

Study on
Biological control of soil-born fungal pathogens using *Trichoderma* species isolated in
Bangladesh

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Summary

Trichoderma spp. can be used as potential biological control agents for a wide range of plant pathogens in agricultural systems because of their high reproductive capacity, ability to survive under harsh conditions, efficiency in the utilization of unfavorable nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms. These properties have made *Trichoderma* a ubiquitous genus that is present at high population densities in all habitats. These direct and indirect *Trichoderma* mechanisms may act coordinately and their importance in the biocontrol process depends on the *Trichoderma* strain, the antagonized fungus, the crop plant and the environmental conditions, including nutrient availability, pH, and temperature. This study was intended to corroborate the positive relationships of molecular and morphological characteristics with the antagonistic ability, as well as the physiological and biochemical features of *Trichoderma* species. Nineteen *Trichoderma* isolates, collected from different locations in Bangladesh, were characterized using phenotypic, biochemical and molecular means. Additionally, their antifungal actions were assessed *in vitro*. The isolates were divided into three groups: *Trichoderma asperellum*, *Trichoderma virens* and *Trichoderma harzianum*. Dual culture and culture filtrate assays against 6 phytopathogens revealed that 9 of the 19 isolates showed significant antifungal activities. The isolate *T. harzianum* TR05 showed the highest inhibition against *Fusarium oxysporum*, *Rhizoctonia solani*, *Fusarium circinatum* and *Phomopsis vexans*, followed by *T. asperellum* TR08 and *T. virens* TR06. TR08 had the highest inhibition against *Sclerotium rolfsii* and *Pythium aphanidermatum*, followed by TR05 and TR06. These findings were in agreement with the activities of their extracellular hydrolytic enzymes, including chitinase, β -1,3-glucanase and proteinase. In a pot experiment under greenhouse conditions, tomato seeds inoculated with TR05 (seed treatment) showed the lowest disease incidence and the highest germination. They also gave superior results in the following plant growth factors: root length, shoot length, fresh weight, dry weight and seedling vigor. In soil treatments, TR08 showed the lowest disease incidence and had superior plant growth factor values. Our results suggest that isolates TR05 and TR08 have the potential to be effective biocontrol agents against phytopathogenic fungi.

ABBREVIATIONS

BCA	Biological Control Agent
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide
EDTA	Ethylenediaminetetraacetic Acid
EF	Elongation Factor
ITS	Internal Transcribed Spacer
ITS1	Internal Transcribed Spacer 1
ITS2	Internal Transcribed Spacer 2
MEGA	Molecular Evolutionary Genetics Analysis
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
rDNA	Ribosomal Deoxyribonucleic Acid
TBE	Tris-Borate-EDTA
<i>Tef1</i>	Translation Elongation Factor 1-Alpha Gene
UV	Ultraviolet

CHAPTER I

INTRODUCTION

Phytopathogenic microorganisms have coexisted with crops and plants since the very beginning of agricultural evolution. Even though this coexistence is a natural phenomenon, it has adversely affected agriculture from time to time. With technological progress, physico-chemical methods have been adopted to mitigate phytopathogenic impacts on agriculture. Crude (ash, raw extracts of certain plants and lime) and chemical pesticides, as well as physical insect traps (Vincent et al. 2003) have been used as control methods. However, the integration of biological controls with pre-existing methods has further revolutionized agricultural pest management. Currently, there is a worldwide movement to use eco-friendly methods for protecting crops from pests and diseases (Rao et al. 1998). The biological control of pathogens using microorganisms is considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods (Eziashi et al. 2007; Shalini and Kotasthane 2007). The potential of the antagonistic microorganisms to reduce the intensity of crop damage by soil-borne plant pathogens has also been reported by Lewis and Larkin (1997).

The plant growth-promoting and antagonistic fungi *Trichoderma* (Ascomycota, Hypocreales) contains a large number of strains that are beneficial to agriculture mainly as biological control agents (BCAs). They provide important benefits to agriculture, including their ability to protect crops from diseases and to increase crop yields under field conditions (Harman et al. 2004). They are non-pathogenic, soil-borne, free-living microorganisms that are commercially used as biopesticides and biofertilizers (Harman et al. 2004). Among the most frequently isolated, soil imperfect filamentous fungi, *Trichoderma* spp. are well known for their biocontrol ability against a wide range of plant pathogens (Harman et al. 2004) and for their plant growth enhancement (Hoyos-Carvajal et al. 2009). Several strains of *Trichoderma* spp. are effective biocontrol agents (BCAs) of various soil-borne plant pathogens, such as *R. solani*, *S. rolfsii*, *P. aphanidermatium*, *F. oxysporum*, *F. culmorum*, *Aspergillus* spp., *Penicillium* spp. and *Phomopsis* spp. under greenhouse and field conditions (Chet and Baker 1981; Cook and Baker 1983; Papavizas 1985; Sivan and Chet 1992). Several *Trichoderma* species reduce the incidence of soil-borne pathogens under natural conditions (Sivan et al. 1984; Lynch 1990; Papavizas, 1992). However, their efficacy largely depends on the physical, chemical and biological soil conditions. *Trichoderma* species colonize numerous plant roots,

decompose plant residues and are involved in the biodegradation of fertilizers and pesticides (Anand et al. 2006).

To date, several mechanisms, including mycoparasitism, competition for space and nutrients, colonization, antibiosis, inactivation of the pathogen's enzymes, secretion of antibiotics, and fungal cell wall-degrading enzymes, have been suggested infighting against phytopathogens (Howell 2003; Harman et al. 2004; Renio et al. 2008). Additionally, *Trichoderma* produces extracellular lytic enzymes, such as chitinases, glucanases and proteases, at a constitutive level (Viterbo et al. 2002), and it can discover the presence of another fungus by sensing the molecules released from the host using enzymatic degradation (Harman et al. 2004). This observation, together with chitin and β -1,3 glucan being the main structural components of fungal cell walls, suggests that the chitinases and β -1,3 glucanases produced by some *Trichoderma* isolates are key enzymes in cell wall lysis during mycoparasitic actions, which are most frequently considered to play important roles in biocontrol (Kullnig et al. 2000; Kubicek et al. 2001). However, proteases produced by *Trichoderma* play a role in the host lysis by attacking lipids and proteins, which are also part of the cell wall's skeleton. Additionally, some strains of *Trichoderma* are capable of producing antifungal antibiotics (Ghisalberti and Rowland 1993). Secondary metabolites may also act as inhibitors to pathogens (Grondona et al. 1997, Humphris et al. 2002) and induce resistance in plants (De Meyer et al. 1998).

The genus *Trichoderma* contains many economically important species. Some of the species, including *Trichoderma reesei* and *Hypocrea jecorina* are producers of industrial enzymes (Kubicek and Penttila 1998) and some are producer of antibiotics (Sivasithamparam and Ghisalberti 1998). The most common strains of the *Trichoderma* genus that are used as biological control agents are *T. virens*, *Trichoderma viride*, *Hypocrea lixii*, *Trichoderma atroviride*, *Hypocrea atroviridis*, *T. asperellum* (Jeger et al. 2009) and, above all, *T. harzianum* (Grondona et al. 1997). Thus, there is an increasing interest in this genus because of its biological properties against soil-borne plant pathogenic fungi and potential commercialization as a biopesticide, biofertilizer and soil amendment (Harman et al. 2004; Lorito et al. 2004).

Most species of the genus grow rapidly in artificial cultures and produce large numbers of small green or white conidia from conidiogenic cells situated at the ends of widely branched conidiophores. This characteristic allows for the relatively easy identification of *Trichoderma* as a genus, but the species concepts are difficult to interpret and there is considerable

confusion over the application of specific names. Although *Trichoderma* spp. have been known since 1865 (Bisby 1939), the taxonomy and species identification were vague until 1969 (Rifai 1969). In fact, Druzhinina and Kubicek (2005) have extensively reviewed species concepts and biodiversity in *Trichoderma* fungi and mentioned that *Trichoderma* fungi are difficult to distinguish morphologically.

Knowledge concerning the behavior of *Trichoderma* spp. as antagonists, as well as their morphological, physiological and biochemical characteristics is important not only for their effective use but to allow for specific characterizations based on molecular data. The molecular characterizations of *Trichoderma* spp. and the genetic diversity among the species have been examined in studies focused on isozyme analyses (Stasz et al. 1989), PCR and sequencing (Kuhls et al. 1995), random amplified polymorphic DNA-like PCR fingerprinting and universally primed PCR (Cumagun et al. 2000), inter-simple sequence repeat analysis, and internal transcribed spacer (ITS) analysis of ribosomal DNA (rDNA). Additionally, a single gene is not sufficient for the actual identification of *Trichoderma* species (Bissett et al. 2003; Chaverri et al. 2004). Although the ITS region is generally used for molecular characterization, closely related species of *Trichoderma* cannot be separated accurately based on this region. For example, several species belonging to *Trichoderma*, including *T. viride*, share the same ITS sequence (Lieckfeldt et al. 1999). Therefore, the *translation elongation factor 1-alpha* gene (*tef1*) may be used because of the greater ability to differentiate species within and among closely related species than ITS rDNA (Samuels 2006).

However, the use of *Trichoderma* as a BCA or the growth of this antagonistic organism is sometimes limited by inhospitable soil caused by physiological and environmental conditions; therefore, the strains show varying effects on pathogens (Betina and Farkas 1998). The growth and sporulation of *Trichoderma* species also vary with environmental factors, such as temperature, pH, nitrogen and carbon sources, and light and darkness (Miller and Reid 1961). Pesticides and organic compounds are widely used (3 million tons of pesticides used yearly worldwide) to control plant pathogens in many countries. Applications of fungicides and fumigants are often applied in greater quantities than herbicides and insecticides in agricultural production. Although the chemical control of soil-borne pathogens provides a certain degree of control, it also has adverse effects on beneficial microorganisms. Moreover, chemical methods are not economical in the long run because they pollute the atmosphere, damage the environment, leave harmful residues, and can lead to the development of resistant strains among the target organisms (Naseby et al. 2000). In fact,

pesticides and organic compounds are not completely degraded, resulting in toxic residues entering the food chain (Lynch 1990). In Bangladesh, diseases cause enormous crop losses; the estimated crop loss by disease is ~10–15% annually (Fakir 2002). As a result, farmers use fertilizers to supply plant nutrients and pesticides for crop protection. Many of these treatments contain metallic ions or toxic elements as contaminants.

To combat soil-borne pathogens, farmers use different cultural management and chemical fungicides. However, the control of soil-borne pathogens with chemicals is very expensive and is almost impractical in several areas, including Bangladesh. In addition, the unwise use of chemicals in agriculture causes environment pollution and health hazards, destroying the natural balance and beneficial micro-flora of the soil. Consumers are also becoming increasingly concerned about the chemical pollution of the environment and pesticide residues in food, and farmers will be faced with the development of pathogen resistance to chemical fungicides. Therefore, there is a need for the development of efficient alternative measures to chemicals in combating diseases and inocula build up in the soil.

To date, a number of BCAs have been registered and are available as commercial products, including strains belonging to the bacterial genera *Agrobacterium*, *Pseudomonas*, *Streptomyces* and *Bacillus*, and fungal genera *Gliocladium*, *Trichoderma*, *Ampelomyces*, *Candida* and *Coniothyrium*. Fungal-based BCAs have gained wide acceptance after bacteria (mainly, *Bacillus thuringiensis*), primarily because of their broader spectra of disease control and production yield (Copping and Menn 2000). In this context, *Trichoderma* sp. have been the cynosure of many researchers in the biological control field (Heraux et al. 2005; Ortiz and Orduz 2001). Furthermore, *Trichoderma* spp. share almost 50% of the fungal BCAs market, mostly as soil and/or growth enhancers and this makes them interesting candidates (Whipps and Lumsden 2001).

At present, the reduction or elimination of synthetic pesticide applications in agriculture is highly desirable. One promising mean to achieve this goal is to use new tools based on BCAs for disease control alone, or to integrate BCAs with reduced doses of chemicals, resulting in the minimal impact of the chemicals on the environment (Chet and Inbar 1994; Harman and Kubicek 1998). Currently, the role of BCAs is a well-established fact and, when antagonistic fungi play an important part, they have become increasingly crucial, either as complements to, or replacements of, their chemical counterparts (Chet 1993; Whipps and Lumsden 2001).

Although several fungicides based on *Trichoderma* formulations have been commercialized in the last few years worldwide, there is still an appreciable interest in finding and formulating more efficient products based on this fungus. Particularly, in Bangladesh, only one strain of *Trichoderma* sp. has been used commercially as a BCA (Meah 2007; Hossain and Hossain 2010; Kashem et al. 2011). In spite of the enormous amount of research on *Trichoderma* spp. as BCAs, their physiological, biochemical and molecular characterizations, as well as their environmental requirements, have not been studied in detail. Moreover, one strain is insufficient to produce high potential bio-pesticides against a wide range of pathogens. Hence, there is an immense need to discover more *Trichoderma* species with potential antimicrobial activities against the wide range of pathogens that cause severe economic damage in Bangladesh agriculture under various environmental conditions. Therefore, the present study was undertaken to obtain the following objectives:

- i. The screening of effective strains of *Trichoderma* that are compatible with fertilizers and pesticides.
- ii. To study influences of the temperature, pH and nutritional requirements of the *Trichoderma* isolates on their growth and sporulation.
- iii. To biochemically and molecularly characterize *Trichoderma* isolates to determine genetic variation.

CHAPTER II

MATERIALS AND METHODS

2.1. Experimental period

The experiments were carried out from April 2012 to September 2014.

2.2. Collection of soil samples

Thirty soil samples were collected from various regions of Bangladesh during 2012–2013 (Table 1). Each soil sample was taken randomly from the rhizosphere of the crop at a 15–20 cm soil depth. Initially, five primary soil samples were randomly collected from different agricultural fields. A working sample was prepared by mixing the five primary samples. At the time of collection, the surface of the soil was scraped to remove dry topsoil and other superficial plant debris. Each composite sample was placed in a cellophane bag with proper labeling and stored at 4°C.

2.3. Isolation of *Trichoderma* species from soil samples

Trichoderma spp. were isolated from soil samples using the serial dilution technique according to Askew and Laing (1993).

2.3.1. Sterilization of the working area

Since bacteria and fungi are always present as contaminants in soil, it is important to exclude them, as much as possible, from the surface of the working area and the equipment to be used. The surface of the working area was disinfected with cotton soaked in methylated spirits (70%). Hands were also disinfected using the same process. The glassware (test tubes, Petri dishes, pipettes, and beakers) was sterilized in a dry oven.

2.3.2. Preparation of the working sample

For every dilution of the soil samples, a composite working sample was prepared after the soil samples were collected from different agricultural fields.

2.3.3. Making a suspension (soil dilution)

- i. Soil (1 g) was placed in a test tube containing 9 ml of sterile water and stirred thoroughly for a few min to obtain a uniform 10^0 dilute soil suspension. This was used as the stock suspension.

- ii. The stock suspension (1 ml) was transferred with a sterile pipette into a second test tube containing 9 ml sterile water, which was then shaken thoroughly and a 10^{-1} dilute soil suspension was obtained.
- iii. Repeating the same process, a 10^{-3} dilution was obtained.

Table 1. *Trichoderma* isolates newly collected from different locations in Bangladesh[¶].

Isolates	Location	Sampling point	Crop
TR02	Dinajpur	25.650° N, 88.683° E	Lentil
TR03	Gazipur	24.000° N, 90.430° E	Groundnut
TR04	Jamalpur	24.920° N, 89.960° E	Soybean
TR05	Mymensingh	24.754° N, 90.403° E	Chickpea
TR06	Chandpur	23.214° N, 90.636° E	Soybean
TR07	Comilla	23.450° N, 91.200° E	Lentil
TR08	Bhola	22.690° N, 90.653° E	Tomato
TR09	Brahmanbaria	23.953° N, 91.117° E	Soybean
TR10	Satkhira	22.350° N, 89.080° E	Rice
TR11	Jessor	23.170° N, 89.200° E	Linseed
TR12	Khulna	22.817° N, 89.550° E	Blackgram
TR13	Sylhet	24.898° N, 91.871° E	Seasame
TR14	Barisal	22.700° N, 90.367° E	Rice
TR15	Rangpur	25.733° N, 89.250° E	Wheat
TR16	Bagerhat	22.667° N, 89.800° E	Chickpea
TR17	Norsingdi	23.920° N, 90.730° E	Rice
TR18	Sherpur	25.000° N, 90.000° E	Tomato
TR19	Tangail	24.300° N, 89.920° E	Soybean
TR20	Feni	23.017° N, 91.392° E	Soybean

[¶] In addition to the listed isolates, *Trichoderma* strain TR01, described as T25 in Parvin et al. (2011), was examined as a positive control in this study.

2.3.4. Isolation of *Trichoderma* species

- i. Each sterile Petri-plate was filled with 15 ml of warm (~45°C) melted PDA medium (pH 5.6).
- ii. One ml of the 10⁻³ soil dilution was placed at the center of a PDA plate and spread using a glass rod. Four Petri-plates were each inoculated with 1ml of diluted sample. This was repeated for every soil sample.
- iii. The inoculated PDA plates were incubated at 25°C for 5–6 days.
- iv. After incubation, plates were observed for *Trichoderma* colony formation. The growing margin of a *Trichoderma* colony was cut into 5 mm blocks using a cork borer. The blocks were carefully placed on new PDA plates to obtain pure cultures of *Trichoderma*. The new plates were incubated as before. The well-developed pure cultures were sub-cultured to PDA plates for preservation at 4°C for further use. In this way, 19 colonies that were assumed to be *Trichoderma* spp. were isolated based on observations according to Samuels et al. (2014), and were then named as TR02–TR20 (Table 1).

In addition to the isolates, *Trichoderma* sp. TR01, which was previously reported as strain T25 (Parvin et al. 2011), was used as the positive control for the following examinations.

2.4. Morphological features of the isolates

The isolates of *Trichoderma* spp. were characterized morphologically. Nineteen isolates were purified and were maintained on PDA medium at 25±1°C (incubation) to study their morphological characteristics (Singh and Singh 2004).

2.4.1. Morphological characteristics

The following morphological characteristics of the isolates were observed on PDA after 6 days of incubation:

- Colony color
- Radial growth
- Growth habit
- Colony consistency
- Conidia and conidiophores
- Sporulation density
- Chlamydospore production

2.4.2. Measurement of sporulation density

Because *Trichoderma* is a fast growing fungus, its growth and sporulation could be completed within 5–6 days. The spore density was measured using the following formula: Number of spores per cubic ml suspension = (Number of spores counted × dilution) / (Number of smallest square counted) × 4,000.

2.4.2.1. Procedure used for calculating the spore density

- i. To determine the spore density, 100 ml of sterile water was placed in a test tube and 10 ml was poured into PDA plates containing 10-day-old cultures of *Trichoderma* spp.
- ii. The conidial suspension was gently scraped smoothly with a scalpel or slide to collect conidia. The suspension was placed into a beaker and stirred continuously with a glass rod.
- iii. The volume of the beaker containing the conidial suspension was increased to 500ml with sterile water, and one drop of Tween-20 was added to it and dispersed by stirring. From this solution, one drop of suspension was placed on the center of haemocytometer and a cover slip was placed on it. Finally, spores were counted under a microscope at 40x magnification.
- iv. To determine the spore density the blocks and lines of the haemocytometer were adjusted under the microscope so that 25 blocks remained at the center, and each block was divided into 16 sub-blocks. After that 4 blocks were selected randomly and the spores present in 16 sub-blocks of each block were counted. Finally, spores from the 4 blocks were multiplied to equal the amount in 25 blocks, estimating the number of spores/ml.

Then, the average number of spores per unit cell was used in the above mentioned formula, and the number of spores per ml was determined.

2.5. Molecular features of the isolates

All *Trichoderma* spp. isolates were characterized molecularly using the rDNA of ITS1, ITS2 and *tefl*.

2.5.1. Preparation of broth culture to harvest mycelia

For DNA extraction, mycelial cultures were individually grown in 250-mL conical flasks containing 100mL of liquid culture medium. The culture medium was prepared using potato

dextrose broth (PDB; Difco). The conical flasks containing mycelia were kept for 6 days at $25\pm 1^{\circ}\text{C}$. After this incubation, mycelia were harvested through filter paper in a Buchner funnel, washed with distilled water, frozen and lyophilized. After harvesting, each mycelia isolate was wrapped with aluminum foil sheets and kept at 4°C until just prior to the genomic DNA isolation.

2.5.2. DNA extraction

The total genomic DNA was extracted from each *Trichoderma* isolate following the protocol of the ISOPLANT kit (Nippon Gene Co. Ltd., Toyama, Japan).

2.5.2.1. Contents of ISOPLANT kit (For 100 extractions)

Solution I: Extraction Buffer	30 ml
Solution II: Lysis Buffer	15 ml
Solution III: Sodium Acetate (pH 5.2)	15 ml
TE: 10 mM Tris-HCl (pH 8.0) and 1mM EDTA	10 ml
RNase A: 1 mg/ml	100 μl

2.5.2.2. Protocol for genomic DNA extraction using the ISOPLANT kit

The genomic DNA of each *Trichoderma* isolate was extracted using their harvested mycelia using the following the steps:

- i. Placing 50 μl TE Buffer (pH 8.0) plus the fungal sample (mycelia) in 1.5 ml Eppendorf tubes.
- ii. Centrifuging the tubes at $6,000 \times g$ at 4°C for 2 min.
- iii. The upper part of the sample was gently removed by pipetting and discarded. Only the lower part of the sample was retained.
- iv. Adding 300 μl of Solution-I to the sample and vortexing for 1–2 s.
- v. Again adding 150 μl of Solution-II and vortexing for 5–6 s.
- vi. Then heating the mixture at 50°C for 15 min.
- vii. Adding 150 μl of Solution-III and vortexing for 1–2 s.
- viii. Then, the mixture was kept on ice for 15 min.
- ix. Again, the mixture was centrifuged at $12,000 \times g$ at 4°C for 15 min.

- x. A sample of 300 μ l of solution (150 μ l+150 μ l; two times) from the upper part was transferred to another 1.5 ml Eppendorf tube.
- xi. Adding 700 μ l of cool ethanol (95–99%) and mixing thoroughly by pipetting up and down.
- xii. Again, the mixture was centrifuged at 12,000 $\times g$ at 4°C for 10 min.
- xiii. The solution was poured into a beaker.
- xiv. Washing the tube with 300 μ l of cool ethanol (70%) and pouring the solution into the beaker.
- xv. The 1.5 ml Eppendorf tube, containing the pellet, air dried for 5–6 hours.
- xvi. Adding 50 μ l of TE buffer (pH 8.0) into the 1.5 ml Eppendorf tube.
- xvii. Finally, 50 μ l of genomic DNA solution was obtained and stored at –20°C.

2.5.3. Primers used

2.5.3.1. For the ITS region (ITS1 and ITS2)

ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and

ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3')

2.5.3.2. For *tefl*

EF1-728F (5'-CATCGAGAAGTTCGAGAAGG-3') and

EF1-986R (5'-TACTTGAAGGAACCCTTACC-3')

2.5.4. PCR mixture for each reaction

Components	amount (μ l)
Sterile water	5.6 μ l
Buffer	12.5 μ l
dNTPs	5.0 μ l
Primer-1	0.2 μ l
Primer-2	0.2 μ l
KOD FX NEO Enzyme	0.5 μ l
Template DNA	1.0 μ l
Total	25 μ l

2.5.5. Components of the KOD FX NEO Enzyme

KOD FX Neo (1.0 U/ μ l)	200 μ l \times 1
2 \times PCR Buffer for KOD FX Neo	1.7 ml \times 3
2 mM dNTPs	1 ml \times 2

2.5.6. PCR amplifications

2.5.6.1. PCR amplifications of the ITS region (ITS1 and ITS2)

An ITS region of the rDNA was amplified by PCR with universal primers ITS4 and ITS5 (White et al. 1990) in a Takara PCR Thermal Cycler Dice (Ohtsu, Japan) using the following program: initial denaturation for 2 min at 94°C; 30 cycles of denaturation for 10 s at 98°C, primer annealing for 30 s at 51°C and extension for 30 s at 68°C; followed by a final extension period of 7 min at 68°C.

2.5.6.2. PCR amplifications for *tef1*

In addition, a 0.3-kb fragment of *tef1*, containing the fourth large intron, was amplified by the primer pair EF1-728F and EF1-986R (Druzhinina et al. 2004) using the following program: initial denaturation for 2 min at 94°C; 35 cycles of 10 s at 98°C, 30 s at 55°C and 30 s at 68°C; followed by a final extension period of 7 min at 68°C.

2.5.7. Electrophoresis

Each PCR product was electrophoresed on 1% agarose mini-gels containing 0.5 g/ml ethidium bromide in Tris-acetate EDTA buffer.

2.5.7.1. Preparation of agarose gel

2.5.7.1.1. Reagent used

- Agarose powder
- 5 \times TBE buffer (pH 8.3); Compositions (for 1 l)
 - Tris: 54 g
 - Boric acid: 27.5 g
 - EDTA: 4.65g
- Ethidium Bromide

2.5.7.1.2. Preparation of 5× TBE buffer (1,000ml)

- i. Tris base (54 g) was placed in a 1,000ml measuring cylinder and 800 ml ddH₂O was added to it.
- ii. Then, the mixture was stirred for 30 min.
- iii. After stirring, 27.5 g boric acid was added and stirred until completely dissolved.
- iv. Then, 20 ml 0.5M EDTA (pH8.0) was added.
- v. Finally, ddH₂O was added to a volume of 1,000 ml.

2.5.7.1.3. Preparation of 1X TBE buffer (1,000ml)

- 100 ml 5× TBE was added to 900 ml ddH₂O.

2.5.7.1.4. Preparation of 1% Agarose gel (100 ml)

- i. The electrophoresis buffer 1× TBE (100 ml) was placed in a conical flask and 1.0g of agarose was added.
- ii. The top of the conical flask was covered with an aluminum foil sheet to prevent excessive evaporation and the mixture was heated in a microwave oven with occasional swirling to generate a uniform suspension until no agarose particles were seen and the agarose solution become transparent.
- iii. The gel was kept at room temperature for 10–15 min to cool to 50°C.
- iv. The gel was then poured into a mold and 1 µl ethidium bromide (0.5 g/mL) was added for staining and mixed well by gentle stirring.
- v. Two combs were placed in the gel.
- vi. Within 30 min the gel was solidified.
- vii. The combs were removed from the gel.
- viii. The gel was submerged into 1× TBE buffer.
- ix. The gel was then ready for DNA sample loading.

2.5.7.2. DNA sample preparation for electrophoresis

2.5.7.2.1. Reagents used

- 10 ml 6× loading dye

- 25 mg xylene cyanol
- 25 mg bromophenol blue
- 3.3 ml glycerol, and
- 6.7 ml TE buffer
- DNA marker (DNA ladder)

2.5.7.2.2. Procedure for DNA sample preparation for electrophoresis

- i. For each sample, 1 µl loading dye was placed on a piece of aluminum foil using a 0.5–10 µl adjustable micropipette.
- ii. Finally, 5 µl DNA was added to it and mixed well using the same micropipette.
- iii. Then, 5 µl of a DNA ladder was loaded into the first wells of both gel lanes.
- iv. The samples were then loaded slowly to the appropriate gel wells.
- v. The gel tank was covered.
- vi. The power supply was then connected and turned on and the DNA moved from negative to positive electrodes (black to red, respectively). Electrophoresis was carried out at 120 V for 20 min (Bio-red Power /Pac 200).
- vii. After the bromophenol blue dye had reached three-fourths of the gel length, the electrophoresis was stopped and the power supply was disconnected.

2.5.8. Documentation of the DNA samples

After electrophoresis, the gel was carefully removed from the gel apparatus and placed in the dark chamber of the ultraviolet light box (UV Transilluminator). Images of the DNA gel run were taken.

2.5.9. Sequencing

Following the manufacturer's instructions, the PCR products were purified using EXOSAP-KIT (USB, Ohio, USA). The PCR amplicons corresponding to the ITS region of rDNA and *tef1* were then sequenced by FASMAC Co. Ltd. (Kanagawa, Japan). All of the isolates were characterized, and the ITS and *tef1* sequences were used for a phylogenetic analysis.

2.5.10. Phylogenetic analysis

The ITS and *tefl* nucleotide sequences were submitted to the basic local alignment search tool (BLAST) search engine in the National Center for Biotechnology Information database (<http://blast.ncbi.nlm.nih.gov>) to identify the *Trichoderma* isolates. The ITS and *tefl* sequences were aligned with the closest sequences identified by the BLAST algorithm using CLUSTALW. A phylogenetic analysis was carried out using the MEGA 6 program, and a Neighbor-joining tree was constructed using the Kimura-2-parameter distance model (Kimura 1980). Confidence values were assessed from 1,000 bootstrap replicates of the original data.

2.5.11. Nucleotide sequence accession numbers

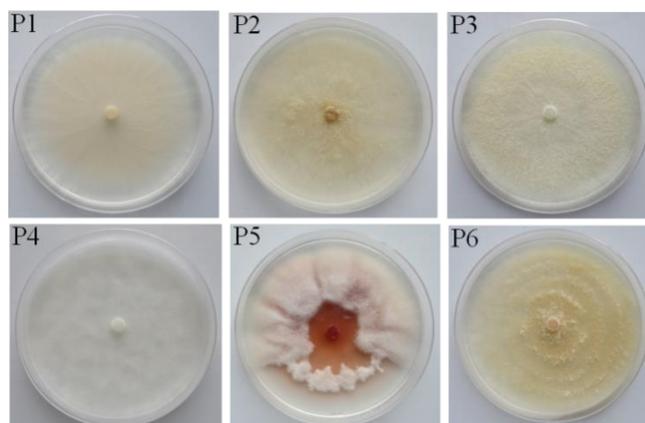
The sequences obtained in this study have been deposited in the DNA Data Bank of Japan under accession numbers AB935948–AB935987.

2.6. Antagonism of the isolates against pathogens

The antagonism of the *Trichoderma* isolates against the pathogens mentioned below, were determined using dual culture (Grondona et al. 1997) and culture filtrate assays.

2.6.1. Selected pathogens

Six pathogens, *F. oxysporum* f.sp. *lycopersici* MAFF305914, *R. solani* MAFF241953, *S. rolfsii* MAFF103049, *P. aphanidermatum* MAFF241101, *F. circinatum* MAFF236397 and *P. vexans* MAFF305185, were purchased from the National Institute of Agrobiological Sciences, Japan. These pathogens are shown in Photograph 1.



Photograph 1. Different pathogens (at 12 days old on PDA): P1= *Fusarium oxysporum*, P2= *Rhizoctonia solani*, P3= *Sclerotium rolfsii*, P4= *Pythium aphanidermatum*, P5= *Fusarium circinatum*, and P6= *Phomopsis vexans*

2.6.2. Dual culture assay

First, the antagonism of the *Trichoderma* isolates against the six pathogens was assayed on PDA plates using a dual culture technique (Grondona et al. 1997). Mycelial discs (5 mm in diameter) were taken from actively growing *Trichoderma* and pathogenic fungal plates. The discs were placed on either end of the same Petri dish at an equal distance from the periphery. A Petri dish containing only the target pathogen at the same position without *Trichoderma* sp. was used as a control. Inoculated plates were incubated at $25 \pm 1^\circ\text{C}$ for 6 days. After the incubation period, the radial growth diameters of the pathogens were measured, and the percent inhibition of radial growth was calculated using the following formula (Singh et al. 2002): $I = (C-T)/C \times 100$, where I = inhibition (%), C = colony diameter on the control plate (mm), and T = colony diameter on the treated plate (mm). The area of intermingling contact between *Trichoderma* and pathogens was also microscopically observed.

2.6.3. Culture filtrate assay

The efficacy of culture filtrates, as well as non-volatile metabolites produced by the *Trichoderma* isolates, against the growth of pathogens was also examined. Mycelial discs (5 mm in diameter) were excised from young *Trichoderma* cultures and were inoculated into 100mL PDB in 250-mL conical flasks. After incubation at $25 \pm 1^\circ\text{C}$ for 6 days, the culture supernatants were collected by centrifugation at 3,500 rpm for 30 min and were subsequently filtered through 0.45- μm Millipore filters (Toyo Roshi Kaisha Ltd., Tokyo, Japan) to obtain cell-free culture filtrates under aseptic conditions. Just before pouring, the cell-free culture filtrates were mixed with molten PDA in the proportion of 1:4. The pathogens (5-mm discs) were inoculated into Petri dishes containing PDA amended with cell-free culture filtrates. PDA amended with the same amount of sterilized distilled water was used as a control. After incubation at $25 \pm 1^\circ\text{C}$ for 6 days, the radial growth diameters of pathogens were measured and the percent inhibition of radial growth was calculated using the formula mentioned above.

2.7. Physiological features of the isolates

The physiological features of the *Trichoderma* isolates were determined using several parameters.

2.7.1. Temperature

The growth and sporulation of the *Trichoderma* isolates were tested at 4, 35, and 40°C after incubation for 6 days on PDA. The thermal resistance of the spore suspensions was determined after incubation for 5 min at 75°C (Grondona et al.1997). A positive result was

the formation of a colony on PDA within 6 days. Conflicting results were repeated again, when necessary, to increase their consistency.

2.7.2. pH

The growth of the *Trichoderma* isolates at pH 2, 4, 5.6, 10 and 12 were examined in PDB medium containing 0.05g/l bromocresol purple (Bridge 1985). A positive result was the formation of a hyphal mat in PDB within 6 days. Conflicting results were repeated again, when necessary, to increase their consistency.

2.7.3. Nitrogen sources

The growth and sporulation of the *Trichoderma* isolates were examined on PDA containing ammonium oxalate, sodium nitrate or potassium nitrate as the sole nitrogen source (3.0g/l). The assimilation of creatine, urea and glycine as the sole nitrogen source was also examined using PDB (2.0g/l). A positive result was the formation of a colony on PDA or a hyphal mat in PDB within 6 days. Conflicting results were repeated again, when necessary, to increase their consistency.

2.7.4. Carbon sources

The use of lactose, sucrose, soluble starch and ammonium oxalate as the sole carbon sources was examined using PDA (30g/l). The use of glucose, galactose, xylose, mannitol, ammonium tartrate, ammonium oxalate, malic acid, lactic acid, citric acid and tannic acid was also examined using PDB (10g/l). Plates and liquid cultures were incubated at $25 \pm 1^\circ\text{C}$ for 6 days for evaluation (Bridge 1985; Grondona et al. 1997). A positive result was the formation of a colony on PDA or a hyphal mat in PDB within 6 days. Conflicting results were repeated again, when necessary, to increase their consistency.

2.8. Biochemical features of the isolates

The following biochemical features of *Trichoderma* isolates were examined on PDA according to Bridge (1985) and Grondona et al. (1997):

2.8.1. Hydrolysis of aesculin

Aesculin hydrolysis was tested on PDA, with the sucrose content reduced to 5g/l and with the addition of 5 g/l aesculin and 0.2 g/l ferric citrate. The blackening of the colony and the surrounding medium denoted a positive reaction.

2.8.2. Hydrolysis of starch

Starch hydrolysis was tested on PDA with the sucrose replaced by 10 g/l soluble starch. A positive reaction was indicated by the appearance of a clear zone around the colony after flooding with Gram's iodine solution.

2.8.3. Hydrolysis of cellulose

Cellulose hydrolysis was tested on PDA containing 40g/l ball-milled cellulose. A positive result was a clear zone around the streak inoculation within 15 days.

2.8.4. Hydrolysis of casein

Casein hydrolysis was tested on PDA containing 10 g/l glucose and 10g/l skim milk (Difco) that was adjusted to pH 5.6. A positive result was a clear zone around the streak inoculation after flooding with 10% (w/v) HgCl₂.

2.8.5. Hydrolysis of polypectate

Hydrolysis of polypectate was tested at pH 6 and pH 8 on a modified PDA medium containing 6 ml 10% (w/v) CaCl₂·2H₂O per liter and 0.05 g/L bromothymol blue. The medium was adjusted to pH 6 or pH 8 with 1M NaOH after the addition of 35 g/l polygalacturonic acid. A positive result was denoted by the liquefaction of the gel.

2.8.6. Hydrolysis of Tween 80

Tween 80 hydrolysis was tested using a method based on the utilization of two components, 100g/l aqueous Tween 80 solution and a basal medium containing mycological peptone (10 g/l), NaCl (5 g/l), CaCl₂·2H₂O (0.1 g/l), agar (15 g/l) and bromocresol purple (0.05 g/L). The basal medium was adjusted to pH 5.6 with 1 M NaOH and the two components were autoclaved separately. The basal medium and the Tween 80 solution were cooled and mixed in a 9: 1 proportion and poured into petri dishes. A positive result was the appearance of white crystals in the medium.

2.8.7. Hydrolysis of gelatin

Gelatin hydrolysis was tested as the ability to liquefy PDA medium that had been solidified with 120 g/l gelatin. This test was chilled for 1h at 4°C after incubation before assessing.

2.8.8. Reduction of tetrazolium

The reduction of tetrazolium was tested on PDA containing 0.064 g/l tetrazolium salt. A positive result was the appearance of a red color in the colony.

2.8.9. Reduction of tellurite

The reduction of tellurite was tested on PDA containing 0.032 g/l potassium tellurite. A positive result was the blackening of the colony.

2.9. Hydrolytic enzyme activities of the isolates

Several hydrolytic enzyme activities of the *Trichoderma* isolates were determined.

2.9.1. Extraction of lytic enzymes

The *Trichoderma* isolates were grown in PDB at $25 \pm 1^\circ\text{C}$ for 6 days. After incubation, the mycelia mat was transferred to a 100-ml conical flask containing 25 ml 100 mM phosphate buffer (pH 5.5) supplemented with 50 mM sodium chloride. For enzyme induction, 10 g/l of chitin, laminarin or casein was added to the conical flask, and the pH was adjusted to 5.5. The cultures were shaken in an orbital shaker at 150 rpm at $27 \pm 1^\circ\text{C}$ for 8 h. After being transferred to 50-ml centrifuge tubes, the cultures were centrifuged at 12,000 rpm for 10 min. Finally, the supernatants were collected and stored at -20°C for further use to assay enzyme activities.

2.9.2. Chitinase activity

The chitinase activity was assayed in the presence of 200 μl 5 g/l chitin in 10 mM sodium acetate buffer (pH 5.2) with 100- μl culture supernatants incubated for 1 h at 50°C . The produced sugar N-acetylglucosamine was estimated by the dimethylaminobenzaldehyde method (Reissig et al. 1955). One unit of chitinase activity was defined as the amount of enzyme necessary to release 1 μmol reducing sugar per min.

2.9.3. β -1,3-glucanase activity

The β -1,3-glucanase activity was detected using 100 μl of 40 g/l laminarin in 50 mM sodium acetate buffer (pH 5.2) with 100- μl culture supernatants. After incubation at 37°C for 10 min, the glucose released by β -1,3-glucanase was measured by the dinitrosalicylic acid method (Sadasivam and Manickam 1992). One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 μmol reducing sugar per min.

2.9.4. Proteinase activity

The proteinase activity was determined in the presence of 500 μl enzyme solution and 500 μl of 3.6 g/l casein with 2.0 ml of 100 mM acetate buffer (pH 3.6). After incubation for 1 h at 50°C , the reaction was stopped with 3 ml of 50 g/l trichloroacetic acid. The mixtures were

then centrifuged at 5,000 rpm for 10 min, and 500- μ l supernatants were used to estimate the released free amino acids using the ninhydrin method (Lee and Takahashi 1966). One unit of protease activity was defined as the amount of protein necessary to produce 1 μ g free amino acids per min.

2.10. Green house experiment

The effectiveness of the three *Trichoderma* strains against *S. rolfsii* MAFF103049 on tomato seedlings was examined in a greenhouse under seed treatment and soil treatment conditions.

2.10.1. *Trichoderma* strain

Three *Trichoderma* strains, *T. harzianum* TR05, *T. virens* TR06 and *T. asperellum* TR08, were used in this study.

2.10.2. Selected pathogen

As a pathogen, *S. rolfsii* MAFF103049, obtained from the National Institute of Agrobiological Sciences, Japan, was used after its pathogenicity was confirmed by artificial inoculations on tomato seedlings.

2.10.3. Seed treatment

The effectiveness of the three *Trichoderma* strains against *S. rolfsii* MAFF103049 on tomato seedlings was examined in a greenhouse under seed treatment conditions. The *Trichoderma* and *S. rolfsii* MAFF103049 inoculations were performed in the following combinations:

SE₁ = TR05 + pathogen

SE₂ = TR06 + pathogen

SE₃ = TR08 + pathogen, with

SE₀ = Control and

SE₄ = pathogen only.

Three replications were performed for each treatment. First, 12-day-old PDA-grown cultures of the isolates TR05, TR06 and TR08 were mixed with sterile deionized water, and 30-ml fungal suspensions were prepared. Each spore density in the suspension was 7×10^8 spore/ml, as determined using a haemocytometer under a light microscope. Tomato seeds were sterilized in a 1% sodium hypochlorite solution for 3 min and rinsed thoroughly in sterile distilled water. Inoculation with the *Trichoderma* strains was then performed by dipping the seeds in the fungal suspension for 30 min. Control seeds were soaked in an equal volume of

deionized water. The treated and control seeds were directly sown into trays (12" × 8" × 3") filled with autoclaved commercial culture soil (0.8 kg/tray) at the rate of 50 seeds per tray.

S. rolfsii MAFF103049 that had been established on corn meal in a conical flask was applied to the tray at the rate of 5 g/kg soil at 7 days before the seed sowing. Trays were placed on a bench in a greenhouse. After 2 weeks, seedling emergence was monitored to determine the effectiveness of the *Trichoderma* treatments on germination.

The percent disease incidence was determined 4 weeks after sowing using the following formula: Percent disease incidence = (Number of infected plants/Total number of plants) × 100. The tomato seedlings were then removed from each tray and the roots were gently washed using tap water. The effects of *Trichoderma* isolates on the growth of tomato seedlings were evaluated and recorded as follows: shoot lengths, root lengths and dry weights measured after drying for 5 days at 45°C. The seedlings vigor was calculated using the following formula: Vigor index = (Root length + Shoot length) × Seed germination percentage.

2.10.4. Soil treatment

The effectiveness of the three *Trichoderma* strains against *S. rolfsii* MAFF103049 on tomato seedlings was examined under soil treatment conditions. *Trichoderma* strains and *S. rolfsii* MAFF103049 inoculation were conducted according to the following:

SO₀ = control

SO₁ = only TR05

SO₂ = TR05 + pathogen

SO₃ = only TR06

SO₄ = TR06 + pathogen

SO₅ = only TR08

SO₆ = TR08+ pathogen, and

SO₇ = only pathogen.

Three replications were performed for each treatment. The *Trichoderma* isolates and *S. rolfsii* MAFF103049 were cultured on corn meal in conical flasks separately. Each pot (6" diameter × 8" height) was filled with 1.3 kg of autoclaved commercial culture soil. Seven days before transplanting tomato seedlings, the soil was infected with *S. rolfsii* MAFF103049 at the rate of 5 g/kg soil. The *Trichoderma* isolates were then inoculated into the pots at the rate of 5 g/kg soil at 3 days before transplanting. The pots were watered for 3 days. Six 14-day-old

seedlings were transplanted into each pot and were kept on a bench in a greenhouse. After 4 weeks, the tomato seedlings were removed from the pots and the roots were gently washed using tap water. The effects of the *Trichoderma* isolates on disease incidence, growth of tomato seedlings and seedling vigor were evaluated and measured as mentioned above.

2.11. Statistical analysis

Tukey's test was performed to analyze statistical differences in antifungal activities among *Trichoderma* isolates using the statistical software KyPlot version 2.0 beta 15.

CHAPTER III

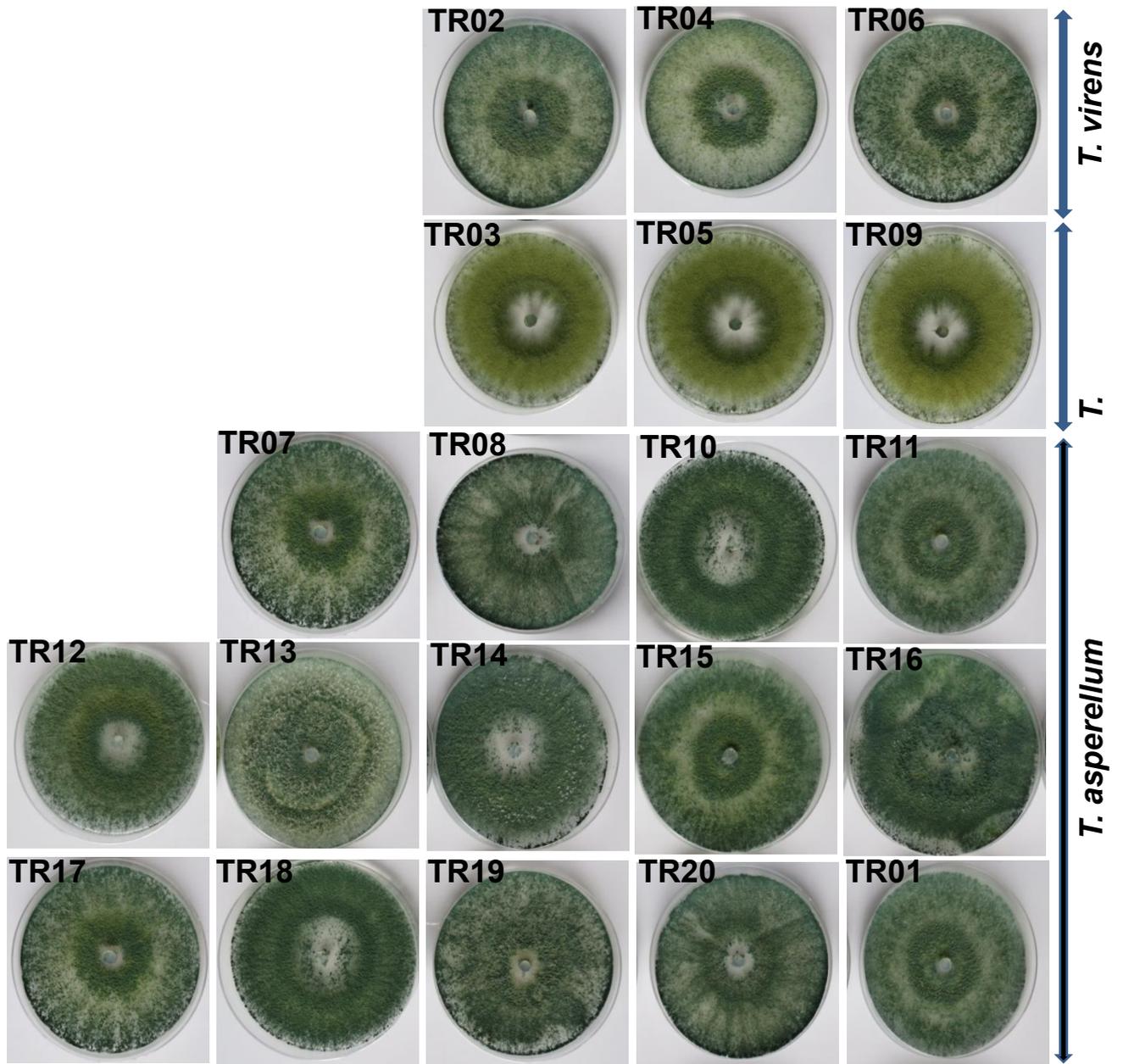
RESULTS

3.1. Morphological features of the isolates

We obtained 19 monoconidial isolates that were assumed to be *Trichoderma* spp. from 30 soil samples collected from various regions of Bangladesh by transferring single conidia germinated on PDA (Table 1).

3.1.1. Grouping of the isolates

All of the *Trichoderma* isolates could be grouped into three different species as shown in Table 2. Among the 19 isolates, 13 isolates, TR07, TR08 and TR10–TR20, had similar morphological characteristics on PDA to TR01, which corresponded to *T. asperellum* (Samuels et al. 2014). The isolates TR02, TR04 and TR06 were also morphologically distinguishable and showed similarities to *T. virens* (Bissett 1991). The others, TR03, TR05 and TR09 were presumed to be *T. harzianum* (Rifai 1969). The morphogenetic variations of different *Trichoderma* isolates are shown in Photograph 2.



Photograph 2. Morphogenetic variations of the *Trichoderma* isolates.

Table 2. Morphological characterization of the *Trichoderma* isolates.

Isolates	TR01, TR07, TR08, and TR10-20	TR02, TR04, and TR06	TR03, TR05, and TR09
Colony on PDA			
- Color	Dark green	Dull to blackish green	Yellowish green to pale green
- Shape	Regular shaped	Irregular shaped	Regular shaped
- Consistency	Very compact	Medium compact	Compact
- Size at 6days	≥ 7.8 cm	≥ 8.2 cm	≥ 8 cm
- Radial growth	Medium fast	Fast	Fast
Conidia			
- Color	Light green	Dark green	Light green
- Shape	Globose to sub-globose	Elliptical	Sub-globose
- Sporulation density	0.74×10^{10} spores/mL	1.11×10^{10} spores/mL	0.98×10^{10} spores/mL
- Chlamydospores	6.8 μm, frequently	7.4 μm, abundant	8.2 μm, abundant
Conidiophore	Regular branching with paired	Irregular branching with broad	Irregular branching with narrow
Estimated species	<i>T. asperellum</i> -like	<i>T. virens</i> -like	<i>T. harzianum</i> -like
References	Samuels et al. (2014)	Bissett (1991)	Rifai (1969)

3.1.2. Morphological characteristics of the isolates

The morphological characteristics of the *Trichoderma* isolates are shown in Table 2.

3.1.2.1. Colony color

The TR01, TR07, TR08 and TR10–TR20 isolates produced dark green colonies while the isolates TR02, TR04 and TR06 formed dull to blackish green colonies. The others, TR03, TR05 and TR09, produced yellowish green to pale green colonies.

3.1.2.2. Radial growth

The radial growth of TR01, TR07, TR08 and TR10–TR20 was medium to fast, and the others isolates, TR02, TR04, TR06, TR03, TR05 and TR09, grew rapidly.

3.1.2.3. Growth habit

The TR01, TR03, TR05, TR07, TR08, TR09, and TR10–TR20 isolates were had regular growth shapes, while TR02, TR04 and TR06 showed irregular growth shapes.

3.1.2.4. Colony consistency

The colony consistency of the TR01, TR07, TR08 and TR10–TR20 isolates was very compact and that of TR02, TR04 and TR06 was medium compact. The others, TR03, TR05 and TR09, had compact colonies.

3.1.2.5. Sporulation density

The TR01, TR07, TR08 and TR10–TR20 isolates were found to produce 0.74×10^{10} spores/ml, while the isolates TR02, TR04 and TR06 produced 1.11×10^{10} spores/ml. The isolates TR03, TR05 and TR09 produced 0.98×10^{10} spores/ml.

3.1.2.6. Chlamyospore production

The TR01, TR07, TR08 and TR10–TR20 isolates frequently produce 6.8 μm chlamyospores, while TR02, TR04, and TR06 isolates produced abundant 7.4 μm chlamyospores. The isolates of TR03, TR05, and TR09 produced abundant 8.2 μm chlamyospores.

3.1.2.7. Conidia and conidiophores

The TR01, TR07, TR08 and TR10–TR20 isolates were found to produce globose to subglobose-shaped light green conidia and regular branching with paired conidiophores, while the isolates TR02, TR04 and TR06 had elliptical-shaped dark green conidia and

irregular branching with broad conidiophores. The others, TR03, TR05 and TR09, produced subglobose-shaped light green conidia and irregular branching with narrow conidiophores.

3.2. Molecular features of the isolates

All isolates of *Trichoderma* spp. were characterized molecularly on the basis of the rDNA of ITS1, ITS2 and *tef1*.

3.2.1. Molecular characterization

3.2.1.1. Characterization based on the ITS region

As a result of sequencing the ITS region and subsequent BLAST algorithm-based searches, 14 isolates (TR01, TR07, TR08 and TR10–TR20) were determined to be *T. asperellum*, three isolates (TR02, TR04 and TR06) were determined to be *T. virens*, and the other three isolates (TR03, TR05 and TR09) were determined to be *T. harzianum* (Table 3). Based on the sequences of the ITS region, all of the isolates are 100% homologous against their respective closest relatives, except TR04 and TR18 (99%) (Table 3).

3.2.1.2. Characterization based on *tef1*

As a result of sequencing *tef1* and subsequent BLAST algorithm-based searches, 14 isolates (TR01, TR07, TR08 and TR10–TR20) were identified as *T. asperellum*, three isolates (TR02, TR04 and TR06) were identified as *T. virens*, and the other three isolates (TR03, TR05 and TR09) were identified as *T. harzianum* (Tables 4). Based on the *tef1* sequences, TR03, TR05, TR06, TR09, TR16 and TR20 matched their respective closest relatives at a 99% homology level, and the remaining isolates showed 100% homology (Table 4).

3.2.2. Phylogenetic Neighbor-joining trees

To determine the diversity and genetic distances among the isolates, phylogenetic Neighbor-joining trees were constructed using the sequence data of the ITS region and *tef1* of the *Trichoderma* isolates and several known *Trichoderma* strains found in GenBank.

Table 3. Closest relatives of the *Trichoderma* isolates based on internal transcribed spacer sequences.

Isolates	Accession no.	Closest relatives (accession no.)	Alignment	Homology
TR01	AB935948	<i>Trichoderma asperellum</i> TR696 (KC993073)	544/544	100
TR02	AB935949	<i>Trichoderma virens</i> TR039 (HQ608079)	525/525	100
TR03	AB935950	<i>Trichoderma harzianum</i> TR068 (KC993075)	553/553	100
TR04	AB935951	<i>Trichoderma virens</i> CIBT06 (EU280076)	548/549	99
TR05	AB935952	<i>Trichoderma harzianum</i> IMI 304056 (AJ224016)	553/553	100
TR06	AB935953	<i>Trichoderma virens</i> DAOM 233974 (EU280090)	549/549	100
TR07	AB935954	<i>Trichoderma asperellum</i> CPK2722 (FJ412053)	537/537	100
TR08	AB935955	<i>Trichoderma asperellum</i> TR044 (KC993074)	538/538	100
TR09	AB935956	<i>Trichoderma harzianum</i> CY216 (HQ608036)	553/553	100
TR10	AB935957	<i>Trichoderma asperellum</i> 362/02 (HQ857124)	547/547	100
TR11	AB935958	<i>Trichoderma asperellum</i> 356/02 (HQ857123)	537/537	100
TR12	AB935959	<i>Trichoderma asperellum</i> 400/01 (HQ857121)	537/537	100
TR13	AB935960	<i>Trichoderma asperellum</i> TR696 (KC993073)	537/537	100
TR14	AB935961	<i>Trichoderma asperellum</i> DAOM 233975 (EU280132)	537/537	100
TR15	AB935962	<i>Trichoderma asperellum</i> CIBT05 (EU280109)	537/537	100
TR16	AB935963	<i>Trichoderma asperellum</i> GJS 99-6 (DQ109538)	537/537	100
TR17	AB935964	<i>Trichoderma asperellum</i> Tr48 (AJ230669)	544/544	100
TR18	AB935965	<i>Trichoderma asperellum</i> TR696 (KC993073)	525/528	99
TR19	AB935966	<i>Trichoderma asperellum</i> CPK2722 (FJ412053)	537/537	100
TR20	AB935967	<i>Trichoderma asperellum</i> GJS 99-6 (DQ109538)	537/537	100

Table 4. Closest relatives of the *Trichoderma* isolates based on *tefl* sequences.

Isolates	Accession no.	Closest relatives (accession no.)	Alignment	Homology
TR01	AB935968	<i>Trichoderma asperellum</i> PPRC S34 (FJ436191)	278/278	100
TR02	AB935969	<i>Trichoderma virens</i> DAOM 237548 (EU280065)	258/258	100
TR03	AB935970	<i>Trichoderma harzianum</i> NR 6929 (AF348103)	267/268	99
TR04	AB935971	<i>Trichoderma virens</i> DAOM 237548 (EU280065)	261/261	100
TR05	AB935972	<i>Trichoderma harzianum</i> PPRC SP8 (FJ436162)	264/265	99
TR06	AB935973	<i>Trichoderma virens</i> DAOM 237548 (EU280065)	256/257	99
TR07	AB935974	<i>Trichoderma asperellum</i> PPRC S10 (FJ436185)	280/280	100
TR08	AB935975	<i>Trichoderma asperellum</i> CIB T48 (EU279962)	254/254	100
TR09	AB935976	<i>Trichoderma harzianum</i> CIB T23 (EU279989)	252/253	99
TR10	AB935977	<i>Trichoderma asperellum</i> PPRC S5 (FJ436183)	278/278	100
TR11	AB935978	<i>Trichoderma asperellum</i> CIB T25 (EU279957)	258/258	100
TR12	AB935979	<i>Trichoderma asperellum</i> PPRC S1 (FJ436181)	271/271	100
TR13	AB935980	<i>Trichoderma asperellum</i> PPRC W6 (FJ436148)	258/258	100
TR14	AB935981	<i>Trichoderma asperellum</i> PPRC W7 (FJ436140)	229/229	100
TR15	AB935982	<i>Trichoderma asperellum</i> PPRC SS2 (FJ436125)	260/260	100
TR16	AB935983	<i>Trichoderma asperellum</i> PPRC SS12 (FJ436138)	271/272	99
TR17	AB935984	<i>Trichoderma asperellum</i> CIB T25 (EU279957)	265/265	100
TR18	AB935985	<i>Trichoderma asperellum</i> PPRC W7 (FJ436140)	232/232	100
TR19	AB935986	<i>Trichoderma asperellum</i> PPRC S1 (FJ436181)	278/278	100
TR20	AB935987	<i>Trichoderma asperellum</i> PPRC S10 (FJ436185)	268/272	99

3.2.2.1. Phylogenetic tree based on the ITS sequences

The phylogenetic tree based on the ITS sequences separated the different *Trichoderma* isolates into three groups (Figure 1). Group I consisted of 14 isolates that were identified as *T. asperellum* and was supported by a bootstrap value of 99%. Group II includes three strains representing *T. virens* and was supported by a bootstrap value of 87%. Group III represents *T. harzianum* (three isolates) and was supported by a bootstrap value of 94%. Among the isolates, the transition/transversion ratio was 0.85 and the evolutionary divergence was 0.043. The genome sizes of the amplicons are shown in Photograph 3(a).

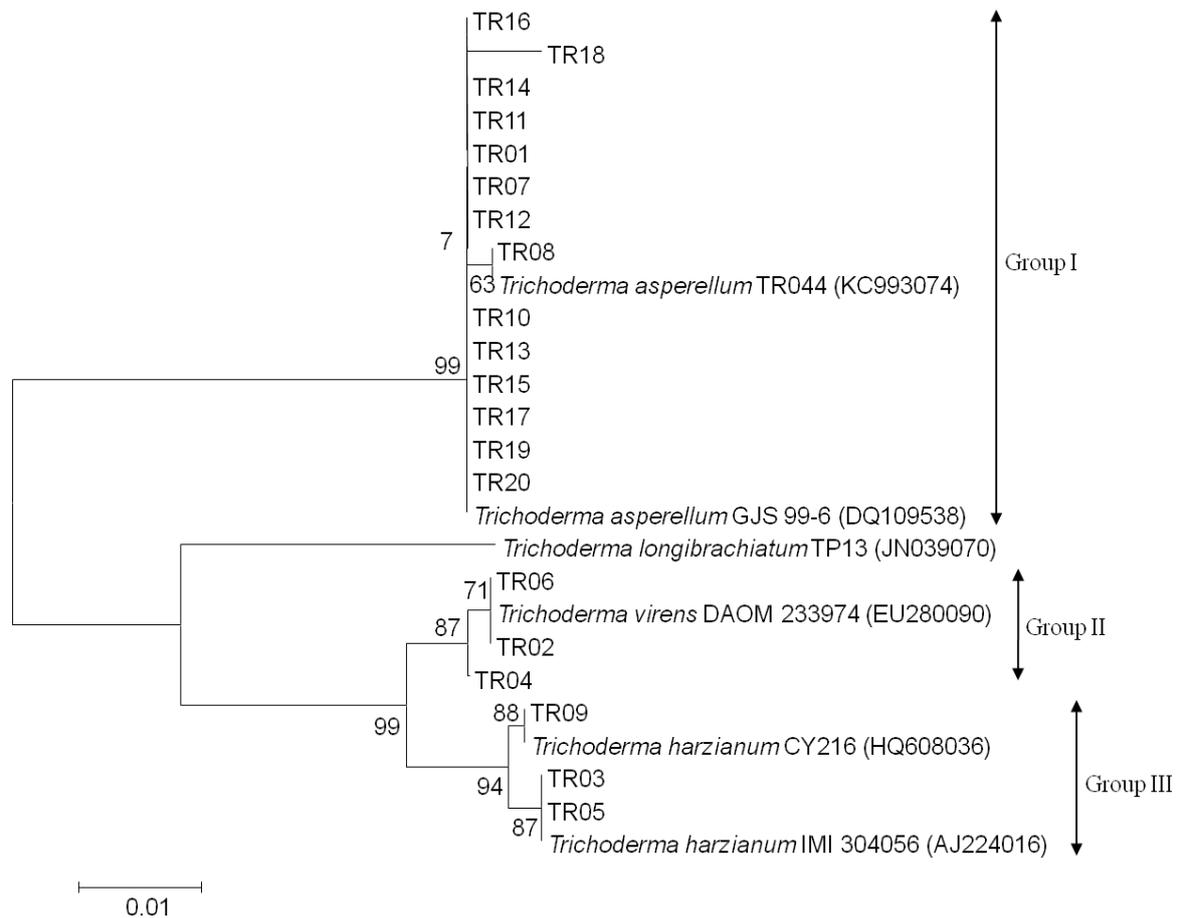


Figure 1. Phylogenetic relationships of the *Trichoderma* isolates and the typical known *Trichoderma* spp. inferred by analysis of internal transcribed spacer 1 and 2 sequences.

3.2.2.2. Phylogenetic tree based on the *tef1* sequences

The tree based on the *tef1* sequences also clearly divided the *Trichoderma* isolates into three groups (Figure 2). Group I (*T. asperellum*, 14 isolates) was supported by a bootstrap value of 99% and consists of two subgroups supported by bootstrap values of greater than 71%. Group II includes three strains representing *T. virens* and was supported by a bootstrap value of 99%. Group III represents *T. harzianum* (three isolates) and was supported by a bootstrap value of 80%. In this case, the transition/transversion ratio was 1.51 and the evolutionary divergence was 0.275. The genome sizes of the amplicons are shown in Photograph 3(b).

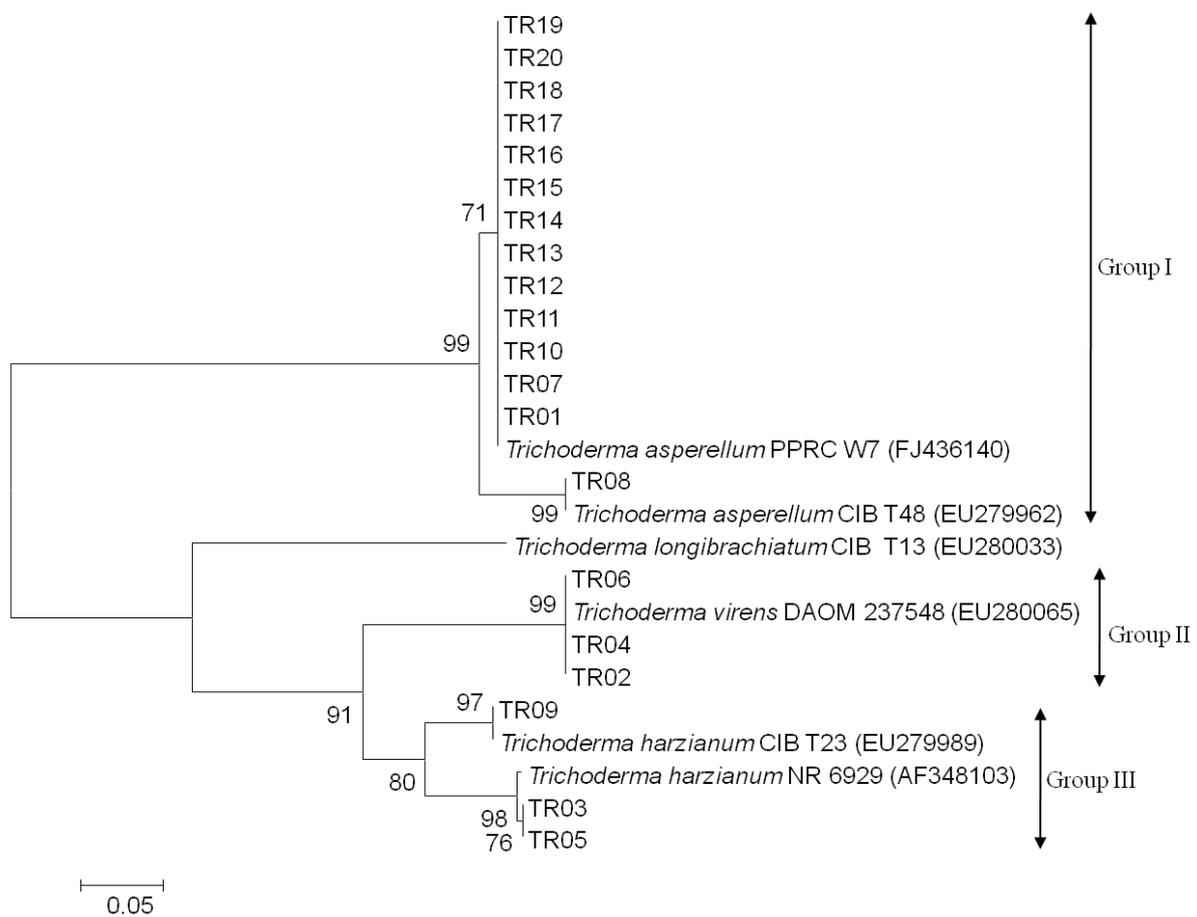
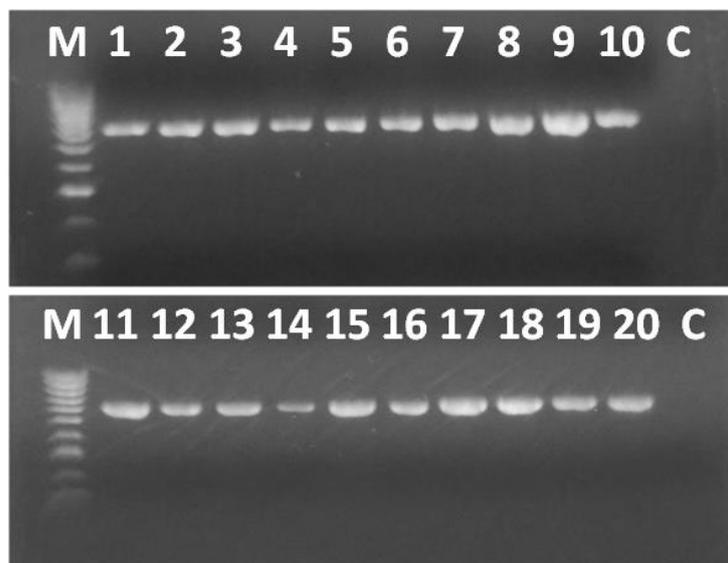
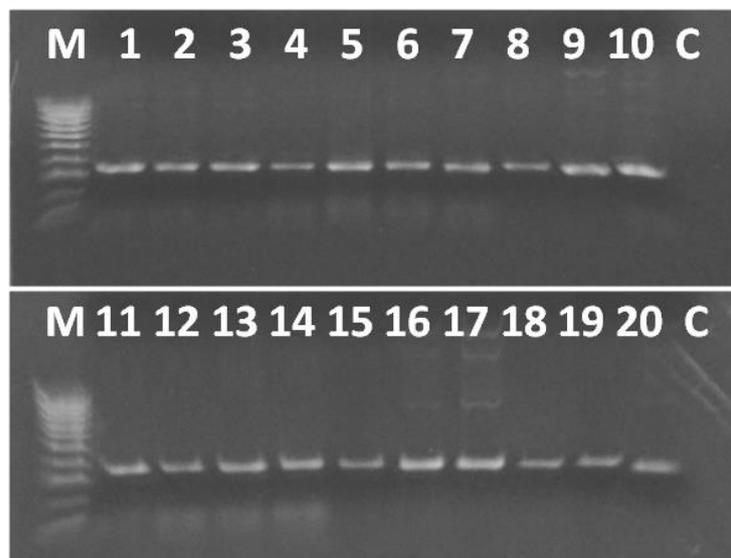


Figure 2. Phylogenetic relationships of the *Trichoderma* isolates and the typical known *Trichoderma* spp. inferred by analysis of *tef1* sequences.

a. ITS region



b. *tef1*



Photograph 3. PCR amplification patterns of *Trichoderma* isolates using the ITS4 and ITS5 primers for ITS region (a) and the EF1-728F and EF1-986R primers for *tef1* (b). Lanes 1–20: TR01-TR20 isolates, respectively, M: a molecular size marker containing 100, 200, 300/311, 400, 500, 600, 700, 800, 900 and 1013 bp; Hyper Ladder 100bp, Bioline Reagents, UK) and C: a negative control.

3.3. Antagonism of the isolates against pathogens

The antagonism of the *Trichoderma* isolates against pathogens was determined using the dual culture and culture filtrate assays.

3.3.1. Antagonism of pathogens as determined by the dual culture assay

All of the *Trichoderma* isolates were evaluated against selected soil-borne phytopathogens using the dual culture assay. Of the 19 isolates, TR02–TR10 showed antifungal antagonistic activities against those pathogens. Figure 3 shows the percentage of pathogen growth inhibition of different isolates.

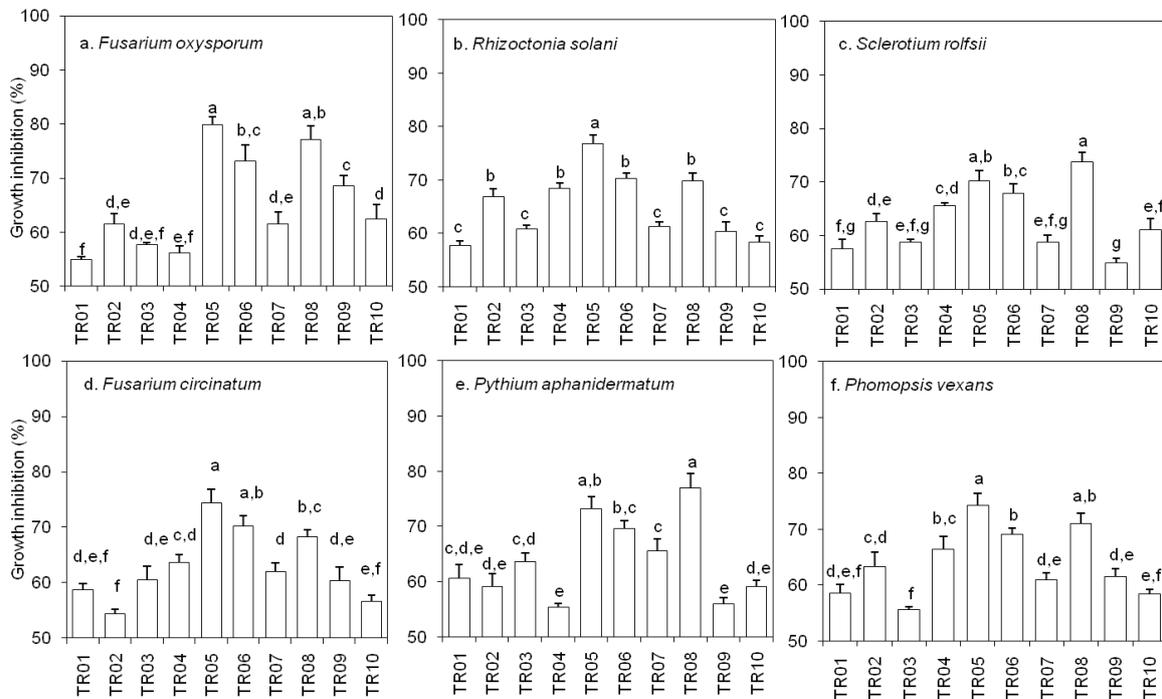


Figure 3. Percent growth inhibition of pathogens by dual culture assay of the *Trichoderma* isolates. Bars indicate standard deviations (n = 3). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

3.3.1.1. Inhibition of *F. oxysporum* f.sp. *lycopersici*

TR05 showed the highest inhibition (79.9%) against *F. oxysporum*, which was statistically similar to the inhibition by TR08 (77.1%), which was followed by that of TR06 (73.1%). The lowest inhibition (55.0%) was found in TR10, which was statistically similar to the inhibition by TR03 (57.7%) and TR04 (56.1%) (Figure 3a).

3.3.1.2. Inhibition of *R. solani*

TR05 also showed the highest inhibition (76.9%) against *R. solani*, followed by TR02 (66.9%), TR04 (68.4%), TR06 (70.3%) and TR08 (69.9%). The lowest inhibition was displayed by TR01 (57.8%), which was statistically similar to the inhibition by TR03 (60.9%), TR07 (61.4%), TR09 (60.4%) and TR10 (58.4%) (Figure 3b).

3.3.1.3. Inhibition of *S. rolf sii*

The maximum inhibition against *S. rolf sii* was recorded by TR08 isolates (73.7%), which was statistically similar to the inhibition by TR05 (70.2%). The minimum inhibition was recorded by TR09 isolates (54.8%), which was statistically similar to the inhibition by TR01 (57.5%), TR03 (58.8%) and TR07 (58.7%) (Figure 3c).

3.3.1.4. Inhibition of *F. circinatum*

In the case of *F. circinatum*, TR05 showed the highest inhibition (74.3 %), which was statistically similar to the inhibition by TR06 (70.3%). The lowest inhibition was displayed by TR02 (54.4%), which was statistically similar to the inhibition by TR01 (58.7%) and TR10 (56.6%) (Figure 3d).

3.3.1.5. Inhibition of *P. aphanidermatum*

The highest inhibition (76.9%) against *P. aphanidermatum* was observed by TR08, which was statistically similar to the inhibition by TR05 (73.2%). The lowest inhibition was displayed by TR04 (55.4%) and TR09 (55.9%), which were statistically similar to the inhibition levels of TR10 (59.1%), TR02 (59.1%) and TR01 (60.6%) (Figure 3e).

3.3.1.6. Inhibition of *P. vexans*

The maximum inhibition against *P. vexans* was obtained with TR05 (74.3%), which was statistically similar to the inhibition by TR08 (71.1%), followed by TR06 (69.2%). The minimum inhibition was obtained with TR03 (55.7%), which was statistically similar to the inhibition by TR10 (58.5%) and TR01 (58.7%) (Figure 3f).

3.3.2. Antagonism of pathogens as determined by the culture filtrate assay

Results from the culture filtrate assay also showed variations in the percentage of pathogen growth inhibition caused by different isolates (Figure 4).

3.3.2.1. Inhibition of *F. oxysporum* f.sp. *lycopersici*

TR05 showed the highest inhibition (87.8%) against *F. oxysporum*, followed by TR06 (81.1%) and TR08 (79.2%). The lowest inhibition was displayed by TR01 (54.5%) and TR04 (57.7%) (Figure 4a).

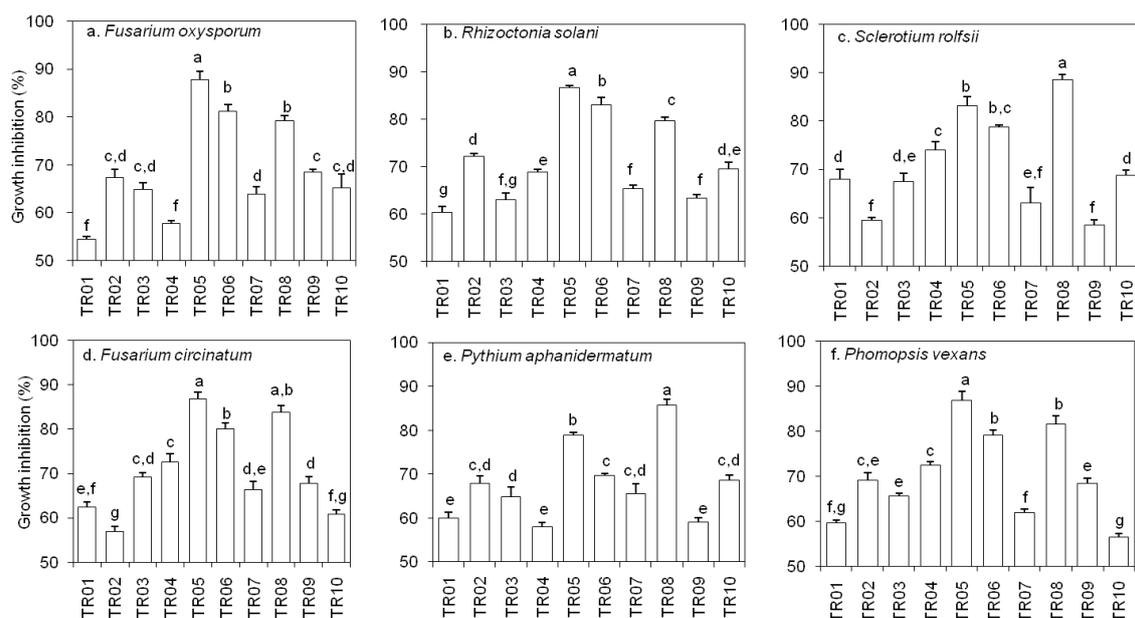


Figure 4. Percent growth inhibition of pathogens by culture filtrate assay of *Trichoderma* isolates. Bars indicate standard deviations (n = 3). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

3.3.2.2. Inhibition of *R. solani*

TR05 recorded the highest inhibition (86.7%) against *R. solani*, followed by TR06 (83.1%) and TR08 (79.7%). The lowest inhibition was recorded by TR01 (60.4%), which was statistically similar to the inhibition by TR03 (63.1%) (Figure 4b).

3.3.2.3. Inhibition of *S. rolfsii*

The maximum inhibition (88.5%) against *S. rolfsii* was displayed by isolate TR08, followed by TR05 (83.1%), which was statistically similar to the inhibition by TR06 (78.7%). The minimum inhibition was displayed by TR09 (58.5%) and TR02 (59.4%), which were statistically similar to the inhibition by TR07 (63.0%) (Figure 4c).

3.3.2.4. Inhibition of *F. circinatum*

The highest inhibition against *F. circinatum* was observed by TR05 (86.8%), which was statistically similar to the inhibition by TR08 (83.7%), followed by TR06 (80.0%). The lowest inhibition was observed with TR02 (57.0%) which was statistically similar to the inhibition by TR10 (60.8%) and TR01 (62.5%) (Figure 4d)

3.3.2.5. Inhibition of *P. aphanidermatum*

In the case of *P. aphanidermatum*, TR08 displayed the maximum inhibition (85.7%), followed by TR05 (78.9%). The minimum inhibition was displayed by TR04 (58.1%), which was statistically similar to the inhibition by TR09 (59.1%) and TR01 (59.9%) (Figure 4e).

3.3.2.6. Inhibition of *P. vexans*

The highest inhibition against *P. vexans* was displayed by TR05 (86.8%), followed by TR08 (81.7%) and TR06 (79.1%). The lowest inhibition was displayed by TR10 (56.4%), which was statistically similar to the inhibition by TR01 (59.7%) (Figure 4f).

3.4. Physiological features of the isolates

After assessing the biocontrol properties exhibited by the *Trichoderma* isolates as described above, TR01–TR10, which showed antifungal activities, were selected for physiological characterization.

3.4.1. Temperature

The *Trichoderma* isolates showed variation in growth and sporulation at different temperatures (Table 5). While all of the isolates grew at 35°C, only TR01, TR05, TR06 and TR08 were able to grow and sporulate at 40°C. None of the isolates grew at 4°C. Sporulation of TR05 and TR08 showed resistance to 75°C.

3.4.2. pH

The *Trichoderma* isolates also showed variation in their growth and sporulation at different pH levels (Table 5). All of the isolates were found to grow and sporulate at pH 5.6. However, none of the isolates grew at pH 2 and pH 12, and only three isolates, TR05, TR06 and TR08, were capable of growing and sporulating on medium at pH 4 and 10.

3.4.3. Nitrogen sources

All of the isolates were capable of growing with ammonium oxalate, potassium nitrate and sodium nitrate as nitrogen sources on solid medium and in liquid medium, while none of them, except TR05, TR06 and TR08, grew with creatine and urea as the nitrogen sources. All of the isolates except TR04, TR07 and TR09 were also found to grow and sporulate with glycine (Table 5).

Table 5. Physiological features of the *Trichoderma* isolates.

Isolates	TR01	TR02	TR03	TR04	TR05	TR06	TR07	TR08	TR09	TR10
Effect of temperature										
• 4°C	-	-	-	-	-	-	-	-	-	-
• 35°C	+	+	+	+	+	+	+	+	+	+
• 40°C	+	-	-	-	+	+	-	+	-	-
Effect of medium pH										
• pH2	-	-	-	-	-	-	-	-	-	-
• pH4	-	-	-	-	+	+	-	+	-	-
• pH5.6	+	+	+	+	+	+	+	+	+	+
• pH10	-	-	-	-	+	+	-	+	-	-
• pH12	-	-	-	-	-	-	-	-	-	-
Spore resistance at 75°C	-	-	-	-	+	-	-	+	-	-
Nitrogen utility										
• Ammonium oxalate	+	+	+	+	+	+	+	+	+	+
• Sodium nitrate	+	+	+	+	+	+	+	+	+	+
• Urea	-	-	-	-	+	+	-	+	-	-
• Creatin	-	-	-	-	+	+	-	+	-	-
• Glycine	+	+	+	-	+	+	-	+	-	+
• Potassium nitrate	+	+	+	+	+	+	+	+	+	+

Carbon utility

• Lactose	+	+	+	+	+	+	+	+	+	+
• Sucrose	+	+	+	+	+	+	+	+	+	+
• Soluble starch	-	-	-	-	+	+	-	+	-	-
• Ammonium oxalate	+	+	+	+	+	+	+	+	+	+
• Glucose	+	+	+	+	+	+	+	+	+	+
• Galactose	+	+	+	+	+	+	+	+	+	+
• Xylose	-	-	-	-	+	+	-	+	-	-
• Mannitol	-	-	-	-	+	+	-	+	-	-
• Ammonium tartrate	+	+	+	+	+	+	+	+	+	+
• Malic acid	-	-	-	-	+	-	+	+	-	-
• Lactic acid	+	+	+	+	+	+	+	+	+	+
• Citric acid	-	-	-	-	+	+	-	+	-	-
• Tartaric acid	-	-	-	-	+	+	-	+	-	-

+, able to grow and sporulate; -, no growth and sporulation

3.4.4. Carbon sources

All of the isolates were capable of growing and sporulating on media containing lactose, sucrose, ammonium oxalate, glucose, galactose, lactic acid and ammonium tartrate. In addition, only three isolates, TR05, TR06 and TR08, could grow and sporulate on media containing soluble starch, xylose, mannitol, malic acid, citric acid and tannic acid as the carbon source (Table 5).

3.5. Biochemical features of the isolates

All of the isolates hydrolyzed media containing aesculin and Tween 80, indicating that they possessed β -glucosidase and esterase activity, respectively. Additionally, all of the isolates were capable of hydrolyzing media with cellulose, tetrazolium and casein. Only three isolates, TR05, TR06 and TR08, were found to hydrolyze starch and gelatin, indicating that they had amylase and protease activities. In addition, TR01, TR04, TR05, TR06 and TR08 were produced mycelia and spores on media containing polypectate and tellurite (Table 6).

Table 6. Biochemical features of the *Trichoderma* isolates.

Isolates	TR01	TR02	TR03	TR04	TR05	TR06	TR07	TR08	TR09	TR10
Hydrolysis of										
• Aesculin	+	+	+	+	+	+	+	+	+	+
• Starch	-	-	-	-	+	+	-	+	-	-
• Gelatin	-	-	-	-	+	+	-	+	-	-
• Cellulose	+	+	+	+	+	+	+	+	+	+
• Casein	+	+	+	+	+	+	+	+	+	+
• Polypectate	+	-	-	+	+	+	-	+	-	-
• Tween 80	+	+	+	+	+	+	+	+	+	+
Reduction of										
• Tetrazolium	+	+	+	+	+	+	+	+	+	+
• Tellurite	+	-	-	+	+	+	-	+	-	-

+, positive reaction; -, negative reaction

3.6. Hydrolytic enzyme activities of the isolates

Chitinase, β -1,3-glucanase and proteinase activities were determined using the secreted culture filtrates (Figure 5).

3.6.1. Chitinase activity

The highest level of chitinase activity was observed from TR08 (12.8 U/ml), followed by TR06 (10.6 U/ml) and TR05 (7.63 U/ml). The lowest level was observed from TR04 (1.00 U/ml), which was statistically similar to the activities of TR01 (1.70 U/ml) and TR07 (1.83 U/ml) (Figure 5).

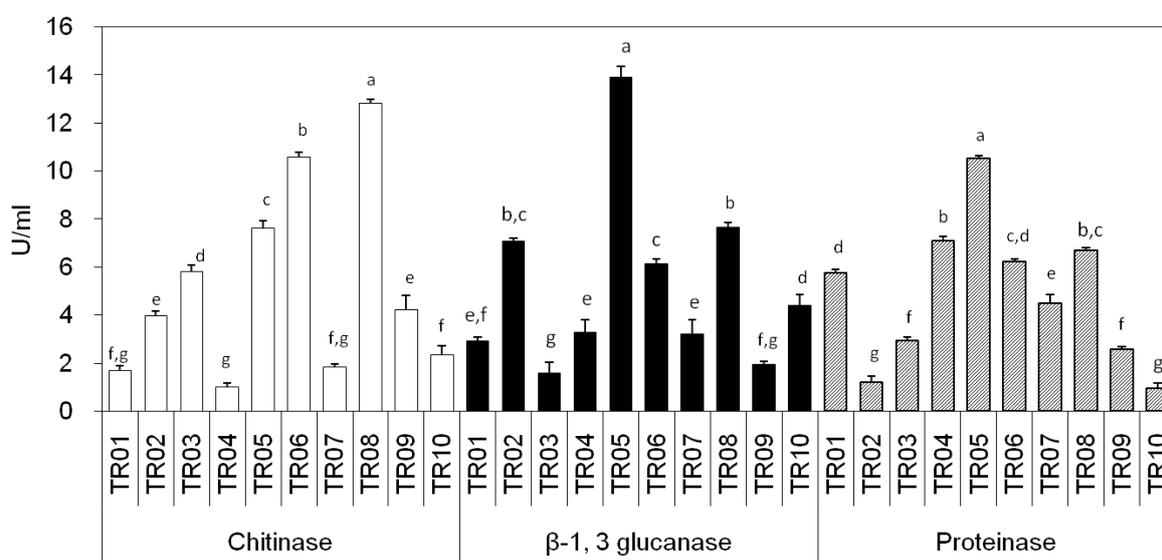


Figure 5. Hydrolytic enzyme activities of the *Trichoderma* isolates. Bars indicate standard deviations ($n = 3$). Different letters indicate significant differences according to Tukey's test ($P < 0.05$).

3.6.2. β -1,3-glucanase activity

The maximum level of β -1,3-glucanase (13.9 U/ml) was observed from TR05, followed by TR08 (7.67 U/ml) and TR02 (7.07 U/ml). The minimum level of β -1,3-glucanase was observed from TR03 (1.60 U/ml), which was statistically similar to the level of β -1,3-glucanase from TR09 (1.93 U/ml) (Figure 5).

3.6.3. Proteinase activity

The highest level of proteinase activity was recorded from TR05 (10.5 U/ml), followed by TR04 (7.10 U/ml) and TR08 (6.70 U/ml). The lowest level was recorder by TR10 (0.97 U/ml), which was statistically similar to the level of TR02 (1.20 U/ml) (Figure 5).

3.7. Greenhouse experiment

Based on the antagonism and the hydrolytic enzyme activities in the *in vitro* investigations, the effectiveness of three *Trichoderma* strains, *T. harzianum* TR05, *T. virens* TR06 and *T. asperellum* TR08, against *S. rolfsii*, which causes collar rot on tomato seedlings, were assayed in a greenhouse using seed and soil treatments.

3.7.1. Effects of seed treatment

The effects of the *Trichoderma* isolates showed variations in the disease incidence caused by *S. rolfsii*, the growth of tomato seedlings and seedling vigor using the seed treatment (Figure 6).

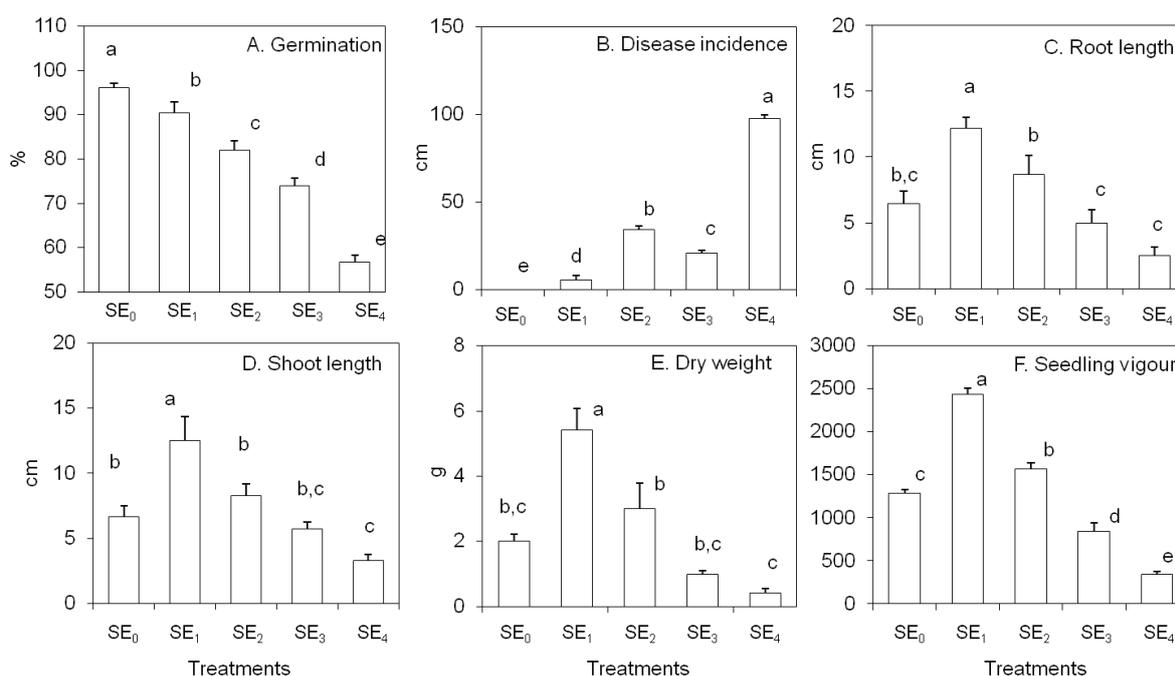


Figure 6. Effects of the seed treatment with *Trichoderma* TR05, TR06 and TR08 on tomato infected by *Sclerotium rolfsii* MAFF103049 (at four weeks): SE₀ = control, SE₁ = TR05 + pathogen, SE₂ = TR06 + pathogen, SE₃ = TR08 + pathogen and SE₄ = only pathogen. Bars mean standard deviations (n=3). Different letters indicate significant differences according to Tukey's test (P<0.05).

3.7.1.1. Germination

The highest germination percentage (96.0%) was found for the control (SE₀) and SE₄ (only pathogen) had the lowest percentage (56.7%). TR05 (SE₁) showed a statistically higher germination percentage (90.3%) than TR06 (SE₂:82.0%) and TR08 (SE₃: 74.0%) (Figure 6A).

3.7.1.2. Disease incidence

The highest disease incidence (97.8%) was recorded for SE₄ (only pathogen) and the lowest percentage (0.00%) was recorded for the control (SE₀). TR05 (SE₁) showed a statistically lower disease incidence (5.36%) than TR06 (SE₂: 34.20%) and TR08 (SE₃: 20.8%) (Figure 6B).

3.7.1.3. Root length

The greatest root length (12.2cm) was obtained in SE₁ (TR05), followed by TR06 (SE₂: 8.70cm). The shortest root length (2.5cm) was obtained in SE₄ (only pathogen), which was statistically similar to TR08 (SE₃: 4.97cm) (Figure 6C).

3.7.1.4. Shoot length

The maximum shoot length (12.5cm) was observed in TR05 (SE₁), followed by TR06 (SE₂: 8.27cm) and control (SE₀: 6.67cm). The minimum shoot length (3.30cm) was observed in SE₄ (only pathogen), which was statistically similar to TR08 (SE₃: 5.73cm) (Figure 6D).

3.7.1.5. Dry weight

The greatest dry weight (5.40g) was for TR05 (SE₁), followed by TR06 (SE₂: 3.0g). The lowest dry weight (0.41g) was for SE₄ (only pathogen), which was statistically similar to the control (SE₀: 2.00g) and TR08 (SE₃: 0.99g) (Figure 6E).

3.7.1.6. Seedling vigor

The highest seedling vigor value (2,430) was observed for TR05 (SE₁), followed by TR06 (SE₂: 1,568), TR08 (SE₃: 838) and the control (SE₀: 1,287). The lowest value (340) was observed in SE₄ (only pathogen) (Figure 6F).

3.7.2. Effects of soil treatment

The results obtained by soil treatments with the isolates TR05, TR06 and TR08 also showed variations in different plant growth factors (Figure 7).

3.7.2.1. Disease incidence

The highest disease incidence (95.2%) was with SO₇ (treated with only pathogen). TR08 (SO₆) showed a lower disease incidence (9.78%) than TR05 (SO₂: 16.4%) and TR06 (SO₄: 32.4%). SO₀, SO₁, SO₃ and SO₅ showed no incidence because they were not inoculated with the pathogen (Figure 7A).

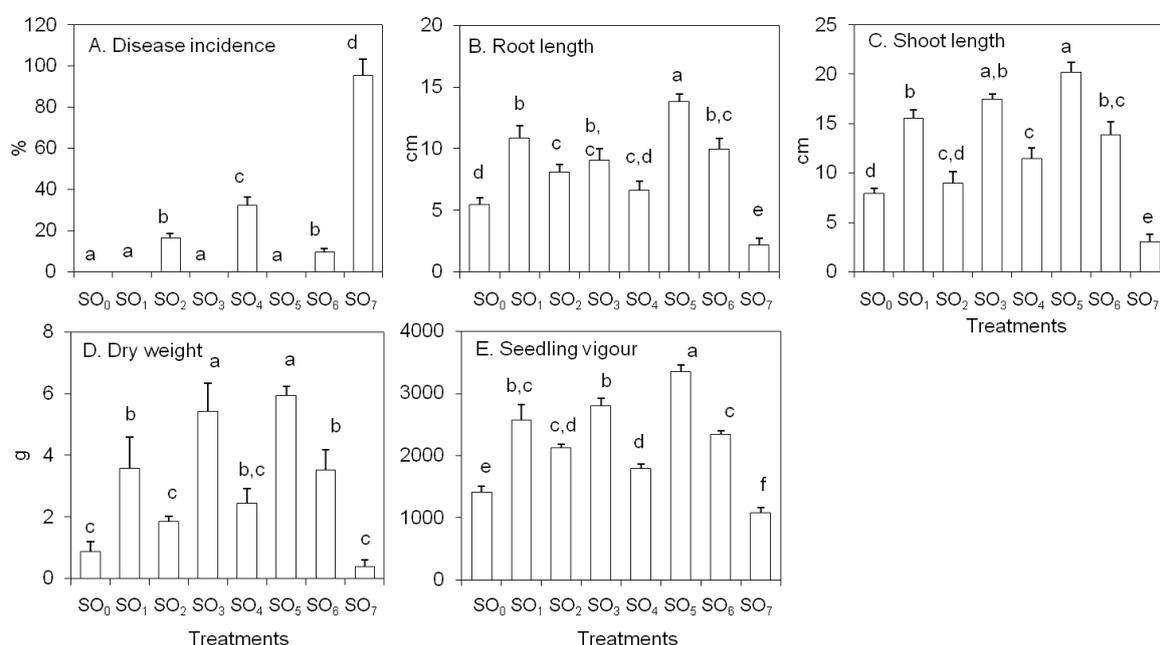


Figure 7. Effects of the soil treatment with *Trichoderma* TR05, TR06 and TR08 on tomato infected by *Sclerotium rolfsii* MAFF103049 (at four weeks): SO₀ = control, SO₁ = only TR05, SO₂ = TR05 + pathogen, SO₃ = only TR06, SO₄ = TR06 + pathogen, SO₅ = only TR08, SO₆ = TR08+ pathogen and SO₇ = only pathogen. Bars mean standard deviations (n=3). Different letters indicate significant differences according to Tukey's test (P<0.05).

3.7.2.2. Root length

The greatest root length (13.8cm) was recorded in SO₅ (only TR08), followed by SO₁ (10.8cm) and SO₆ (9.93cm), which was statistically similar to SO₃ (9.1cm). The shortest root length (2.2cm) was recorded in SO₇ (only pathogen) (Figure 7B).

3.7.2.3. Shoot length

The maximum shoot length (20.2cm) was observed in SO₅ (only TR08), which was statistically similar to SO₃ (17.5cm), followed by SO₁ (15.6cm) and SO₆ (13.8cm). The minimum shoot length (3.03cm) was observed in SO₇ (only pathogen) (Figure 7C).

3.7.2.4. Dry weight

The greatest dry weight (5.93g) was found in SO₅ (only TR08), which was statistically similar to SO₃ (5.43g), followed by SO₁ (3.56g) and SO₆ (3.53g). The lowest weight (0.4g) was found in SO₇ (only pathogen), which was statistically similar to SO₀ (0.87g) and SO₂ (1.86g) (Figure 7D).

3.7.2.5. Seedling vigor

The highest seedling vigor value (3,350) was recorded for SO₅ (only TR08), followed by SO₃ (2,800) and SO₁ (2,570). The lowest seedling vigor (1,080) was recorded for SO₇ (only pathogen) (Figure 7E).

CHAPTER IV

DISCUSSION

Trichoderma spp. are widely used in agriculture as BCAs because of their ability to reduce the incidence of diseases caused by plant pathogenic fungi, particularly many common soil-borne pathogens (Ashrafizadeh et al. 2005; Dubey et al. 2007). The current study was undertaken to corroborate the antagonistic ability of *Trichoderma* isolates by determining their morphological, physiological, biochemical and molecular features.

4.1. Morphological features of the isolates

Among the morphological features studied, TR02–TR10 possessed antifungal activities and were capable of producing abundant chlamydospores, which play a vital role in the sporulation, survival and establishment of the fungus in the soil as a potential BCA, with diameters greater than 6.8 μm (Chet 1987). Variations among the other morphological characteristics, such as colony color, radial growth, growth habit, consistency, conidia and conidiophores, and spore density, were also observed (Table 2). These findings are supported by Kucuk and Kivanc (2003), who reported that morphological characteristics were generally highly variable. This is in accordance with Druzhinina and Kubicek (2005) who reported that morphological analyses are highly prone to error and, consequently, roughly 50% of the *Trichoderma* spp. deposited in culture collection under names obtained by morphological analyses alone is incorrect. Thus, using gene sequence analyses is becoming more popular and can be used to complete most of the phylogenetic analyses (Szekeres 2005; Jom-in and Akarapisan 2009). The fungal morphological differences when grown on PDA may be related to nutritional factors, including environmental and genetic factors influencing fungal growth and development. Domschet al. (1993) stated that fungal growth is affected over a large environmental range. Pauliz and Belanger (2001) also reported that BCAs are sensitive to environmental conditions, and an unfavorable medium has been cited as a reason for failure or inconsistent performance.

Based on morpho-taxonomic, quantitative and qualitative characteristics, three species, *T. asperellum*, *T. virens* and *T. harzianum*, were identified from 30 locations in Bangladesh. Even though the classification of the *Trichoderma* isolates was performed using pedestal morpho-taxonomic characterizations, it was difficult to separate them at the species level. Waalwijk et al. (1996) reported the difficulties of speciation based on morphology alone. Seaby (1996) agreed with this statement and pointed out that morphological characterizations

are subject to environmental conditions and can significantly vary from culture to culture. Based on phenotypes, our morphological and taxonomic considerations may be subjected to ambiguities that are influenced by environmental conditions. Therefore, to authenticate the morphological identification and to resolve the ambiguities, molecular analyses should be used.

4.2. Molecular features of the isolates

The molecular characteristics of the ITS region and *tefl* gene of the *Trichoderma* isolates revealed that the 19 *Trichoderma* isolates could be divided into three groups (Figures 1 and 2). The results matched the morphological characterization well. However, the phylogenetic analysis using the ITS region did not divide the different *Trichoderma* species clearly (Figure 1). This may be owing to less variation among the isolates, a low transition/transversion ratio and evolutionary divergence. A similar result was reported by Hoyos-Carvajal et al. (2009), who suggested that the rDNA of the ITS region could not differentiate some species and presented several misidentifications because of the presence of non-orthologous copies of the ITS region in some *Trichoderma* isolates. However, the phylogenetic analysis based on *tefl* illustrated more differentiation among the *Trichoderma* isolates within and among groups of closely related species (Figure 2). This reflects the higher level of variability in *tefl* than in the rDNA of the ITS region (Kullnig-Gradinger et al. 2002; Hermosa et al. 2004; Druzhinina et al. 2005; Samuels 2006).

4.3. Antagonism of the isolates as determined by the dual culture assay

In this study, significant inhibitory effects on the growth of the selected phytopathogenic fungi were observed using dual culture and culture filtrate assays. In the dual culture assay, the inhibition zone, with or without contact between pathogens and the *Trichoderma* isolates, can be explained by the overgrowth of the pathogens, mycoparasitism (Howell 2003), competition for space and nutrients (Elad et al. 1999), antibiosis (Howell 1998), inactivation of the pathogen's enzymes (Roco and Perez 2001), secretion of some diffusible non-volatile antibiotics by *Trichoderma* species (Kerkeni et al. 2007; Hajieghrari et al. 2008), and the secretion of cell wall-degrading enzymes (Harman et al. 2004). Mycoparasitism may involve morphological changes, such as coiling and the formation of appressorium-like structures, which serve to penetrate the host (McIntyre et al. 2004). Monteiro et al. (2010) also reported that *T. harzianum* ALL42 was capable of overgrowing and degrading *R. solani* and *M. phaseolina* mycelia, coiling around the hyphae with the formation of appressoria- and hook-

like structures. Moreover, for mycoparasitism, *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes, such as chitinases, β -1,3 glucanases and proteases (Haran et al. 1996). Because the skeletons of pathogenic fungal cell walls contains chitin, glucan and proteins, enzymes that hydrolyze these components must be present in a successful antagonist for them to play a significant role in the pathogen's cell wall lysis (Lorito et al. 1994; Carsolio et al. 1999). Howell (2003) also reported that *Trichoderma* uses cell-wall carbohydrates to attach to pathogen lectins. These features may play key roles in the suppression of fungal pathogens and the deterioration of cell wall integrity just prior to contact.

4.4. Antagonism of the isolates as determined by the culture filtrate assay

In the culture filtrate assay, *Trichoderma* species could secrete some metabolites, such as trichodermin, trichovirdin, dermadin and heptalic acid (Nakkeeran et al. 2002). Some researchers also reported the extracellular production of antibiotics by several *Trichoderma* species (Siddiquee et al. 2009; Rajendiran et al. 2010). Cell-free culture filtrates of *Trichoderma* species could increase the significantly inhibition of pathogen growth as the concentration of the filtrates increased in the culture media (Nahed 2008). Such inhibitory effects have also been reported by Suarez et al. (2004), and likely indicate that the antagonistic fungi produce metabolites that inhibit pathogen growth. Siameto et al. (2010) reported that a junction is formed between *F. oxysporum* f.sp. *Lycopersici* and *T. harzianum* in culture filtrates of the isolates, which had inhibitory effects on the radial growth of the pathogens and mycelial accumulation, suggesting an action of non-volatile antibiotics in the filtrates. We observed similar results in which *Trichoderma* isolates produced metabolites that induced cellular changes in the pathogens mycelia. Based on this observation, we hypothesize that the metabolites from the *Trichoderma* isolates, rather than the loss or decrease in the nutrient contents, are inducing cell death in pathogens. These properties may play an important role in the suppression or deterioration of cells or in controlling fungal pathogens.

4.5. Physiological features of the isolates

Based on the physiological features studied, all of the isolates exhibited different growth and sporulation rates at different temperatures. While all of the isolates grew at 35°C, only TR01, TR05, TR06 and TR08 were able to grow and sporulate at 40°C. This agrees with the findings of Sharma et al. (2005), who reported that temperature had a profound effect on

fungal growth. Additionally, none of the *Trichoderma* species grew above 40°C. Singh et al. (2011) reported similar results. Singh et al. (2014) also stated that although all of the *Trichoderma* species were sufficiently produced at different temperatures, 20°C, 25°C, 30°C and 35°C, they grew best at a temperature range of 25°C to 30°C. We showed that none of the isolates grew at 4°C. This result corroborated that of Jayaswal et al. (2003), who reported that at lower temperatures (below 20°C) *Trichoderma* growth and sporulation were inhibited.

For pH, none of the isolates could grow on medium at extreme pH values (pH 2 and 12) but all of the isolates grew at pH 5.6. This agrees with the findings of Papavizas (1985), who reported that the maximum linear growth of the isolates occurred when the pH was 6.5. Verdin et al. (2004) reported that most fungi do not grow at very low pH values. Singh et al. (2014) also reported that the most favorable pH range for *Trichoderma* was between 5.5 and 7.5.

All of the isolates were capable of growing on media containing ammonium oxalate, potassium nitrate and sodium nitrate as nitrogen sources, but some of them did not grow on creatine, glycine or urea. This result partially agrees with the findings of Jayaswal et al. (2003) who reported that growth and sporulation were greatly affected by various nitrogen sources; however, both were favored on ammonium forms of nitrogen compared with nitrite or nitrate forms. Urea did not support either growth or sporulation. The differences in growth may be explained by the increased pH of the medium due to the accumulation of ammonia, suggesting that the isolates are liable to suffer from autolysis caused by ammonium compounds (Bridge 1985).

All of the isolates were able to grow and sporulate on media containing glucose, sucrose, ammonium oxalate, glucose, galactose, lactic acid, and ammonium tartrate as the sole carbon source. However, some of the isolates did not grow on soluble starch, xylose, mannitol, malic acid, citric acid and tannic acid, which was also reported by Grondona et al. (1997). They found that all of the *Trichoderma* isolates were able to assimilate glucose, ethanol and lactose as a sole carbon source, although the responses of the strains to other carbon sources varied. These variations might have resulted from differences in the assay conditions, such as pH, temperature and media (Manczinger and Polner 1987).

4.6. Biochemical features of the isolates

The enzymatic activities of each *Trichoderma* isolate examined in this study showed wide variations (Table 6). Lynch et al. (1981) also reported that the development of isolates

showed variations depending on enzyme activity levels. Three isolates, TR05, TR06 and TR08, hydrolyzed aesculin, Tween 80, gelatin and starch. This indicates that they are capable of producing cellulase, esterase, protease, β -1,3-glucanase, chitinase enzymes and extracellular hydrolases, which are mainly involved in the degradation of cell wall-associated glucans in plant pathogens (Howell 2003; Woo et al. 2006).

4.7. Hydrolytic enzyme activities of the isolates

Among the *Trichoderma* isolates obtained in this study, TR05, TR06 and TR08 showed higher enzyme activities than the others, as shown in Figure 5. TR05 was the highest producer of β -1,3-glucanase and proteinase, probably causing the maximum inhibition against *F. oxysporum*, *R. solani*, *F. circinatum*, and *P. vexans* in the dual culture and culture filtrate assays (Figures 3 and 4, respectively). β -1,3-glucanases hydrolyzes the O-glycosidic linkages of β -glucan chains of these pathogens and inhibits spore germination. In addition, the high protease activity of TR05 may contribute to the degradation of the filamentous fungal cell wall because proteases can break down pathogen cell walls by attacking lipids and proteins, or by acting as proteolytic inactivators of pathogen-produced enzymes. Yan and Qian (2009) also reported that some proteases, including aspartyl protease, serine protease and subtilisin-like protease, had biocontrol functions. However, TR08 was the highest producer of chitinase, probably causing the maximum inhibition against *S. rolf sii* and *P. aphanidermatum* in the dual culture and culture filtrate assays (Figures 3 and 4, respectively). The inhibiting properties of TR08 and TR06 may be due to their high chitinase activities (Figure 5), which can degrade the cell walls of plant pathogens (Gruber and Seidl-Seiboth 2012). As an example, an isolate of *T. harzianum* strain TH 250 produced high levels of chitinase and β -1,3-glucanase when grown on mycelia of *R. solani*, whereas it produced low levels of these enzymes on mycelia of *S. rolf sii* (Elad et al. 1982; Grondona et al. 1997). A similar mechanism could be operating between *Trichoderma* isolates TR06 and TR08, or that other compounds with biological activities could be produced by these strains, causing the fungal growth inhibition (Sivasithamparam and Ghisalberti 1998). Moreover, a direct relationship has been presented between the antagonistic capacity and enzyme activities of *Trichoderma* as reported by Davet (1987). We observed that different isolates showed different levels of enzyme activities and caused a different rate of inhibition against different pathogens. This indicates that *Trichoderma* species exhibit variations among the strains in relation to the production of lytic enzymes, biocontrol activity and host range (Sivan and Chet 1992).

4.8. Effects of seed treatment

This study reveals that seeds treated with TR05, TR06 and TR08 had a highly reduced disease incidence as tomato seedlings (Figure 6). *T. harzianum* TR05 was the most effective isolate in controlling the tested diseases. A possible explanation of this phenomenon includes the seed coat bearing inocula of *Trichoderma* sp. to provide systemic protection against many seed-borne diseases (Linda, 2000). Additionally, *Trichoderma* is also known to provide plants with useful molecules, such as glucoseoxidase, and growth stimulating compounds that can increase their vigor, resulting in pathogen resistance (Brunner et al. 2005). *Trichoderma* can also produce antibiotics, such as gliotoxin, viridin and cell wall degrading enzymes, as well as biologically active heat-stable metabolites, such as ethyl acetate. These substances are known to be involved in disease incident suppression. Moreover, *T. viride* and *T. harzianum* were reported as the best antagonists against several soil- and seed-borne plant pathogens (Poddar et al. 2004). This study indicated that inoculations of TR05, TR06 and TR08 not only suppressed collar rot diseases but also enhanced the germination percentage, root and shoot growth, dry weight and vigor of tomato seedlings compared with the infected control. Among the three *Trichoderma* isolates, TR05 also exhibited a significant enhancement of the above mentioned plant growth factors after the seed treatment (Figure 6). Some researchers reported that *Trichoderma* increases plant growth and productivity (Harman 2006; Manju and Mall 2008). In this study, the TR05 isolate had the highest germination percentages, which has also been reported in different plants (Hanson 2000; Mishra and Sinha 2000). We corroborated that *T. harzianum* and *T. viride* enhanced seed germination, and root and shoot lengths (Dubey et al. 2007), as well as the frequency of healthy plants. In a similar study, Lo and Lin (2002) also screened different *Trichoderma* strains for plant growth and root growth of the bitter melon, loofah and cucumber and noticed that *Trichoderma* strains significantly increased seedling height 26 to 61%, root exploration 85 to 209%, leaf area 27 to 38% and dry weight 38 to 62%, 15 days after sowing. Seedling vigor was also significantly affected by the application of TR05 isolates (Figure 1). The vigor index showed differences similar to those of the germination percentages. A seed treatment with TR05 increased the vigor index compared with the control. Mukhtar (2008) investigated that a seed treatment with *T. harzianum* resulted in the highest germination index in okra, and that *T. harzianum* can be useful in enhancing the germination percentage, as well as the seedling vigor, of okra seeds. Begum et al. (2010) evaluated five *Trichoderma* strains for their efficacy in suppressing *Alternaria* fruit rot disease of chili and in promoting plant growth and yield. They observed

that an application of *T. harzianum* IMI392432 significantly suppressed the disease and improved the seed germination percentage, vigor, growth and yield. Other investigators have also reported that seeds treated with *T. viride*, *T. harzianum* and *Trichoderma pseudokoningii* inoculant extracts, showed increased seed germination rates and seedling vigor, and reduced the incidence of seed-borne fungal pathogens compared with the control (Zheng and Shetty 2000; Bharath et al. 2006).

4.9. Effects of soil treatment

In the present study, a management strategy was developed based on eliminating or reducing the inocula causing this disease. Therefore, soil treatment formulations developed by the effective isolates of TR05, TR06 and TR08 were evaluated under pot conditions against this disease. The findings clearly indicated that TR08 was effective in reducing the disease incidence and that it provided the highest seed germination rate, shoot and root lengths and seedlings vigor value compared with those obtained with any individual treatment (Figure 7). Similar observations were also recorded by earlier workers on other host pathogen systems. BCA TR08 isolates used in the present study may be better able to colonize the rhizosphere by inhibiting the pathogen community (Bennett and Whipps 2008). Moreover, there are many mechanisms that may clarify the role of an antagonism in the suppression of pathogen growth and in disease control. This could be through mycoparasitism (Haran et al. 1996), antibiosis (Ghisalberti and Rowland 1993), induction of resistance in plants (Yedidia et al. 1999), or competition for nutrients and/or space (Inbar et al. 1994). These conditions may have a stimulatory effect on plant growth because they modify the soil conditions. In addition to providing antimicrobial actions, they also stimulate the biological activity of resident antagonistic microbial populations, thus promoting plant growth (Harman et al. 2004).

CHAPTER V

CONCLUSIONS AND FUTURE WORK

Trichoderma spp. can be used as potential biological control agents (BCAs) for a wide range of plant pathogens in agricultural systems due to their high reproductive capacity, ability to survive under harsh conditions, efficiency in the utilization of unfavorable nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi and efficiency in promoting plant growth and defense mechanisms. These properties have made *Trichoderma*, a ubiquitous genus present at high population densities in any habitat and act as BCAs through different synergistic mechanisms. However, it is difficult to predict the degree of synergism and the behavior of a BCA in a natural pathosystem. Based on environmental conditions, the selection of BCAs begins with a characterization of the biocontrol strains of *Trichoderma*, which is important since the exact identification of strains to the species level is essential in using the full potential of fungi in specific applications.

The integration of physiological, biochemical and molecular attributes, together with morphological criteria, has been used in this study to characterize a group of *Trichoderma* isolates from different regions of Bangladesh. All of the *Trichoderma* isolates could be grouped into three different species (*T. asperellum*, *T. virens*, and *T. harzianum*) based on the different morphological characteristics. The results matched the molecular characterization well. As a result of sequencing, the ITS region and *tefl* nucleotide and subsequent BLAST algorithm-based searches, 14 isolates (TR01, TR07, TR08 and TR10–TR20) were identified as *T. asperellum*, three isolates (TR02, TR04 and TR06) were identified as *T. virens*, and the other three isolates (TR03, TR05 and TR09) were identified as *T. harzianum*. To determine the diversity and genetic distances among the isolates, the phylogenetic analysis were done using the sequence data of the ITS region and *tefl* of the *Trichoderma* isolates and several known *Trichoderma* strains found in GenBank. The phylogenetic analysis based on *tefl* illustrated better differentiation among the *Trichoderma* isolates within and among groups of closely related species than the rDNA of the ITS region due to reflects more variability among the isolates and a high transition/transversion ratio and evolutionary divergence.

Among the physiological features studied, it is notable that only three isolates, TR05, TR06 and TR08, were capable of growing and sporulating on medium at pH 4 and 10, and able to grow and sporulate at 40°C. In addition, TR05, TR06 and TR08, could grow and sporulate on media containing soluble starch, xylose, mannitol, malic acid, citric acid and tannic acid as

the carbon source, and, were capable of growing with ammonium oxalate, potassium nitrate, creatine, glycine and urea as the nitrogen sources. In case of biochemical features studied, three isolates, TR05, TR06 and TR08, were found to hydrolyze the media containing starch, gelatin, aesculin, Tween 80, cellulose, casein, tetrazolium, polypectate and tellurite.

The *Trichoderma* isolates native to Bangladesh, including *T. harzianum* strain TR05, *T. virens* strain TR06 and *T. asperellum* strain TR08, were identified as potential BCAs. These isolates possessed significant antagonistic properties both in dual culture and culture filtrate assay against *F. oxysporum*, *R. solani*, *F. circinatum*, *P. vexans*, *S. rolfsii*, and *P. aphanidermatum*, which cause severe agricultural damage in Bangladesh. These findings were also in agreement with the activities of their extracellular hydrolytic enzymes, including chitinase, β -1,3-glucanase and proteinase. In a pot experiment under greenhouse conditions, *T. harzianum* TR05 and *T. asperellum* TR08 were identified as potential BCAs through the seed and soil treatments, respectively, against collar rot of tomato seedlings. They showed the lowest disease incidence levels and the highest germination rates, as well as providing superior results in the following plant factors: root length, shoot length, fresh weight, dry weight and seedling vigor.

Although our results clearly showed that considerable success in biocontrol can be achieved under laboratory conditions, the outcome has not been tested under field conditions. Hence, the following plan of work will be done in the near future:

- i. To produce a large scale *Trichoderma*-based bio-pesticides for our farmers in the near future.
- ii. To develop technologies that allows BCAs to spread and proliferate under different field conditions.
- iii. To improve the BCA strains that are capable of becoming established and surviving under adverse field conditions.

CHAPTER VI

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ACKNOWLEDGEMENTS

All praises for the "Almighty Allah", the most gracious, most merciful and the supreme ruler of the universe to whom all praises go, Who has blessed the author with life, time and energy and enable his to complete this research work and this thesis for the degree of Doctoral Program (PhD) in Life and Food Science, Niigata University, Japan.

The author would like to wish with pleasure to express his heartfelt respect and the deepest, profound gratitude and indebtedness to his reverend academic advisor, Dr. Naoki Harada, Department of Applied Biological Chemistry, Faculty of Agriculture, Niigata University, Japan for his keen interest, scholastic guidance, valuable suggestions, helpful comments, constructive criticism and constant inspiration, providing facilities and supports during the entire tenure of the research work and writing up this thesis.

The author also expresses his deep sense of gratitude and indebtedness to his other academic advisors Prof. Dr. Masanori Nonaka and Prof. Dr. Takuji Ohyama, Faculty of Agriculture, Niigata University, Japan and Prof. Dr. M. Delwar Hossain, Department of Plant Pathology, Bangladesh Agricultural University (BAU), Mymensingh for their kind help, valuable advice and factual comments in upgrading the research work.

The author is pleased to extend his gratefulness and indebted to all laboratory mates of Soil Science Laboratory, Faculty of Agriculture, Niigata University, Japan, especially, Muzahid san, Narisawa san, Suzuki san, Manami san and Sayaka san for their continuous and cordial cooperation during the entire period of the study.

Cordial appreciation and thanks are extended to Global Circus Program, Niigata University, Japan for providing financial supports.

Finally, the author desires to express immense gratitude to his beloved wife Aysha Akter and respected parents for their constant inspiration and blessing.

The Author