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Molecular Characterization of *Trichoderma virens* Isolated from Mushroom Compost and Its Efficacy against *Fusarium* f. sp. *lycopersici* Causing Wilt of Tomato

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KEY WORDS

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ABSTRACT A new strain of *Trichoderma virens* was isolated from mushroom compost which causes green mold disease from the mushroom production unit of Lovely Professional University, Punjab, India. Based on 5.8S rRNA, partial 18S, and 28S rRNA genes, ITS1, ITS4 sequence analysis, and morphological characteristics, the fungal isolate was identified as *T. virens*. The universal primers (ITS-1 and ITS-4) were used to amplify the 28S rRNA gene fragment that produced a sharp band of about 600 bp on the agarose gel. The amplified gene fragment was then sequenced and showed 100% similarity with *T. virens*. GC/MS analysis of the strain revealed the presence of several antimicrobial compounds, such as n-nonadecanol-, propanoic acid, 2-methyl-butanoic acid, and 2-methyl-dodecane. The current study confirmed the potent antimicrobial and chitinolytic activity against *Fusarium* and its potential as a bio-control agent for managing plant diseases.

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INTRODUCTION

Conventional methods of identification of the genus *Trichoderma* using the morphological and cultural approach have been used, including colony appearance and microscopic morphological features, arrangement of conidiophores, phialides, and pigmentation of hyphae (Seaby, 1996). These characteristics allow relatively easy identification of genus, but the species confirmations are very difficult due to the intertwining of morphological traits and phenotypic phenomena, particularly between *Trichoderma* anamorphs (Grondona *et al*., 1997; Druzhinina *et al*., 2006; Hassan *et al*., 2014).

Molecular approaches are routinely used to identify *Trichoderma* species, which are often more efficient, accurate, and reliable than those based on morphological characteristics (Hafizi *et al*., 2013). Molecular methods based on sequence analysis of multiple genes can help classify and identify *Trichoderma* isolates and new species

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(Kindermann *et al*., 1998; Xu, 2020). The use of internal transcribed spacer (ITS) region Deoxyribonucleic acid (DNA) sequence analysis has now become a standard routine and potential method for the identification, characterization, classification, and phylogenetic analysis of many fungi at the species level (Sallam *et al*., 2019).

Wilt is the main challenge in protected and open field-grown tomato production (Camargo *et al*., 2006, Abdallah *et al*., 2016; Srinivas *et al*., 2019). It is caused by the destructive fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici* (Loganathan *et al*., 2009). Fusarium wilt is a monocyclic, root-inhabiting soil and seed-borne pathogen which remains in soil both as a saprophyte and pathogenic for several years (Haware *et al*., 1992, Patil *et al*., 2017, Xiong *et al*., 2018). The management strategies are highly dependent on the application of fungicides. Regular application of fungicides potentially increases the consequence of toxicity of environmental concern and a significant chance of development of resistance. Therefore, biocontrol agents (BCAs) are considered more sustainable, reduce harmful residues entering the food chain, are safer for implementation, and are economical in cost, depending on the strength of protective antagonistic organisms against plant pathogens.

Many species of *Trichoderma* as fungal pathogens include *Trichoderma asperellum*, *Trichoderma viride*, *Trichoderma harzianum,* and *T. virens* (Hermosa *et al*., 2000; Longa *et al*., 2009; Chaverri *et al*., 2015). The application of beneficial microbes has become a component of integrated plant disease management (Monte, 2001; Kancelista *et al*., 2013; Smolińska *et al*., 2014a,b). Antagonism of *Trichoderma* against different pathogens has been reported against *Sclerotium rolsfii* (Mukherjee and Tripathi, 2000), and *Fusarium ciceris*, *Macrophomina phaseolina*, and *Rhizoctonia solani* (Mukhopadhyay and Pan, 2012a). Li *et al*. (2018) showed that *Trichoderma* inhibited *F. oxysporum* growth by producing volatile compounds.

However, the first step in such studies should be species identification of promising *Trichoderma* strains. Correct species identification is necessary due to *Trichoderma* similarity in morphology and complex taxonomy (Hermosa *et al*., 2000). Morphology alone is insufficient in accurate *Trichoderma* species identification. Recently, 228 species of *Trichoderma* and several not yet taxonomically characterized species were distinguished (Jaklitsch and Voglmayr, 2015). The aim of the present study was the molecular identification of new *Trichoderma* strains collected from compost to select plant growth-promoting fungi or BCA strains suitable for application in agricultural and horticultural production. In this context, the novel *Trichoderma* strains were identified by molecular characterization and explored as an effective and sustainable approach for managing the biocontrol of devastating plant pathogens.

MATERIALS AND METHODS

Microbial Cultures

Trichoderma virens, *Pseudomonas fluorescens*, and *F. oxysporum* cultures were used in the present study. *Trichoderma* was isolated from mushroom compost collected from the Department of Plant Pathology, Lovely Professional University Punjab, India. The isolation was carried out on *Trichoderma* selective medium by serial dilution (Johnson and Crul, 1972; Nirenberg, 1976).

Morphological Identification

Sub-sample of fully sporulated *Trichoderma* culture was mounted on slides, fixed with lactic acid, and stained with lactophenol cotton blue. The slides were examined under a compound microscope. Using the taxonomic keys, the strain was identified based on the morphological characteristics of conidiophores and conidia.

DNA Isolation of *Trichoderma*

Pure culture of the target fungal isolate was grown in potato dextrose broth medium on BOD cum rotary shaker at 25°C for 7 days. Fungal mycelium was filtered and crushed in liquid nitrogen with the help of a sterilized mortar and pestle. Genomic DNA was isolated using the standard protocol of the Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) method. Purification was done using DNeasy Plant Mini Kit according to the manufacturer's instructions with minor modifications. Polyvinylpyrrolidone and polyvinylpolypyrrolidone were used, according to Vandroemme *et al.* (2008), and proteinase K and RNase A were added to the lysis buffer. Both the purity and quantity of DNA were checked by agarose gel electrophoresis, determined with the NanoDrop 8000 spectrophotometer and the DNA concentration was adjusted to 10 ng/µL.

The isolated strain was then identified at the molecular level using PCR amplification of the specific gene sequence with universal ITS-1 (forward) and ITS-4 (reverse) primers. The total genomic DNA was extracted from an isolate of *Trichoderma* based on CTAB mini extraction method as was described by Crowhurst *et al.* (1995).

The ITS region (ITS) was amplified by PCR using primers ITS1 and ITS 4. The PCR reaction mixture was prepared in a final volume of 25 µL (1X); it contained: H_2O (17.8 µL), buffer without $MgCl_2$ (2.5 µL), 10 nm dNTPs (0.5 µL), $MgCl_2$ (1 µL), ITS-1 (0.5 µL), ITS-4 (0.5 µL), Taq Polymerase (0.2 µL), and 2 µL of DNA (samples diluted 1:10). The amplification program consisted of 1 cycle of 95°C for 4 min, 32 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 2 min, with a final cycle of 72°C for 5 min. All PCR reactions were carried out in a thermal cycler. The PCR

product was purified by QIAGEN gel extraction kit using the protocol described in the manufacturer's manual. DNA sequencing of the 28S rDNA fragment A pair of universal ITS primers ITS-1 (forward) and ITS-4 (reverse) was used for sequencing of the amplified product, and this step was carried out at the Merck Laboratory (Bangalore, India).

Phylogenic Tree

The basic local alignment search tool (BLAST) was used to find the regions of local similarities between *Trichoderma* nucleotide sequences obtained from National Center for Biotechnology Information (NCBI), an open database resource providing genome sequences of various organisms. The phylogenic tree was constructed using the FASTA format of different *Trichoderma* species. The construction of the phylogenic tree was prepared by using Mega 5.2 software.

Preparation of Talc-based Bioformulation

Talc-based formulations of *T. virens* were prepared at the Department of Plant Pathology, LPU, for seed treatment of tomatoes as per the procedure prescribed by (Jeyarajan *et al*., 1994). *Trichoderma* was grown in the liquid medium, mixed with talc powder in a ratio of 1:2 and dried to 8% moisture under shade. Tomato seeds were treated at 4–5 g/kg seed.

Fungal Cultures and Bioassay

The fungal plant pathogen *F. oxysporum* f. sp. *lycopersici* causing vascular wilt in tomatoes was isolated from the naturally infected plant of an agriculture research farm at Lovely Professional University, Punjab, India, using a standard isolation technique with some modification (Riker and Riker, 1936). The pathogen was identified macroscopically and microscopically (Leslie and Summerell, 2006). The pathogen was tested for pathogenicity (Venkatesh *et al*., 2013). These cultures were grown and maintained on a PDA medium for further experiments.

Screening of antagonistic activity of *T. virens* against *Fusarium*, *Trichoderma* isolates were examined using the dual culture technique as described previously (Furuya *et al*., 2011). *Trichoderma* isolates and *Fusarium* (9 mm diameter) were kept towards each other in Petri plates, respectively. Incubation was carried out at 28°C. The antagonistic activity of *Trichoderma* isolate was measured by examining the growth inhibition of the tested pathogen colony with the naked eye.

Radial growth of FOL strains was measured, and inhibition of average radial growth was calculated with the growth of the controls as follows: $I = (C-T/C) \times 100$. Where I = inhibition (%); C = radial growth of pathogen (mm) alone (control); T = radial growth of pathogen (mm) in the presence of *Trichoderma* isolates (Edington *et al*., 1971).

Fluorescent *Pseudomonads*

The isolate of *P. fluorescens* was obtained from the rhizosphere of healthy tomato plants. The rhizosphere was tested for its antagonistic activity against *F. oxysporum* after the dual culture technique. Both K.M.B. and PDA were used in the screening process. Culture discs of seven mm in size of the pathogen were placed at the center of the sterilized Petri plates containing sterilized media. The respective bacterial isolate was then streaked 2 cm away from the pathogen at the center in a triangular pattern. The inhibition zone was measured after 5 days of incubation.

Fungicides

Three fungicides, copper oxychloride 50% WP, carbendazim, and silicon were evaluated against the mycelia growth of the pathogen. Fungicides were used at the rate of 500, 1000, 1500, and 2000 ppm concentrations in autoclaved PDA medium by poisoned food technique (Dhingra and Sinclair, 1985). PDA medium (20 mL) was poured into each sterilized Petri dish and solidified. After solidification, a 7 mm disc of the 7-day-old fresh culture of FOL was cut using a sterile cork borer and placed in the center of a Petri dish containing different concentrations of fungicides and kept at 25 ± 1°C in BOD. Treatments were replicated thrice along with control. The radial growth of *F. oxysporum* f. sp. *lycopersici* was measured.

Cultivation of *T. virens*

Two 7 mm fungus discs were inoculated into 1000 mL-Erlenmeyer flasks containing 250 mL of sterile one-fifths strength potato dextrose broth (PDB). The stationary cultures were incubated for 30 days at 28 ± 2°C (12 h darkness, 12 h light). The procedure was replicated three times (Vinale *et al*., 2006).

Secondary Metabolites and Detection of VOCs

The culture broth of *Trichoderma* species was extracted by solvent ethyl acetate (polar solvent) using a modified method of Siddiquee *et al*. (2012). The extracted VOCs were identified via GC-MS device. A section of *T. virens* was inoculated into minimal broth media containing KH_2PO_4 (7 g/L); K_2HPO_4 (2 g/L); $MgSO_4 \cdot 7H_2O$ (0.1 g/L); $(NH_2)SO_4$ (1 g/L); yeast extract (0.6 g/L); and glucose (10 g/L). The inoculated culture broth was incubated for 31 days at 25°C. After completion of the incubation period contents of the flask were filtered. The filtered culture broth was mixed with ethyl acetate and kept in a separating funnel overnight. The organic phase was separated from the media phase, and the separation of *Trichoderma* metabolites from the organic phase was done with the help of a rotatory evaporator. The obtained residue was dried, dissolved in a

solvent, and kept at −20°C till use. The obtained residue was resuspended in solvent (acetone) for the characterization of metabolites by GC-MS

Statistical Analysis

Disease incidence was calculated using a formula:

Disease incidence = Number of infected plants/Total number of plants × 100

Twelve tomato plants were randomly selected for each treatment and were measured. Statistical data analysis of disease incidence was performed using analysis of variance and least significant difference with open-source software tools (https://www.omicshare.com/tools/Home/Soft/letter sig, accessed on August 8, 2021).

The field experiment was conducted during the rabi season of 2020–2021 to assess the possibility of managing the disease in susceptible cultivar Pusa ruby by different fungicides. Three sprays of each fungicide were applied *in vivo* and observations were recorded. The data on the management of the disease was recorded.

RESULTS

Morphological Identification of *T. virens*

Morphological characterization was conventionally used to identify *Trichoderma* species, and it remains a potential method to identify them. Species-level identification of *Trichoderma* isolate was made based on the color of the colony, formation of chlamydospores, conidiophores, and phialides characters, and shape of conidia as the main characters to identify the species. *Trichoderma* isolate showed hyaline conidiophores arising in clusters from aerial mycelium, branching toward the tip, each branch terminating in a penicillus of 3–6 closely appressed and divergently branched phialides toward the apex, with a sterile stipe. They also exhibited effuse conidiation without the formation of any pustule.

Molecular Characterization of *T. virens*

Pure genomic DNA of the *Trichoderma* strain was isolated and used for nucleotide sequencing. The isolated DNA, after PCR analysis, was run through gel electrophoresis. The PCR product resulted in a band size of 600 bp. ITS-rDNA partial gene was successfully amplified using universal primers ITS 1 and ITS 4. The sequences obtained were subjected to BLAST analysis to identify the new isolate and were submitted to the NCBI GenBank with accession numbers MN452801. The tested fungal stain showed (100%) sequence similarity homology with *T. virens* and clustered with similar groups. The results of the phylogenic analysis of *T. virens* are given in Fig. 1.

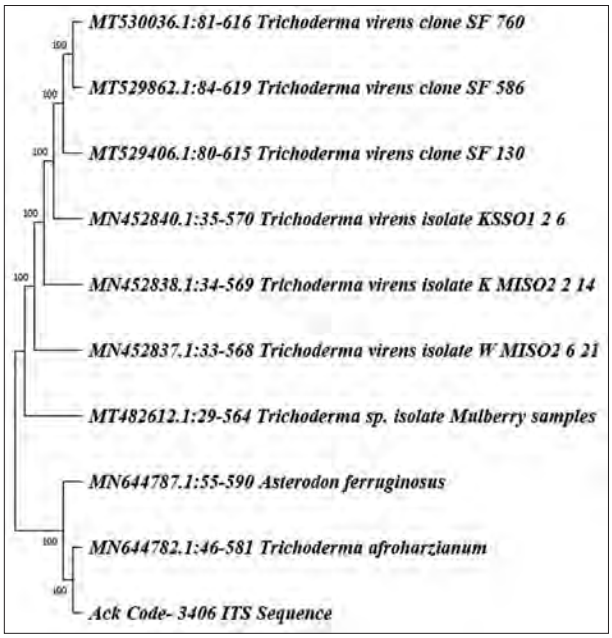

Fig. 1. Phylogenic analysis of *Trichoderma virens*.

Identification of Bioactive Compounds

GC/MS analysis of the fungal strain revealed several antimicrobial compounds with potent antimicrobial properties. The details of the compounds identified are given in Table 1.

The Antagonistic Action of *T. virens* and *Pseudomonas fluorescence*

The antagonistic action of *T. virens* and *Pseudomonas fluorescence* was observed against *F. oxysporum* f. spp. *lycopersici* on PDA (potato dextrose agar medium at 28°C) using a dual culture technique. The average percentage of inhibition (%) of the mycelial growth for FOL showed that *T. virens* significantly inhibited the mycelia growth of the test pathogen (Fig. 2). Among the two biocontrol antagonists, *T. virens* showed a higher percentage of inhibition (85.01%) in comparison to *Pseudomonas fluorescence* (71.14%) when challenged with *Fusarium.* These results suggest that *T. virens* is superior to *Pseudomonas fluorescence* against *F. oxysporum* f. sp. *lycopersici* (FOL).

Field Efficacy of Bioagents and Fungicides for the Management of Disease

The field experiment was conducted during the rabi season of 2020–2021 to assess the possibility of managing the disease in susceptible cultivar Pusa ruby by different fungicides. Three fungicide sprays were applied *in vivo*, and observations were recorded.

Table 1. Identification of antimicrobial compounds from *Trichoderma virens*

S. No.	Name of compound	Chemical formula	Peak area %	Activity
1	n-Nonadecanol-1	$C_{19}H_{40}O$	4.46	Anti-plant pathogenic activity
2	Propanoic acid, 2-methyl	C_4H_8O	3.22	Anti-plant pathogenic activity
3	Butanoic acid, 2-methyl	$C_5H_{10}O$	3.99	Anti-plant pathogenic activity
4	Dodecane	$C_{12}H_{26}$	0.83	Anti-plant pathogenic activity
5	1,4-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	$C_{16}H_{22}O_4$	15.30	Anti-plant pathogenic activity
6	2,5-Piperazinedione, 3,6-bis (2-methylpropyl)-	$C_{12}H_{22}N_2O$	5.57	Anti-plant pathogenic activity
7	Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4-hydro	$C_{34}H_{50}NiO_6$	1.80	Anti-plant pathogenic activity

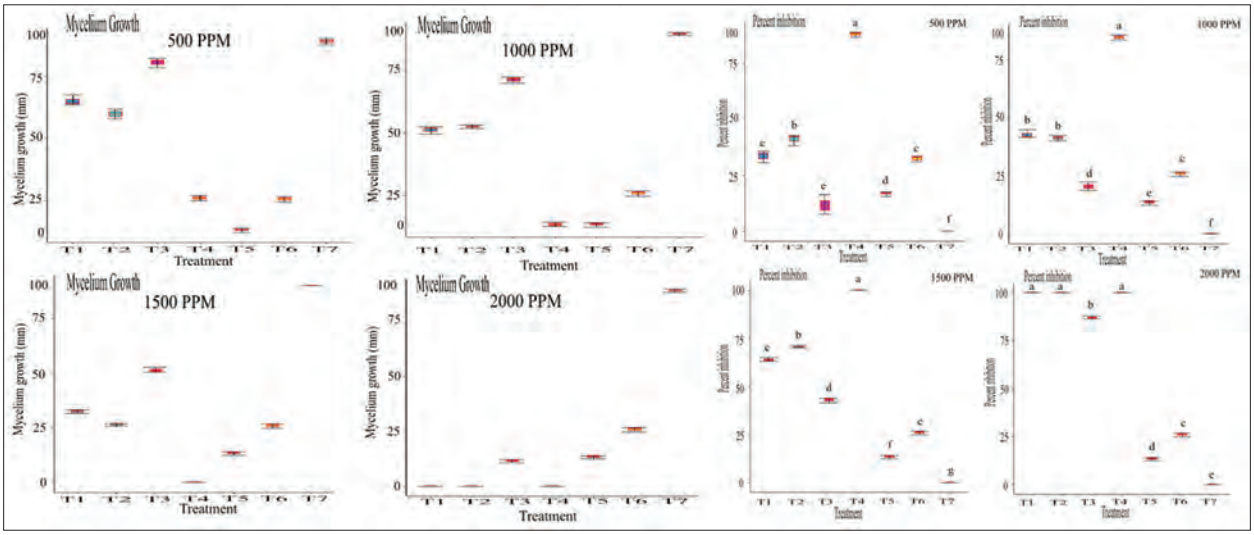

Fig. 2. Box plot represents the mycelium growth inhibition and per cent inhibition at different concentrations.

The last disease incidence was observed with plants treated with *T. virens* followed by carbendazim under field conditions. *P. fluorescens* resulted in 25% disease incidence and was less effective than *T. virens*. The highest disease incidence was recorded in control treatment, with a disease incidence of 76.66% (Fig. 3, Table 2).

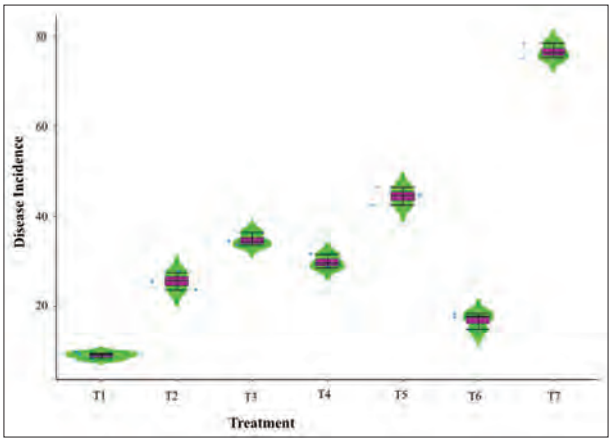

Fig. 3. Violin plot represents the disease incidence (%).

DISCUSSION

A proper selection of BCAs begins with correct identification. The current study identified the virus strain *Trichoderma* when isolated from mushrooms and confirmed the effective strain as a biocontrol agent. *Trichoderma* species can act as BCAs and prevent seed and soil-borne plant diseases. Earlier, the preliminary classification of the *Trichoderma* strain was based on colony morphology and microscopic observations, which were found insufficient, inaccurate, and non-reliable to confirm the concept of species (Seaby, 1996). Therefore, the precise identification of fungal strains at the species level is the first step and is required for utilizing the full potential of fungi, mycological investigation and their specific applications (Lieckfeldt, 1999). DNA barcoding in fungal identification has drastically

Table 2. Effect of inoculum concentration on disease severity of tomato plants at 4 weeks after inoculation with *Fusarium oxysporum* f. spp. *lycopersici*

Treatment	Disease incidence
T1	9.01±0.63^{g}
T2	25.58±1.99^{e}
T3	34.71±1.44^{c}
T4	29.85±1.46^{d}
T5	44.41±1.95^{b}
T6	16.85±1.86^{f}
T7	76.66±1.63^{a}
CV	4.76
SE (m)	2.6

P<0.001, *Significant letter on the basis of DMRT test ($p < 0.05$), ± is the standard error of the mean

improved species identification while reducing labor costs (Schoch *et al*., 2012; Vu *et al*., 2019; Ahluwalia *et al*., 2012).

DNA barcoding is now routinely used to identify *Trichoderma* species, which are often more efficient, accurate, and reliable than those based on morphological characteristics (Hafizi *et al*., 2013). ITS region has been widely used as a genetic marker during the last 15 years for exploring fungal diversity in environmental samples (Lindahl *et al*., 2007, O'Brien *et al*., 2005, Zinger *et al*., 2009; Peay *et al*., 2010). Therefore, the use of ITS region DNA sequence analysis has now become a standard routine and potential method for the identification, characterization classification, and phylogenetic analysis of many fungi at the species level (Sallam *et al*., 2019). More than 100,000 fungal ITS sequences generated by conventional Sanger sequencing are deposited in the International Nucleotide Sequence Databases and other databases (Nilsson *et al*., 2009), providing extensive reference material for the identification of fungal taxa.

The sequencing of the ITS region of the rDNA remains one of the most reliable methods for identifying fungi at the species level (Kullnig-Gradinger *et al*., 2002). By comparing the obtained sequence of the ITS region to the sequences deposited in the GenBank database, the representative isolate was confirmed as *T. virens* with a homology percentage of 100%.

This research found that fungal and bacterial antagonists can control the growth of *F. oxysporum* f. spp. *lycopersici* under lab and field conditions, respectively. Information on using fungi and bacteria as bioagents against plant pathogens have been increased in recent decades. Two antagonists, *T. virens* and *Pseudomonas fluorescence,* were examined against *F. oxysporum* f. sp. *lycopersici,* as described in the methods section. The biological control of plant pathogens and diseases has been studied for many years, and the introduction of beneficial microorganisms into the soil or the rhizospheric zone has been proposed for the biological control of soil-borne plant pathogens (Joffe, 1986; Cook, 1993). The results of the dual culture indicated that *T. virens* and *Pseudomonas fluorescence* significantly inhibited the mycelial growth of the pathogen. Of the two antagonists, *T. virens* showed a higher percentage of inhibition (85.01%) compared to *Pseudomonas fluorescence* which showed (71.14%) when challenged with *Fusarium.* The degree of the antagonist was found to vary for each bio-control agent (Rahman *et al*., 2009). Altinok and Erdogan, 2015, tested *T. virens* against FoL and found the same result, which supports our results. In addition, several studies have been conducted on *Trichoderma* and *Pseudomonas* species and found that these can produce the antifungal compound that effect *Fusarium* wilt (Duijff *et al*., 1999; Al-Ani, 2018). Chemical-free strategies for preventing plant diseases are of considerable interest due to the negative impacts of fungicides on the environment and health (Reuveni, 1995). Therefore, it is likely that there will be better dependence on beneficial microorganisms as BCAs of soil plant pathogens in the future (Hall, 1995; De Cal *et al*., 2009). On the other hand, the range of activity of microorganisms as BCAs is usually narrower than that of chemical pesticides (Baker, 1991; Janisiewicz, 1996). In addition, the inconsistent performance of valuable microorganisms in commercial agriculture has narrowed their application as BCAs for controlling plant pathogens (Backman *et al*., 1997; De Cal *et al*., 2009).

In conclusion, the information obtained from the morphological study alone was insufficient to precisely identify *Trichoderma* because they have relatively few morphological characteristics and a limited variation that may cause overlapping and misidentification of the isolates. Both morphological and molecular approaches are essential methods to identify *Trichoderma* isolates. Furthermore, the *Trichoderma* isolates displayed robust antagonistic activity against specific tomato pathogens. The dual culture assays suggested that *Trichoderma* spp. can be used as a biological control agent in agriculture. From the ecological point of view, such local isolates may be highly significant under field experiments for better utilization as commercial products.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial, or otherwise.

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