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Screening of native *Trichoderma harzianum* isolates for their ability to control *Verticillium* wilt of strawberry

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Abstract

In this research, the antagonistic potential of native *Trichoderma harzianum* isolates toward important strawberry pathogen *Verticllium dahliae* was studied. For this purpose, fungi were isolated from the rhizosphere and soil from five different strawberry production sites in Estonia over two growing seasons and were investigated under laboratory and greenhouse conditions. *T. harzianum* colonies were recovered using a selective medium after 12 days and confirmed through species-specific primers. In the laboratory, although all isolates of *Trichoderma* produced volatile and non-volatile metabolites, seven isolates which had the strongest inhibitory effects on mycelial growth of pathogen were selected for greenhouse assays. In the greenhouse, the disease severity was measured in a split plot treatment design with seven antagonist isolates applied to the two main treatment factors (soil and root), in which all levels of each factor were used in combination with all other factor levels. The result of greenhouse experiments showed that there was no significant difference among *T. harzianum* treatments but, among selected *T. harzianum* isolates, TU79 was the most effective isolate for inhibiting the effects of strawberry *Verticillium* wilt. In the cross-interaction between the antagonist isolates and their treatments, the minimum disease severity was significantly recorded when both soil and roots were treated with TU79 isolate. However, there was no statistically significant difference when this isolate was applied to the soil alone. The results of this study demonstrated that native *T. harzianum* isolates collected from Estonian fields have potential biocontrol ability so that they may be extensively used to control *Verticillium* wilt in strawberry nurseries.

Key words: non-volatile metabolites, *Trichoderma harzianum*, *Verticillium* wilt, volatile metabolites.

Introduction

Strawberry (*Fragaria* × *ananassa* Duch.) is one of the world's most commercially important fruit crops (Suga et al., 2013). According to Food and Agriculture Organization (FAO) statistics (FAOSTAT, 2013), global strawberry production was 4,516,810 tons in 2012, and in Estonia, strawberry is grown for fruit and plant production and it is developing into a promising horticultural crop for small growers. Hence, a lot of effort has been made to uphold strawberry production in the different regions of the country. A considerable limiting factor is strawberry diseases that severely influence both fruit and plant production and are frequently challenging to control. Most strawberry cultivars are susceptible to major fungal diseases. *Verticillium* wilt, caused by soil inhabiting *Verticillium dahliae* Kleb, is one of the most important diseases worldwide, resulting in great economic losses in many crops like strawberry and can be severe on some cultivars, even at low inoculum densities (Mirmajlessi et al., 2015). The fungus is distinguished from other closely related fungi by producing multicellular and melanized structures known as microsclerotia that persist in the soil, promoting survival between crops (Klosterman et al., 2009). Because of long viability of microsclerotia, the control of the pathogen is difficult even where non-susceptible hosts have been grown, suggesting that non-hosts may serve as a pathogen reservoir.

Intensive use of fungicides has caused drastic problems of chemical residues in the environment. Due to the change of attitude in the current European policy regarding crop protection, the European Commission has approved a legislative agreement (Directive 2009/128/EC), which regulates the use of plant protection products and establishing the integrated control and non-chemical means as a fundamental strategy to fight against diseases, pests and weeds. Therefore, the use of synthetic pesticides is being progressively diminished, and it is supplemented by an increased reliance on the use of microorganisms as biocontrol. In plant pathology, biological suppression of plant diseases has been increasingly recognized as a promising alternative way to achieve sustainable agricultural systems as it is safe to use and environmentally friendly, preventing pollution and health hazards resulting from the conventional use of chemicals (Zheng et al., 2011). Biocontrol systems

frequently use natural living microorganisms known as antagonists that are capable of reducing the effects of undesirable microorganisms. Antagonism implies direct interaction between two microorganisms that share the same ecological niche. Such antagonists can compete with pathogens for nutrients, diminish pathogens by parasitism, inhibit growth of pathogens through antibiosis or even induce systemic resistance in plants (Shishido et al., 2005). Earlier studies demonstrated that the volatile and non-volatile compounds of a variety of fungal microorganisms inhibit the activity of pathogenic fungi. For instance, Padder and Sharma (2011) showed the efficacy of some fungal isolates including *Trichoderma viride*, *T. harzianum*, *T. hamatum* and *Gliocladium virens* in inhibiting the *in vitro* and *in vivo* growth of *Colletotrichum lindemuthianum*, the causal agent of cowpea anthracnose by using volatile and non-volatile extracts. Basically, the efficacy of biological control depends on the development of the antagonist in the rhizosphere zone and colonizing the plants roots, and so the survival qualities of antagonists can be intensely affected by environmental conditions (Larkin, Fravel, 2002).

Trichoderma spp. have been used for many years as antagonists and have a great contribution to the biological control of many fungal plant pathogens, e.g., *Rhizoctonia solani* (Naeimi et al., 2010), *Botrytis cinerea* (Cheng et al., 2012), *Verticllium dahliae* (Xiaojun et al., 2014) and *Phytophthora ramorum* (Widmer, 2014). Basically, mycoparasitism, competition for space and nutrients, antibiosis through the production of inhibitory metabolites and induction of the plant's systemic resistance have been described as antagonistic mechanisms of *Trichoderma* spp. (Harman et al., 2004). Each of these mechanisms may play a key role during antagonism. Among *Trichoderm*a species, *T. harzianum* Rifai has been applied as an antagonist agent and it behaves efficiently under different environmental conditions to protect crops against diseases. Although some *Trichoderma* species are among the most investigated fungal biocontrol agents as biofungicide in plant disease management, full-scale application of biological control has not been widespread.

The objectives of the current study were (i) to obtain different *Trichoderma harzianum* isolates from the rhizosphere of field-grown strawberries as a potential antagonists against *Verticillium dahliae*; (ii) to assess the possible role of volatile and non-volatile compounds produced by *T. harzianum* isolates for control of *Verticillium* wilt of strawberry under greenhouse conditions.

Material and methods

Verticllium dahliae isolates. During 2014–2015, soil samples were collected from several strawberry fields, located in different production sites in Estonia, that were suspected of being infected with *Verticillium* wilt (Table 1). *V. dahliae* was isolated from the soil by wet-sieving plating method as previously described by Harris et al. (1993) with some modifications. Briefly, soil samples were air dried for two weeks at room conditions, mixed and ground with a mortar and pestle. Samples were then sifted using a 20-mesh sieve (Tyler equivalent) to eliminate large and unbreakable debris. Twenty g of each sieved soil sample was shaken at 250 rpm and dispersed in distilled water for one hour. Then the soil was wet sieved through 60 and 400 mesh sieves, sequentially, and residue retained in the 400 mesh sieve was suspended in 100 ml distilled water. Aliquots of 1 ml from each suspension were scattered onto 90-mm Petri plates of Czapek's Dox agar supplemented with streptomycin (100 mg l^{-1}) and incubated in the dark at 26°C. After two weeks' incubation, the plates were carefully washed with distilled water to remove remaining soils. Afterwards, drained plates were scanned for the existence of typical star-shaped colonies of *V. dahliae* using a stereomicroscope Olympus SZX10 (Japan). Hyphal tips grown out from each piece of tissue were picked and transferred to fresh potato dextrose agar (PDA) plates until further use.

Table 1. Detection of *Verticillium dahliae* in strawberry fields

Isolate code	Location (coordinates)	Symptoms in field
SV-05	Vasula (58°47′ N, 26°73′ E)	plant collapse and death
SV-07		yellow wilt
SV-11		wilting, initially older leaves only
SV-14		poor growth and stunting
SV-15		poor growth and stunting
SV-17		yellow wilt
SV-19		plant collapse and death
SV-20		yellow wilt
SV-21		green dry necrosis
SR-32	Rohu (59°09′ N, 26°48′ E)	yellow wilt
SR-36		wilting, initially older leaves only
SR-38		plant collapse and death
SR-39		no symptoms
SR-41		yellow wilt
SR-43		no symptoms
SM-46	Marjamaa (58°90′ N, 24°42′ E)	plant collapse and death
SM-48		poor growth and stunting
SM-49		poor growth and stunting
SM-50		yellow wilt
SM-53		green dry necrosis
SM-55		plant collapse and death
SU-63	Unipiha (58°26′ N, 26°58′ E)	no symptoms
SU-65		yellow wilt
SU-67		yellow wilt
SU-68		poor growth and stunting
SU-69		no symptom
ST-76	Utsu (58°40′ N, 26°80′ E)	no symptoms
ST-78		no symptoms
ST-79		yellow wilt

Trichoderma harzianum isolates. Soil samples were collected from the surface (10–20 cm) layer around healthy plant roots and their rhizosphere zone from different sites of strawberry fields, placed in plastic bags and stored at 4°C until processing. The samples were air dried (at 27°C), sifted with 20-mesh sieve to eliminate large debris. One gram of each sample was added to 40 mL distilled water and shaken by hand for one minute. The suspensions were diluted to 10^{-3} and then 0.5 mL aliquots were removed and spread on plates containing *Trichoderma* selective medium described previously by Elad et al. (1982). The medium consisted of a basal medium including 1.0 g K_2HPO_4, 0.3 g $MgSO_4$ ($7H_2O$), 1.0 g NH_4NO_3, 3 g glucose, 0.18 g KCl, 20 g agar and 0.18 g rose Bengal. Cultures were incubated at 27°C under continuous fluorescent light. Monoconidial cultures were identified 12 days after incubation by using the identification key provided by Bissett (1991). To confirm the existence of *T. harzianum*, genomic DNA of each isolate was extracted and amplified by conventional polymerase chain reaction (cPCR) using specific primers THITS-F2 (CGGGTTTTTATAATCTGAGCC)

and THITS-R3 (CATTCAGAAGTTGGGTG) to *T. harzianum* (Miyazaki et al., 2009).

Pathogenicity test. All isolates of *V. dahliae* collected from different strawberry fields were subjected to the pathogenicity test on the susceptible strawberry cultivar 'Sonata', using the root dip and soil inoculation methods.

Root dip inoculation. Nine-day-old cultures of *V. dahliae* isolates were washed with sterile distilled water from each plate. The inoculum concentration was adjusted to 10^6 conidia ml^{-1} by a spectrophotometer. The strawberry seedlings were uprooted, washed under running tap water and submerged in a conidial suspension. After two hours, the inoculated seedlings were planted in 20 cm pots containing a mixture of perlite, peat moss and vermiculite (1:1:1 by volume) and kept on a greenhouse bench at 24 ± 1°C with 12 hour day length provided by fluorescent light. Then, appearance of wilt symptoms was observed 30 days after inoculation.

Soil inoculation. Isolates of *V. dahliae* were applied after forming microsclerotia on PDA plates. Strawberry seedlings were inoculated with the whole agar piece removed from the plate beneath the roots in pots four weeks after planting. Three replications of four plants for each isolate were designed. The plants were kept under the same conditions as described above. Wilt incidence was observed 30 days after inoculation and its severity was rated on an arbitrary 0–5 point ranking scale, where 0 = healthy (no visual symptoms), 1 = 1% to 20% diseased (marginal and interveinal browning of outer leaves), 2 = 21% to 40% diseased, 3 = 41% to 60% diseased, 4 = 61% to 80% diseased and 5 = 81% to 100% diseased (Mirmajlessi et al., 2012). Pathogenicity of each isolate was calculated as follows:

$$\sum\left(\frac{\text{Disease index} \times \text{number of inoculated root samples in each index}}{\text{Maximum index} \times \text{total number of inoculated root samples}}\right) \times 100.$$

Pathogenicity tests were repeated three times using a randomized complete block design and virulence was measured using disease severity.

In vitro testing of antagonistic effects of T. harzianum isolates on V. dahlia. Inhibition of mycelial growth. T. harzianum isolates were tested *in vitro* for their antagonistic ability over *V. dahliae* in dual culture. Among all collected *V. dahliae*, one isolate was used as it exhibited the highest level of pathogenicity towards strawberry plants. After colony diameter reached 1.5 cm, a mycelial disc (5 mm in diameter) of each *T. harzianum* isolate and selected *V. dahliae* were placed together (5 cm apart) in a 9 cm Petri dish containing PDA medium and incubated at 26°C. Three replications were used per each *Trichoderma*-*Verticillium* combination. The experiment was assessed based on the inhibitory effect of the antagonist on radial growth of *V. dahliae* four days after incubation using the following formula:

$$\text{Growth inhibition (\%)} = (\text{CDC} - \text{CD} / \text{CDC}) \times 100,$$

where CDC is the average diameter of the *V. dahliae* colony as control, CD – the average diameter of the *V. dahliae* colony in culture medium affected by *T. harzianum*.

Effects of volatile metabolites. To determine the effects of volatile extracts released by *T. harzianum* isolates on the growth of *V. dahliae*, 5 mm diameter discs removed from the leading edge of a 10-day-old culture of selected *V. dahliae* were transferred into the centre of PDA plates and incubated at 26°C for 7 days prior to the interaction test. Discs from each of the *T. harzianum* isolates, removed from the leading edge of cultures grown on PDA, were placed on fresh PDA medium before the interaction occurred. The lids of Petri dishes were removed when a plate containing *V. dahliae* was placed over a plate containing *T. harzianum* and each pair was sealed by adhesive tape. The plates were incubated at 26°C for two weeks. The diameter of *V. dahliae* colonies affected by antagonist metabolites was measured and the percentage growth inhibition was then calculated as described above. The control set included cultures of *V. dahliae* paired with uninoculated PDA plates.

Effects of secondary metabolites. To evaluate effects of secondary metabolites (non-volatile) released by the *T. harzianum* isolates on *V. dahliae*, 5 mm mycelial discs were removed from the leading edge of 10-day-old culture of *T. harzianum* and transferred to the centre of PDA plates overlaid with sterilized cellophane membranes (Courtauld films, 50-µm thickness). After 72 hours, the cellophane and fungus were removed and a 5 mm disc of *V. dahliae* was then transferred on the centre of each plate. All plates were incubated at 26°C for two weeks and the percentage of growth inhibition was calculated as described above. The plates with cellophane film but without antagonist were employed as controls. The *T. harzianum* isolates that most highly inhibited *V. dahliae* growth were chosen for *in vivo* experiments.

In vivo experiments. Plant inoculation. V. dahliae inoculum was prepared by growing the fungus on PDA medium at 25°C. After eight days, colony spores were washed from the plates with sterilized-distilled water and the resulting conidial suspension was adjusted to 10^5 conidia ml^{-1} using a spectrophotometer. Strawberry seedlings at the trifoliate leaf stage were uprooted, cleaned from soil with running tap water and superficially wounded to ensure the entrance of the pathogen. Seedlings were root-dipped in fungal inoculum for one hour and then transplanted to a sterile soil and kept at 24 ± 1°C in the greenhouse. Control plants were non-inoculated and the inoculum was replaced with distilled water.

Trichoderma harzianum inoculums. For preparation of antagonist inoculum, each *T. harzianum* isolate was individually prepared on PDA medium at 25°C by adding two mycelial plugs of *Trichoderma* isolates taken from three-day old cultures into flasks containing water-soaked wheat bran which was sterilized (121°C, 15 psi, 10 minutes) and cooled before inoculation. Inoculated flasks were then incubated at 26°C for two weeks. The number of *T. harzianum* in each gram of wheat bran was then measured using a hemocytometer (Sigma, United Kingdom). One gram of inoculated wheat bran was then suspended in 12 ml sterile distilled water and the amount of conidia was measured by counting conidia in one ml of this suspension. *T. harzianum* inoculum was added to the soil of each pot at 10^7 conidia per g soil. For root treatment, strawberry seedlings were soaked in this inoculum for two hours.

Antagonist ability of Trichoderma harzianum on strawberry Verticillium wilt in the greenhouse. The antagonist effect of *T. harzianum* isolates for biological control of *V. dahliae* was investigated in a greenhouse. The experiment was performed by using antagonist treatments on root, soil and both root and soil, and consisted of *T. harzianum* isolates with pathogen, pathogen alone and *T. harzianum* without pathogen. Treatments without antagonist and pathogen were considered as control to ensure the health of plants. After approximately two months of inoculation, plants

were assessed for *Verticillium* wilt symptoms using the 0–5 point ranking scale described above. The entire experiment was repeated twice in a split plot treatment trial in which each experiment consisted of a randomized complete block design with three replications and four plants per replication. All pots were kept on a greenhouse bench at 24 ± 1°C. All statistical analyses were performed by analysis of variance (*ANOVA*) using statistical software *MSTATC*, version 1.42. Means were separated by Duncan's multiple range test ($P \leq 0.05$).

Results

Isolation of Verticillium dahlia. In this study, 29 isolates of *V. dahliae* were obtained from soil of strawberry fields from different areas of Estonia (Table 1). Identification of *V. dahliae* was based on morphological structures such as production of dark microsclerotia with hyaline and septate mycelium along with ovoid and single-celled conidia on PDA (Fig. 1).

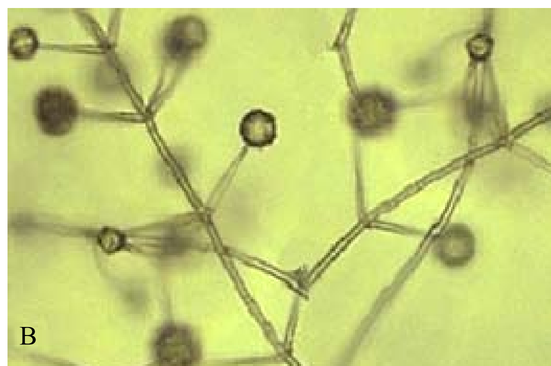
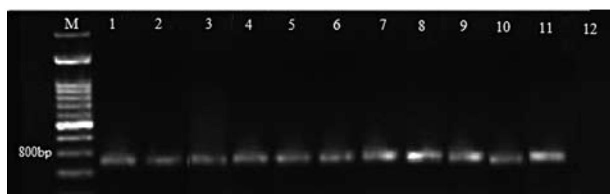


Figure 1. Dark microsclerotia (A) and microscopic view of *Verticillium dahliae* with hyaline and septate mycelium (B)

Isolation of Trichoderma harzianum. Eleven isolates of *T. harzianum* (TU62, TU63, TU67, TU68, TU70, TU72, TU74, TU75, TU76, TU79 and TU80) were obtained from the rhizosphere of strawberry plants only from the Unipiha area. The species-specific primer (THITS-F2/THITS-R3) was examined using cPCR against genomic DNA from pure culture of *T. harzianum* isolates. PCR products were generated from all DNA extracted using specific primers, demonstrating the existence of amplifiable template. The specific DNA fragments of the expected size were clearly observed by agarose gel electrophoresis for all isolates that confirmed presence of *T. harzianum* in this study (Fig. 2).



Note. Lane M – 100-bp DNA Ladder, lanes 1–11 – specific DNA fragment (approximately 830 bp) amplified from the genomic DNA of *Trichoderma harzianum*, lane 12 – distilled water as negative control; agarose gel electrophoresis of PCR products amplified using the primer pair THITS-F2/THITS-R3.

Figure 2. Primer pair specificity

Pathogenicity test of Verticillium dahliae isolates. All isolates of *V. dahliae* were inoculated on strawberry seedlings using root dip and soil inoculation procedures and the percentage of disease severity was recorded. All isolates were found to infect strawberry plants as shown in Table 2.

Combining results of the two inoculation methods indicated that disease severity of isolates varied considerably from 6.8% to 79.5% (Table 3). The isolates SV-17 and SV-19 collected from Vasula area showed the lowest (6.8%) and highest (79.5%) disease severity, respectively. In summary, the disease severity was <20% in six isolates, between 20% and 80% in 22 isolates and only one isolate revealed a disease severity of around 80%.

Moreover, a combined disease severity resulting from the use of two inoculation methods on strawberry

Table 2. Pathogenic variability of *Verticillium dahliae* isolates on strawberry plants (cv. 'Sonata')

Isolate code	Disease severity %	
	root dip inoculation	soil inoculation
SV-05	31 bc	33.7 bcd
SV-07	56.6 ab	44.2 abcd
SV-11	56.6 ab	68.1 ab
SV-14	56.6 ab	44.2 abcd
SV-15	56.6 ab	44.2 abcd
SV-17	11 d	4.3 ef
SV-19	76.6 a	86.3 a
SV-20	56.6 ab	20 de
SV-21	56.6 ab	44.2 abcd
SR-32	56.6 ab	54.3 abc
SR-36	31 bc	20 de
SR-38	56.6 ab	33.7 bcd
SR-39	56.6 ab	68.1 ab
SR-41	31.0 bc	54.3 abc
SR-43	56.6 ab	26.6 cd
SM-46	31 bc	54.3 abc
SM-48	56.6 ab	54.3 abc
SM-49	56.6 ab	26.6 cd
SM-50	31 bc	44.2 abcd
SM-53	56.6 ab	54.3 abc
SM-55	31 bc	44.2 abcd
SU-63	31 bc	20 de
SU-65	56.6 ab	28.1 cd
SU-67	31 bc	44.2 abcd
SU-68	31 bc	8.1 ef
SU-69	31 bc	28.1 cd
ST-76	31 bc	8.1 ef
ST-78	31 bc	8.1 ef
ST-79	31 bc	8.1 ef
Control	0 e	0 g

Note. Means in the same column followed by different letters differ from each other significantly at $P \leq 0.05$ (Duncan's multiple range test); control – without *V. dahliae*.

Table 3. Combined pathogenic variability of *Verticillium dahliae* isolates on strawberry plants (cv. 'Sonata') inoculated by root dip and soil inoculation procedures

Isolates	Disease severity %	Isolates	Disease severity %
SV-19	79.5 a	SV-20	32.5 de
SV-11	58.4 ab	SV-05	32.4 de
SR-39	58.2 ab	SM-55	32.3 de
SM-53	55.3 abc	SR-43	32 de
SR-32	52.5 abcd	SM-50	32.1 de
SV-21	52 abcd	SU-67	31.4 de
SM-48	51.1 abcd	SU-69	25.3 def
SV-15	47.9 abcd	SU-63	23.5 def
SV-07	43.6 bcd	SR-36	17 ef
SV-14	39.1 cde	ST-78	16.2 ef
SR-38	38.8 cde	ST-76	16 ef
SM-49	38.6 cde	SU-68	13.9 efg
SM-46	38.4 cde	ST-79	11.2 fg
SR-41	37.3 cde	SV-17	6.8 g
SU-65	35.8 cde	control	0 h

Note. Means in the columns followed by different letters differ from each other significantly at $P \leq 0.05$ (Duncan's multiple range test); control – without *V. dahliae*.

seedlings showed that the root dip inoculation method presented higher disease severity (44.2%) than the soil inoculation method (Table 4). As the most pathogenic isolate, SV-19 was chosen for further investigation.

Table 4. Combined disease severity of two inoculation procedures

Inoculation method	Disease severity %
Root dip	44.2 a
Soil	37.1 b

Note. Means in the same column followed by different letters differ from each other significantly at $P \leq 0.05$ (Duncan's multiple range test).

Antagonistic effects of T. harzianum isolates on V. dahlia. Dual culture. In this assay, 11 isolates of *T. harzianum* were tested against *V. dahlia* isolate SV-19 to evaluate mycelial growth inhibition. In dual culture, most *Trichoderma* isolates grew very fast and showed significant inhibition ability over *V. dahliae*, covering the whole *V. dahliae* colony after four days (data not shown). In two cases (TU62 and TU76), however, an inhibition zone was observed at the contact area of the growing colonies without overgrowth. The percentage of *V. dahliae* colony growth inhibition by all *Trichoderma* isolates is shown in Table 5.

Volatile metabolites. Volatile metabolites deriving from different *T. harzianum* isolates exhibited a wide range of inhibitory effect on *V. dahliae*, varying from 15.8% to 88.3%, with isolates TU76 and TU79 showing minimum and maximum effect on the pathogen, respectively (Table 5).

Table 5. Evaluation of dual culture, volatile and non-volatile metabolites tests on mycelial growth inhibition of *Verticillium dahliae* SV-19

Trichoderma harzianum isolates	Mycelial inhibition %			Average
	volatile metabolites	non-volatile metabolites	dual culture	
TU62	24.1 cde	40.6 ab	30.1 cd	31.6 cd
TU63	83.1 ab	35.1 abc	60.8 bcd	59.7 abc
TU67	52.9 bcd	28.1 bcd	62.3 bcd	47.8 abcd
TU68	67.5 bc	36.6 abc	75.5 bc	59.9 abc
TU70	53.3 bcd	11.2 de	61.2 bcd	41.9 bcd
TU72	79.8 abc	20.7 cd	88.1 ab	62.9 ab
TU74	81.7 ab	22.2 cd	85.9 abc	63.3 ab
TU75	55.2 bcd	30.4 abcd	93.1 a	59.6 abc
TU76	15.8 de	50.5 a	17.9 de	28.1 cde
TU79	88.3 a	18.2 cde	92.1 a	66.2 a
TU80	80.3 abc	31.1 abcd	85.6 abc	65.7 a
Control	0 f	0 f	0 f	0 f

Note. Means in the same column followed by different letters differ from each other significantly at $P \leq 0.05$ (Duncan's multiple range test).

Secondary metabolites. Evaluation of the inhibitory effect of non-volatile metabolites of *T. harzianum* isolates over *V. dahliae* indicated a wide range of mycelial growth inhibition which varied from 11.2% to 50.5% (Table 5). Generally, based on the results of dual culture, volatile and non-volatile metabolites, seven isolates (TU63, TU68, TU72, TU74, TU75, TU79 and TU80) which showed mycelial inhibition of more than 50% were chosen for the greenhouse assays.

Antagonistic ability of T. harzianum isolates on strawberry V. wilt in the greenhouse. The seven selected isolates of *T. harzianum* that achieved suitable biocontrol efficacy in *in vitro* experiments were further evaluated for their antagonistic effects on strawberry *Verticillium* wilt in greenhouse experiments. As shown in Table 6, treatments affected by different *T. harzianum* isolates resulted in a mean disease severity of ≈38%, locating in one statistical group. However, the lowest mean disease severity was related to the soil treatment (38.4%). On the other hand, different *Trichoderma* isolates decreased

Table 6. The effects of different treatments and isolates of *Trichoderma harzianum* on disease severity of *Verticillium* wilt in the greenhouse

Treatments	Mean disease severity %
Root	38.7 a
Soil	38.4 a
Root and soil	38.5 a
*V. dahliae**	75.6 a
TU63 + P	55.8 b
TU68 + P	56.2 b
TU72 + P	43.2 bc
TU74 + P	42.5 bc
TU75 + P	55.8 b
TU79 + P	26.8 cd
TU80 + P	37.5 bcd
Without pathogen	0 e

Note. * – fungal inoculum as positive control, P – *V. dahliae* isolate SV-19 as pathogen; means in the same column followed by different letters differ from each other significantly at $P \leq 0.05$ (Duncan's multiple range test).

disease severity in comparison with the control (no *Trichoderma* inoculation), but the effects of six of the *Trichoderma* isolates were statistically indistinguishable ($P < 0.05$). Only isolate TU79 showed a significantly reduced disease severity (≈27%) by its treatment on root or soil.

The cross-interaction between the antagonist isolates and the type of treatment (root, soil and both root and soil) showed a varied range of disease severity from 14.2% to 75.6%. The maximum disease severity, indistinguishable from the positive control, was found when the root was treated with isolate TU75. Also, the minimum disease severity was observed when inoculate TU79 was applied either to the soil or to both soil and root (Table 7).

Table 7. Interaction between all *Trichoderma harzianum* isolates and their treatments regarding disease severity of *Verticillium* wilt in the greenhouse

Fungal isolates	*T. harzianum* treatments	Mean disease severity %
*V. dahliae**	–	75.6 a
TU75 + P	root	75.6 a
TU63 + P	root	55.3 b
TU72 + P	root	55.3 b
TU68 + P	soil	55.3 b
TU68 + P	root, soil	55.3 b
TU75 + P	root, soil	55.3 b
TU63 + P	root, soil	51.4 bc
TU63 + P	soil	51.4 bc
TU68 + P	root	51.4 bc
TU74 + P	soil	46.3 bcd
TU72 + P	soil	46.3 bcd
TU72 + P	root, soil	46.3 bcd
TU75 + P	soil	46.3 bcd
TU80 + P	root	31.5 cd
TU74 + P	root	28.1 cde
TU74 + P	root, soil	28.1 cde
TU80 + P	root, soil	23.3 de
TU79 + P	root	23.3 de
TU80 + P	soil	23.3 de
TU79 + P	soil	14.2 ef
TU79 + P	root, soil	14.2 ef
Without pathogen	–	0 g

Note. * – fungal inoculum as positive control, P – *V. dahliae* isolate SV-19 as pathogen; means in the same column followed by different letters differ from each other significantly at $P \leq 0.05$ (Duncan's multiple range test).

Discussion

The use of microorganisms to control plant diseases is an exciting part of applied biology. Endogenous agents including *Trichoderma* spp. have been considered as a favourable resource for biocontrol of soil-borne pathogens. Since they mostly live in the rhizosphere they can affect the competitive ability of target pathogens like *V. dahliae*. Indeed, the efficacy of *Trichoderma* spp. as biocontrol agents against phypathogens has been demonstrated in previous studies (Naeimi et al., 2010; Cheng et al., 2012; Widmer, 2014). The present investigation examined multiple criteria to assess the antagonistic activity of several strains of *T. harzianum* against *V. dahliae*, the causal agent of *Verticillium* wilt of strawberry.

In this study, 29 isolates of *V. dahliae* were collected from various strawberry growing areas in Estonia. The isolates were similar in morphological characteristics but differed in disease severity. The results of the pathogenicity tests showed that *V. dahliae* isolate SV-19 was the most pathogenic, leading to the highest disease severity (79.51%), while SV-17 showed the lowest severity (6.8%) when seedlings were inoculated by root dipping or soil inoculation methods. Both isolates originated from the same (Vasula) area but generated different wilt symptoms on strawberry plants, reflecting genetic diversity in the *V. dahliae* population. Significant genetic diversity among *V. dahliae* isolates is a common phenomenon that has been reported by Berbegal et al. (2010) who showed a degree of host specificity and different virulence from certain hosts. Also, our results indicated that root inoculation using spore suspension induced greater disease severity than infections initiated by microsclerotia in the soil. However, both inoculation methods were efficacious on plants when analyzed separately. Gordon et al. (2005) illustrated that root dip inoculation method was more consistent over time than soil inoculation method on different strawberry genotypes. As in root dip inoculation the root system is directly subjected to the pathogen propagules, systemic infections are more rapidly predictable than soil inoculations. So, infection will not occur until growing roots encounter microsclerotia in the soil, which may be affected by annual temperatures. However, development of disease may vary between plants inoculated by different methods (Xiaojun et al., 2014).

In the *in vitro* test, all *T. harzianum* isolates obtained from healthy strawberry rhizospheres successfully inhibited radial growth of the *V. dahliae* (SV-19) by growing faster than the pathogen on PDA in comparison with untreated controls. Competition for space and nutrients could be one of the most general mechanisms applied by biocontrol agent to inhibit mycelial growth of the pathogen. In a similar study, the antagonistic activity of *Trichoderma* species against *Fusarium oxysporum* f. sp. *pisi* has been proved using competition as an effective mechanism where *Trichoderma* and *Fusarium* showed varying degree of inhibition on each other, indicating competitive ability of *Trichoderma* isolates (Sharma, 2011). However, in our study, an inhibition zone through lysis and deformation of *V. dahliae* mycelium was observed at the contact area of the growing colonies without overgrowth only by two isolates of *T. harzianum* (TU62 and TU76), demonstrating the attendance of diffusible inhibitory substances. Besides, production of extracellular enzymes such as chitinase, cellulase and β-glucanase is another biocontrol mechanism that apply by *Trichoderma* towards fungal pathogens, which degrade the fungal cell walls (Hassan, 2014). There was no correlation between antagonist capability to produce antifungal antibiosis and growth percentage of *V. dahliae*. Overall, growth inhibition occurred by either production of clear zone between the two colonies or by direct overgrowth on the pathogen in dual cultures. Indeed, the production of fungal metabolites as an indirect mechanism could not be the primary biocontrol mechanism and it might be through inhibition, competition or direct killing of the pathogen mycelium. These direct and indirect mechanisms may have effect on the biocontrol process that depends on the *Trichoderma* strain, the pathogen and the environmental conditions such as pH, nutrient availability and temperature (Benítez et al., 2004).

In addition, antifungal activity of *T. harzianum* isolates by production of volatile and non-volatile

metabolites was also tested in our study. Previous investigations showed that antimicrobial metabolites of *Trichoderma* spp. are able to inhibit growth of a wide range of plant pathogens such as *Staphylococcus aureus*, *Bipolaris sorokiniana*, *Bacillus subtilis*, *Streptococcus faecalis*, *Clavibacter michiganensis*, *Rhizoctonia solani*, *Botrytis cinerea*, *Curvularia lunata*, *Colletotrichum lagenarium*, *C. gloeosporioides*, *C. falcatum* and *C. acutatum* (Xiao-Yan et al., 2006; Porras et al., 2009). It has also been found that there is a large variety of antifungal compounds such as harzianopyridone, harzianolide, azaphilone, diterpenes, peptaibols, butenolides, furanones, pyrones and pyridones produced by *T. harzianum* which play an important role in controlling phytopathogens (Vinale et al., 2006; Siddiquee et al., 2012). In this study, it was observed that volatile or non-volatile metabolites of *T. harzianum* isolates produced were effective in inhibiting the mycelial growth of tested *V. dahliae* in the culture medium which is in agreement with the general definition of antibiosis as the mechanism mediated by metabolites.

This study also demonstrated that *Verticillium* wilt of strawberry is controlled efficiently by treating the strawberry soil or root with spore suspensions of *T. harzianum* under greenhouse conditions, whereas severity of disease was significantly reduced in comparison with control plants. In a similar study, intensity of *Verticillium* disease was reduced when roots of susceptible strawberry seedlings were soaked with *T. harzianum* and *T. viride* isolates, and the treatment was even more effective than the standard fungicide Topsin M (Meszka, Bielenin, 2009). Many studies regarding the efficacy of *Trichoderma* spp. in reducing disease severity of *Verticillium* wilt on different hosts have been published, indicating that *Trichoderma* can be a dominant bio-agent in management strategies. However, decreased severity of *Verticillium* wilt was found when plants had been treated with other antagonists (Naraghi et al., 2010; Zheng et al., 2011).

Conclusion

The present study showed the capability of volatile and non-volatile metabolites of *Trichoderma harzianum*, isolated from field-grown strawberries in the Unipiha area, Estonia in reducing the mycelial growth of *Verticillium dahliae*. Both volatile and non-volatile metabolites could be also involved in controlling *Verticillium* wilt under greenhouse conditions. The outcomes may have practical applications in fields naturally infested by *V. dahliae* with the aim of protecting biological resources as well as confining the use of chemical compounds. Field experiments, efficient formulation and studies on the mechanism of interactions between biocontrol agent, pathogen and plant should be the main goals of future research.

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Trichoderma harzianum vietinių izoliatų tinkamumas kontroliuoti braškių verticiliozę

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Santrauka

Tirtas *Trichoderma harzianum* vietinių izoliatų antagonizmas braškių patogenui *Verticillium dahliae*. Tuo tikslu Estijoje penkiose braškių auginimo vietose du vegetacijos laikotarpius grybai buvo išskirti iš rizosferos bei dirvožemio ir ištirti laboratorijoje bei lauko sąlygomis. *T. harzianum* kolonijos buvo išskirtos naudojant selektyvinę terpę po 12 dienų ir identifikuotos naudojant rūšiai būdingus pradmenis. Visi *Trichoderma* izoliatai gamino lakius ir nelakius metabolitus. Tyrimui šiltnamyje buvo pasirinkti septyni izoliatai, kurie pasižymėjo stipriausiu patogeną slopinančiu poveikiu. Šiltnamyje ligos pažeidimas matuotas skaidytų laukelių variantų metodu, naudojant septynis izoliatus dviem pagrindiniams variantų veiksniams (dirvožemiui ir šaknims). Šiltnamio tyrimo duomenys parodė, kad slopinant braškių verticiliozę nebuvo reikšmingo skirtumo tarp *T. harzianum* izoliatų, bet tarp pasirinktų *T. harzianum* izoliatų efektyviausias buvo TU79. Esant kryžminei sąveikai tarp antagonistinių izoliatų ir jų variantų minimalus ligos pažeidimas buvo esminis, kai dirvožemis ir šaknys buvo paveikti TU79 izoliatu. Tačiau esminis skirtumas nebuvo nustatytas, kai šis izoliatas buvo naudojamas vien tik dirvožemiui. Tyrimo rezultatai parodė, kad vietiniai *T. harzianum* izoliatai, surinkti Estijos laukuose, pasižymi antagonistiniu poveikiu *V. dahliae* ir todėl gali būti plačiai naudojami verticiliozės kontrolei braškių augynuose.

Reikšminiai žodžiai: lakūs metabolitai, nelakūs metabolitai, *Trichoderma harzianum*, *Verticillium dahliae*.

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