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Antagonistic activity and molecular characterization of biological control agent *Trichoderma harzianum* from Saudi Arabia

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Abstract

Trichoderma harzianum is one of the most commonly used fungal species used in the biological control of plant pathogenic fungi. Its ability to control biological pathogens is strengthened by the production of analytic enzymes. In the identification of these fungal species, it has been noted that morphological characteristics alone are insufficient. Hence, the aim of this study was to identify 12 *Trichoderma* isolates based on their molecular markers and to evaluate the antagonistic activity of these *Trichoderma* isolates against several plant pathogens. The 12 *Trichoderma/Hypocrea* isolates were harvested from the rhizosphere of healthy tomato plants in Abha region, Saudi Arabia. These isolates were identified by both morphological and molecular techniques, such as the sequencing of the 5.8S-ITS region. Furthermore, based on the taxonomy and phylogeny of these isolates, the genetic similarities based on the inter-simple sequence repeat-PCR markers separated the 12 *Trichoderma* isolates into 2 major clusters. The 12 isolates were then used in confrontational assays with *Rhizoctonia solani*, *Pythium ultimum*, and *Alternaria solani*. Most of the *Trichoderma* isolates showed a strong antagonistic activity, which could also be observed with the use of SEM. This study highlights the possibility of using *T. harzianum* in IPM programs as an effective biological agent against several pathogenic fungi.

Keywords: *Trichoderma*, Morphological identification, Molecular characterization, Antagonistic activity, Biological control

Background

Due to the harmful effects of chemical pesticides on both human health and the environment, biological control agents have recently been used more frequently as an alternative in the control of plant pathogens (Vinale et al. 2008). Biological control agents act against plant pathogens in several ways, by mycoparasitism, antibiotic-mediated suppression, production of lytic enzymes and other byproducts, competition for nutrients, or induction of host resistance mechanisms (Pal and McSpadden Gardener 2006). Several biological control agents are now commercially available for separate use in disease control, or in combination with chemical pesticides, thus reducing the amount of pesticides necessary in the fight against plant pathogens. Both bacterial and fungal strains can be used as biological control agents (Melnick et al. 2008; Validov et al. 2009; Hassan et al. 2014). The most commonly used fungal species in biological control are the *Trichoderma* species (Singh et al. 2013). These fungi are commonly found in the soil-borne plant root ecosystem (Vinale et al. 2008). *Trichoderma* species are non-sexual organisms and co-exist with other soil inhabiting fungi with teleomorphs belonging to the genus *Hypocrea* (Ascomycota). Many difficulties exist in determining the morphology of *Trichoderma*, such as the intertwining of morphological traits and phenetic phenomena, especially among *Trichoderma* anamorph forms (Druzhinina et al. 2006; Hassan et al. 2014). The initial approach to understanding the diversity of *Trichoderma* was made by Rifai (1969), who introduced the concept of 9 "species groups" in *Trichoderma*; however, these groups could include multiple species that cannot be distinguished based on morphological characteristics alone. In an attempt to counter this lack, research over the past 20 years in *Trichoderma* biodiversity has focused on the development of a variety of molecular markers

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for species differentiation, including RAPDs, ISSR, RFLP, AFLP, and nucleotide sequencing techniques.

Hence, the objective of the present study was to identify *Trichoderma* isolates based on their molecular markers and to evaluate the antagonistic activity of these *Trichoderma* isolates against *Rhizoctonia solani*, *Pythium ultimum*, and *Alternaria solani*.

Material and methods

Isolation and identification of fungal isolates

Soil samples were collected from healthy tomato plant rhizospheres from Abha farms, Saudi Arabia. Isolation of the *Trichoderma* isolates was done, using the dilution plate method, as described by Hassan and El-Awady (2011).

Morphological identification of *Trichoderma*

The morphological identification of the *Trichoderma* isolates was carried out based on the appearance and pigmentation of the respective colonies, growth rate, and microscopic characteristics such as branching patterns of conidiophores, the arrangement of phialides, and the shapes and sizes of conidia, as described previously by Kumar and Sharma (2011).

Antagonistic activity of *Trichoderma* spp. in dual culture

The antagonistic activity of the *Trichoderma* isolates was assessed against several soil borne pathogens; *Rhizoctonia solani*, *Pythium ultimum*, and *Alternaria solani*. The isolates were obtained from the Scientific Research Center, Biotechnology and Genetic Engineering Unit, Taif University, Saudi Arabia. Antagonistic activity was achieved in dual culture, as described previously by Fahmi et al. (2012). Percent inhibition of radial growth of pathogen was calculated as follows: percentage of inhibition = [(R1−R2)/R1] × 100, where R1 is the radius of the pathogen away from the antagonist and R2 is the radius of the pathogen.

Total genomic DNA extraction

Trichoderma isolates were cultured on Czapek Dox broth at 28 °C for 5 days, after which total genomic DNA for each strain was extracted, using the Norgen Plant/Fungi DNA Isolation Kit (Sigma, Thorold, Canada) as previously reported by Hassan et al. (2019). The extracted genomic DNA was subsequently used for sequencing analyses.

Sequence analysis of 5.8S-ITS region

The sequencing of the 5.8S region for all fungal isolates was done at Macrogen Co., Seoul, South Korea. Multiple nucleotide alignment of the ITS regions was performed using BioEdit version 7.2.5 software. The obtained sequences with about 600 bp were aligned by known sequences of the 5.8S-ITS region obtained from GenBank and subsequently used for the construction of a phylogenetic tree (MEGA 7.10) as described previously by Kumar et al. (2016).

Inter-simple sequence repeat (ISSR) analysis

For ISSR analysis, the total genomic DNA of the *Trichoderma* isolates was subjected to PCR amplification as described by Lakhani et al. (2016). Ten ISSR primers labeled as ISSR-1 to ISSR-10 and their sequencing were as the following: ISSR-1: $(GA)_8TT$; ISSR-2: $(GA)_8TC$; ISSR-3: $(AC)_8TT$; ISSR-4: $C(GA)_7G$; ISSR-5: $GAC(GA)_7$; ISSR-6: $TAG(CA)_7$; ISSR-7: $CAG(CA)_7$; ISSR-8: $(AG)_8$ T; ISSR-9: $(AG)_8$ C: and ISSR-10: $(AG)_8$ G, which were used in separate single PCR reactions (Lakhani et al. 2016; Hassan et al. 2019). The similarity matrix was estimated based on a simple matching coefficient that was estimated by means of Jaccard's coefficient (Rohlf 2000).

Specimen preparation for scanning electron microscopy (SEM)

Parasitism of the hyphal cells of *R. solani*, *P. ultimum*, and *A. solani* by *Trichoderma* was studied by SEM as described previously by Hassan (2014). To obtain the sites of interaction of the hyphae with the pathogens used in the study, *Trichoderma* and each pathogen were grown on PDA at 28 °C for 48 h, respectively. *Trichoderma* and the respective pathogens grew towards each other allowing their hyphae to interact with one another. After 48 h of incubation, a light microscope was used to observe the early stage of the interaction. The interaction sites were marked, and 1 cm^2 agar block was removed from the plate for SEM preparation. Mycelial samples from the region of interaction were fixed for 24 h with vapors of glutaraldehyde and osmium tetroxide (3:1), air-dried for 48 h, and then coated with gold.

Data analysis

The analysis of the variance of the antagonistic activity was done using one-way analysis of variance, using SPSS software var. 16. Duncan's multiple range test, as reported by Snedecor and Cochran (1989), was used to detect variations among treatments at 5% probability. Means within a column, followed by the same letter, were not significantly different.

Results and discussion

Morphological characterization of *Trichoderma*

Fifty fungal isolates were collected from the rhizosphere of different tomato plants in the Abha region of southern Saudi Arabia. Twelve isolates were identified as *Trichoderma* spp. (labeled T.1–T.12, respectively). After 4 days of incubation at 25 °C, the growth and sporulation patterns of the *Trichoderma* isolates showed significant

Table 1 Colony morphology, pigmentation, and sporulation of *Trichoderma* isolates

Strains	Morphology	Pigmentation	Spore coloration
T1 (*T. harzianum*)	Dark green dense mycelium	Dark green	Light green
T2 (*T. harzianum*)	White and green mycelium	Green	Light green
T3 (*T. harzianum*)	Thick dense green mycelium	Green	Light green
T4 (*T. harzianum*)	Dark green dense mycelium	Dark green	Light green
T5 (*T. harzianum*)	Dark green dense mycelium	Dark green	Light green
T4 (*T. harzianum*)	Dark green dense mycelium	Dark green	Light green
T7 (*T. harzianum*)	Dark green dense mycelium	Dark green	Light green
T8 (*T. harzianum*)	White and green mycelium	Green	Green
T9 (*T. harzianum*)	Dark green dense mycelium	Dark green	Light green
T10 (*T. harzianum*)	Dark green dense mycelium	Dark green	Light green
T11 (*H. lixii*)	Sparse yellowish brown	Dark yellow	White green
T12 (*H. lixii*)	Sparse yellowish brown	Dark yellow	White green

differences (Table 1). The conidial wall patterns and shape were rough and subglobose with a green to dark green color. The growth characteristics of the reverse of the colony and the mycelial color patterns varied significantly between the *Trichoderma* isolates, ranging from colorless to yellow and watery white to white. Green conidial production was observed in the growth of the isolates, and these were denser in the center with dark green conidia distributed throughout the culture, along with the formation of 1–2 concentric rings around the culture. With the use of previous data (Hassan et al. 2014), *Trichoderma* isolates were identified as *T. harzianum* and telomorphic *Hypocrea lixii*. Morphological characterization was conventionally used in the identification of *Trichoderma* species, and it remains a potential method to identify *Trichoderma* species (Gams and Bissett 2002; Anees et al. 2010; Hassan et al. 2014). Therefore, morphological features of the *Trichoderma* isolates were included in this study. The microscopic features that are frequently studied include the shapes and sizes of conidia and the branching patterns of conidiophores (Samuels et al. 2002; Anees et al. 2010; Hassan et al. 2014). In this study, the description of the shapes of conidia posed a concern in the identification process

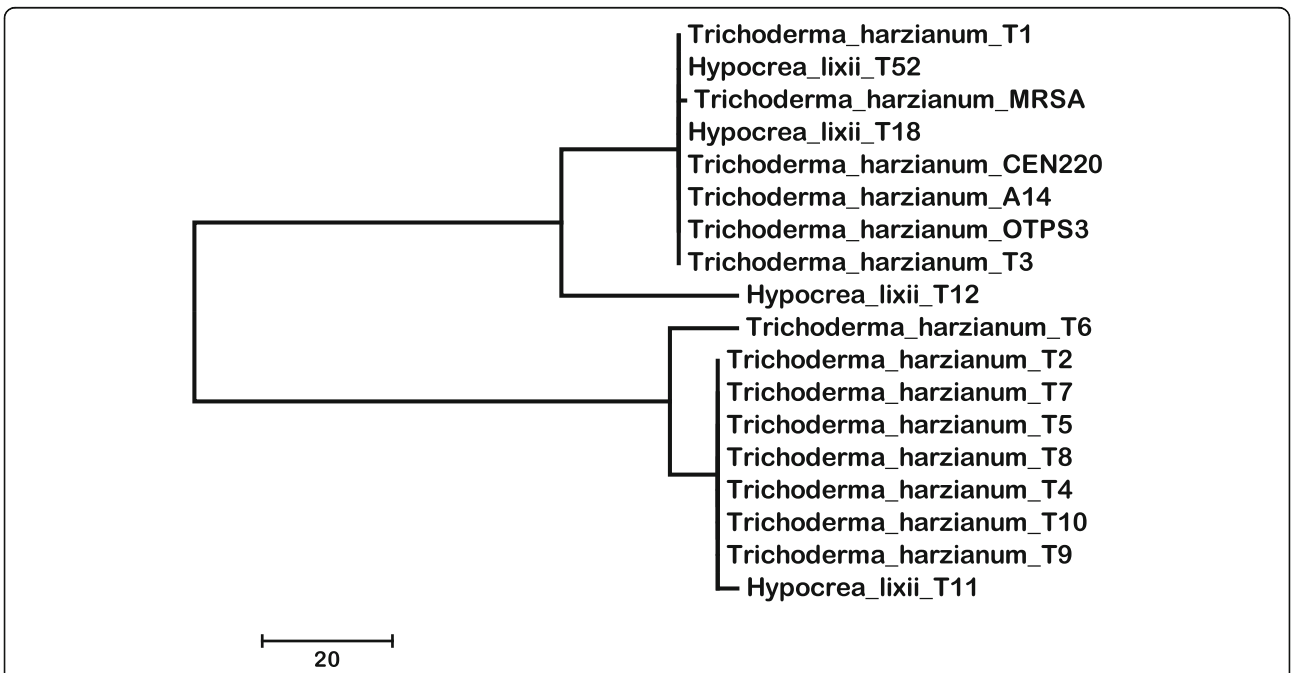

Fig. 1 Phylogenetic tree and diversity of the 5.8S-ITS region in 12 *Trichoderma* sp. isolates compared with reference *Trichoderma* strains. The phylogenetic tree was generated using parsimony neighbor-joining and maximum likelihood analysis

of most of the isolates due to the different terms used in literature for describing the shapes of the conidia, which could lead to possible confusion.

Sequence analysis of 5.8S-ITS region

Genomic DNA of all *Trichoderma* isolates was successfully extracted and used for nucleotide sequencing. The universal primers ITS 1 and ITS 4 were used to amplify the internal transcribed spacer (ITS) regions of the rDNA of all the *Trichoderma* isolates used in this study. The sequences obtained were subjected to a BLAST analysis to identify the isolates and were submitted to the National Center for Biotechnology Information (NCBI) GenBank (accession numbers MK680282–MK680291). There was a substantial difference between the number of base pairs of the ITS sequences of the *T. harzianum* (442 bp) and *H. lixii* (466 bp) isolates. The isolates of *T. harzianum* showed (98%) homology in the nucleotide sequence. Conversely, the *H. lixii* isolates showed nucleotide divergence of (2.2%) in the ITS region. Isolates T.2 and T.3 show a 100% homology in nucleotide sequence (Fig. 1). To elucidate the genetic relatedness between the *Trichoderma* isolates, a phylogenetic tree was constructed based on the sequence analysis of the ITS regions, using the neighbor-joining method in the MEGA 7.1 software. A random sequence was used as an out-group to demonstrate where the root of the tree was situated. A bootstrap analysis of the ITS region with 1000 bootstrap repeats showed 2 main clusters (Fig. 1). The majority of the *T. harzianum* isolates were included in the second cluster with a bootstrap value of 100%. However, isolate T.6 formed a sub-cluster in this main cluster. The first main cluster consisted of the remaining *T. harzianum* isolates and isolate T.12 identified to be *H. lixii*, with a bootstrap value of 99%. The information obtained from the morphological study alone was insufficient to precisely identify *Trichoderma* spp. because they have relatively few morphological characteristics

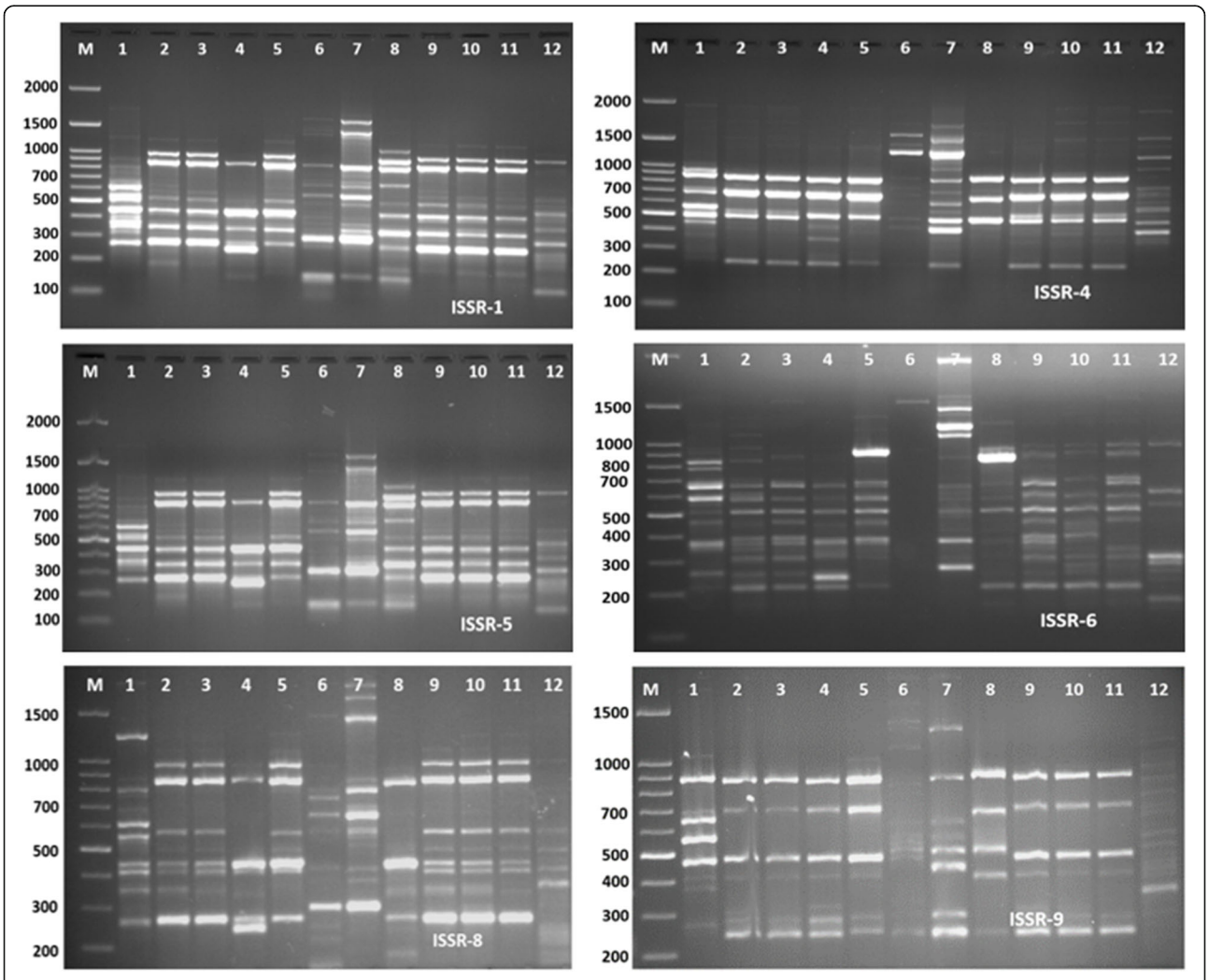

Fig. 2 ISSR-PCR profile of the 12 *Trichoderma* isolates generated with 6 ISSR primers. *M* is 100 bp DNA ladder

and a limited variation that may cause overlapping and misidentification of the isolates (Anees et al. 2010). In addition to this limitation of morphological characteristics, they are also influenced by culturing conditions such as temperature and quality of used media (Hassan et al. 2014). Therefore, it was necessary to include molecular techniques in the identification of these organisms. The ITS region is one of the most reliable loci for the identification of a strain at the species level (Kullnig-Gradinger et al. 2002; Hassan et al. 2019). Thus, in this study, DNA sequencing of the 5.8S-ITS region and ISSR-PCR analysis were done for all isolates. By comparing the sequences of the 5.8S-ITS region to the sequences available on GenBank, all of the *Trichoderma* isolates could be identified to species level with homology of at least 99%.

Molecular characterization using the ISSR-PCR markers

For the 12 ISSR markers tested from the respective isolates in this study, 10 primers (ISSR1-ISSR10) were used for the amplification of all the isolates. The primers generated 191 ISSR bands, and the size of the amplicons ranged from 50 to 2200 bp (Fig. 2). The PCR reaction with primer ISSR-2 produced the maximum number of bands (23 bands), while the reaction for ISSR-7 produced the minimum number of bands (15 bands). Marker ISSR-2 produced the maximum number of polymorphic loci (19 loci out of 23 loci), followed by primers ISSR-6 and ISSR-10 (17 loci each) (Table 2). The polymorphism percentage ranged from 10.5 to 38.9%, and the mean value of the Jaccard's similarity coefficient of the ISSR markers was 0.761. Based on the results, the genetic characteristics of the isolates could be grouped into two main clusters (Fig. 3). Cluster 1 included strain T.1, T.3, and T.12, while cluster 2 was divided into two sub-clusters. Sub-cluster 1 included the strains T.2, T.4, T.5, T.7, T.8, T.9, T.10, and T.11, while sub-cluster 2 included only strain T.6 (Fig. 3). The obtained results indicated that the ISSR primers amplify random regions in the genome of *Trichoderma* spp., as occurs in the RAPD technique, and no relationship could be established between the amplified regions (Mehta et al. 2002; Hassan et al. 2014). It is also likely that both coding and non-repetitive regions were targeted for amplification in these fungi. Nevertheless, the ISSR-PCR is a highly reproducible method for the characterization of bacterial or fungal species (Hiett and Seal 2009; Hassan et al. 2014). The ISSR-PCR approach was useful for assessing the genetic diversity both of *Trichoderma* isolates obtained from different geographic locations (Mehta et al. 2002) and between different *Trichoderma* species (Hassan et al. 2019). Moreover, Lakhani et al. (2016) reported that the ISSR approach is a robust method to detect polymorphisms among different *Trichoderma* species.

Table 2 Polymorphism level detected by the 10 ISSR primers that have been used for fingerprinting PCR analysis in *Trichoderma* isolates

Primers Name	TB	PB	MB	PPB (%)	NSB	PSB
ISSR-1	19	5	14	26.3	2.00	10.5
ISSR-2	23	4	19	17.3	0.00	0.00
ISSR-3	20	7	13	35.0	0.00	0.00
ISSR-4	16	5	11	31.2	0.00	0.00
ISSR-5	18	7	11	38.9	0.00	0.00
ISSR-6	21	4	17	19.1	0.00	0.00
ISSR-7	15	5	10	33.3	1.00	6.70
ISSR-8	21	6	15	28.6	2.00	9.50
ISSR-9	19	6	13	31.5	2.00	10.5
ISSR-10	19	2	17	10.5	0.00	0.00
Total/mean	191	51	140	38.4^{m}	7.00	3.72^{m}

TB total bands, *PB* polymorphic bands, *MB* monomorphic bands, *PPB* percentage of polymorphic bands, *NSB* number of specific bands, *PSB* specific band ratio, *m* mean

Antagonistic ability of *Trichoderma* spp. in dual culture

The antagonistic ability of all 12 *Trichoderma* isolates were used in confrontational assays with 3 plant pathogens (*Rhizoctonia solani*, *Pythium ultimum*, and *Alternaria solani*) on PDA at 28 °C for 4 days. The negative control plates were inoculated by either plant pathogen species or *Trichoderma* isolates (Table 3). In all the dual culture plates tested, the contact zone between the fungi was curved, with concavity oriented towards the plant pathogen. The average percentage of inhibition (%) of the mycelial growth for each of the 3 pathogens is presented in Table 3. All the *Trichoderma* isolates had the ability to inhibit the mycelial growth of the 3 pathogens. The percentage reduction in the growth of these pathogens ranged between 36.5 and 81.4%. Among all *Trichoderma* isolates, T.6 showed a significantly higher percentage of inhibition (81.4%) when challenged with *R. solani*, followed by T.7 (79.6%), while T.2 showed the lowest percentage of inhibition (52.0%). *P. ultimum* was inhibited significantly more by T.6 (61.2%) followed by isolate T.10 (58.3%), whereas T.1 and T.2 had the lowest percentage of inhibition (36.5%). Mycelial growth of *A. solani* was significantly inhibited by isolate T.6 (62.8%), followed by T.5 (57.4%), and T.10 had the lowest percentage of inhibition (42%) (Table 3). The curvature of the contact area between the *Trichoderma* colony and the pathogenic fungi colony in the same PDA plate depends on the growth rate of the colonies. If a colony displays a greater growth rate than the other colonies, a curve is likely to be observed in the contact zone. However, if the 2 colonies have the same growth rate, a straight line is expected, when the mycelia come in contact with both fungi (Shruti and Manda 2012;

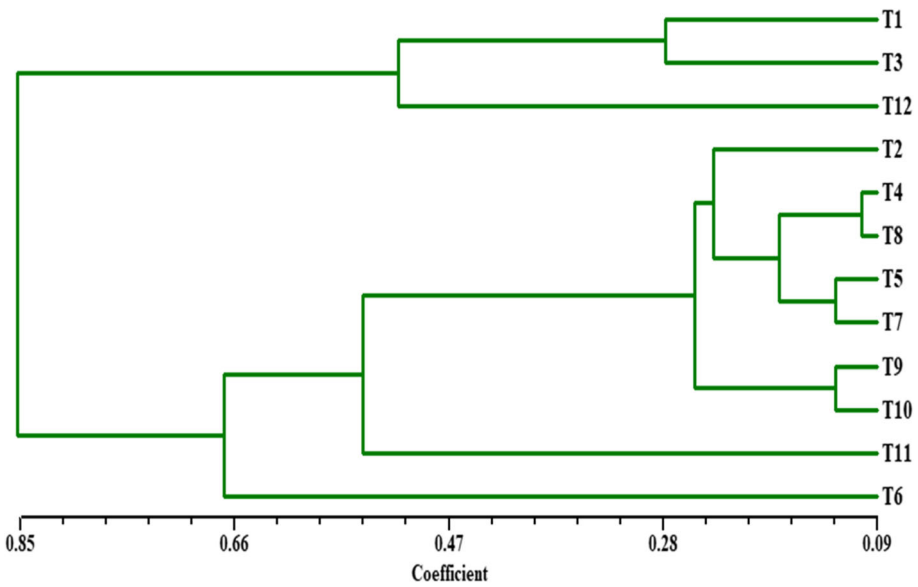

Fig. 3 Dendrogram analysis based on 10 ISSR-PCR primers that have been used for fingerprinting PCR analysis of 12 *Trichoderma* sp. isolates

Kushwaha and Verma 2014; Hassan et al. 2014). The potency of *Trichoderma* may be attributed to the ability to produce toxic hydrophilic metabolites or lytic enzymes (Shruti and Manda 2012; Hassan 2014; Parmar et al. 2015). These enzyme components, such as glucanase and chitinase, are released by *Trichoderma* strains at low levels. Therefore, it can act against pathogenic fungi before interacting with the 2 mycelia, thus increasing the antagonistic capacity of *Trichoderma* (Kushwaha and Verma 2014; Fahmi et al. 2012). Mycoparasitism is an important and complex process in which *Trichoderma* spp. extends lytic enzymes towards its host or prey and attaches to and coils around the organism's hyphae, sometimes penetrating them (Parizi et al. 2012; Hassan 2014).

Table 3 Antagonistic potential of *Trichoderma* isolates against *Rhizoctonia solani*, *Pythium ultimum*, and Alternaria solani

Trichoderma isolates	Antagonistic potential inhibition %		
	R. solani	*P. ultimum*	*A. solani*
T.1	66.7^{g}	36.5^{f}	54.3^{c}
T.2	52.0^{i}	36.5^{f}	43.8^{f}
T.3	72.4^{f}	48.3^{c}	51.5^{d}
T.4	72.4^{f}	48.3^{c}	54.6^{c}
T.5	72.4^{f}	45.2^{d}	57.4^{b}
T.6	81.4^{a}	61.2^{a}	62.8^{a}
T.7	79.6^{b}	42.9^{e}	45.8^{e}
T.8	74.5^{d}	45.2^{d}	54.6^{c}
T.9	72.8^{e}	48.4^{c}	54.6^{c}
T.10	76.7^{c}	58.3^{b}	42.0^{g}
T.11	60.2^{h}	48.3^{c}	45.8^{e}
T.12	72.4^{f}	42.9^{e}	51.5^{d}

*Mean values within a column followed by the same letter are not significantly different at $P \leq 0.05$

SEM observation of mycoparasitism of *Trichoderma*

With SEM, the coils and the points of interaction could be visualized more clearly (Fig. 4). Coiling of the 3 pathogens' hypha was observed when confronted with most *Trichoderma* isolates, but only isolate T.6 was selected for visualization via SEM because of the highest potential in antagonistic activity. After 3 days of inoculation, the electron micrographs were visualized, and the results showed a complete colonization of *R. solani* with the *Trichoderma* isolate. The contact zone revealed that the parasitic hyphae grew by forming coiling structures and producing spores. During the confrontation, *Trichoderma* isolates formed aspersorium-like structures without penetrating the cell walls of either *P. ultimum* or *A. solani* (Fig. 4c, f). This indicated that the antagonistic effect of *Trichoderma* resulted in the breakdown of pathogen hyphae was due to the presence of lytic enzymes. Lytic enzymes might lead to the degradation of the pathogen's cell wall, especially due to the high concentrations of chitinase which is produced by *Trichoderma* spp. (Hassan 2014). This corresponds to what was observed in this study, where partial degradation of the cell walls was visualized in the later stages of the confrontational challenge. The possible effects of the degrading cell walls on the plant pathogens could be further studied by using different approaches, such as ultrastructural and/or histochemical studies.

Conclusion

Both morphological and molecular approaches are important methods to identify *Trichoderma* isolates. Furthermore, the *Trichoderma* isolates displayed a strong

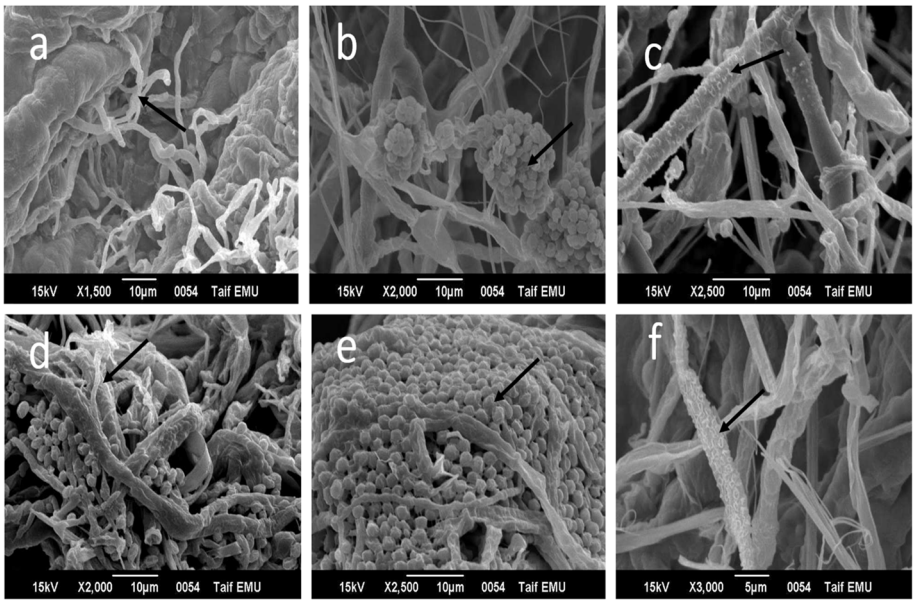

Fig. 4 Scanning electron micrographs displaying the antagonistic activity of *Trichoderma harzianum* isolate (T.6). **a**, **b** Coiling and spore formation of T.6 in the mycelium over *Rhizoctonia solani* and **c**, **d** T.6 spore formation without penetration into the cell wall and coiling of *Trichoderma* of *Pythium ultimum*. **e**, **f** Spore formation of *Trichoderma* T.6 over *A. solani* mycelia and seemed to be capable of degrading the cell walls of *A. solani*

antagonistic activity against certain tomato pathogens. The dual culture assays and SEM suggested that *Trichoderma* spp. can be used as a biological control agent in agriculture. These isolates may further be evaluated in field experiments and the production of commercial fertilizers.

Abbreviations

AFLP: Amplified fragment-length polymorphism; ISSR: Inter simple sequence repeats; ITS: Internal transcribed spacer; PCR: Polymerase chain reaction; RAPD: Random amplification of polymorphic DNA; RFLP: Restriction fragment length polymorphism; SEM: Scanning electron microscope

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Authors' contributions

This study was conceived and designed by YM, MFA, and MH. The laboratory work was carried out by MH, MF, and AK. Data were analyzed by MF, ME, and YM. The manuscript was written by AK and ME. The manuscript was revised by MFA and MF. All authors have accepted the final version of the manuscript.

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Availability of data and materials

Data and materials in this study can be used as a reference by other researchers.

Ethics approval and consent to participate

Not applicable. Ethical approval is not required for this study.

Consent for publication

Not applicable.

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