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Influence of chitinase production on the antagonistic activity of *Trichoderma* against plant-pathogenic fungi

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Abstract

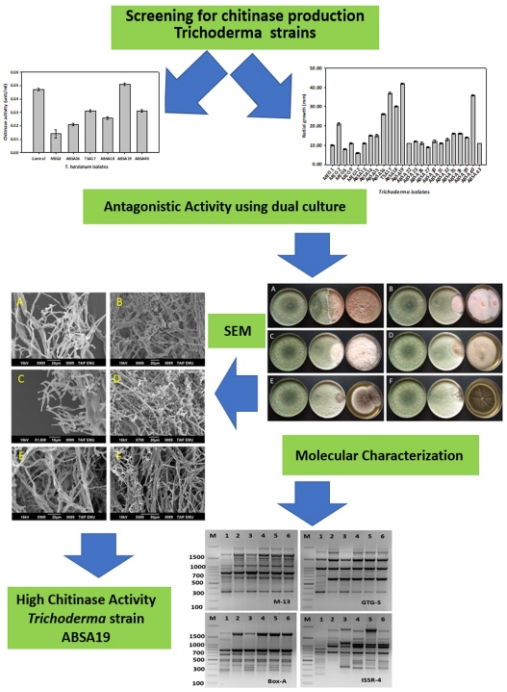
Aim: The present study aimed to investigate the antagonistic activity of *Trichoderma* strains with high chitinase production against some plant pathogenic fungi.

Methodology: A total of twenty-four *Trichoderma* isolates were extracted from soil samples collected at different geographical locations in Egypt and Saudi Arabia. These isolates were screened for their chitinase production ability using colloidal chitin media. Six isolates with high chitinase ability were identified as *T. harzianum* by internal transcribed spacer (ITS) region sequencing. These strains were tested for their ability to degrade chitin into N-acetyl-β-D-glucosaminidase. Finally, the biocontrol activities of these *Trichoderma* strains against pathogenic fungi were tested by dual culture technique.

Results: The maximum activity among the chitinolytic enzymes was 0.051 units ml^{-1} in *Trichoderma* strain ABSA19, which showed an activity comparable to the control: pure enzyme, (0.047 units ml^{-1}). The lowest activity (0.014 units ml^{-1}) was found in *Trichoderma* strain MEG2. In dual culture, *Trichoderma* strain ABSA19 exerted the highest antagonistic activity in addition to hyphal intertwining and degradation using scanning electron microscopy. Finally, 11 inter simple sequence repeats (ISSR) primers were used to evaluate the genetic polymorphism among *Trichoderma* strains, which yielded 133 bands with fragment sizes of 130-3400 bp, 47 (35.3%) of which were polymorphic.

Interpretation: The current study confirms the existence of several useful *Trichoderma* strains with the highest chitinase activity and antagonistic activity against some pathogenic plant fungi that may have a super potential for application in biological control of plant diseases.

Key words: Antagonistic activity, Chitinase assay, Genetic polymorphism, Internal transcribed spacer, *Trichoderma* strains



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Introduction

Trichoderma spp. are among the most important fungi used for the biological control of plant pathogenic fungi in soil. The important roles of these fungi are ability to synthesize many enzymes, such as chitinase, cellulase and protease, which enable them to control plant diseases (Hassan *et al.,* 2013, 2014; Mazrou *et al.,* 2020b). *Trichoderma* species exert multiple methods of biological control that can be employed against plant pathogens. These methods include mycoparasitism, antibiotic-mediated suppression, production of lytic enzymes and other byproducts, competition for nutrients, or induction of host resistance (Navazio *et al.,* 2007; Goswami *et al.,* 2008; Vinale *et al.,* 2009; Karlsson *et al.,* 2010; Hassan *et al.,* 2019). Chitinases are chitin-degrading enzymes that are widely distributed in nature and hydrolyze the β-1,4-glycosidic bonds between the N-acetyl glucosamine residues of chitin (Hassan 2014; Aggarwal *et al.,* 2015). The chitinases from *Trichoderma* species belong to glycosyl hydrolase family, and can be grouped into categories III and V. Various chitinase genes from *Trichoderma* spp. have been reported and characterized worldwide with emphasis on their roles in biological control strategies (Binod *et al.,* 2007; Hassan *et al.,* 2014; Abd-El-Kareem *et al.,* 2019). Substrates like solid chitin, colloidal chitin, or red chitin can be used to evaluate chitinase activity. In addition, chitinase activity can easily be estimated by colorimetric quantification method (Hassan, 2014). However, since chitinase activity is exhibited as a reaction to pathogens, the colorimetric method cannot be readily applied for the identification of strains with low chitinase production.

Therefore, simpler and faster tests, such as those using colloidal chitin in solid media, need to be developed. These assays should be designed so that chitinase activities of known microbes can be visualized, for instance by the formation of a clear halo due to chitin digestion (Schickler *et al.,* 1988; Rojas-Avelizapa *et al.,* 2001; Agrawal and Kotasthane 2012; Mazrou *et al.,* 2020b). The most sensitive detection techniques usually require more expensive substrates. Therefore, these assays are better suited for characterizing all proteins expressed in each microbial strain, as opposed to the selective detection of chitinolytic strains. Recently, some researchers (Parmar *et al* ., 2015 ; Mazrou *et al.,* 2020b) have proposed a sensitive, reproducible, easy, and cost-effective method for evaluating chitinolytic properties of *Trichoderma* isolates from India and Egypt against some pathogens. In view of the above, the aim of the present study was to find novel biocontrol strains of *Trichoderma* and investigate the relationship between antagonistic activity of *Trichoderma* strains and chitinolytic enzyme production.

Materials and Methods

Isolation and identification of *Trichoderma* strains: Twenty-four *Trichoderma* isolates were extracted from soil samples collected from different geographical locations in the Menoufia Governorate of Egypt and the cities of Taif and Abha in the Kingdom of Saudi Arabia. The rhizospheres from healthy tomato plants were used in accordance with the protocol outlined in Hassan *et al.* (2014). The chitinase detection medium used for inducing chitinase activity was prepared according to the method described by Agrawal and Kotasthane (2012) using *Rhioctonia* solani as a negative control and *Bacillus* as a positive control. Chitinase detection medium was supplemented with 4.5 g of colloidal chitin. Colloidal chitin was prepared from commercial chitin (Himedia, Mumbai, India). As reported by Roberts and Selitrennik (1988), the supplemented colloidal chitin acts as the sole carbon source in the chitinase assay medium. The chitinase detection medium was poured into ten different Petri plates (five represent negative control and five represent positive control) and inoculated with fresh fungal cultures. All treatments were incubated for two days at 28°C, and the formation of colored zones was tracked to test the *Trichoderma* isolates for chitinase activity.

Chitinase activity in *Trichoderma* isolates: Chitinolytic assays to check for intracellular chitinase production were performed twice in a total of ten-250 ml Erlenmeyer flasks, five as positive and five as negative control. The colloidal chitin broth medium was inoculated with fresh cultures of *Trichoderma* isolates and incubated for five days at 28°C with continuous shaking at 200 rpm. The total chitinolytic and N-acetyl-β-D-glucosaminidase activities of the filtrates obtained from ten cultures were then checked using ELISA reader 800™ TS Absorbance Reader (BioTek, Vermont, USA). These assays were performed while measuring the amount of reducing saccharides released from the colloidal chitin medium using a chitinase assay kit, catalog number CS0980 (Sigma-Aldrich, Louis, USA) according to the manufacturer's instructions.

Tests of antagonistic activity against pathogens with chitin cell walls: Biocontrol activities of *Trichoderma* strains with high chitinase activity (strains MEG2, ABSA16, TSA17, ABSA18, ABSA19 and ABSA40) were checked twice against the following pathogenic fungi in three replicates: *Colletotrichum gossypii, Fusarium oxysporum, Fusarium fujikuroi, Rhizoctonia* solani, *Aspergillus calidoustus* and *Alternaria brassicicola* by the dual culture technique using Czapeck-Dox agar medium as described by Fahmi *et al.* (2012). Pathogenic fungi were obtained from GeneBank of Biotechnology and Genetic Engineering Unit, Scientific Research Deanship, Taif University, Saudi Arabia.

Sample preparation for scanning electron microscopy (SEM): The parasitism exhibited by hyphal cells of *Trichoderma* strains towards those of the tested pathogens was studied in detail by SEM at the Electronic Microscope Unit, Faculty of Science, Taif University, according to protocols described in 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 hr, respectively. *Trichoderma* and the respective pathogens grew towards each other allowing their hyphae to interact with one another. After 48 hr of incubation, the early stages of interaction was studied under a light microscope. 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 hr with vapors of glutaraldehyde and osmium tetroxide (3:1), air-dried for 48 hr, and then coated with gold.

Extraction of DNA from *Trichoderma* isolates: *Trichoderma* isolates with high chitinase activity were transferred to Czapeck-Dox broth medium and maintained at 28°C for three days. Total DNA was then extracted from selected isolates using the Nour plant and fungi DNA extraction kit (Sigma, Canada) according to the manufacturer's instructions.

Molecular identification: Strain identification was based on the internal transcribed spacer 1 and 2 (ITS1 and ITS2) regions of the ribosomal RNA gene cluster sequences according to the method described by Hassan *et al.* (2014). All sequences were obtained by PCR and direct amplicon sequencing from the 5' and 3' ends, using a 3130 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) at Macrogen Co., Seoul, South Korea. The sequencing data were compared with the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/), using the nucleotide BLAST program to identify homology between the PCR fragments and sequences in the GenBank database. The sequences were deposited in the National Center for Biotechnology Information (NCBI) GenBank database under accession numbers MK680260, MK680282, MK680283, MK680284, MK680285 and HM545086.

Inter simple sequence repeats (ISSR) analysis: A total of 11 ISSR primers (Lakhani *et al.,* 2016) were used for *Trichoderma* genotyping, (Table 1). PCR amplifications were performed according to the method described by Hassan *et al.* (2019). ISSR-PCR was performed in reaction mixture containing 1 µl (20 ng) of genomic DNA, 12.5 µl of GoTaq® Green Master Mix (Promega, Madison, WI, USA), 1 µl of each primer (20 pmol) and deionized distilled water (up to a total volume of 25 µl). For DNA amplification, the tube contents were heated at 94 °C for 10 min in a C1000 Touch™ Thermal Cycler (Bio-Rad, Munich, Germany). Subsequently, Taq polymerase was added, followed by 35 cycles of 1 min at 94 °C, 1.5 min at 52 °C and 2.5 min at 72 °C, with a final 7-min extension at 72 °C. Amplified DNA products were analyzed by electrophoresis in 2% agarose gels run in Tris/borate/EDTA buffer. The gels were stained with ethidium bromide (5 µg·ml−1). A 100 bp DNA Ladder RTU (GeneDireX®, München, Germany) was used as a standard. DNA was visualized by UV illumination, and then photographed, using a Bio-Rad Gel Doc 2000.

Table 1: ISSR Primers name and sequencing used in *T. harzianum* genotyping

Primers name	Primers sequences (5'————3')
ISSR-2	GAG AGA GAG AGA GAG AA
ISSR-4	GAG AGA GAG AGA GAG ATT
ISSR-11	AGA GAG AGA GAG AGA GTT
ISSR-13	AGA GAG AGA GAG AGA GG
ISSR-16	GAG AGA GAG AGAG AGA C
ISSR-18	TCT CTC TCT CTC TCT CC
ISSR-29	ACA CAC ACA CAC ACA CT
ISG-4	AGA GAG AGA GAG AGA GG
M-13	GTT TTC CCA GTC ACG AC
Box-A1	CTA CGG CAA GGC GAC GCT
GTG-5	GTG GTG GTG GTG GTG

Data analyses: The mean chitinase and antagonistic activities of six different strains against pathogens was compared using One-way analysis of variance (ANOVA) as implemented in SPSS software, version 16. Mean treatment comparisons was conducted using Duncan's Multiple Range (Snedecor and Cochran 1989) at P = 0.05.

Results and Discussion

Trichoderma species are known as soil-borne fungi. Some of these species have important biotechnological applications due to their production of many enzymes, including cellulase and chitinase, which can act as biocontrol agents (Fahmi *et al.,* 2016; Hassan *et al.,* 2019). The antagonistic potential of *Trichoderma* species against diverse fungal phytopathogens has been reported by several researchers over the years (Fahmi *et al.,* 2012; Hassan *et al.,* 2014; Mazrou *et al.,* 2020b). *Trichoderma* spp., particularly *T. harzianum,* shows significant variation among the strains with respect to their hostile activity and host range (Hassan, 2014; Parmar *et al.,* 2015). Thus, new strains of *Trichoderma* spp. from the rhizosphere of host plant that have a possible biocontrol activity against some

Table 2: Comparison between *Trichoderma* isolates and similar strains in NCBI database and accession numbers

Trichoderma strains	*Trichoderma* identification	Accession numbers	Similarity value (%)	Similar strain	Accession numbers*
MEG2	*T. harzianum*	MK680260	98	T. h-LWU_41	MK299140
ABSA16	*T. harzianum*	MK680282	100	T. h-Cul2	Mk673510
TSA17	*T. harzianum*	MK680283	100	T.h-T50	FJ884179
ABSA18	*T. harzianum*	MK680284	100	T. h-Cul2	Mk673510
ABSA19	*T. harzianum*	MK680285	100	T. h-AT73	Mk615748
ABSA40	*T. harzianum*	HM545086	100	T. h-SVPP-20	Ku215928

*Strains accession numbers obtained from NCBI

Table 3: Antagonistic potential of six *Trichoderma* isolates against *Colletotrichum gossypii, Fusarium oxysporium, Rhizoctonia solani, Fusarium fujikuroi, Aspergillus calidoustus* and *Alternaria brassicicola*

Trichoderma	Antagonistic potential inhibition %					
isolates	***C. gossypii***	***F. oxysporium***	***R. solani***	***F. fujikuroi***	***A. calidoustus***	***A. brassicicola***
MEG2	70.8^{d}	83.7^{c}	79.8^{e}	81.2^{c}	87.8^{c}	89.2^{d}
ABSA16	74.6^{c}	83.7^{c}	83.1^{b}	82.9^{b}	85.5^{e}	90.6^{c}
TSA17	70.8^{d}	81.8^{d}	82.9^{c}	80.8^{d}	86.1^{d}	90.6^{c}
ABSA18	74.6^{c}	85.6^{b}	81.4^{d}	80.8^{d}	88.5^{b}	88.1^{e}
ABSA19	78.9^{a}	86.5^{a}	84.0^{a}	85.7^{a}	90.5^{a}	92.5^{a}
ABSA40	76.8^{b}	85.6^{b}	81.4^{d}	81.2^{c}	88.5^{b}	91.2^{b}

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

Table 4: Total number of scorable bands, polymorphism percentage and band size of ISSR markers obtained by 11 primers among *T. harzianum* samples

Primers name	Total scorable fragments	Polymorphic fragments	Polymorphism (%)	Fragment size range (bp)
ISSR-2	11	2	18.2	490-1800
ISSR-4	18	8	44.4	210-3000
ISSR-11	11	4	36.4	400-2900
ISSR-13	9	3	33.3	200-2950
ISSR-16	12	6	50.0	130-2950
ISSR-18	14	5	35.7	300-2900
ISSR-29	12	7	58.3	250-2750
ISG-4	7	2	28.6	260-2900
M-13	14	3	21.4	320-2990
Box-A1	11	2	18.2	295-1700
GTG-5	14	5	35.7	310-2990
Total	133	47		

pathogens of the host in question must be explored to generate an industrially promising strain. This study was carried out to screen 24 native isolates of *Trichoderma* spp. (from the rhizospheric soil samples collected from Menoufia Governorate in Egypt and the cities of Taif and Abha in Saudi Arabia) for chitinase and biocontrol activities. All the *Trichoderma* strains were screened for chitinase production ability. Nonetheless, only six isolates secreted large amounts of chitinase, as assessed by colloidal chitin hydrolytic activity assay performed on agar plates. The highest chitinase production ability was detected in six *Trichoderma* strains (MEG2 from Menoufia, Egypt; TSA17 from Taif, Saudi Arabia; and ABSA16, ABSA18, ABSA19 and ABSA40 from Abha, Saudi Arabia), which were selected for determining chitinase activity. Among the *Trichoderma* isolates, strain ABSA19 formed the largest zone of inhibition, which exceeded 43 mm in diameter followed by TSA17 (37 mm) (Fig. 1 and S. 1).

These six isolates were identified using ITS region sequencing. The obtained sequences of the ITS regions were then subjected to BLAST search at NCBI database. These six strains with high chitinolytic activity were all identified as *T. harzianum.* Their sequences were deposited in the NCBI GenBank database (GenBank accession No. MK680260, MK680282, MK680283, MK680284, Mk680285 and Hm545086). Nucleotide comparisons of ITS regions among *Trichoderma* strains and other similar strains retrieved from NCBI revealed 100% identity between *T. harzianum* (ABSA16 and ABSA18) and *T. harzianum* Cul2 from the GenBank (Table 2). On the other hand, strains TSA17 and MEG2 exhibited 100% and 98% similarity with strain T50 and LWU-41 from the GenBank, respectively. Therefore, it is important to identify *Trichoderma* isolates to the species level. This is especially important because identification of these species at morphological level has been reported to be unreliable (Sharma *et al.,* 2009; Shahid *et al.,* 2013) due to high degree of similarity in morphology across strains. Molecular identification of *Trichoderma* strains to species level by ITS region sequencing was recently introduced as an effective alternative method for their accurate identification (Gajera and Vakharia 2010; Błaszczyk *et al.,* 2011; Hassan *et al.,* 2019; Mazrou *et al.,* 2020b). The ITS region is one of the most reliable loci for identifying strains at species level (Hassan *et al.,* 2019; Mazrou *et al.,* 2020a). Thus, in this study, DNA sequencing of the 5.8S-ITS region was carried out for all isolates. By comparing the

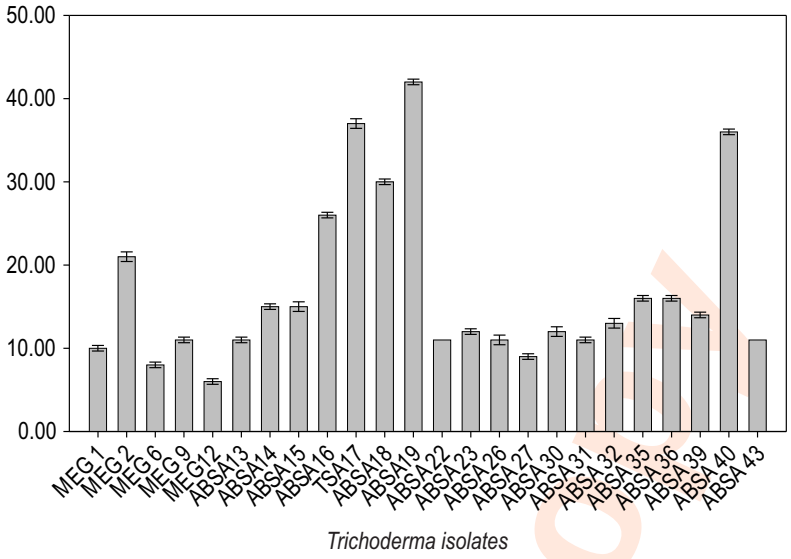

Fig. 1: Mycelial radial growth (mm) of *Trichoderma* isolates based on their chitinase production ability using colloidal chitin media. Mean standard deviation is expressed in error bar (n = 3).

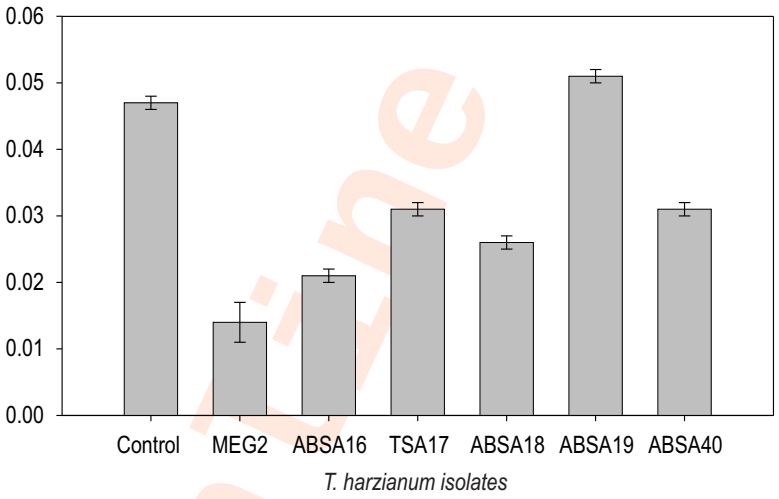

Fig. 2: Determination of total chitinase activity of six *T. harzianum* strains isolated from different location in Egypt and Saudi Arabia.

sequences of the 5.8S-ITS region to the sequences available on GenBank, all the *Trichoderma* isolates could be identified to species level with homology of at least 99% (Filizola *et al.,* 2019).

The total chitinase activity ranged between 0.014 and 0.051 units ml^{-1} across strains (Fig. 2). Strain ABSA19 showed the highest total chitinase activity of 0.051 units ml^{-1} among strains. This activity was comparable to that of the control (containing 0.050 unit ml^{-1} chitinase enzyme, Sigma, USA). The second highest chitinase activity of 0.031 units ml^{-1} was attributable to strains TSA17 and ABSA40. ABSA16 and ABSA18 strains had a relatively low chitinase activity of 0.021 and 0.026 units ml^{-1}, respectively. The lowest total chitinase activity (0.014 units ml^{-1}) was observed in strain MEG2. Induction of chitinase hydrolytic activity detected herein indicates that this chitinase may be involved in the mycoparasitism of *T. harzianum* toward plant-pathogenic fungi. In this context, chitin and β-1,3-glucans are known to be the structural components of fungal cell wall, suggesting that chitinase and β-1,3-glucanase produced by *Trichoderma* species may act as cell lysis enzymes during mycoparasitism (Mohamed *et al.,* 2010; Hassan 2014; Urbina-Salazar *et al.,* 2018). Furthermore, the antagonistic effects of six

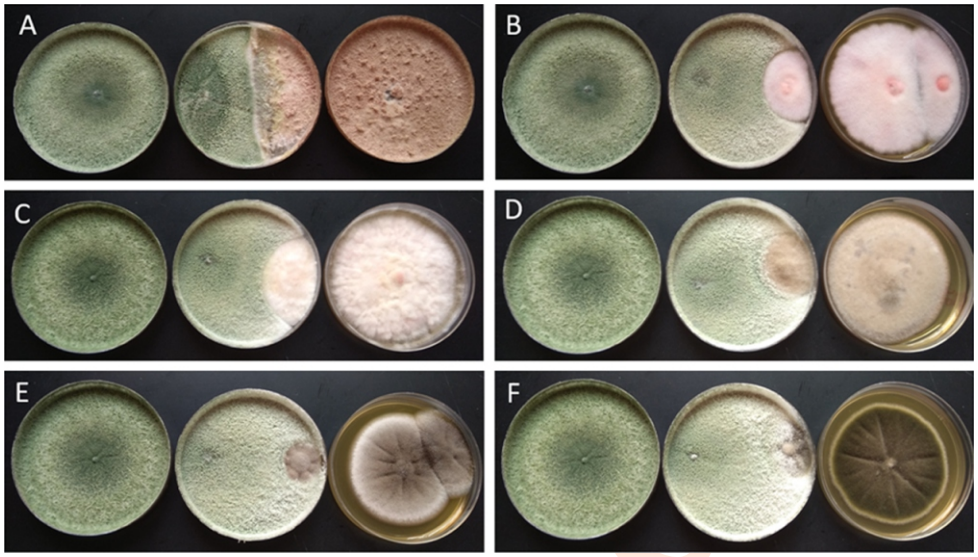

Fig. 3: Antagonistic activity of ABSA19 *Trichoderma* strain against: (A) *Colletotrichum gossypii;* (B) *Fusarium oxysporium;* (C) *Rhizoctonia solani;* (D) *Fusarium fujikuroi;* (E) *Aspergillus calidoustus* and (F) *Alternaria brassicicola* (on the left: pure culture of *Trichoderma* isolates, on the right: pure culture of pathogens and on the middle: the dual culture of pathogen and *Trichoderma* strain).

Trichoderma strains (MEG2, ABSA16, TSA17, ABSA18, ABSA19 and ABSA40) against six tomato plant pathogens (*C. gossypii, F. oxysporum, F. fujikuroi, R. solani, A. calidoustus,* and *A. brassicicola*) were evaluated. In all the dual culture plates tested, the contact zone showed curved pattern, with the concavity oriented toward the plant pathogen (Fig. 3). The negative control plates were inoculated only with the plant pathogen while the negative control plate inoculated only with *Trichoderma* strains. The average rates of percent inhibition (%) of mycelial growth by the strains toward all six pathogens are listed in Table 3. All the *Trichoderma* isolates inhibited the mycelial growth of the pathogens, with percent reduction in pathogen growth varying between 70.8 and 92.5%. The ABSA19 strain showed the highest inhibition of *C. gossypii* mycelial growth as compared to other isolates, with a percent inhibition of 78.9%. The close second was strain ABSA40 (76.8% inhibition), whereas TSA17 and MEG2 caused the lowest percent inhibition of 70.8%.

The growth of *F. oxysporum* was inhibited significantly by ABSA19 (86.5%) as compared to other strains, followed by ABSA18 and ABSA40 (85.6%). Moreover, mycelial growth in *R. solani* was mostly inhibited by isolates ABSA19 (84.0%), followed by ABSA16 (83.1%). On the other hand, isolate ABSA19 showed the highest percent inhibition of *F. fujikuroi* mycelial growth as compared to other isolates, with a percent inhibition of 85.7%, followed by isolate ABSA16 (82.9%). *A. calidoustus* growth was significantly inhibited by ABSA16 (90.5%), followed by isolate ABSA40 (88.5%). The growth of *A. brassicicola* was inhibited significantly by isolate ABSA19 (92.5%) rather than other isolates, followed by isolate ABSA40 (91.2%), while ABSA18 caused the lowest percent inhibition of 88.1%. All the strains showed antagonistic activity ranging from moderate to strong against plant pathogens. The ABSA19 strain manifested highest activity against all the pathogens and showing highest chitinase activity. The results support those obtained in the dual culture assay antagonistin abilities of these *Trichoderma strains*. The potency of *Trichoderma* may be attributed to the ability to produce toxic hydrophilic metabolites or lytic enzymes (Hassan 2014; Parmar *et al.,* 2015; Mazrou *et al.,* 2020b). 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 two mycelia, thus increasing the antagonistic capacity of *Trichoderma* (Fahmi *et al.,* 2012; Filizola *et al.,* 2019). Enzymes chitinase and glucanase are produced by *Trichoderma* species, but these enzymes are mainly expressed in the presence of their respective substrates (chitin and glucan) (Gajera and Vakharia 2012; Rana *et al.,* 2012; Hassan 2014; Abd-El-Kareem *et al.,* 2019).

A more detailed picture of the development of coils and initiation of interactions among the tested pathogens and *Trichoderma* strains was obtained by SEM (Fig. 4). Coiling of pathogen hyphae was observed in ABSA19 *Trichoderma* strains, but only ABSA19 was selected for visualization via SEM. Scanning electron micrographs taken three days after inoculation showed complete colonization of *C. gossypii* with the ABSA19 strain (Fig. 4a). Examination of contact zone revealed that the parasitic hyphae had reached and grown on the surface of the pathogen via coiling and subsequent spore formation on *F. oxysporum, F. fujikuroi,* and *R. solani* (Fig. 4B - D). The observed growth pattern was followed by the formation of aspersorium-like structures that did not penetrate the cell walls of *A. calidoustus*

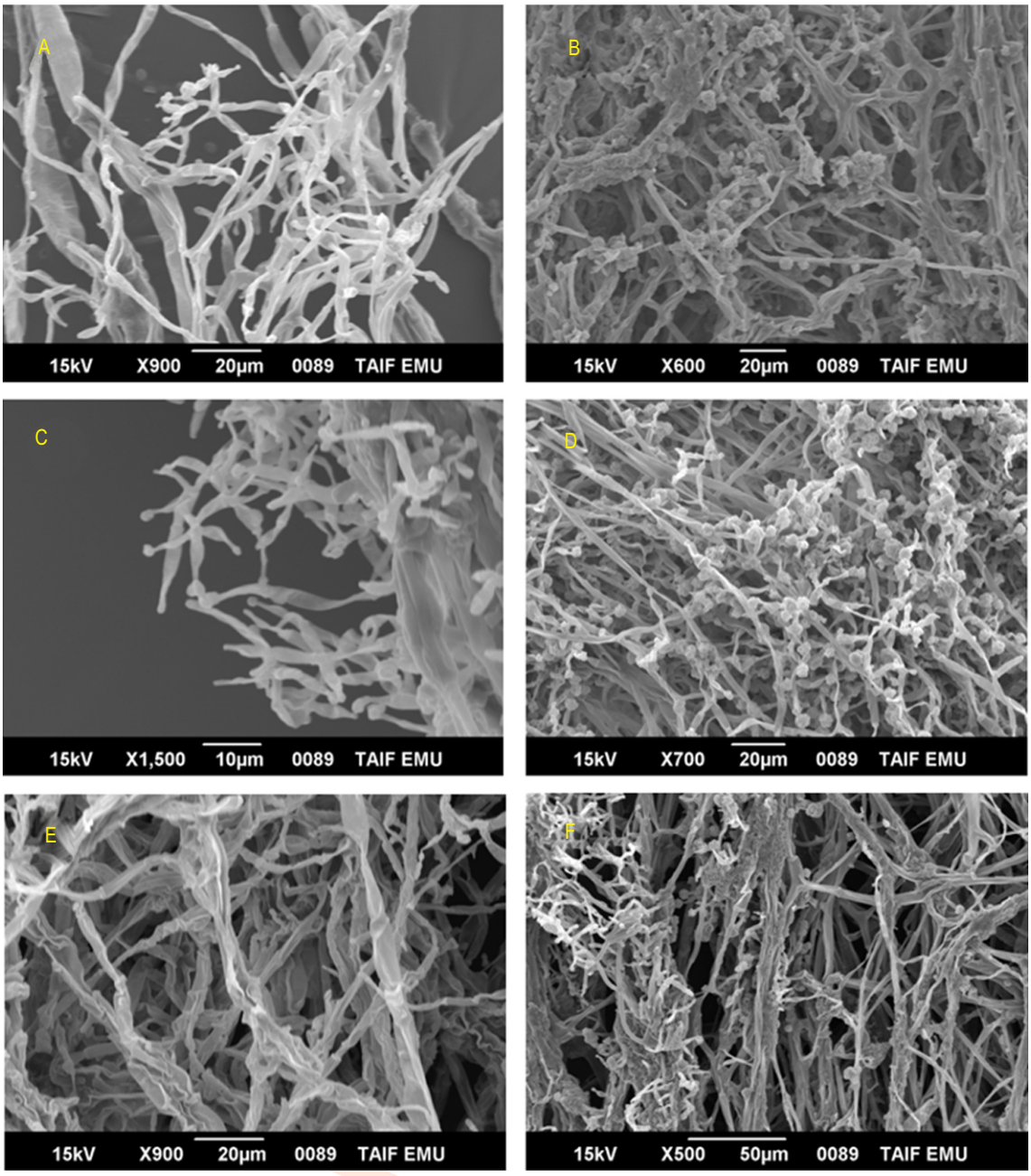

Fig. 4: Scanning electron micrographs showing antagonistic activity of ABSA19 strain. Coiling of *Trichoderma* isolates, spores formation over lytic enzymes of pathogens and seem to be capable of degrading the pathogen cell walls of (A) *Colletotrichum gossypii;* (B) *Fusarium oxysporium;* (C) *Fusarium fujikuroi;* (D) *Rhizoctonia solani;* (E) *Aspergillus calidoustus* and (F) *Alternaria brassicicola.*

and *A. brassicicola* (Fig. 4E, F). Pathogens invaded by parasitic hyphae started appeareing to disintegrate. In the current study, each fungal pathogen of plants responded differently to diffusible or volatile metabolites of strain ABSA19 in dual culture, is in agreement with the findings of Cooney and Lauren (1998), who reported that *T. harzianum* produces larger amount of 6-pentyl-αpyrone in response to specific pathogens. This effect could be due to direct interaction and response of the antagonist to the presence of the pathogen. Additionally, it is possible that some pathogens can produce extracellular enzymes, proteins, or metabolites that cause the antagonist to abundantly generate antifungal metabolites. Accordingly, scanning electron micrographs were utilized to determine the damage inflicted on the pathogens' hypha. Coiling of pathogen hyphae was observed

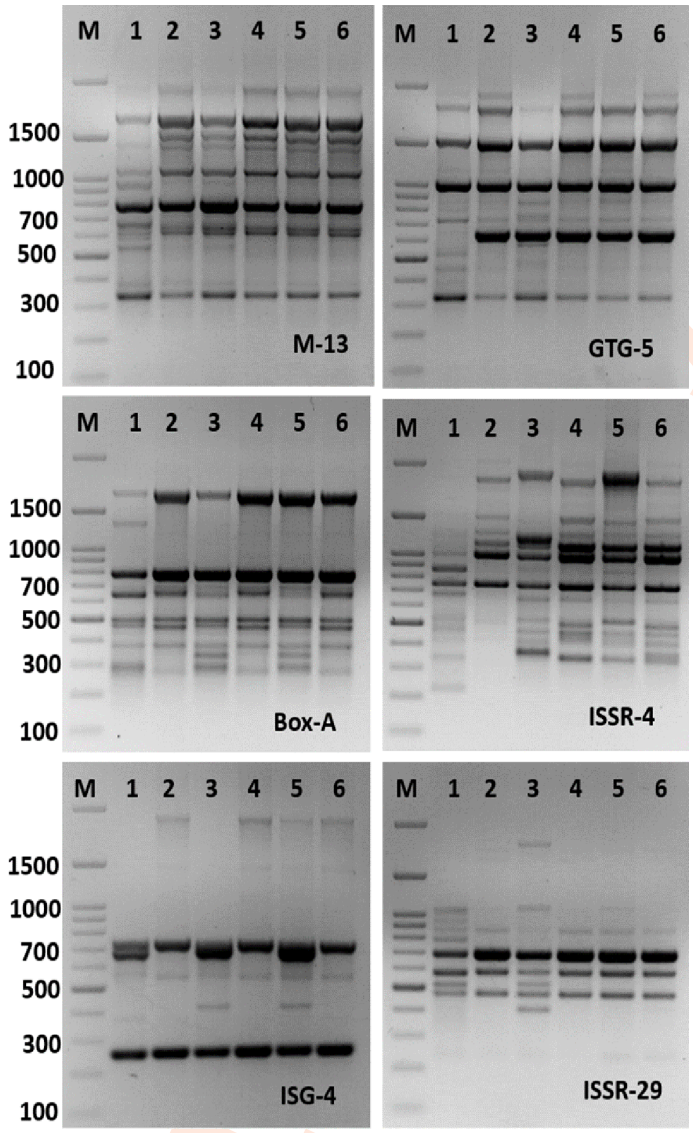

Fig. 5: ISSR-PCR profile of six *T. harzianum* samples (1=MEG2, 2=ABSA16, 3=TSA17, 4=ABSA18, 5=ABSA19 and 6=ABSA40) generated with the respective ISSR primers: M-13, GTG-5, Box-A, ISSR-4, ISG-4 and ISSR-29. The first lane in each panel corresponds to 100-bp molecular weight markers.

for most *Trichoderma* strains, but only *Trichoderma* strain ABSA19 was selected for visualization via SEM. The collapse of parasitic hypothecium has been reported as an effect of cellular enzymes on some pathogens, along with observations of coagulation, *Trichoderma* infiltration, and spore formation. In addition, the host's hyphae were found to completely disappear seven days after the inoculation (Hassan, 2014; Mazrou *et al.,* 2020b). Lytic enzymes from *Trichoderma* strains can produce large amount of chitinase seem to be capable of degrading pathogens' cell wall. Mycoparasitism is an important and complex process in which *Trichoderma* spp. grow toward their hosts and attach and coil around the host hyphae, sometimes penetrating them (Zarei *et al.,* 2011). Partial degradation of host cell wall was observed at later stages of this process (Hassan, 2014). The effects of cell wall degrading enzymes on the host have been reported previously using different ultrastructural and/or histochemical approaches (Hassan, 2014; Abd-El-Kareem *et al.,* 2019; Mazrou *et al.,* 2020b).

Finally, molecular characterization of *Trichoderma* strains was implemented by ISSR markers. ISSR markers have been used to study the genetic diversity and population dynamics of various *T. harzianum* strains (Hassan *et al.,* 2014; Lakhani *et al.,* 2016). A total of 133 fragments across the six isolates were

obtained, of which 47 were polymorphic (35.3%). A representative sample of six ISSRs profiles for six isolates are presented in (Fig. 5). The fragment size ranged between 130 and 3000 bp, with ISSR-4 showed the highest number fragments (18, Table 4), while ISG-4 yielded the lowest number of fragments (7, Table 4). Two specific fragments were detected in *Trichoderma* strain MEG2 with primers BoxA1 and ISSR-4 with molecular weight 1400 and 210 bp. Moreover, two additional specific fragments were detected in strain TSA17 with primer ISSR-29, with molecular weight 1800 and 400 bp. and one specific fragment was observed in TSA17 and ABSA19 using ISG-4 with about molecular wight 400 bp. ISSR analysis has previously been used to explore the genetic diversity among species and strains of *Trichoderma* (Lakhani *et al.,* 2016; and Hassan *et al.,* 2019; Mazrou *et al.,* 2020a).

In this study, ISSR results indicated that *Trichoderma* strains were differed and collected from different geographic areas. Lakhani *et al.* (2016) developed ISSR markers to discriminate genetic diversity of two strains of *Trichoderma* spp. and their protoplast fusants. They found that the percent polymorphism for all strains was 88%. The data obtained in the present study are consistent with the results of Hassan *et al.* (2014), who conducted the ISSR analysis, and the percent genetic polymorphism reached as much as 58.7% between *Trichoderma* species. The strains of *T. harzianum* with high chitinase activity displayed strong antagonistic activity against certain tomato pathogens, including *Colletotrichum gossypii, Fusarium oxysporum, Fusarium fujikuroi, Rhizoctonia solani, Aspergillus calidoustus* and *Alternaria brassicicola* using dual culture assays. The results obtained suggested that ABSA19 strain may be used as a biological control agent in agriculture and it may further be used in field experiments and also for the production of commercial fertilizers.

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