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INTEGRATED ASSESSMENT OF THE MYCOPARASITIC AND PHYTOSTIMULATING PROPERTIES OF *TRICHODERMA* STRAINS AGAINST *RHIZOCTONIA SOLANI*

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Abstract

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In this work, biocontrol efficacy of the two *Trichoderma harzianum* sensu stricto and *Trichoderma atroviride* (teleomorph: *Hypocrea atroviridis*) isolates against root rot disease agent *Rhizoctonia solani* (teleomorph *Thanatephorus cucumeris*) and related biostimulation effect on beans (*Phaseolus vulgaris*) were investigated. Efficacy of the agents was compared with commercially available products; T-22 Planter-Box [*Trichoderma harzianum* Rifai Irk KRL-AG2 WP], Trichoflow [*Trichoderma harzianum* FL], Sim Derma [*Trichoderma harzianum* KUEN 1585] and fungicide Thiram 80% WP. Consequently, *Trichoderma harzianum* sensu stricto 10.T.TR.7 was found to be the most effective isolate that decreased the disease severity caused by *Rhizoctonia solani* AG 4 HG-I about 65%, by comparison with the other treatments in the study. Moreover, this isolate also stimulated the growth of bean plants by increasing seedling height, root length and root weight by 11.3%, 37.5% and 94.4%, respectively. *T. atroviride* isolate was not so effective in preventing root rot disease of beans, while it caused 26.7% increase in plant fresh weight. Neither commercially available biocontrole products, nor fungicide treatment significantly decreased root rot caused by *R. solani*.

Key words: Biocontrol, *Hypocrea*, phytostimulation, root rot, *Rhizoctonia solani*, *Trichoderma* spp.

Introduction

Bean (*Phaseolus vulgaris* L.) is one of the major legume crops growing in Samsun, Turkey. With its 105.436 tones production, Samsun comes first in the country with respect to bean cultivation (Erper et al., 2011). Because of the warm and humid weather conditions of the province, fungal diseases can cause significant yield losses. Among those, damping-off and root rot is very common on legume crops and *Rhizoctonia* group fungi were found to be one of the main agents of the diseases. These fungi are soilborne pathogens, which attack a wide range of plants and are distributed all over the world (Ogoshi, 1996).

Rhizoctonia solani Kühn (teleomorph: *Thanatephorus cucumeris* [Frank] Donk) is the well-known member of *Rhizoctonia* group fungi and affects many important agricultural and horticultural crops worldwide. *R. solani* strains are ubiquitous and cosmopolitan saprotrophs in soil and plant pathogens as well with more than 500 hosts (Grosch et al., 2007). Based on hyphal anastomosis reaction, isolates of *Rhizoctonia* are divided into 13 anastomosis groups (AGs) designated as AG 1 through 13 (Carling et al., 2002). *R. solani* AG 4 is the most

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common pathogen and can cause several types of damage, including hypocotyl rot, root rot and web blight on bean in Turkey and all over the world (Sneh et al., 1991; Eken and Demirci, 2004). AG 4 was divided into subgroups; AG 4 HG-I, AG 4 HG-II and AG 4 HG-III (Kuramae et al., 2003). Among *R. solani* anastomosis groups, AG 4 and AG 4 HG-I subgroup are especially the most common fungal pathogens in bean growing areas in the Black Sea Region of Turkey (Karaca et al., 2002; Cebi-Kilicoglu and Ozkoc, 2010; Erper et al., 2011).

Control of soil borne pathogens are limited and generally some chemical fungicides are applied to control these pathogens. Application of chemical fungicides has been replaced by biocontrol agents (BCAs) because of the emergence of fungicide-resistant strains and public concerns regarding the health and environmental impacts of these chemicals. In the recent years, several potential biocontrol organisms have been isolated, characterized and commercialized, and thus, biocontrol of plant diseases has received more consideration in plant disease control. The efficacy of this control method has mainly been demonstrated against pathogenic soil fungi and fungi-like organisms such as *R. solani*, *Fusarium* spp. *Sclerotium rolfsii* and *Pythium* spp. (Cundom et al., 2003; Grosch et al., 2007; John et al., 2010).

Trichoderma (teleomorph: *Hypocrea*, Hypocreales, Ascomycota, Dikarya) are among the most frequently isolated soil fungi and present in plant root ecosystems. It is well documented that some strains promote plant growth, increase nutrient availability, improve crop production and enhance disease resistance. The competition with pathogens, parasitism and the production of antifungal compounds are the most important mechanisms in biocontrol activity (Savazzini et al., 2009; John et al., 2010). These fungi are opportunistic, avirulent plant symbionts, function as parasites and antagonists of many phytopathogenic fungi, and so protect plants from diseases (Harman, 2000; Harman et al., 2004; Hohmann et al., 2011). *Trichoderma* spp. are among the most commonly studied fungal BCAs. These species commercially marketed as biofungicides such as T-22 Planter-Box [*Trichoderma harzianum* Rifai Irk KRL-AG2 WP] and Trichoflow [*Trichoderma harzianum* FL], Sim Derma [*Trichoderma harzianum* KUEN 1585] as microbial fertilizer and soil amendments. The antagonistic potential of some *Trichoderma* spp. against *R. solani* has been demonstrated on bean (*P. vulgaris* L.) (Barakat et al., 2007; Grosch et al., 2007). Cundom et al. (2003) investigated the possibility of using regional *Trichoderma* spp. isolates to reduce melon seedling death caused by *R. solani* in warm and wet climates in the northwest of the Corrientes province, Argentina.

The objectives of the present work were to evaluate the mycoparasitic effects and phytostimulation properties of *Trichoderma harzianum* and *T. atroviride* (teleomorph: *Hypocrea atroviridis*) isolates, obtained from bean growing areas in the Black Sea Region, and compare with that of the three commercial *T. harzianum* products, and fungicide Thiram, against pathogenic *R. solani* AG 4 HG-I isolate *in vivo*.

Materials and Methods

Fungal cultures

R. solani AG 4 HG-I isolate M-62 which causes root rot disease on bean plants was used in this study. M-62 isolate was previously characterized (colony morphology, anastomosis reactions, rDNA-ITS RFLP and rDNA-ITS phylogeny) and its ITS1-5.8S-ITS2 sequence was deposited into NCBI GenBank with the accession number HE805671 (Cebi-Kilicoglu and Ozkoc, 2010 and unpublished data). *Trichoderma harzianum* sensu stricto (Druzhinina et al., 2010) isolate 10.T.TR.7 and *T. atroviride* isolate 10.T.TR.5 were screened out among 44 *Trichoderma* spp. isolates, which were obtained from bean growing areas in the Black Sea Region of Turkey during the routine disease surveys in 2006 and 2010. Single conidia cultures of each fungus were maintained on 3.9 % potato dextrose agar (PDA, Oxoid) and PDA slants stored at +4 °C served as stock cultures for further use.

Molecular characterization of *Trichoderma* isolates

Regarding the molecular identification of *T. harzianum* sensu stricto and *T. atroviride* isolates, commercial DNA extraction kits (Qiagen, Germany) were used according to the manufacturer's instructions. Molecular taxonomic diagnosis was based on the analysis of the universal fungal DNA barcode marker: the internal transcribed spacers 1 and 2 of the ribosomal RNA gene cluster (ITS1 and 2) complemented with the large intron of the *tef1* (translation elongation factor 1 alpha), using primer combinations SR6R and LR1 for ITS1 and 2, and EF1728F (Chaverri et al., 2003) and TEF1LLErev (5'-AACTTGCAGGCAATGTGG-3') for *tef1,* respectively. PCR fragments were purified (PCR purification kit, Qiagen, Hilden, Germany), and sequenced at MWG (Ebersberg, Germany). ITS sequences of *T. harzianum* and *T. atroviride* were deposited into GeneBank (NCBI, www.ncbi.nlm.nih.gov) with the accession numbers JN387048 and JN387049, respectively. *tef1* accession numbers are listed in Table 1. All isolates are stored at −80°C in 50% glycerol in the Collection of Industrially Important Microorganisms of Vienna University of Technology (TUCIM) under the collection codes TUCIM N156 (for *T. harzianum* 10.T.TR.7) and TUCIM N154 (for *T. atroviride* 10.T.TR.5), respectively.

Commercial products

In order to compare the effects of *T. harzianum* s.s. (isolate 10.T.TR.7) and *T. atroviride* (isolate 10.T.TR.5) isolates for the

biocontrol of *R. solani* AG 4 HG-I (isolate M-62), three biocontrol products; T-22 Planter-Box (*Trichoderma harzianum* Rifai Irk KRL-AG2 WP), Trichoflow (*Trichoderma harzianum* FL) and Sim Derma (*Trichoderma harzianum* KUEN 1585) and a synthetic fungicide, Thiram 80% WP were used in the study.

Inoculum of Trichoderma isolates

In order to prepare the inoculum of *T. harzianum* s.s. and *T. atroviride* isolates, wheat bran/peat mixture (1:1, v/v) adjusted to 40% moisture (w/w) was autoclaved in autoclavable polyethylene bags (30x30 cm) for 1 h at 121°C on three successive days. The substrate mixture was inoculated with a conidial suspension of each of the two isolates of *Trichoderma,* pre-grown on PDA and was further incubated in an illuminated chamber for two weeks at 30°C. The polyethylene bags were shaken daily to ensure even colonization of the mixture by the fungus. The preparations contained $1x10^8$ CFU/g (Sivan et al., 1984).

Inoculum of Rhizoctonia solani

Pieces of agar (15 mm in diameter) cut from the growing edge of a 5-day-old colony of *R. solani* AG 4 HG-I (isolate M-62) grown on PDA were transferred to the bottles (9x15 cm) with sand mix (115 g sand, 35 g corn meal and 20 ml potato broth) and autoclaved for 1 h at 121°C on 3 successive days. Bottles were incubated at 25°C for 3 weeks. They were shaken daily to ensure even colonization of the sand mix by the fungus (Sneh et al., 1991).

Biocontrol assay

Bean seeds (*P. vulgaris* L. 'cv. Gina'-MAY SEED) were surface sterilized by submerging in 1% NaOCl for 5 min, rinsed with sterilized distilled water (SDW) and blotted dry with sterile paper towels. The experiment was carried out in plastic boxes (9x9x10 cm) each containing 250 g of sterilized soil mixture. Inocula of 10.T.TR.7 and 10.T.TR.5 isolates each with or without pathogen (M-62 isolate) inoculum were mixed in pots (5 g/kg soil) with sterilized soil mixture of sandy loam soil/manure/peat (2:1:1, v/v/v) which was autoclaved for 1 h at 121°C on 3 successive days. Likewise, pathogen inoculum was added to sterilized soil mixture in a rate of 2% (Sneh et al., 1991) in all treetments. Additionally, three commercial biological products and the fungicide were applied

Table 1
Results of identification based on the sequence similarity search for the strains used in this study

Taxon	GeneBank Nr. *tef1*	Strain Nr	Origin	Substratum	Reference
Trichoderma harzianum s.s.	JN387050	10.T.TR.7, TUCIM N156	Turkey	soil	this study
based on the 100% length of the 4th intron is identical to					
	FJ463396	GJS 04-71	Italy	*Castanea sativa* twig	n.a.
	EF392752	DAOM 233352	Russia	soil	n.a.
	EF488114	UNISS 14-2	Sardinia	soil	Migheli et al., 2009
type culture	EF191337	JB RO111	Romania	n.a.	n.a.
	EF116561	Kazan 30	Russia	soil	Alimova, Druzhinina, unpublished
	EF116560	Kazan 28	Russia	soil	
	AF348101	CBS 226.95	UK	garden soil	Samuels et al., 2002
	AY605830	TUB F-477	Russia	soil	Druzhinina et al., 2010
Hypocrea atroviridis/ *T. atroviride*	JN387051	10.T.TR.5, TUCIM N154	Turkey	soil	this study
based on the 100% length of the 4th intron is identical to					
	AB558906	Th002	Colombia	n.a.	n.a.
type culture (*T. atroviride*)	AF456890	G.J.S. 95-38	New Zealand	kiwifruit orchard soil	Dodd et al., 2003
	FJ860611	CBS 119499	Austria	corticated twigs of *Prunus padus*	Jaklitsch et al., 2009

according to the manufacturer's advices, each with or without M-62 isolate inoculum, into the pots with sterilized soil mixture. Commercial products and their doses were: T-22 Planter-Box (*T. harzianum* Rifai Irk KRL-AG2 WP) 7.5g/kg seed, Trichoflow (*T. harzianum* FL) 10g/m^2 and Sim Derma (*T. harzianum* KUEN 1585 10g/kg seed and Thiram 80% WP 200g/100kg seed. The pathogen M-62 isolate was also evaluated alone in the experiment. In control treatments, bean seeds were planted into sterilized soil mixture. All pots were transferred to the greenhouse with 24±2°C temperature. After 3 weeks of incubation, plants were removed from the pots and roots were washed. Disease severity ratings were made by using 0-5 scale where 0= no lesions on hypocotyl, 1= lesions ≤ 2.5 mm long, 2= lesions 2.5-5.0 mm long, 3= lesions ≥ 5.0 mm long, 4= lesions girdling plant and visible wilting on leaves, and 5= severe damping-off or dead seedlings (Cardoso and Echandi, 1987). Fresh and dry weight, seedling height, root length and root weights of plants were also determined at the end of the experiment. Symptomatic roots were aseptically plated on PDA to complete Koch's postulates. All experiments were conducted twice.

Experimental design and data analyses

All experiments were conducted in a complete randomized design with fourteen treatments and four replications. Analysis of variance was implemented using the program *Minitab* (version 12, "Minitab", USA) and Duncan's multiple range test at $P < 0.05$ significance level was used to compare treatment means.

Results and Discussion

Molecular identification of the strains

The ITS1 and 2 (rRNA gene cluster) oligonucleotide barcode program TrichOKey (Druzhinina et al., 2005) identified the isolate 10.T.TR.7 as *T. harzianum* and the isolate 10.T.TR.5 as *T. atroviride* with high reliability. As both of these species are known to have the complex infraspecific structure (Chaverri et al., 2003; Druzhinina et al., 2010; *for T. harzianum* and Dodd et al., 2003, for *H. atroviridis*) the 4th large intron of *tef1* gene was used to find the exact phylogenetic position of both isolates inside their species. For this purpose, the TrichoMARK tool (www.isth.info) was first used to split the 1.26 kb tef1 fragments into exact phylogenetic markers (i.e. the most diagnostic regions) and 4th large intron to the NCBI BLAST. Table 1 shows that 10.T.TR.7 is identical (100% length of the query intron sequence) to at least 50 records in GenBank which all correspond to the clonal phylogenetic species *T. harzianum* sensu stricto. As the phylogenetic position of strains bearing this allele of tef1 has been resolved in Druzhinina et al. (2010), no further phylogenetic analyses are required. Similarly, the *tef1* allele of 10.T.TR.5 isolate is identical (100% length of the query intron sequence) to members of *H. atroviridis* subclade A, that contains both, the type strain of *T. atroviride* and the holotype strains of *H. atroviridis* (Dodd et al., 2003).

Effect of Trichoderma isolates on root rot disease

Data of the two pathogenicity tests were combined because of the lack of significant differences between the two tests and with the studied variables (Tables not shown). *T. harzianum* isolate 10.T.TR.7 was detected to be the most effective biocontrol agent against *R. solani* AG 4 HG-I isolate M-62, when compared to all other treatments used (P<0.05). This isolates reduced root rot and post emergence damping-off disease caused by *R. solani* about 65%. However, *T. atroviride* isolate 10.T.TR.5 was not as effective in preventing root rot of beans as far as 10.T.TR.7 isolate. None of the commercially available biopreparations used in this study significantly decreased root rot caused by *R. solani* AG 4 HG-I. Moreover, there was no statistically significant differences among the pots inoculated with *R. solani* alone and with T-22 Planter-Box (P<0.05) (Figure 1). Previous studies had also results showing the preventive effects of *Trichoderma* species against pathogens. John et al. (2010) used *T. viride* versus

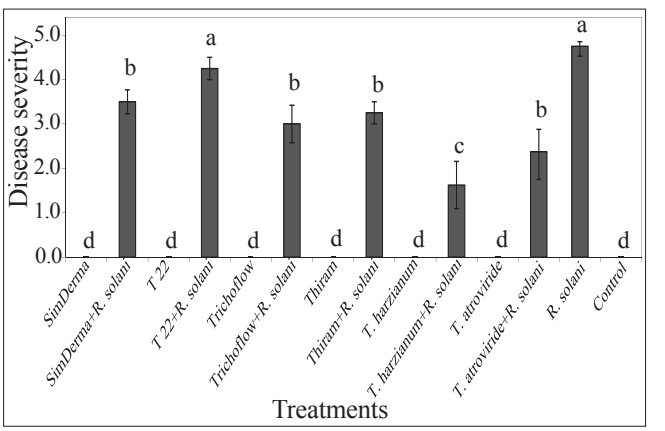

Fig. 1. Root rot disease severity (0–5 scale, in which 0= no lesions on hypocotyl, 1= lesions ≤2,5 mm long, 2 =lesions 2.5-5.0 mm long, 3= lesions ≥ 5.0 mm long, 4= lesions girdling plant and wilting visible on leaves, and 5= seedlings damped-off or dead) on bean plants in greenhouse pots, 21 days after the treatments of two *Trichoderma* isolates (*T. harzianum* sensu stricto and *T. atroviride*), three biopreparations (T 22 Planter-Box, Trichoflow, Sim Derma) and Thiram 80% WP inoculations, with or without *Rhizoctonia solani* AG 4 HG-I. Bars indicate ± standard error (*n=4*). Means followed by a common letter do not significantly differ from each other according to Duncan's Multiple Range Test

F. oxysporum f. sp. *adzuki* and *Pythium arrhenomanes* and *Trichoderma* treatment reduced the severity of the infection caused by the pathogens. Similarly, Barakat et al. (2007) observed that application of *Trichoderma* isolates as a conidial suspension ($3x10^7$) greatly reduced the disease index on bean plants caused by *R. solani* in different rates, and the most effective *T. harzianum* isolate, Jn14 reduced the disease by 65%. Additionally, results showed that *T. harzianum* (Jn14) and *T. hamatum* (T36) were the most effective isolates at 25°C and inhibited *R. solani* mycelial growth by 42% and 78% respectively, due to their fungitoxic metabolites production. The effect of *Trichoderma* on bean seedlings growth was obvious; height was nearly doubled (160-200%), while fresh and dry weights increased for 133% and 217%, respectively. Thiram is also widely used as a seed dressing on a wide range of crops where it provides good control of root rot, damping-off and many other soil and seed-borne diseases. In this study, Thiram had limited effect on preventing root rot disease of bean caused by *R. solani* and did not statistically found better than Sim Derma, Trichoflow and *T. atroviride* isolate (10.T.TR.5) (P<0.05) (Figure 1).

Effect of Trichoderma isolates on plant growing

At the end of the experiment, bean plants were evaluated in terms of plant growing, namely fresh and dry weight, seedling height, root length, and root weight. Results of the study showed that *T. harzianum* isolate 10.T.TR.7 induced plant growth when applied to pots with or without the pathogen. Moreover, when it was applied with *R. solani* (M-62), in comparison with the control, it increased seedling height, root length and weight by 11.3%, 37.5% and 94.4%, respectively (Table 2). Average height of the bean seedlings inoculated with *R. solani* isolate M-62 was 14.80±9.19 cm, while that of *T. harzianum* s.s. isolate (10.T.TR.7) treated bean plants was 49.06±14.82 cm. However, when two isolates were inoculated together, average height of the bean plants was 55.09±11.52 cm (Table 2).

Although *T. atroviride* isolate 10.T.TR.5 was not adequately effective in the protection of bean plants when applied with *R. solani* AG 4 HG-I, it increased fresh weight and dry weight of the plants by 26.7% and 8.6%, respectively, when applied alone. However, differences in plant growth parameters caused by this isolate was not statistically significant that, all means except fresh weight of the seedlings arranged in the same groups with control plants (P<0.05) (Table 2).

Various researchers mentioned similar results on the enhanced growth of plants with *Trichoderma* inoculations. For instance, Barakat and colleagues (2007) found the positive effect of *Trichoderma* on seedling height, fresh and dry weights on bean plants. The effect of *Trichoderma* on the

Table 2
Effects of *Trichoderma* isolates, biopreparations, and fungicide Thiram on root weight and length, dry weight, fresh weight and seedling height of bean plants inoculated with or without *Rhizoctonia solani* AG 4 HG-I

Treatments	Root		Dry weight, g	Seedling	
	Weight, g	Length, cm		Fresh weight, g	Seedling height, cm
Sim Derma	0.22[a]±0.06 b[b]	17.38±1.52 bc	0.39±0.05 abc	4.02±0.21 b	54.00±7.29 a
Sim Derma+*R. solani* AG 4HG-I	0.15±0.04 b	11.31±1.30 ef	0.23±0.07 ef	1.98±0.19 ef	31.13±11.62 de
T-22 Planter-Box	0.21±0.06 b	16.88±1.52 bcd	0.34±0.11 cde	3.10±0.28 cd	54.13±11.84 a
T-22 Planter-Box +*R. solani* AG 4 HG-I	0.24±0.12 b	11.94±1.47 ef	0.21±0.05 f	1.40±0.17 f	24.50±9.71 ef
Trichoflow	0.34±0.09 a	18.71±1.61 b	0.41±0.07 abc	4.16±0.26 ab	46.76±8.48 abc
Trichoflow+*R. solani* AG 4 HG-I	0.17±0.05 b	12.74±1.29 def	0.23±0.07ef	2.53±0.21 de	35.48±10.31 cde
Thiram	0.21±0.06 b	16.30±1.28 bcd	0.48±0.11 ab	3.92±0.24 b	46.47±10.25 abc
Thiram+*R. solani* AG 4 HG-I	0.14±0.06 b	13.81±1.13 cde	0.35±0.12 bcd	2.72±0.29 de	38.06±13.59 bcd
T. harzianum sensu stricto	0.22±0.07 b	17.25±1.85 bc	0.40±0.20 abc	3.90±0.34 b	49.06±14.82 ab
T. harzianum sensu stricto + *R. solani* AG 4 HG-I	0.35±0.11 a	22.69±1.42 a	0.43±0.09 abc	3.92±0.24 b	55.09±11.52 a
T. atroviride	0.24±0.07 b	17.88±1.09 bc	0.50±0.11 a	4.78±0.15 a	54.19±14.22 a
T. atroviride+*R. solani* AG 4 HG-I	0.17±0.07 b	8.44±0.75 fg	0.25±0.06 def	2.44±0.15 de	31.81±7.74 de
R. solani AG 4 HG-I	0.14±0.10 b	4.49±1.60 g	0.23±0.18 ef	1.34±0.33 f	14.80±9.19 f
Control	0.18±0.08 b	16.50±1.06 bcd	0.46±0.11 abc	3.77±0.27 bc	49.50±9.79 ab

[a] Values represent the means of 8 replications for each treatment; four per experiment, ± Standard error.
[b] Means in a column followed by the same letter are not significantly different from each other according to Duncan's multiple range test (P < 0.05).

growth of bean seedlings was obvious that, plant height was nearly doubled (160-200%), while fresh and dry weights increased by 133% and 217%, respectively. Similarly, John et al. (2010) showed that *Trichoderma* improved root and shoot dry weights of soybean plants inoculated with *Fusarium* spp. and *Pythium* spp. In another study, Hohmann et al. (2011) showed that two *Trichoderma* isolates (*T. hamatum* LU592 and *T. atroviride* LU132) had ability to promote the growth and health of commercially grown *Pinus radiata* seedlings. It was previously documented that some strains of *Trichoderma* spp. could promote plant growth, increase nutrient availability, improve crop production and enhance disease resistance (Harman et al., 2004). Namely, *Trichoderma* spp. were mentioned as opportunistic filamentous fungi that could affect plant growth by promoting abundant and healthy plant roots, possibly via the production or control of plant hormones (Harman, 2000). It was also reported that some *Trichoderma* spp. released secondary metabolites, such as auxin inducing substances, during *Trichoderma*-plant interaction and that might be a cause for the enhanced growth (Vinale et al., 2008).

Results of the present study showed that three commercially available biopreperations used in the study were not much effective against M-62 isolate. However, these biopreparations were also observed to enhance the growth parameters of the plants non-inoculated with the pathogen. Especially, Trichoflow treatment increased fresh weight, root length and root weights by 10.3%, 13.3% and 88.8%, respectively. Trichoflow was also the most effective biopreparation when applied with *R. solani* (Table 2).

Conclusions

As a result of the present study, two promising *Trichoderma* strains, isolated from the bean fields in the Black Sea Region, Turkey, could be selected as potential biological control agents against root rot disease caused by *R. solani* AG 4 HG-I; especially *T. harzianum* s.s. isolate 10.T.TR.7 as the biocontrol agent, and *T. atroviride* isolate 10.T.TR.5 as a plant growth promoter. These isolates should also be evaluated for their biocontrol activities against other soil-borne plant pathogens of different crops. The selection of the isolates is only the beginning, in the development of a product to control soil borne pathogens such as *R. solani* AG 4 HG-I. Moreover, the production and especially formulation of a biological control agent is very important.

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