

Characterization of Local *Trichoderma* spp. as Potential Bio-Control Agents, Screening of in vitro Antagonistic Activities and Fungicide Tolerance

Potansiyel Biyokontrol Ajanları Olarak Yerel *Trichoderma* spp. Türlerinin Karakterizasyonu, in vitro Antagonistik Aktivitelerinin ve Fungisit Toleransların İncelenmesi

Research Article

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ABSTRACT

Enlightening effects of biocontrol agents as *Trichoderma* spp. to provide disease control by combating pathogens is an important alternative in agricultural crop production. To that end, 9 species of *Hypocrea/Trichoderma* having green ascospores isolated from the soil that tea plants cultivated in are identified by ITS sequences and were found to be close relative of *Trichoderma* sect. *Pachybasium* (77%). Ethyl acetate extracts of fungal isolates exhibited the antimicrobial activity against to *Vibrio* sp, *Serratia marcescens*, *Mycobacterium smegmatis* and *Bacillus cereus* but no antifungal activity was detected. The highest level of inhibitory activity was observed against to *M. smegmatis* by *Trichoderma* *harzianum* ID4A, ID4B and ID6B. In dual culture test, all *Trichoderma* strains were found to be showing the highest inhibitory activity against to the plant pathogens *Botrytis cinerea*, *Sclerotinia sclerotiorum* and *Rhizoctonia solani* (AG3), but relatively low activity against to the entomopathogen fungi. Volatile metabolites of *Trichoderma* spp. caused maximum reduction in mycelial growth and sclerotial production. Tested strains showed the highest tolerance to the fungicide Dikozin, whereas the lowest tolerance was against to the Captan regardless of the dosage.

Key Words

Trichoderma, bio-control agent, Antimicrobial activity, Fungicide.

ÖZ

Trichoderma spp. gibi biyokontrol ajanlarının etkilerini incelemek tarımsal üretimde patojenlerle mücadele yoluyla hastalık kontrolünü sağlamada önemli bir alternatiftir. Bu amaçla, çay bitkilerinin topraklarından izole edilmiş yeşil askosporlara sahip 9 *Hypocrea/Trichoderma* türü, ITS sekansları ile tanımlanmış ve türlerin % 77 oranında *Trichoderma* sect. *Pachybasium* ile yakın akraba olduğu belirlenmiştir. Fungal izolatların etil asetat ekstraktlarının, *Vibrio* sp., *Serratia marcescens*, *Mycobacterium smegmatis* ve *Bacillus cereus*'a karşı antimikrobiyal aktivite sergilediği tespit edilmesine karşın, izolatlarda antifungal aktivite gözlenmemiştir. En yüksek inhibisyon aktivitesinin, *Trichoderma harzianum* ID4A, ID4B ve ID6B tarafından *M. smegmatis*'e karşı oluşturulduğu ortaya çıkarılmıştır. Çift kültür testinde, tüm *Trichoderma* suşlarının *Botrytis cinerea*, *Sclerotinia sclerotiorum* ve *Rhizoctonia solani* (AG3) bitki patojenlerine karşı en yüksek inhibitör aktivite gösterdiği, fakat entomopatojen mantarlara karşı nispeten düşük etkide oldukları bulunmuştur. *Trichoderma* spp. uçucu metabolitleri, misel artışı ve sklerotik üretimde maksimum azalmaya neden olmuştur. Test edilen suşların, dozajdan bağımsız olarak en yüksek toleransı Dikozin fungusitine karşı, en düşük toleransı ise; Captan'a karşı gösterdiği belirlenmiştir.

Anahtar Kelimeler

Trichoderma, biyokontrol ajanı, antimikrobiyal aktivite, fungusit.

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INTRODUCTION

Trichoderma is one of the most common fungal biocontrol agents in agriculture for the management of plant diseases caused by an extensive spectrum of fungal pathogens [1]. Some studies showed that several strain of *Trichoderma* had significant remedial effect on plant diseases caused by pathogens such as *Botrytis cinerea*, *Fusarium oxysporum*, *F. culmorum*, *F. moniliforme*, *Sclerotium rolfsii*, *Sclerotium sclerotonia*, *Rhizoctonia solani* and *Aspergillus flavus* [2].

Trichoderma spp. are primarily present in all agricultural soils including decaying wood. In the past studies, nine species were distinguished based on the morphological features according to Rifai [3]. This first classification has been considered preliminary, since some aggregates formed among morphologically indistinguishable species and have made identification harder [4]. Consistent results have been obtained through the sequence analyses of ITS1, ITS2, and of genes such as β -tubulin and hydrophobin or all combined [5]. Major mechanism involved in the biocontrol activity of *Trichoderma* spp. is the ability of competition for space and nutrients in the rhizosphere flora, production of diffusible and/or volatile antibiotics, prevention of abiotic stresses such as salt, heat and drought and finally mycoparasitism [6].

Trichodermin was a secondary metabolite isolated from the fermented broth of *T. harzianum* and showed antifungal activity against mycelial growth of phytopathogenic fungi, such as *Botrytis cinerea*. In contrast to other fungi, *Trichoderma* spp. has been reported to have limited applications in biocontrol of pathogenic bacteria [7]. In addition, it has been reported that *Trichoderma* spp. is able to produce antibacterial compounds that act against human-pathogenic bacteria such as *E. coli*, *S. aerus*, *K. pneumoniae* [8].

The combined use of biocontrol agents and chemical pesticides has attracted much attention by either farmers or producers in order to obtain synergistic or additive effects in the control of soilborne diseases up to now [9]. *Trichoderma* spp. strains with greater biocontrol efficiency also have better tolerance against pesticides to be used as major component in Integrated Pest Management

(IPM). To date, fungicides and insecticides are extensively used by farmers to control insects, pests and diseases in agro-economically important crops such as cotton, rice, maize and tomato [10], however agriculture needs to be sustained by more cleaner and safer processes to pursue an eco-friendly approach. In this manner, identifying biocontrol agents and understanding the mechanisms underlying the antagonistic effects of *Trichoderma* spp. especially on plant pathogens are important in creating effective and safe bio-control strategies.

In the present study, we aimed to determine the physical properties of strains with molecular characterization and their culture effect and the biocontrol potential. In this regard, *Trichoderma* spp. isolated from the soil which indigenous tea plants cultivated in İkizdere, Rize, Turkey identified with molecular tools and the capabilities of strains such as sporulation properties under different growth temperatures, heat tolerance, antibacterial and antifungal activities and fungicide tolerance were examined.

MATERIAL and METHODS

Culture Medium and Inoculum Development

Trichoderma spp. strains formerly isolated from the indigenous tea cultivation area in Rize province of Turkey were used in this study [11]. Strains were stored in Recep Tayyip Erdogan University, Microbiology and Molecular Biology Laboratory culture collection unit for further use. Control strain *T. harzianum* KUENS 1585 was purchased by the Simbiyotek Company in Istanbul. *Trichoderma* spp. strains were grown on Potato Dextrose Agar (PDA) (Merck, Germany) for 7 days. *Hyphae* and spores gained from pure cultures were transferred into 500 mL flasks containing 100 mL of Malt Extract Broth and were shaken at 150 rpm in a rotary shaker (GFL, Germany) at 28 °C for 10 days. *Mycelia* were harvested by filtration, washed with distilled water and powdered in liquid nitrogen.

DNA Extraction and PCR Amplification of Fungi

Genomic DNA extraction was carried out using DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Primers ITS4 and ITS5 described by Kanematsu and Naito [12] were used to amplify the 5.8S

rDNA gene with the ITS-1 and ITS-2 regions. PCR amplification of ITS1-5.8S-ITS2 genes of fungal isolates were performed with universal primers of ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') as forward primer and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') as reverse primer [13]. PCR amplifications were established in a total volume of 50 μ L. The components of reaction were 5 μ L 10 \times Taq-DNA polymerase buffer, 200 μ M of dNTPs, 0.05 nmol of the primer pairs, 2.5 unit Taq-DNA polymerase (Fermentase), and 0.05 μ g genomic DNA. After the primary denaturation at 95 °C for 5 min, 95 °C for 1 min, 55 °C for 55 s, 72 °C for 2 min, with an extension step at 72 °C for 10 min were operated with thirty-five cycles in thermocycler (Eppendorf, Germany). Separated PCR products on 1% agarose gel, visualized with ethidium bromide and photographed under UV light.

Characterisation of physical properties

Studies were performed based on the effects of temperature on the sporulation and mycelial growth. Optimum temperature for growth [14] and effect on volatile metabolite production of *Trichoderma* spp. strains were also investigated according to Keszler et al. [15]. Effect of temperatures (15-32°C) on the linear hyphal growth of *Trichoderma* spp. strains were studied in vitro on PDA medium in 9 cm Petri plates for each temperature. A bit (5 mm) of pure culture of *Trichoderma* spp. strains were placed at the center of Petri plates, replicated thrice with the help of sterile cork borer. The inoculated Petri plates were kept at 15, 20, 24, 28 and 32°C in the incubator and daily observation on mycelial growth of *Trichoderma* spp. strains were recorded at every 24 hrs up to 15 days. Radial development after the first 48 h culture was taken into account. On the 4th, 7th and 15th days of fungi cultures, the number of spores was determined. Spore suspensions were prepared by adding 15 ml of sterile distilled water to mature (7th and 15th days) fungal colonies on PDA plates to dislodge the spores from the mycelium. The spores were counted using a hemocytometer (Neubauer, Germany) as mentioned before [14].

A 1×10^7 cfu mL⁻¹ spore suspension was prepared to determine the tolerance of *Trichoderma*

spp. spores to different temperatures. The spor suspension was distributed to ependorfs (1 mL) and each ependorf was exposed to heat in a heater for 15 min at 45, 55, 65 and 75 °C. Subsequently, 100 μ L was added to the surface of the DRBC agar and spreading was carried out by baguette. Incubation was allowed for 3 days at 28 °C [22].

In vitro Antagonistic Activity Assays

In vitro antagonistic activities were tested against to plant (*Sclerotinia sclerotiorum*, *Rhizoctonia solani* (AG3), *Botrytis cinerea*), human (*Aspergillus flavus*, *Aspergillus niger* RSKK4017) and insect pathogens (*Beauveria bassiana* ARSEF 8664, *Isaria fumosorosea* ARSEF 8333, *Metarhizium anisopliae* ARSEF 8433). All fungi were revived and cultured on PDA at 28 \pm 1 °C for seven days. The antagonistic activity of *Trichoderma* spp. strains were tested in vitro using 85 mm petri dishes containing 20 mL of PDA medium with pH 5.5. Mycelial discs (5 mm in diameter) of *S. sclerotium*, *R. solani*, *B. cinerea*, *A. flavus* and *A. niger* were placed on one edge of a petri dish containing PDA, while mycelial discs of *Trichoderma* spp. strains were placed on the opposite edge of the plate. Control petries consisted of the individual cultures of the pathogen. Each dual culture (pathogen-antagonist) had three replicates under 28 \pm 1 °C. After 4, 7 and 14 days, the plates were evaluated for antagonistic activity of the strains, considering the ability of the microorganisms to reduce pathogen colony expansion. The equation for the percentage inhibition of radial growth (PIRG) applied as below:

$$\%PIRG = \frac{R_1 - R_2}{R_1} \times 100 \quad (1)$$

PIRG = Percentage inhibition of radial growth;

R1 = Radial growth of control;

R2 = Radial growth of *Trichoderma* spp. in the presence of the antagonist

The production of volatile compounds from *Trichoderma* spp. were determined as follows; two Petri dishes containing PDA were individually inoculated with a disc of *S. sclerotium* and *Trichoderma* spp. and bottom parts were adjusted and attached by tape. The control sets did not contain the *Trichoderma* spp. The plates were randomized and incubated at 28 \pm 2°C for 7 days.

The diameters of *Sclerotonia sclerotium* colony cultures were measured daily. Three replicate plates were set for each treatment [16].

Antimicrobial Activity Tests

Trichoderma spp. were cultivated and maintained on slants of PDA media for 7 days at 28 ± 1 °C. Conidia were scrapped from mycelia which grown on slants and cultivated on potato dextrose broth (PDB) media. Conidial densities in the suspension were determined by using the hemocytometer under a light microscope. Fungal inoculum (1×10^7 spore/mL) of each strain was prepared by blending PDA-grown cultures of the fungus with sterile distilled water for 14 days. A spore suspension of each strain was 10^7 spores inoculated in 250 mL flasks containing 50 mL of PDB incubated for 21 days at 28 ± 1 °C [17]. Fermentation broth was filtered and extracted by 50 mL 100% ethyl acetate, agitated for 60 min and then centrifuged at 1500 g for 30 min. After separating two phases, organic phase was collected and dried at 40 °C under vacuum using a rotary evaporator. Extracts were dissolved in 1 mL of DMSO which was then used to screen for their antimicrobial activity by agar diffusion assay method.

All test microorganisms except for plant pathogen fungi obtained from Abant Izzet Baysal and Gaziosmanpasa University were obtained from the Hifzisiha Institute of Refik Saydam (Ankara, Turkey). The antimicrobial activities were evaluated against eight selected gram negative bacteria; *Escherichia coli* ATCC25922, *Klebsiella pneumonia* ATCC13883, *Pseudomonas aeruginosa* ATCC43288, *Yersinia pseudotuberculosis* ATCC911, five Gram positive bacteria; *Bacillus cereus* 709 ROMA, *Staphylococcus aureus* ATCC25923, *Enterococcus faecalis* ATCC29212, *Bacillus subtilis* ATCC6633, *Listeria monocytogenes* ATCC43251, an acid-fast bacterium; *Mycobacterium smegmatis* ATCC607, yeast like fungi; *Candida albicans* ATCC60193, *Candida tropicalis* ATCC13803 and *Saccharomyces cerevisiae* RSKK 251, two plant pathogenic fungi; *Botrytis cinerea*, *Rhizoctonia solani* AG3 and a saprophyte fungus; *Aspergillus niger* RSKK4017. *Salmonella* sp., *Proteus vulgaris*, *Vibrio* sp. and *Serratia marcescens* were clinically isolated strains.

Simple susceptibility screening test via agar-well diffusion method was used. Each bacterium was

suspended in Mueller Hinton (MH) (Difco, Detroit, MI) broth, whereas target fungi were suspended in the yeast extract broth. The microorganisms were diluted approximately to 10^6 cfu mL⁻¹ with the aid of the McFarland 0.5 standard. All fungi species were cultivated on PDA and *M. smegmatis* was cultivated on Brain Heart Infusion Agar (BHIA) (Difco, Detroit, MI). Those were flood-inoculated onto the surface of MHA, BHIA and PDA and then air-dried. Five-millimeter diameter wells were created from the agar cutting with a sterile cork-borer and 50 µL of the *Trichoderma* spp. extracts were placed into the wells. Plates that consist bacteria incubated for 24-48 h at 35°C, whereas the fungi plates were incubated for 3-5 days at 28 °C. *M. smegmatis* was grown for 3 days on BHIA plates at 35 °C [18]. Antimicrobial activity was evaluated by measuring inhibition zone against to the target microorganism. Streptomycin (10 µg), ampicillin (10 µg) and fluconazole (5 µg) were standard drugs used in the experiment. Ethyl acetate and dimethyl sulfoxide were used as negative control.

Assay of Fungicide Tolerance

Fungicides used by farmers extensively were selected to assess their effect on the growth of the *Trichoderma* spp. isolates. To that end, *Trichoderma* spp. isolates were exposed to three fungicides; Captan, Dikozin and Cuprenax. Three different fungicides Dikozin (75% Mancozeb), Captan (Captan M 50 WP) and Cuprenax (Copper oxychloride) were added to PDA medium with a final concentration of 2.5, 5.0 and 10.0 mg mL⁻¹. A mycelial block in 5 mm diameter from one week of *Trichoderma* spp. culture was cut and placed into the center of the plates. After 7 days, *Trichoderma* spp. mycelial radial growth zone were measured [19]. Calculations were conducted based on the formula for the percentage inhibition of radial growth (PIRG) in duplicate.

RESULTS and DISCUSSION

The identification were performed based on the macroscopic and microscopic properties of nine *Trichoderma* spp. strains formerly isolated from the soil which tea plants cultivated in 100-1000 meter high. A commercial strain was tested for its properties as control (*T. harzianum* KUEN 1585). Isolates of *Trichoderma* spp. were characterized based on their 700 bp fragment of the ITS1-5.8S-

Table 1. Examined *Hypocrea* and *Trichoderma* spp. isolates and GenBank accession numbers of target sequences.

Strain no	Species	Similarity	Gen Bank. No
(%)	ITS 1- 5.8- ITS2		
ID4A	<i>Hypocrea lixii</i> BHU 221	100%	JN604833.1
	<i>Trichoderma harzianum</i> A12	99%	KC139308.1
	<i>Hypocrea lixii</i> BHU18	99%	JN604835.1
	<i>Hypocrea lixii</i> BHU110	99%	JN604836.1
	<i>Hypocrea lixii</i> BHU226	99%	JN604834.1
ID4B	<i>T. harzianum</i> NBAII-Th13	99%	JX644593.1
	<i>Trichoderma harzianum</i> T12	99%	KC609759.1
	<i>Trichoderma</i> <i>harzianum</i> IBSD-T142	99%	JX518931.1
	<i>Trichoderma</i> <i>harzianum</i> IBSD-T47	99%	JX518904.1
	<i>Trichoderma</i> <i>harzianum</i> IBSD-T11	99%	JX518894.1
ID6B	<i>Trichoderma</i> <i>harzianum</i> FUE15	99%	KC200074.1
	<i>Trichoderma</i> <i>harzianum</i> T65-NI	99%	U78881.1
	<i>Hypocrea lixii</i> BHU51	99%	JN618343.1
	<i>Hypocrea lixii</i> BHU159	99%	JN618342.1
	<i>Hypocrea lixii</i> BHU166	99%	JN618339.1
ID7C	<i>Trichoderma</i> <i>harzianum</i> NR6929	98%	AF194011.1
	<i>Trichoderma</i> <i>harzianum</i> 2930	98%	AJ224016.1
	<i>Trichoderma harzianum</i> A14	98%	KC139307.1
	<i>Hypocrea lixii</i> ATCC 20847	98%	FJ545255.1
	<i>Hypocrea lixii</i> IB32E1	98%	FN598939.1

ITS2 gene. According to the cluster analysis based on the ITS sequence of the genomic rRNA gene of 10 potential biocontrol agents, most *Trichoderma* spp. isolates were found to be very similar to *Trichoderma* sect. *Pachybasium* (77%), whereas the others were similar to *Trichoderma* sect. *Trichoderma* spp. section (Table 1). Three groups of similar isolates were identified by cluster analysis. Seven isolates were found identical to *T. harzianum*, one isolate to ID17E of *T. hamatum* and one to ID20G *T. atroviride*. According to the sequencing data, seven strains were found similar

to *T. harzianum* (99%), one strain is to *T. hamatum* (94%) and a strain is to *T. atroviride* (97%).

Factors in determining the survivals of certain fungi on natural environment are important. One of these factors is the growing ability at different temperatures. Growth properties of *Trichoderma* spp. strains at different temperatures (2,4,10,15, 20, 24, 28, 32, 37 and 42 °C) were investigated and no growth was observed on the strains at 2 to 10 °C. In addition to that, increasing the temperature above 37-42 °C inhibited mycelial growth (Table

Table 1. Examined *Hypocrea* and *Trichoderma* spp. isolates and GenBank accession numbers of target sequences (continue).

ID9A	<i>Trichoderma aureoviride</i> T77	100%	HQ596945.1
	<i>Trichoderma aureoviride</i> T59	100%	HQ596942.1
	<i>Hypocrea lixii</i> JBSEB241	100%	EF191311.1
	<i>Hypocrea lixii</i> NMMX3008	99%	JQ040357.1
	<i>Trichoderma harzianum</i> GJS 05-93	99%	FJ442645.1
ID11C	<i>Hypocrea lixii</i> DAOM 222136	100%	JN942884.1
	<i>Trichoderma citrinoviride</i> T200	99%	HQ596983.1
	<i>Hypocrea lixii</i> DAOM 229959	99%	AY605733.1
	<i>Trichoderma harzianum</i> TRO40	99%	AF443925.1
	<i>Trichoderma harzianum</i> GJS 92-61	99%	HQ608080.1
ID11D	<i>Hypocrea lixii</i> GJS 04-227	100%	FJ442266.1
	<i>Hypocrea lixii</i> GJS 91-138	100%	AF443917.1
	<i>Hypocrea lixii</i> DAOM231402	99%	AY605732.1
	<i>Hypocrea lixii</i> JBT1244	99%	AY605741.1
	<i>Trichoderma harzianum</i> P134	99%	JF311950.1
ID17E	<i>T. hamatum</i> HBJZ1001	94%	JQ040347.1
	<i>T. hamatum</i> CQJB2001	94%	JQ040344.1
	<i>T. hamatum</i> T090	94%	HQ608116.1
	<i>T. hamatum</i> DAOM237553	94%	EU280136.1
	<i>T. hamatum</i> GHJ-5	94%	GQ331987.1
ID20G	<i>Trichoderma atroviride</i> DR19	97%	KC311841.1
	<i>Trichoderma atroviride</i> EGE-K-65	97%	JX119037.1
	<i>Trichoderma atroviride</i> ATCC20476	97%	JQ745258.1
	<i>Trichoderma atroviride</i> T39	97%	FJ975597.1
	<i>Trichoderma atroviride</i> KUC5026	97%	GQ241294.1
T.h1585	<i>Hypocrea lixii</i> T 22	99%	GU570562.1
	<i>Hypocrea lixii</i> BHU199	99%	JN618342.1
	<i>Hypocrea lixii</i> BHU162	99%	JN604838.1
	<i>Hypocrea lixii</i> DLY1202	99%	HQ259304.1
	<i>Hypocrea lixii</i> T18-1	99%	EU744189.1

2). However, it was observed that it maintains the viability of the *Trichoderma* spp. spores. Increasing temperature from 15 to 24°C led to an increase on the growth rates of *Trichoderma*, followed by a plateau. Optimum temperature for mycelial growth was observed between 24 and 28 °C. Strains ID11C and ID11D were found to show the highest growth characteristics in all tested temperatures (from 4 to 32 °C). In contrast, it was noted that ID7C strains exhibited the lowest growth level.

Temperature is considered as one of the most important parameters of biocontrol activity of *Trichoderma* spp [10]. In biocontrol organisms, the presences of tolerance against to temperature fluctuations are important parameters for the maintainability of life of the organism in extreme environments. The common incubation temperature for fungi growth such as *Trichoderma* sp., *Fusarium* sp., *Penicillium* sp. and *Graphium* sp. is 30°C [20]. and researchers also reported that none of the *Trichoderma* species grew at or above 40°C. Therefore it could be concluded that time and incubation temperature were two effective criteria on the sporulation [14]. Some isolated *Trichoderma* strains from the higher altitude (2000-3500 m) of Garhwal Himalayan region in India presented similar results with previous reports [21]. In this study, *Trichoderma* spp. strains were revealed to exhibit the ideal growth at 24 °C and 28°C, however, the maximum temperature for *Trichoderma* spp. growth

was examined at 32 °C when incubated on Tryptic Soy Agar under 4 °C conditions. The fungus was able to grow normally, characterized with heavily induced sporulation within three weeks of the incubation. Induction of sporulation via exposure to low temperatures appeared to be one of the strategies for survival of these species in extreme cold environments such as at 4 to 5 °C.

Trichoderma spp. spores were found to show heat tolerance at a certain range of temperature values (45-75°C) after 10 min exposure (Table 2). In the heat tolerance test of this study , although all strains of *Trichoderma* spp. were detected to be able to germinate spores at the range of 45 to 65°C, only three strains (ID11D, ID4A and ID4B) were observed to be able to protect the vitality of spores at 75 °C. Kucuk and Kivanc [22] indicated that 6 strains out of 7 isolates germinated at 75°C. This result indicated that *Trichoderma* spp. spores are heat-tolerant for a certain period of time at a range of 45 to 75°C. As *Trichoderma* spp. is well accepted being a potential and ecofriendly biological control agent by the community, it is necessary to grow it at suitable conditions before it could be used against to other soil borne plant pathogens or for commercial purposes. In this regard, heat tolerance of *Trichoderma* spp. spores is very important to maintain the viability during storage and after formulation processes.

Table 2. Radial growth of *Trichoderma* spp. at different temperatures on PDA medium for 48 hours and heat tolerance of fungi spores (cfu/mL).

Strain No	Incubation of temperature (°C) and radial growth (cm)					Heat (°C) tolerance of spore (10 min)			
	15	20	24	28	32	45	55	65	75
ID4A	3.5	4.2	7.5	7.3	2.0	≥10 ³	≥10 ³	≥10 ³	2×10 ²
ID4B	2.5	3.7	5.2	6.0	2.0	≥10 ³	≥10 ³	≥10 ³	3×10 ²
ID6B	3.7	4.1	6.2	7.0	2.0	≥10 ³	≥10 ³	≥10 ³	-*
ID7C	3.5	4.5	5.5	4.0	1.5	≥10 ³	≥10 ³	≥10 ³	-
ID9A	3.5	4.2	6.8	7.0	2.0	≥10 ³	≥10 ³	≥10 ³	-
ID11C	4.0	5.1	7.0	9.0	3.5	≥10 ³	≥10 ³	≥10 ³	-
ID11D	4.0	5.3	8.5	9.0	3.5	≥10 ³	≥10 ³	≥10 ³	10 ²
ID17E	4.0	5.1	5.5	7.0	3.0	≥10 ³	≥10 ³	≥10 ³	-
ID20G	3.9	5.5	8.5	6.0	3.0	≥10 ³	≥10 ³	≥10 ³	-
K U E N 1585	3.5	3.0	8.0	6.0	3.0	≥10 ³	≥10 ³	≥10 ³	-

Since optimization of the bio-control agents has vital importance, the best growth temperature and sporulation time were determined for the isolated strains in present work. The strain ID17E was found to have some growth parameters like commercial strain KUEN 1585 and no generated spore was detected under test conditions (Table 3). The highest number of spore was obtained from the strains ID9A, ID11C and ID11D at 28°C on day15, sporulation capacity of the strains were marked similar at 15, 20 and 24°C however, 28°C was significantly different in terms of strain's sporulation ability. The best incubation time and temperature was determined on day 15 at 28°C for *Trichoderma* spp. strains.

It is notorious notion that *Trichoderma* spp. species are effective against various gram positive

and negative bacterial species [17]. In the present study, we analyzed that whether our isolated strains have anti-microbial activity or not. Ethyl acetate extract of *Trichoderma* spp. isolates were found to display antibacterial activity but not antifungal activity (Table 4). All extracts were detected to show antibacterial activity against *Vibrio* sp., *S. marcescens*, *L. monocytogenes*, *B. cereus* and *B. subtilis* except for ID17E and ID20G. The ID17E extract was observed to effective against to human pathogen *S. aureus* and the other strains' (ID4A, ID4B and ID6B) extracts were effective for *M. smegmatis* a causative agent of tuberculosis. These results indicate that the exercised strains are effective against bacterial plant pathogens. It was shown that *T. harzianum* and *T. atroviride* have produced some kind of antibiotics by other studies

Table 3. Spore production of *Trichoderma* spp. at different time intervals and in different temperatures (cfu/mL).

Strain No	Days	Incubation Temperatures and viable spores/mL			
		15°C	20°C	24°C	28°C
ID4A	7	-*	8.60×10 ⁷	1.79×10 ⁸	3.01×10 ⁸
	15	1.81×10 ⁷	2.73×10 ⁸	2.78×10 ⁸	5.73×10 ⁸
ID4B	7	-	1.00×10 ⁸	1.22×10 ⁸	1.95×10 ⁸
	15	2.53×10 ⁷	2.63×10 ⁸	1.63×10 ⁸	3.59×10 ⁸
ID6B	7	-	3.91×10 ⁷	1.70×10 ⁸	7.53×10 ⁷
	15	2.31×10 ⁷	2.06×10 ⁸	3.65×10 ⁸	6.78×10 ⁸
ID7C	7	-	6.00×10 ⁷	4.30×10 ⁶	8.50×10 ⁸
	15	2.75×10 ⁷	6.10×10 ⁸	1.20×10 ⁸	3.20×10 ⁸
ID9A	7	-	1.30×10 ⁸	2.49×10 ⁸	3.28×10 ⁸
	15	2.13×10 ⁷	1.81×10 ⁸	5.77×10 ⁸	3.36×10 ⁹
ID11C	7	-	2.32×10 ⁸	5.60×10 ⁶	1.10×10 ⁸
	15	1.63×10 ⁷	6.10×10 ⁸	6.50×10 ⁸	7.70×10 ⁸
ID11D	7	-	3.41×10 ⁸	2.01×10 ⁸	3.59×10 ⁸
	15	1.63×10 ⁸	4.06×10 ⁸	4.53×10 ⁸	5.37×10 ⁸
ID17E	7	-	-	-	-
	15	-	-	-	-
ID20G	7	-	2.68×10 ⁸	1.76×10 ⁸	2.95×10 ⁸
	15	1.41×10 ⁷	2.82×10 ⁸	1.41×10 ⁸	2.94×10 ⁷
KUEN 1585	7	-	-	1.78×10 ⁷	1.26×10 ⁷
	15	4.06×10 ⁶	4.94×10 ⁶	5.03×10 ⁷	2.43×10 ⁸

*- No growth

Table 4. Inhibition zone (mm) of *Trichoderma* spp. fermentation broth extracts against some microorganisms.

	ID4A	ID4B	ID6B	ID7C	ID9A	ID11C	ID11D	ID17E	ID20G	KUEN	ET.AS.	DMSO	Ant.
										1585			
Ec	-	-	-	-	-	-	-	-	-	-	-	-	10
Pv	-	-	-	-	-	-	-	-	-	-	-	-	18
Ss	-	-	-	-	-	-	-	-	-	-	-	-	18
Vs	12	12	10	10	10	11	10	6	7	8	8	-	10
Sm	12	12	11	10	10	11	10	-	6	7	7	-	10
Kp	-	-	-	-	-	-	-	-	-	-	-	-	18
Yp	-	-	-	-	-	-	-	-	-	-	-	-	18
Pa	-	-	-	-	-	-	-	-	-	-	-	-	18
Sa	-	-	6	-	-	-	6	15	-	-	-	-	35
Ef	-	-	-	-	-	-	-	-	-	-	-	-	10
Lm	8	7	6,5	-	6	6	6	-	6	6	-	-	10
Bc	10	9	6	-	6	7	6	7	-	-	-	-	15
Bs	8	7	-	-	6	6	-	9	-	-	-	-	20
Ca	-	-	-	-	-	-	-	-	-	-	-	-	25*
Ct	-	-	-	-	-	-	-	-	-	-	-	-	25*
Sc	-	-	-	-	-	-	-	-	-	-	-	-	>25*
Ms	17	14	10	-	-	-	-	-	-	-	8	6	35**
An	-	-	-	-	-	-	-	-	-	-	-	-	ND
Bcin	-	-	-	-	-	-	-	-	-	-	-	-	ND
Rs	-	-	-	-	-	-	-	-	-	-	-	-	ND

Ec: *E. coli* ATCC25922, Pv: *P. vulgaris*, Ss: *Salmonella* sp., Vs: *Vibrio* sp., Sm: *S. marcescens*, Kp: *K. pneumonia* ATCC13883, Yp: *Y. pseudotuberculosis* ATCC 911, Pa: *P. aeruginosa* ATCC43288, Sa: *S. aureus* ATCC25923, Ef: *E. faecalis* ATCC29212, Lm: *L. monocytogenes* ATCC43251, Bc: *B. cereus* 709 ROMA, Bs: *B. subtilis* ATCC6633, Ca: *C. albicans* ATCC60193, Ct: *C. tropicalis* ATCC13803, Sc: *S. cerevisiae* RSKK251, Ms: *M. smegmatis* ATCC607, An: *A. niger* RSKK4017, Bcin: *B. cinerea*, Rs: *R. solani* AG3, (-); no effect, , KUEN 1585; *T. harzianum* KUEN 1585, ET.AS: Ethyl acetate, DMSO: Dimethyl sulfoxide, Ant.: Antibiotics, ND: Not determined, *: fluconazole **: Streptomycin.

[15]. When *T. harzianum* strains analyzed for their antagonistic activity, the isolates were found to be effective at various concentrations against to many bacterial species (*S. aureus*, *E. coli*, *Proteus* and *Klebsiella*) [23]. On the other hand, antimicrobial activities of 317 Basidiomycetes isolates were screened against nine human pathogens and the most susceptible micro-organism were reported as *B. subtilis* [24], which is very identical to our results. Tarus et al. [25] demonstrated that *T. harzianum* secondary metabolites (i.e. trisol, 6-phenyl prion and sorbicilin) were effective against to pathogen fungi of tea plants (*Armillaria mella*, *Mucor* and *Nematospora corylii*). That compounds (especially

6-phenyl prion) were found to be effective against an array of microorganisms (*Paecilomyces vericoti*, *Penicillium notatum*, *Nematospora coryli*, *Bacillus brevis*, *B. subtilis*, *Sercinia lutea* and *Enterobacter dissolvens*) with either their antibacterial and or antifungal activities. Liouane et al. [26] were reported that the analysed extract exhibited an intriguing antibacterial activity against to all tested bacteria except for the gram-negative *E. coli* and *P. aeruginosa*, but no antifungal activity was detected for the extracts. In our study, *T. harzianum* was found to show very high antagonistic activity against *A. terreus* and *A. fumigatus*. Clinical bacterial isolates of *S. aureus* and *E. coli* were found to be sensitive to *T. harzianum* extract.

Table 5. Effect of *Trichoderma* spp. at day 4, 7 and 15 on in-vitro growth inhibition of some fungi through Dual Plate Culture Technique.

Strains No	Growth range inhibition some of fungi (%)								
	Days	Rs	Bcin	Scs	An	Af	Bb	If	Ma
ID4A	4	30	49	67	23	8	29	2	9
	7	70	61	70	73	17	62	25	26
	15	64	59	69	73	50	83	51	59
ID4B	4	1	48	58	8	0	3	0	3
	7	33	58	58	72	14	33	20	33
	15	63	56	58	77	52	63	46	63
ID6B	4	16	57	64	8	4	3	1	3
	7	56	61	68	71	20	35	23	35
	15	64	62	69	74	52	63	50	63
ID7C	4	23	56	68	0	8	6	2	6
	7	54	61	71	68	27	41	25	41
	15	64	78	72	71	58	68	50	68
ID9A	4	15	56	61	15	13	12	1	12
	7	40	62	67	77	33	44	32	44
	15	67	78	68	77	60	68	55	68
ID11C	4	59	66	66	8	17	17	14	17
	7	70	68	75	73	43	45	36	45
	15	75	78	75	72	66	70	64	70
ID11D	4	55	73	71	23	17	13	8	13
	7	68	81	75	74	37	47	36	47
	15	69	83	78	74	66	70	64	70
ID17E	4	45	68	72	26	13	17	2	17
	7	68	71	74	71	37	56	34	56
	15	77	70	76	72	62	77	64	77
ID20G	4	39	69	68	15	17	12	11	12
	7	67	74	76	76	47	55	39	55
	15	75	78	79	78	68	76	66	76
K U E N 1585	4	25	58	66	8	8	11	2	11
	7	58	63	69	70	24	46	30	46
	15	71	57	69	71	58	70	50	70

Rs: *R. solani* (AG3), Bcin: *B. cinerea*, Scs: *S. sclerotiorum*, As: *A. flavus*, An: *A. niger*, Bb: *B. bassiana* ARSEF8664, Is: *I. fumosorosea* ARSEF8333, Ma: *M. anisopliae* ARSEF8433.

In dual culture test, isolates of *Trichoderma* spp. showed variable effects against to other tested fungi. All *Trichoderma* spp. strains have high inhibition activities against plant pathogen strain *B. cinerea*, *S. sclerotiorum* and *R. solani* (AG3) (Table 5). *Trichoderma* spp. showed inhibition on tested fungi with a significant difference. There was not significant difference between day 7th and 15th (mean rank 26.39 and 29.34) but there was a significant difference between day 4 and other days (mean rank 44.77). In dual culture of *Trichoderma*-*R. solani* (plant pathogen) the inhibition was detected between 63% and 77% (Table 5). The best inhibition values were calculated as 77%, 75% and 69% for strain ID17E, ID20G and ID11D, respectively. In dual culture of *Trichoderma* - *B. cinerea* (plant pathogen), inhibition was determined between 56% and 83%. The best inhibition values were calculated as 83% for ID11D and 78% for ID20G, ID11C, ID7C and ID9A. In dual culture of *Trichoderma* - *S. sclerotium* (plant pathogen), inhibition was determined between 58% and 79%. The best inhibitory strains were observed as ID20G, ID11D, ID17E and ID11C (79-75%). In *Trichoderma* - *A. niger* and *A. flavus* (opportunistic human pathogen) dual cultures inhibition percentages were calculated as 68%-78% and 50%-68%, respectively. The best effective strains against to *A. niger*, were determined as ID9A (77%) and ID20G (78%). The majority of *Trichoderma* isolates were found not to have significant effects on entomopathogenic fungi of *I. fumosorosea*, *B. bassiana* and *M. anisopliae*. *Trichoderma* spp. isolates were found to inhibit the growth of plant and human pathogens strongly, but there was no observed inhibition on insect pathogens. Therefore it is suggested that *Trichoderma harzianum* strains can be used either against to plant pathogens or in combination with insect pathogens in the environment safely as a biocontrol agent. Based on this knowledge, the analyzed isolates of *Trichoderma* spp. can be classified into 2 distinct groups based on their biocontrol capacity. The first group consists ID11C, ID11D, ID17E and ID20G, were true effective as potential bio-control agents. The second group consists ID4A, ID4B, ID6A, ID7C, ID9A, were effected the activities of tested pathogenic fungi but can not be categorized as good biocontrol agents in this study.

Antal et al. [27] reported that, 14 of *Trichoderma* spp. strains were identified as *T. atroviride*, *T. harzianum* and *T. viride* out of 360. It was stated that these strains had good growth parameters at 5°C, and inhibitory effects against *R. solani* and *F. oxysporum* in dual culture at 10 °C. Soil amendment with formulated *Trichoderma* spp. proved to be effective in controlling *Sclerotium rolfsii*, the causative agent for seedling disease of many crops and many researchers reported antagonistic activity of *Trichoderma* spp. isolates against plant pathogens especially against fungal ones such as *Rhizoctonia solani* and *Sclerotium rolfsii* [28]. Lone et al. [29] reported that *T. harzianum* isolated from the rhizosphere of *Juglans regia* L., caused inhibition the growth of *A. niger* by 75%, *C. sphaerospermum* by 72.2% and *F. oxysporum* by 25%. Isolated *Trichoderma* spp. from chickpea rhizosphere and root endophytic region, 7 isolates were found to show the highest (83.3%) inhibition percentage out of ten isolates tested against *Rhizoctonia bataticola* [30]. It was determined that *Trichoderma* spp. had a significant effect against entomopathogenic biocontrol strains of *B. bassiana*, *I. fumosorosea* and *M. anisopliae* (Table 5). Due to the rapid growth of *Trichoderma* spp. strains, it was observed that the strains limited growth area of entomopathogens, showed vertical growth instead of radial growth and blocked the contact between *Trichoderma* spp. and entomopathogenic strain. In a report of Lopez and Orduz [31] entomopathogenic fungus, *M. anisopliae* (strain M-137), or the antagonist fungus, *T. viride* (strain T-26) or a combination of both entomopathogenic and antagonist fungi were applied to control *A. cephalotes* nests. The mortality of nests was found as 100% with *M. anisopliae* and the combined fungal treatment, and 80% mortality in the treatment with *T. viride*. Combined application of entomopathogens (*B. bassiana*, *M. anisopliae* and *Paecilomyces fumosoroseus*) with mycoparasites (*Clonostachys* spp., *T. harzianum* and *Lecanicillium lecanii*) did not influence their biological control efficacy in vivo, although the re-isolation success of entomopathogens could be significantly reduced, especially from smaller insect species according to Krauss et al. [32]. Our results also supported these data and it was concluded that there is no effect of co-application of *Trichoderma*

spp. strains with entomopathogens. *Trichoderma* spp. strain ID4B, ID11D and ID20G were found not to generate spores when tested to produce volatile metabolites (Table 6). All *Trichoderma* spp. strains were detected to produce volatile metabolite having effect in reducing sclerotia and radial growth of *S. sclerotiorum*.

In a report, volatile and non-volatile compounds produced from *Trichoderma* spp., *T. saturnisporum*, *T. harzianum*, *T. viride*, *T. reesei* were studied against *Colletotrichum capsici*. The volatile compounds produced from all the selected *Trichoderma* species were found to show 30 to 67% inhibition of *C. capsici* [33]. Approximately 250 volatile organic compounds have been identified from fungi up to know and *Trichoderma* spp. species are known to produce numerous volatile organic compounds [34]. Mohiddin et al. [35] reported that 6 isolated *T. viride* inhibited sclerotia production of *S. rolfsii* (48%) and *S. sclerotium* (78%) and developed mycelia by 40%. Fungal volatile organic compounds are important in the functioning of both atmospheric and soil ecosystems [36]. When twelve isolates were tested for the production of volatile and non-volatile metabolites, it was found that some

of these compounds (Isobutyric acid, 1,8-Cineole and 6-Pentyl- α -pyrone) showed antifungal and antibacterial activity and in addition to the biocontrol strategies [37]. In our study, *Trichoderma* spp. produced some volatile metabolites, inhibited production of sclerotia and growth of pathogens at the same time. The best effective strains were determined as ID6B, ID11C, ID11D and ID20G in terms of volatile compound production.

Captan is a broad-spectrum fungicide that belongs to the phthalimide chemical family. There are number of fungicides that contain copper hydroxide and Cuprenax is one of them. Mancozeb (Dikozin) contains zinc and manganese ethylene bis-dithiocarbamate [38]. All of these fungicides are used for greenhouse and/or nursery of plants depends on the formulation. *Trichoderma* spp. strains were tested against to various concentrations of different groups of fungicide (Captan (Phthalimide), Dikozin (dithiocarbamate) and Cuprenax (inorganic)) and the highest tolerance was detected against to Dikozin and the lowest to the Captan (Table 7). It was not determined that there is a significant difference among Cuprenax concentrations (2.5, 5, 10 mg mL⁻¹) on the growth rate of *Trichoderma* spp.

Table 6. Evaluation of volatile metabolites produced by *Trichoderma* spp. isolates against mycelial growth and spore production in *Sclerotonia sclerotium*.

Trichoderma Strain No	<i>Trichoderma</i> spp.		<i>Sclerotonia sclerotium</i>	
	Growth	Spore production	Growth	Sclerotia production
ID4A	+++	++	++	*-
ID4B	++	-	++	-
ID6B	+++	+	+	-
ID7C	+++	++	++	-
ID9A	+++	++	++	-
ID11C	+++	++	+	-
(+); low, (++) good, (+++) very good, (-); negative, *; no sporulation				
ID11D	+++	-	+	-
ID17E	++	-	++	-
ID20G	+++	-	+	-
KUEN 1585	++	-	+	-
Negative control	+++	+++		
Sclerotonia control			+++	+++

Table 7. The growth zone of *Trichoderma* spp. on PDA medium containing fungisit (on day 7, mm).

Strain No	Captan (mg mL ⁻¹)			Dikozin (mg mL ⁻¹)			Cuprenax (mg mL ⁻¹)		
	2.5	5	10	2.5	5	10	2.5	5	10
ID4A	40	20	14	85	85	39	35	35	40
ID4B	40	20	14	85	85	40	35	34	38
ID6B	38	21	19	85	85	35	36	30	29
ID7C	38	20	18	85	85	20	30	30	29
ID9A	28	22	18	85	85	30	34	30	30
ID11C	48	20	19	85	85	80	50	45	40
ID11D	35	35	19	85	85	85	42	40	40
ID17E	28	15	5	85	60	50	39	34	30
ID20G	20	12	9	85	70	40	20	17	15
K U E N 1585	75	35	25	85	85	20	35	35	30

*The zone diameter of the control groups were 85 mm at day 7.

strains. However, there was a significant difference among 2.5 mg mL⁻¹ and other doses of Captan and among 10 mg mL⁻¹ and other doses of Dikozin. It was observed that 10 mg mL⁻¹ of Captan inhibited the growth of *Trichoderma* spp. strains by 80%. Dikozin and Cuprenax inhibited the growth at the same concentration by 50% and 62%, respectively. *Trichoderma* spp. was found to be the most sensitive to Captan, Propiconazole and Tebuconazole in our study. Among tested fungicides the most resistant strains were determined as ID11C and ID11D, the most susceptible strain was determined as ID20G. Thiram, Mancozeb, Tebuconazole were found to be the best effective fungicides and the least effective ones were reported as Pyrocymidone and Captan on *T. harzianum* C52 spore germination in a previous study [39]. Khan and Shahzad [19] reported that *T. harzianum* were able to proliferate under the low doses of various fungicides (Benomyl, Topsin-M, Carbendazim and Cuprocaffro). A similar report on *T. harzianum*'s potential integration as a biological agent was reported by Saxena et al. [40]. The compatibility was assessed at different concentrations and the concentration of Carbendazim, Metalaxyl, Captan, Mancozeb, Thiram and Nemacur were under the safe tolerance limit for *T. harzianum* whereas the corresponding values for *T. virens* were found as 40, 1000, 125, 177, 9, and 700 µg/mL, respectively according to Mohiddin and

Khan.[35] The in-vitro effect of 6 commonly used fungicides on the mycelial growth of *Trichoderma harzianum* PBT 23 were evaluated in another study and Captan, Thiram, Chlorothalonil and Copper hydroxide were reported compatible with the test antagonist up to 100 µg/mL, while Mancozeb was compatible with up to 250 µg/mL. It is reported that these affect the growth of test antagonist adversely [40]. Parab et al. [41] studied the sensitivity of *T. harzianum* against to different fungicides and the results indicated that all systemic fungicides were found to inhibit the growth of *T. harzianum* completely whereas Zineb, Copper hydroxide, Mancozeb and copper oxychloride were found safe for the growth of *T. harzianum*.

Based on our results, although there was a reduction in growth rate, used and over dose concentrations of the strain of interest were detected to sustain growth. The strains ID11C, ID11D and ID20G were characterized as potential bio-control agents that did not affect the vitality.

CONCLUSION

With this study an attempt has been made to grow different species of *Trichoderma* spp. at various temperatures, with their antifungal and antibacterial activity in order to reveal all

the relevant and favorable parameters. The *Trichoderma* spp. species inhibits the growth of pathogens by releasing antibiotics and other volatile compounds that are harmful to the pathogens. We showed that *mycelia* growth inhibition affects *Trichoderma*'s volatile organic compounds without direct physical contact between the pathogen and fungus. The temperature is an important parameter for the growth of pathogen and biocontrol agent because it affects spore germination, mycelial growth and competitive saprophytic ability. It is important using *Trichoderma* spp. as a potential bio-control agent in different geographic regions, because strains maintain their spore vitality at a temperature range of 4-75°C and can grow at 15-37°C. This characteristic provides the adaptation of bio-control agents to temperatures at application fields and sustainability of the adapted strains. Considering the reproductive properties, spore production, fungisit tolerance and pathogen inhibition it can be concluded that ID11D and ID11C might be better bio-control agents. Identifying biocontrol agents and understanding the mechanisms underlying the antagonistic effect of *Trichoderma* spp. on plant pathogens are important in creating effective and safe bio-control strategies. Possible mechanisms of antagonism employed by *Trichoderma* spp. includes nutrient and niche competitions, antibiosis by producing volatile components and non-volatile antibiotics that are inhibitory against a range of soil-borne fungi, as well as parasitism. Different soil isolates of *Trichoderma* spp. can variable in their ability to fight against to different fungal pathogens. Therefore, enlightening the effects of new biocontrol agents to provide disease control is important.

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