual and extreme inhibitions were found in the *Phomopsis–Trichoderma* combination, as *T. harzianum* produced the largest inhibition zones (8.5 mm) when grown in dual cultures with *P. carposchiza* on MEA. The average inhibition of radial growth of *P. carposchiza* ranged from 14% to 36% for variant I, and from 5% to 15% for variant II.

The growth of all fungal pathogens was more inhibited when inoculated in variant I than in variant II (Fig. 5). The interactions observed between *T. harzianum* and fungal pathogens are summarized in Table 4. For the majority of dual cultures, different types of interactions between paired fungi on all media were recorded: mu-

Table 2. Percentage inhibition of radial growth of fungal pathogens in dual cultures with *Trichoderma harzianum* isolates (Th01–03), variant I

Potential antagonist	*Phyllosticta sphaeropsoidea*					*Phomopsis carposchiza*					*Diaporthe padi*				
	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA
IRG (%)															
Th01	0	14	13	-5	0	23	29	36	14	26	36	25	8	23	30
Th02	0	0	13	8	0	24	21	21	15	23	27	34	21	24	27
Th03	20	0	11	0	-10	29	19	21	16	23	36	32	33	14	30
ZI (mm)															
Th01	0	0	0	0	0	3	0	1	6.5	0	0	0	0	0	0
Th02	0	0	0	0	0	2	2.5	0	0	6.5	0	0	0	0	0
Th03	0	0	0	0	0	4	2	2	0	8.5	0	0	0	0	0

IRG = percentage inhibition of radial growth of fungal pathogen; ZI = width of zone of inhibition. Media: PDA = potato dextrose agar; CA = carrot agar; WA = water agar; CDA = Czapek-Dox agar; MEA = malt extract agar. Values are means of 5 replicates.

Table 3. Percentage inhibition of radial growth of fungal pathogens in dual cultures with *Trichoderma harzianum* isolates (Th01–03), variant II

Potential antagonist	*Phyllosticta sphaeropsoidea*					*Phomopsis carposchiza*					*Diaporthe padi*				
	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA
IRG (%)															
Th01	0	7	0	3	7	13	10	7	10	6	28	31	0	12	13
Th02	0	0	0	0	0	14	5	13	11	8	20	23	12	10	30
Th03	13	0	7	0	14	13	14	7	15	6	19	20	21	17	14
ZI (mm)															
Th01	0	0	0	4.5	0	2	0	1	0	0	0	0	0	0	0
Th02	0	0	0	0	0	2	0	0	0	5	0	0	0	0	0
Th03	0	0	0	0	0	4	0	0	0	3	0	0	0	0	0

IRG = percentage inhibition of radial growth of fungal pathogen; ZI = width of zone of inhibition. Media: PDA = potato dextrose agar; CA = carrot agar; WA = water agar; CDA = Czapek-Dox agar; MEA = malt extract agar. Values are means of 5 replicates.

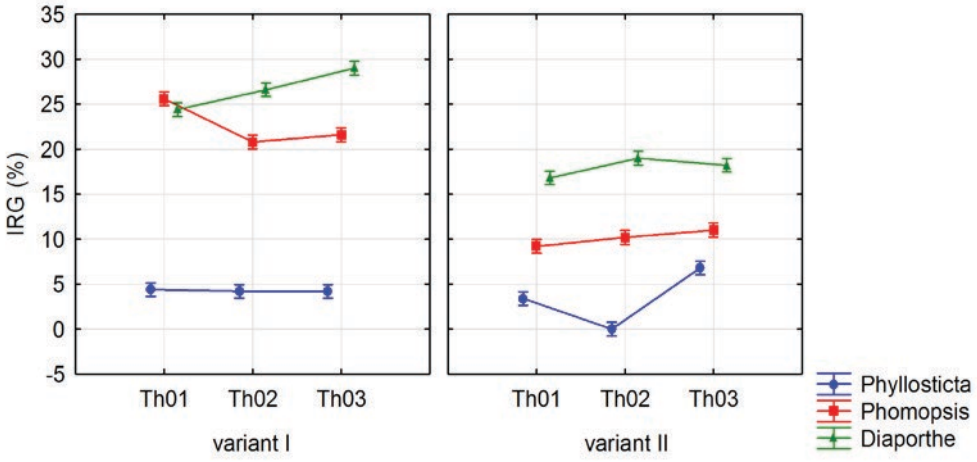

Fig. 3. Influence of *Trichoderma harzianum* isolates (Th01–Th03) on the percentage inhibition of radial growth (IRG) of fungal pathogens. Values are means of 5 replicates; vertical bars represent 95% confidence intervals

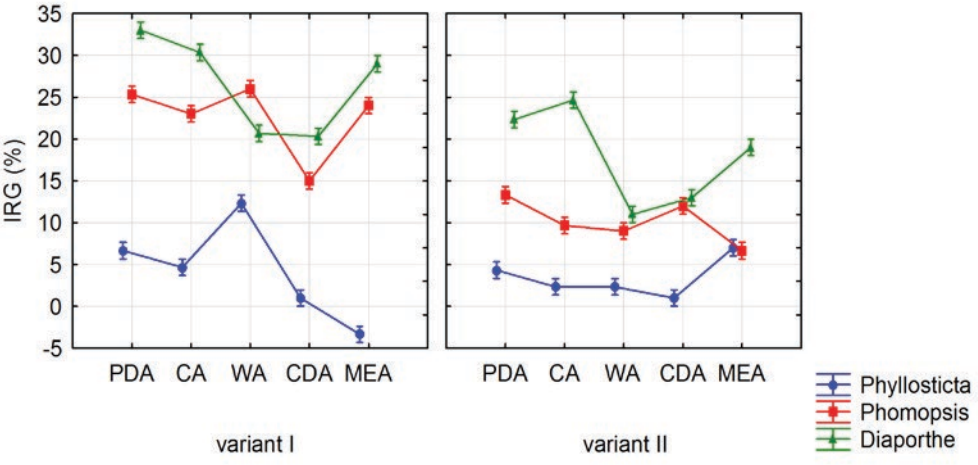

Fig. 4. Influence of 5 nutrient media (PDA = potato dextrose agar; CA = carrot agar; WA = 2% water agar; CDA = Czapek-Dox agar; MEA = malt extract agar) on the percentage inhibition of radial growth (IRG) of fungal pathogens in dual cultures with *Trichoderma harzianum* isolates. Values are means of 5 replicates; vertical bars represent 95% confidence intervals

Table 4. Colony interactions between fungal pathogens and *Trichoderma harzianum* isolates (Th01–03) in dual cultures

Potential antagonist	*Phyllosticta sphaeropsoidea*					*Phomopsis carposchiza*					*Diaporthe padi*				
	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA
Th01	1/2	1	1	1/2	1	3	1	3	4	1	1	1	1	1/2	1
Th02	1/2	1	1	1/2	1	3	3	1	1	4	1	2/1	1	2/1	1
Th03	1/2	1	1	1/2	1	3	3	3	1	4	1	1	1	1	1

Media: PDA = potato dextrose agar; CA = carrot agar; WA = water agar; CDA = Czapek-Dox agar; MEA = malt extract agar. Colony interactions: 1 = antagonist overgrowing fungal pathogen and fungal pathogen stopped; 1/2 = antagonist overgrowing fungal pathogen but fungal pathogen still growing; 2/1 = fungal pathogen overgrowing antagonist but antagonist still growing; 3 = mutual inhibition (about 2 mm distance) or contact inhibition; 4 = extreme inhibition, >4 mm distance (according to Whipps 1987).

Table 5. Microscopic effects on hyphae of fungal pathogens, caused by *Trichoderma harzianum* (isolates Th01–03) during growth in dual cultures

Potential antagonist	*Phyllosticta sphaeropsoidea*					*Phomopsis carposchiza*					*Diaporthe padi*				
	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA
Th01	*l*(*g*)	*Lgc*	*Cpg*	*LGc*	*Glp*	*Glo*	*Clg*	–	*Lg*	*lg*	*lgo*	*Glo*	*LGc*	*LGp*	*Gl*
Th02	–	*g*	*G*(*c*)	*lg*(*c*)	*g*(*c*)	*lg*	*C*(*l*)	*gl*	*LG*	*lg*(*c*)	–	*Clo*	*Glc*	*clg*	*Gp*(*c*)(*l*)
Th03	*Lpg*	*L*	*LG*	*Gpl*	*Gl*	*Lp*	*LCg*	*Gl*	*g*	*lg*	*LG*	*cgl*	*gcl*	*GCL*	*Glp*(*c*)

Media: PDA = potato dextrose agar; CA = carrot agar; WA = water agar; CDA = Czapek-Dox agar; MEA = malt extract agar. Interactions observed on day after contact of colonies: *c* = coiling on surface of fungal pathogen by antagonist; *p* = penetration of hyphae by antagonist; *g* = granulation and vacuolation of cytoplasm of fungal pathogen hyphae; *o* = abnormal branching of fungal pathogen hyphae; *l* = lysis of fungal pathogen hyphae; – = no effect observed. Frequency of occurrence is indicated by letters in: UPPER CASE = very frequent; lower case = frequent; parentheses = occasional.

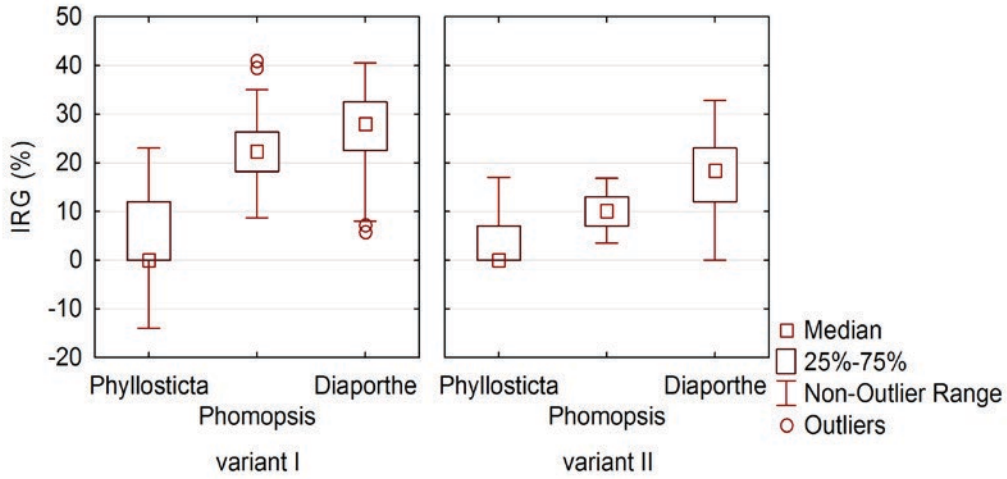

Fig. 5. Percentage inhibition of radial growth (IRG) of fungal pathogens in variants I and II

tual inhibition, extreme inhibition, overgrowing, and growth around (Figs. 6 and 7). Some contrasting interactions were noticed in experiments with one pathogen, e.g. *Trichoderma* isolates Th01 and Th03 showed antagonism by overgrowing *Diaporthe padi* and reducing its growth, but *Diaporthe* overgrew isolate Th02, although *Trichoderma* was still growing.

Antagonism between 2 organisms was recorded when the pathogen stopped growing upon contact with the antagonist and its hyphae begun to lyse back, whereas the antagonist continued its growth over the fungal pathogen colony. On some plates, both organisms stopped growing upon contact, with a small but clearly marked space

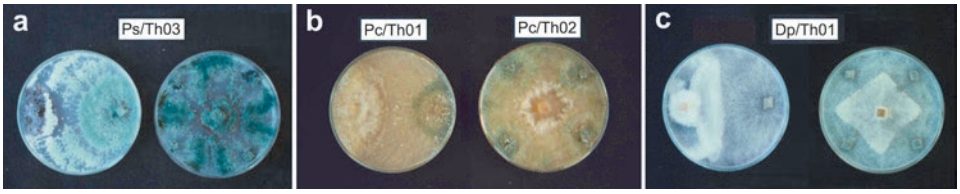

Fig. 6. Interaction of horse-chestnut fungal pathogens and *Trichoderma harzianum* isolates in dual cultures (variants I and II): a = *Phyllosticta sphaeropsoidea* (Ps) with *T. harzianum* (Th03) on potato dextrose agar, interaction type 1/2; b = *Phomopsis carposchiza* (Pc) with *T. harzianum* (Th01, Th02) on carrot agar, variant I with interaction type 1, variant II with interaction type 3; c = *Diaporthe padi* (Dp) with *T. harzianum* (Th01) on potato dextrose agar, interaction type 1

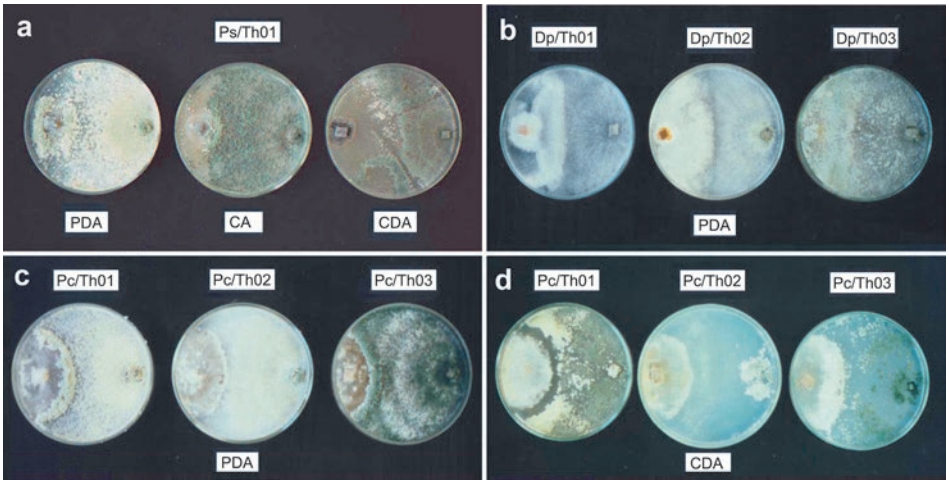

Fig. 7. Interaction of horse-chestnut fungal pathogens and *Trichoderma harzianum* isolates in dual cultures (variant I): a = *Phyllosticta sphaeropsoidea* (Ps) with *T. harzianum* (Th01), interaction types 1 and 1/2; b = *Diaporthe padi* (Dp) with 3 isolates of *T. harzianum*, interaction type 1; c, d = *Phomopsis carposchiza* (Pc) with 3 isolates of *T. harzianum*, interaction types 1 and 3. Media: PDA = potato dextrose agar, CA = carrot agar, CDA = Czapek-Dox agar

between them. The reduced growth rate of the pathogen can indicate either nutrient shortage or production of antifungal metabolites by the antagonist.

Microscopic effects of Trichoderma on hyphae of fungal pathogens

All antagonistic effects of *T. harzianum* on hyphae of the fungal pathogens in dual cultures are given in Table 5. Granulation and vacuolation of the cytoplasm as well as lysis of hyphae of the fungal pathogens were the most frequently observed effects of interaction on all media. *T. harzianum* was not parasitized by any fungal pathogen on any medium.

No growth of *Phyllosticta sphaeropsoidea* and *Diaporthe padi* re-isolated from dual cultures to fresh PDA medium has been recorded (Table 6). The growth of 2 re-isolates of *Phomopsis carposchiza* on fresh medium indicates inadequate antagonistic effect of *T. harzianum* on that species.

DISCUSSION

Antagonists of plant pathogenic fungi have been used to manage plant diseases and 90% of such applications have been carried out with *Trichoderma* species (Monte 2001). In my experiments, the *T. harzianum* isolates differed in their antagonistic potential. Similarly, variation in isolate effectiveness was also observed by Bell et al. (1982) and Whipps (1987). Results of my study indicate that the antagonistic effect of

Table 6. Re-isolations of fungal pathogens from dual cultures on fresh medium (PDA)

Trichoderma isolates	*Phyllosticta sphaeropsoidea*					*Phomopsis carposchiza*					*Diaporthe padi*				
	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA	PDA	CA	WA	CDA	MEA
Th01	3	3	1	3	1	3	3	2	3	1	3	1	1	3	1
Th02	1	1	1	1	1	3	3	3	3	3	1	1	1	1	1
Th03	1	1	1	1	1	3	3	1	1	2	1	1	1	1	1

Media: PDA = potato dextrose agar; CA = carrot agar; WA = water agar; CDA = Czapek-Dox agar; MEA = malt extract agar. Re-isolations evaluated after 10 days of cultivation: 1 = no growth of fungal pathogen after transfer of inoculum disc to fresh PDA; 2 = growth of fungal pathogen observed after transfer of inoculum disc to fresh PDA; 3 = *Trichoderma harzianum* present in fungal pathogen inoculum on PDA.

T. harzianum on the studied horse-chestnut leaf and fruit fungi (*Phyllosticta sphaeropsoidea*, *Phomopsis carposchiza*, *Diaporthe padi*) was reflected in reduced growth of the fungi tested *in vitro*. Chitinolytic enzymes produced by *T. harzianum* are biologically more active than enzymes of other fungi and more effective against a larger spectrum of fungi (Lorito et al. 1993; Zimand et al. 1996). However, according to Dickinson & Skidmore (1976), several common phylloplane fungi cause only slight inhibition of the pathogenic fungus *Septoria nodorum* (Berk.) Berk. in comparison with the fungus *Botrytis*, which totally inhibited germination of its spores.

A combination of hyperparasitism (nutrients taken from the host), competition (for space and nutrients) and/or antibiosis (production of an inhibitory metabolite or antibiotic) is observed in the antagonism of *Trichoderma* species (Whipps & Lumsden 1991; Prokinová 1996; Calistru et al. 1997). Competition between the horse-chestnut pathogens and *T. harzianum* and their mycelial interactions were affected by nutrient media used in this study. Formation of extreme inhibition zones between *Phomopsis* and *Trichoderma* was probably supported by the production of secondary metabolites by both fungi. Chapla et al. (2014) proved that *Phomopsis* sp. produces secondary metabolites for antifungal inhibition and mycotoxicity. The inadequate antagonistic effect of *T. harzianum* on *Phomopsis* led to successful re-isolation of *P. carposchiza* on fresh nutrient agar in my dual culture tests. Similarly, Whipps (1987) recorded the growth of *P. sclerotioides* Kesteren (growing in dual cultures with *T. harzianum*) after transfer of inoculum discs to fresh medium.

Effects of the nutrient-rich and nutrient-poor media used in this study on the growth rates of fungi differed significantly, which is consistent with earlier reports (e.g. Whipps 1987). Antagonist–pathogen interactions are also dependent on temperature (Tronsmo & Dennis 1978; Phillips 1986), pH of the medium (Sy et al. 1984), water potential (Campbell & Clor 1985), production of antifungal metabolites by different strains and species of the antagonist (Pezet et al. 1999), and also other factors relevant in the case of the use of biocontrol agents.

Antagonistic organisms or organic compounds with antagonistic properties towards the fungal genera *Phyllosticta* (asexual stage of *Guignardia*) and *Phomopsis* (asexual stage of *Diaporthe*) are known. Protective application of the extracts of *Hedera helix* L. and *Primula* root reliably control black rot disease of grapevine caused by *Phyllosticta ampelicida* (Engelm.) Aa, the asexual stage of *G. bidwellii* (Ellis) Viala & Ravaz (Koch et al. 2013). Bioactive volatile organic compounds produced by the yeast *Saccharomyces cerevisiae* Meyen ex E.C. Hansen are a promising control method for citrus black spot caused by *P. citricarpa* (McAlpine) Aa, the asexual stage of *G. citricarpa* Kiely (Fialho et al. 2010). Although isolates of *Bacillus subtilis* Ehrenberg and *Trichoderma* spp. significantly inhibited *in vitro* the mycelial growth of *P. citricarpa*, *in situ* treatment was less effective for disease control than chemical fungicide treatment (Kupper et al. 2011). Poovendran et al. (2011) proved an antagonistic effect of *Trichoderma* sp. on a tea plant pathogen, *Phomopsis theae* Petch. Commercial biofungicides based on *Ampelomyces quisqualis* Ces., *Trichoderma album* Preuss, and *Bacillus megaterium* de Bary have been found to be efficient in suppressing *Phyllosticta* leaf spot of banana (Kamhawy 2006). Similarly, the antago-

nistic fungi *Gliocladium roseum* Bainier, *Coniothyrium minitans* W.A. Campb., and *Trichoderma* spp. inhibited *Phomopsis sclerotioides* isolated from infected cucumber roots (Whipps 1987). Endophytic fungal communities are also a source of bioactive molecules, including those able to inhibit or control plant disease pathogens (Orlandelli et al. 2015; Silva-Hudges et al. 2015).

The efficacy of antagonistic fungi in inhibition of horse-chestnut pathogens had not been previously reported, but this study shows the potential of *T. harzianum* for the biocontrol of horse-chestnut leaf blotch and fruit rot. However, my results are preliminary and the plate tests are inadequate indicators of the potential of a microorganism as a biological control agent in the real environment, so further *in vivo* assays are necessary.

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