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Research article

Damping-off disease of big onion (*Allium cepa* L.) in Sri Lanka and evaluation of *Trichoderma asperellum* and *Trichoderma virens* for its control

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Abstract: *Allium cepa* is used as a condiment and reduction of yield due to infectious diseases is a major economical constraint. The present study was aimed at isolation and identification of fungal pathogens associated with damping-off disease of onion in Sri Lanka. *Trichoderma* spp. present in the soil of the same onion fields were isolated with a view to evaluating them as possible bio-control agents of damping-off pathogen(s). The diseased seedlings were collected from fifty-five onion fields in Matale and Anuradhapura districts during the *yala* season. Soil collected from the same onion fields and soil fungi isolated using the Warcup method. *Fusarium* sp. isolated from diseased seedlings was confirmed to be the causative agent of damping-off disease of big onions by following Koch's postulates. The pathogenic *Fusarium* sp. was identified as *Fusarium solani* based on the similarity matches of the Internal Transcribed Spacer region using Basic Local Alignment Search Tool. Two *Trichoderma* spp. showing significantly high ($p \leq 0.05$) reduction of growth of *F. solani* in dual culture assay, higher sporulation capacity and growth rates were identified as *T. asperellum* (Tr.3) and *T. virens* (Tr.1). Polymerase Chain Reaction (PCR) using two primer pairs *i.e.* ITS 1 and ITS 4, FR 1 and NS 1 were used to characterize the seven *Trichoderma* spp. while ITS 1 and ITS 4 were used to characterize *Fusarium* spp. Although a lesser degree of polymorphism was detected using these primers, the random amplified polymorphic DNA analysis had the ability to differentiate *T. asperellum, T. virens* and *F. solani.* The capability of two *Trichoderma* spp. to suppress *F. solani* is through formation of loops and coils and attachment of hyphal tips. They also had the ability to produce Chitinase and volatile metabolites that controlled the growth of *F. solani.*
Keywords: *Fusarium solani* - PCR - *Trichoderma* spp.

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INTRODUCTION

Allium cepa L. or big onion is used as a condiment in many countries of the world. It is an important cash crop grown in Sri Lanka predominantly in Matale and Anuradhapura districts, Mahaweli H region and to a lesser extent in Polonnaruwa district (AESD 2016) In Sri Lanka, Big onion has a year-round consumer demand of around 120,000 MT whereas the annual national production is only about 55,000 to 60,000 MT. Therefore, it is essential that the production is increased and losses due to various factors reduced. One important factor that is reported to reduce yield significantly is the prevalence of diseases that affect the cultivated plants at both nursery and field stages. These diseases are caused mainly by fungi and it has been reported that fungal diseases cause 10–50 % yield loss in onions which might increase up to 100 % loss if left untreated (Wickramaarachchi *et al.* 2004).

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The most common soil-borne seedling disease of big onion seen at the nursery stage is damping-off resulting in severe seedling mortality. Damping-off disease is caused by soil-borne fungal spp. *Fusarium, Pythium* and *Rhizoctonia* either singly or in combination resulting in poor germination and stand of onion seedlings. This disease may manifest before or after the emergence of seedlings *i.e.* pre-emergence or post-emergence damping off respectively (Mishra *et al.* 2014, Dabiré *et al.* 2016). The fungi that cause damping-off are ubiquitous in an onion production field (Brewster 2008). Although the above-mentioned pathogens are known to be the causative agents of damping-off disease of *Allium cepa* in Sri Lanka (Rathnayaka 1992), detailed studies pertaining to the *A. cepa* damping-off causative agent(s) in different onion growing areas have not been reported. Hence, the present study aims at isolating and identifying the causative agent(s) of damping-off from diseased *A. cepa* seedlings and identifying them accurately.

Planting seeds treated with fungicides, Thiram, Homai, soil treatment with fungicides, Brassicol, Benlate, Captan, Cresent and soil solarisation are being carried out conventionally to control damping-off disease (Edirimanna & Rajapakse 2003). However, due to the disadvantages associated with fungicide applications such as the development of resistance by the pathogen when exposed continuously to the chemical, high cost, deleterious effects on soil organisms and most importantly the adverse effects on the environment and associated health hazards, alternative disease management strategies have to be developed. Soil solarization although effective to some extent needs a crop free field for relatively extended periods, depends on weather/climate conditions and sometimes pathogens are seen remain at deeper layers in the soil after treatment. Development of pathogen-resistant cultivars and usage of biological control agents are some of the methods being investigated in agriculture in place of these methods. Pathogen resistant cultivars become obsolete in a short time due to the rapid evolution of phytopathogens (Patel *et al.* 2014).

Biological control of plant pathogens is devoid of most of the above limitations whilst being safe and sustainable. A biological control agent of a plant pathogen is a microorganism that has the ability to keep the pathogenic organism under control and reduce its harmful effects upon a plant. A microbial antagonist can effectively suppress pathogens and reduce the level of disease. A number of microorganisms have been used successfully in the control of fungal pathogens and amongst these *Trichoderma* spp. have been identified as highly effective biological control agents of many soil-borne phytopathogenic fungi including *Fusarium* spp. (Amal *et al.* 2005, Akrami *et al.* 2011, Gveroska & Ziberoski 2011).

Trichoderma spp. possess numerous and effective mechanisms of antagonism such as mycoparasitism, secretion of cell-wall degrading enzymes, antibiosis through the production of antibiotics, competition for space and nutrients, facilitation of seed germination and growth of the plants and inducing resistance in plants (Harman *et al.* 2003).

The release of *Trichoderma* spp. as biocontrol agents into the soil has generated a demand for the development of means to monitor their occurrence or absence and to distinguish between indigenous strains and artificially introduced ones (Knudsen *et al*. 1996). Some of the methods employed to identify and distinguish *Trichoderma* spp. are growth rate, colony appearance as well as microscopic morphological features (Seaby 1996, Shahid *et al.* 2014). However, classification and phenotypic identification of *Trichoderma* spp. have proved to be difficult because morphological characteristics are easily changed by environmental influences (Park *et al.* 2005). Currently, molecular techniques like DNA sequencing (Appel & Gordon 1996), Random Amplification of Polymorphic DNA (RAPD) analysis (Woo *et al.* 1996), Restriction Fragment Length Polymorphism (RFLP) analysis (Meyer *et al.* 1992), internal transcribed sequences (ITS) of the ribosomal DNA (rDNA) analysis have been used to identify and characterize *Trichoderma* isolates more accurately (Cumagun *et al.* 1999).

The present study was aimed at isolating and identifying the causative fungi of damping-off disease of *A. cepa* as up to now detailed studies have not been reported in Sri Lanka. This will provide the background vital to carry out necessary management strategies for damping-off disease. Further, *Trichoderma* spp. were isolated from soils of onion fields with a view to develop them into an inoculum for the control of damping-off disease. *Trichoderma* spp. isolated from various soils were evaluated for their efficacy in controlling the damping-off pathogen in the laboratory and the mechanisms involved with the antagonistic activity towards the damping-off pathogen *Fusarium* sp. were also studied with a view of utilizing the most effective *Trichoderma* isolates for the control of diseases in the field. This has not been attempted in Sri Lanka. The isolated damping-off pathogen *i.e. Fusarium* sp. and *Trichoderma* spp. were identified up to species level using amplification of the ITS region of the rDNA gene sequence and subsequent sequence analysis against available sequences in the GenBank database at NCBI (National Center for Biotechnology Information) using BLAST. Further, the genetic

variability of isolated *Trichoderma* spp. were evaluated using Random Amplified Polymorphic DNA (RAPD) profiles. As it provides a convenient strategy of scanning and comparing the genomes of *Trichoderma* spp., the technique can be utilized for fingerprinting and tracking different *Trichoderma* spp. once they are released to the field as biocontrol agents.

MATERIALS AND METHODS

Collection of seedling and soil samples from the field

Collection of diseased seedling samples and observation of symptoms

Table 1. Sampling fields in Matale district during *yala* season and GPS coordinates.

Site	Location	Elevation	GPS location	
			Latitude	Longitude
G1	Yatigalpoththa, Galewela	208 m	N07^o 48’05.3”	E080^o 35’36.4”
G2	Yatigalpoththa, Galewela	209 m	N07^o 48’09.6”	E080^o 35’33.9”
G3	Thattankotuwa, Pattiwela, Galewela	204 m	N07^o 47’55.9”	E080^o 33’15.3”
G4	Thattankotuwa, Pattiwela, Galewela	197 m	N07^o 47’53.3”	E080^o 33’12.8”
G5	Thattankotuwa, Pattiwela, Galewela	198 m	N07^o 47’53.5”	E080^o 33’13.3”
G6	Pattiwela, Galewela	195 m	N07^o 47’43.5”	E080^o 33’18.6”
G7	Thattankotuwa, Pattiwela, Galewela	195 m	N07^o 47’37.2”	E080^o 33’15.0”
G8	Galewela	220 m	N07^o 47’21.4”	E080^o 33’59.8”
G9	Dewahuwa, Damana	192 m	N07^o 50’31.3”	E080^o 35’00.0”
G10	Muthuporugala, Dandubendiruppa, Dewahuwa	201 m	N07^o 51’38.0”	E080^o 36’39.7”
D1	Athuparayaya, Dambulla	138 m	N07^o 52’53.6”	E080^o 39’42.3”
D2	Athuparayaya, Dambulla	164 m	N07^o 52’52.0”	E080^o 39’37.7”
D3	Athuparayaya, Dambulla	165 m	N07^o 52’56.2”	E080^o 39’51.3”
D4	Athuparayaya, Dambulla	164 m	N07^o 52’52.4”	E080^o 39’39.5”
D5	Athuparayaya, Dambulla	159 m	N07^o 52’57.3”	E080^o 39’16.4”
D6	Kalogahaela, Dambulla	203 m	N07^o 49’28.5”	E080^o 41’33.5”
D7	Kalogahaela, Dambulla	209 m	N07^o 49’32.6”	E080^o 41’35.7”
D8	Pahala Arawula, Dambulla	213 m	N07^o 50’35.2”	E080^o 42’14.9”
D9	Pahala Arawula, Dambulla	210 m	N07^o 50’34.4”	E080^o 42’16.5”
D10	Arawula Junction, Kandalama, Dambulla	198 m	N07^o 50’37.7”	E080^o 42’17.0”
S1	Diganpathana, Kimbissa, Sigiriya	213 m	N07^o 58’50.9”	E080^o 43’07.1”
S2	Diganpathana, Kimbissa, Sigiriya	213 m	N07^o 58’50.9”	E080^o 43’07.1”
S3	Diganpathana, Kimbissa, Sigiriya	206 m	N07^o 59’19.6”	E080^o 43’39.7”
S4	Diganpathana, Sigiriya	199 m	N07^o 59’23.5”	E080^o 43’37.6”
S5	Diganpathana, Sigiriya	202 m	N07^o 59’23.1”	E080^o 43’39.7”
S6	Diganpathana, Kimbissa, Sigiriya	206 m	N07^o 58’53.6”	E080^o 43’19.0”
S7	Diganpathana, Kimbissa, Sigiriya	203 m	N07^o 58’51.8”	E080^o 43’19.9”
S8	Kaudannawa, Kimbissa, Sigiriya	200 m	N07^o 59’28.8”	E080^o 43’30.5”
S9	Diganpathana, Kimbissa,Sigiriya	197 m	N07^o 59’28.2”	E080^o 43’32.9”
S10	Diganpathana, Kimbissa,Sigiriya	196 m	N07^o 59’31.8”	E080^o 43’34.5”

Diseased big onion seedlings at different stages of growth (7 days to 30 days after cultivation) were collected randomly from fifty-five onion fields in the Matale and Anuradhapura districts during the *yala* season (Table 1 & 2). The samples were transported to the laboratory in clean polythene bags and kept in a cold room (9°C) until they were used for further studies. The locations of the sites were recorded through GPS receiver (Garmin) and a map of sites was constructed using ArcMap Version 9.2 (Fig. 1). Macro symptoms present in the diseased seedlings were observed.

Table 2. Sampling fields in Anuradhapura district during *yala* season and GPS Coordinates.

Site	Location	Elevation	GPS location	
			Latitude	Longitude
KO 1	Konwewa	109 m	N08^o 23’05.5”	E080^o 47’21.4”
KO 2	Konwewa	113 m	N08^o 22’46.2”	E080^o 47’30.2”
KO 3	Konwewa	109 m	N08^o 23’23.5”	E080^o 47’07.2”
KO 4	Konwewa	113 m	N08^o 23’39.8”	E080^o 47’06.2”
HO 1	Horowpathana	104 m	N08^o 30’46.8”	E080^o 44’47.4”
HO 2	Horowpathana	103 m	N08^o 30’20.6”	E080^o 44’42.4”
HO 3	Horowpathana	103 m	N08^o 30’06.6”	E080^o 44’50.5”
HO 4	Horowpathana	106 m	N08^o 30’04.9”	E080^o 44’50.1”
RM 1	Rathmalgahawewa	131 m	N08^o 31’19.7”	E080^o 40’34.5”

RM 2	Rathmalgahawewa	128 m	N08° 30’42.9”	E080° 40’31.7”
K1	Kagama	121 m	N08° 03’36.4”	E080° 29’48.0”
K2	Kagama	127 m	N08° 04’07.5”	E080° 29’40.4”
K3	Kagama	123 m	N08° 04’10.9”	E080° 29’38.8”
K4	Kagama	123 m	N08° 04’25.8”	E080° 29’33.2”
K5	Kagama	120 m	N08° 04’16.2”	E080° 29’33.8”
K6	Kagama	119 m	N08° 04’17.3”	E080° 29’20.3”
K7	Kagama	124 m	N08° 03’24.7”	E080° 29’41.7”
K8	Kagama	127 m	N08° 03’58.7”	E080° 30’12.2”
K9	Kagama	130 m	N08° 03’24.2”	E080° 30’21.0”
K10	Kagama	129 m	N08° 03’14.2”	E080° 30’21.3”
B1	Bulnewa	114 m	N08° 04’40.1”	E080° 25’59.1”
B2	Bulnewa	114 m	N08° 04’40.1”	E080° 25’59.1”
B3	Bulnewa	103 m	N08° 04’45.6”	E080° 25’57.3”
B4	Bulnewa	106 m	N08° 04’35.6”	E080° 26’06.2”
B5	Bulnewa	103 m	N08° 04’35.6”	E080° 26’06.3”

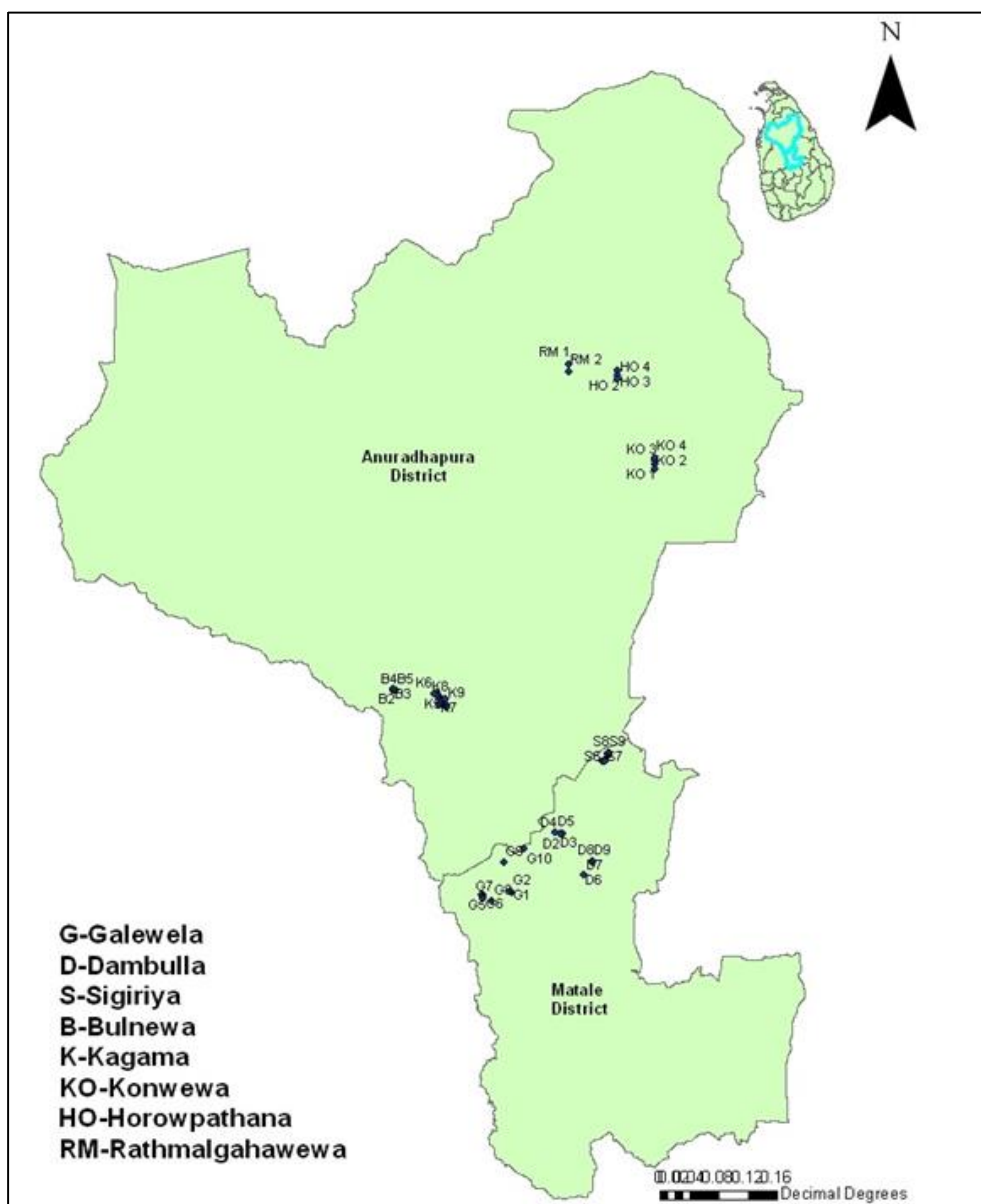

Figure 1. Map of sampling fields.

Collection of soil samples

Six soil samples each of 5 g from the top 5–10 cm depth were collected from each big onion field (Tables 1 & 2) using a sterile spatula. The 6 samples of each field were bulked to form a composite sample of 30 g and transported to the laboratory in clean polythene bags.

Isolation of fungi from diseased seedlings

Diseased seedling samples were washed thoroughly first in tap water and subsequently in distilled water. These were surface sterilized with 70% ethanol for 1 minute and then rinsed three times with sterile distilled water. Seedlings were then dried on a sterilized filter paper and plated under aseptic conditions on Potato Dextrose Agar supplemented with Tetracycline at a concentration of 0.05 g l^{-1} (PDA + Tet.) and were incubated for 5 days at room temperature. Resultant fungal colonies were separately subcultured onto PDA+Tet. plates. Pure cultures were prepared using the hyphal tip method (Tutte 1969) and maintained at room temperature. Identification of the fungal isolates to genus level was done using identification keys (Domsch *et al.* 1993) based on the observed colony and microscopic characteristics.

Confirmation of pathogenicity of fungal isolates using Koch's postulates

The morphologically similar *Fusarium* sp., two *Curvularia* spp. and *Alternaria* sp. isolated from both Matale and Anuradhapura districts were used for confirmation of pathogenicity on *Allium cepa* L. seedlings using Koch's postulates.

After ten days, the number of seedlings showing damping-off symptoms were recorded and disease incidence (%) was assessed according to Tarr (1981):

$$\text{Disease incidence (\%)} = \frac{\text{No. of infected plants}}{\text{Total No. of inoculated plants}} \times 100$$

The means of treatments were analyzed with one-way ANOVA and Tukey's test at 5 % significant level with Minitab 16. Percentage data were transformed into arcsine values.

Molecular characterization of *Fusarium* sp. isolated from seedlings

DNA extraction from Fusarium isolates

Fusarium colonies with similar morphological characters isolated from different regions *i.e.* Matale and Anuradhapura districts grown on Potato Dextrose Agar (PDA) were used in this study.

Hyphae of each *Fusarium* isolate was inoculated to 20 ml of sterile potato dextrose broth (PDB) in a 50 ml flask and allowed to grow for 72 hours at 28±2 °C. The resultant mycelial mat was centrifuged for 5 minutes at 13,000 rpm in a microfuge and the resultant pellet was washed with 500 µl of TE buffer and centrifuged once again. The TE buffer was decanted and 300 µl of extraction buffer (200 mM TrisHCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS) was added to the pellet. The mycelium was crushed with a sterile toothpick. After that, 150 µl of 3 M sodium acetate, pH 5.2 was added and tubes were placed at -20 °C for about ten minutes. Tubes were then centrifuged in a microfuge (10 min 10000 rpm) and the supernatant was transferred to another tube. Then, an equal volume of isopropanol was added and after placing at least 5 minutes at room temperature the precipitated DNA was pelleted by centrifugation in a microfuge (12,000 rpm for 10 min). After a wash with 70 % ethanol, the pellet was dried for a few minutes and resuspended in 30 µl of sterilized distilled water (Cenis 1992).

PCR amplification of rDNA ITS region of Fusarium spp.

Table 3. PCR primers used for amplification.

Primer name	Primer Direction	Primer sequence (5'→ 3')	Source
ITS 1	Forward	TCC GTA GGT GAA CCT GCG G	White *et al.*1990
ITS 4	Reverse	TCC TCC GCT TAT TGA TAT GC	White *et al.*1990
FR 1	Reverse	AIC CAT TCA ATC GGT AIT	Vainio & Hantula, 2000
NS 1	Forward	GTA GTC ATA TGC TTG TCT C	White *et al.*1990

Genomic DNA of the isolates of *Fusarium* was used for the PCR amplification of ITS region DNA fragment in rDNA gene. Polymerase Chain Reaction (PCR) was performed in a total volume of 25 µl, containing 14.95 µl of nuclease-free water, 5 µl 5× *Taq* polymerase buffer, 2mM $MgCl_2$, 1.25 Units of *Taq* polymerase enzyme (Promega, USA), 120 µM dNTPs (Promega, USA), 1 µM reverse and forward primers (IDT, USA) (ITS 1 and ITS 4) (Table 3) and 2 µl of template DNA. PCR was programmed with an initial denaturing at 95°C for 5 min. followed by 30 cycles of denaturation at 95°C for 1 min., annealing at 55°C for 1 min. and extension at 72°C for 2 min. and the final extension at 72°C for 10 min. in a Techne/ Flexigene (England) DNA Thermal Cycler. Amplification products (20 µl) were mixed with 10 × gels loading buffer (5 µl) and then loaded in 1.5 % Agarose gel with 0.5×10^{-3} mg/ml ethidium bromide and 0.5 X Tris Boric acid EDTA (TBE) buffer (Sambrook *et al.* 1989) for examination with horizontal electrophoresis (Bio-Rad, USA).

Sequencing of the ITS region of damping-off pathogen Fusarium sp. for species level identification

Amplified DNA produced using ITS 1 and ITS 4 primers for damping-off pathogen *Fusarium* sp. were sequenced (Macrogen, Korea). The sequences were analyzed using BLAST (Basic Local Alignment Search Tool) on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast) to find the sequences with the highest similarity to query sequences. The sequence has been deposited in GenBank after examining with BioEdit 7.2 sequence alignment software.

*Sequence analysis revealed that pathogenic *Fusarium* sp. as *Fusarium solani*

Isolation and identification of fungal species from soil samples collected from big onion fields

The fungal species present in fifty-five soil samples collected from different big onion fields (Tables 1 and 2) were isolated using the Warcup (1950) method. 0.1 g of sieved, air-dried composite soil from each field was used for each plate. There were 3 replicate plates for each field. Plates were incubated at room temperature in an inverted position for 10 days. From the fungal colonies that grew, those with the characteristics recorded for *Trichoderma* colonies were selected and transferred onto fresh PDA plates. Pure cultures were prepared using the hyphal tip method (Tutte 1969).

In vitro tests for antagonistic activity of the isolated *Trichoderma* spp.

Antagonistic activity against damping-off pathogen Fusarium solani

Seven *Trichoderma* spp. (Tr.1, Tr.2, Tr.3, Tr.4, Tr.5, Tr.6 and Tr.7) isolated from the soil samples collected from onion fields were evaluated under *in vitro* conditions for their antagonistic activity against *Fusarium solani*. One-week-old cultures of both *Fusarium solani* and *Trichoderma* spp. grown on PDA+Tet. plates were used for the test. Each plate was inoculated with a 5 mm diameter disc of *Trichoderma* spp. positioned diametrically opposite a 5 mm disk of the *Fusarium solani* on a 90 mm diameter PDA + Tet plate. In the control treatment, only a 5 mm diameter disk of *Fusarium solani* was placed in the 90 mm diameter PDA+Tet. plate. There were four replicates for each treatment. The plates were incubated at room temperature for six days. After incubation, radial growth of each colony was measured. The efficiency of *Trichoderma* spp. in suppressing radial growth was calculated as follows:

$$\frac{C-T}{C} \times 100$$

Where, C is radial growth of the pathogen in the control and T is radial growth of the pathogen in the presence of *Trichoderma* spp.

The means of treatments were analyzed with one-way ANOVA and Tukey's test at 5 % significant level with Minitab 16.

Determination of the average colony diameter of *Trichoderma* spp. after two days of inoculation

Trichoderma spp. were inoculated onto PDA plates, by transferring 1 cm diameter discs from the growing edges of a pure culture. Radial colony diameters were measured after 2 days of incubation at room temperature. There were 3 replicates. The means were analyzed with one-way ANOVA and Tukey's test at 5 % significant level with Minitab 16.

Molecular characterization of *Trichoderma* spp.

Table 4. RAPD-PCR primers used for amplification.

Primer name	Primer sequence (5'→ 3')	Source
OPD 6	GGGGTCTTGA	Chakraborty *et al.*2010
A-4	AAT CGG GCT G	Shalini *et al.*2006
A-5	AGG GGT CTT G	Chakraborty *et al.*2010
AA-11	AGA CGG CTC C	Shalini *et al.*2006

Above mentioned seven isolates of *Trichoderma* spp. obtained from soil of *A. cepa* L. fields *i.e.* Matale and Anuradhapura districts were studied using ITS 1 and ITS 4 primer set, NS 1 / FR 1 primer set (Table 3) and using RAPD primers *i.e.* OPD 6, A-4, A-5, AA-11 (Table 4).

PCR amplification of rDNA ITS region of *Trichoderma* spp.

The DNA was extracted according to the same procedure described for *Fusarium* sp. Genomic DNA of seven isolates of *Trichoderma* were used for PCR amplification. Primer sequences used in this study for PCR amplification of fungal DNA are presented in table 3. Polymerase Chain Reaction (PCR) was carried out according to the same procedure described for *Fusarium* sp.

Sequencing of the ITS region of *Trichoderma* spp. (Tr. 1 and Tr. 3) for species level identification

Two *Trichoderma* spp. Tr. 3 and Tr.1 were selected as Tr. 3 had the highest antagonistic activity, highest growth rate and highest sporulation capacity while Tr. 1 had higher antagonistic activity, higher growth rate, higher sporulation capacity and was tentatively identified as *Trichoderma virens* according to the micro-morphological structures.

DNA amplified using ITS 1 and ITS 4 primers for the two *Trichoderma* spp. showing higher antagonistic activity in the plate assays, higher growth and higher sporulation rates *in vitro* were sequenced (Macrogen, Korea). The sequences were analyzed using BLAST on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast) to find the sequences with the highest similarity to query sequences. Sequences were deposited in GenBank after examining with BioEdit 7.2 sequence alignment software.

PCR amplification of *Trichoderma* spp. 18S rRNA genes with the NS 1 / FR 1 primer set

Genomic DNA of seven isolates of *Trichoderma* were used for PCR amplification of 18S r RNA genes. Primer sequences used in this study for PCR amplification of fungal DNA are presented in table 3. Polymerase Chain Reaction (PCR) was carried out according to the same procedure described for *Fusarium* sp.

RAPD-PCR analysis of *Trichoderma* spp.

Genomic DNA of seven isolates of *Trichoderma* was used for PCR amplification. Random primer sequences used in this study for RAPD-PCR amplification of fungal DNA are presented in Table 4. RAPD-PCR reaction was done in a total volume of 25 µl, containing 1× *Taq* polymerase buffer, 2 mM $MgCl_2$, 1.25 Units of *Taq* polymerase enzyme (Promega, USA), 120 µM dNTPs (Promega, USA), 0.6 µM primers (IDT, USA) and 2 µl of template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 40 cycles of denaturation at 94 °C for 1 min., annealing at 35°C for 1 min. and extension at 72°C for 2 min. and the final extension at 72°C for 10 min. in a Techne/ Flexigene (England) DNA Thermal Cycler. PCR product (20 µl) was mixed with 10 × gels loading buffer (5 µl) and then loaded in 1.5 % Agarose gel with 0.5×10^{-3} mg/ml ethidium bromide for examination with horizontal electrophoresis (Bio-Rad, USA).

Initially, 2 random 10- mer primers i.e. OPD 6 and A-4 were tested with DNA samples of *Trichoderma* spp. isolates. Then all 4 random 10- mer primers *i.e.* OPD 6, A-4, A-5, AA-11 were tested with DNA samples of *Trichoderma* spp. isolates.

Determination of the growth rate of the pathogen *Fusarium* sp. and *Trichoderma* spp. (Tr. 1 and Tr. 3)

Determination of the growth rate of Fusarium solani

A 1 cm diameter disc cut from the growing edges of a pure culture of *Fusarium solani* isolated from diseased *A. cepa* L. seedlings using a sterile cork borer was inoculated onto a PDA plate. Three replicate plates were prepared and radial colony diameters were measured after 6 days of incubation at room temperature. The growth rate was calculated for each replicate and the mean growth rate of *Fusarium solani* was calculated using these values. Growth rate was calculated according to the following equation:

$$Growth\ Rate = \frac{\text{Colony diameter (mm)}}{\text{Incubated number days}}$$

Determination of the growth rates of Trichoderma spp. (Tr. 1 and Tr. 3)

1 cm diameter discs from the growing edges of pure cultures of *Trichoderma* spp. were cut using a sterile cork borer and inoculated separately onto PDA plates. Radial colony diameters were measured after 3 days of incubation at room temperature. There were 3 replicates per each *Trichoderma* sp. Growth rate at room were calculated for each replicate after 3 days of incubation and mean growth rates were calculated. The growth rate was calculated according to the equation mentioned for *Fusarium solani.* The means were analyzed with one-way ANOVA and Tukey's test at 5 % significant level with Minitab 16.

Determination of the mechanisms of antagonism of *Trichoderma* spp.

Mycoparasitism

Each *Trichoderma* spp. (*Trichoderma asperellum* and *T. virens*) and *Fusarium solani* were inoculated onto the two ends of a slide coated with PDA medium. The inoculated slides were allowed to incubate at room temperature in a moist chamber and observed daily under the high power (10×40) of the light microscope to observe the interaction between the two organisms.

Determination of Chitinase activity

One cm diameter discs of 10 days old cultures of the *Trichoderma asperellum*, *Trichoderma virens* maintained on PDA were inoculated separately on to the Chitinase detection medium (Agrawal & Kotasthane

2009) (Colloidal chitin 4.5 g l^{-1}, $MgSO_4.7H_2O$ 0.3 g l^{-1}, $(NH_4)_2SO_4$ 3.0 g l^{-1}, KH_2PO_4 2.0 g l^{-1}, Citric Acid monohydrate 1.0 g l^{-1}, Agar (Hardy Diagnostics,USA) 15g/l, Bromocresol purple 0.15 g l^{-1}, Tween-80 200 µl, pH was adjusted to 4.7) plates and incubated at room temperature for 3–7 days. Control plates were inoculated with 1 cm diameter PDA discs. There were three replicate plates for each treatment. Chitinase activity was confirmed by the presence of a purple-colored zone surrounding the inoculated culture discs.

Determination of effect of volatile metabolites

The two lower halves of a petri plate containing PDA were inoculated with a 1 cm disc of *Fusarium solani* and *Trichoderma* spp. respectively and both inoculated lower halves were placed facing each other and sealed with parafilm (Dennis & Webster 1971). The petri plates containing PDA without the *Trichoderma* spp. served as a control. Three replicates were maintained for each treatment.

The percent inhibition was obtained using the formula:

$$D_1 - D_2 \times 100$$ (Vincent 1947)

Where, D_1- diameter of radial growth of *Fusarium solani* in control, D_2- diameter of radial growth of *Fusarium solani* in treatment

RESULTS

Symptoms present in diseased seedlings

Fifty-five big onion fields in the Matale and Anuradhapura districts were surveyed during the *yala* season for the seedling diseases prevalent at nursery stage of growth. Damping-off disease was identified to be most prevalent and similar symptoms were observed in both districts. Circular to irregular shaped areas within fields could be observed. Water-soaked, greasy lesions on hypocotyls and roots, formation of stunted, yellow coloured, wilted seedlings were the common symptoms of damping-off seedlings.

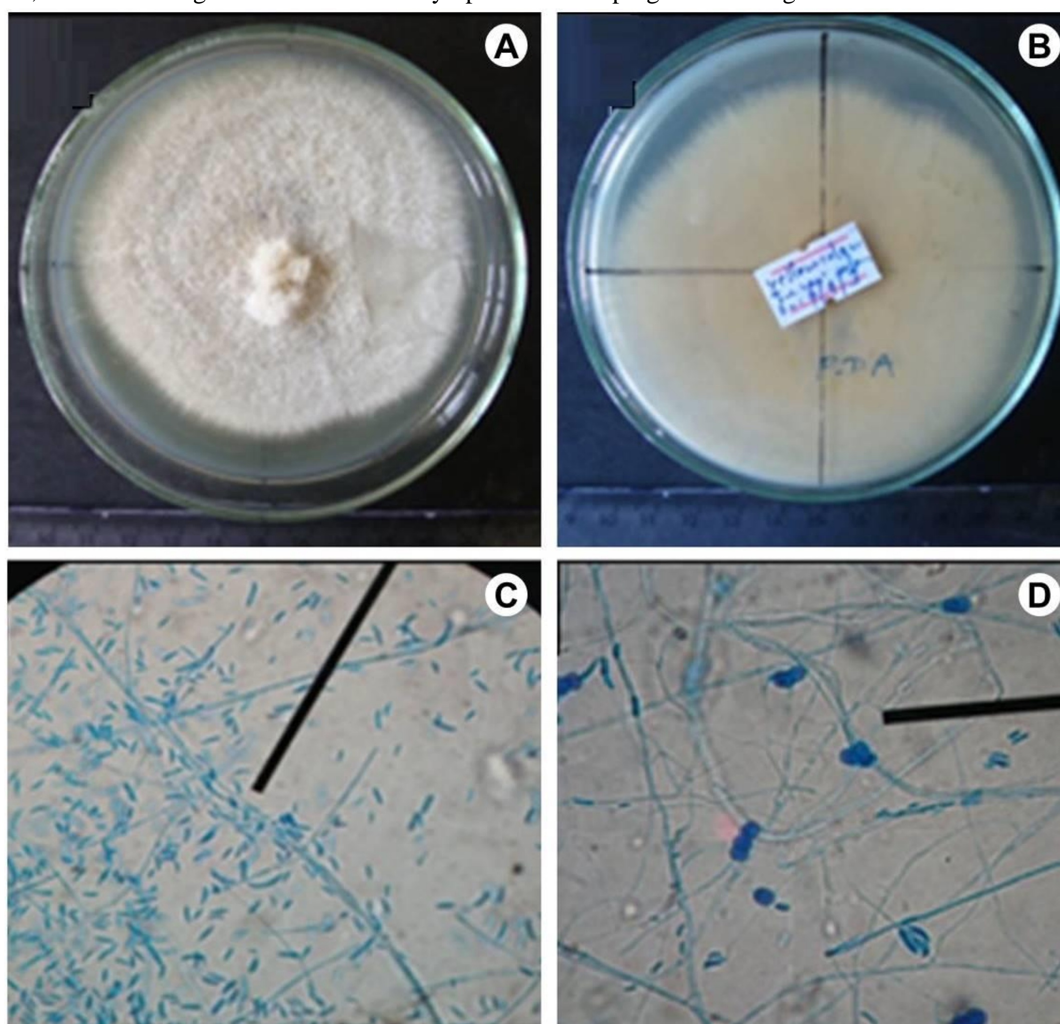

Figure 2. *Fusarium* sp. colony morphology: **A**, *Fusarium* sp. in 7 day old culture; **B**, *Fusarium* sp. in culture-reverse view; Micro morphology of the *Fusarium* sp; **C**, branched, septate hyphae, microconidia (7.56 µm), macroconidia (23.18 µm); **D**, Chlamydospores (10×40×2)

The fungal species present in the diseased seedlings were also isolated and identified. Similar fungal genera *i.e. Aspergillus, Curvularia, Fusarium, Penicillium, Alternaria, Mucor, Sclerotium* were associated with the seedling samples collected from both Matale and Anuradhapura districts. *Fusarium* colonies were white to cream coloured with aerial mycelia (Fig. 2). The mycelia were branched, septate and produced two types of conidia. Macroconidia three-septate, fusiform, moderately curved, microconidia - are abundant, cylindrical to oval. Globose, smooth or rough walled chlamydospores were borne singly or in pairs on short lateral hyphal branches (Fig. 2).

Confirmation of pathogenicity of fungal species isolated from *Allium cepa* L. seedlings (*Fusarium* sp., *Alternaria* sp., *Curvularia* sp.1 and *Curvularia* sp. 2)

Disease incidence

Table 5. Effect of soil inoculation with selected fungal genera on disease incidence on potted *Allium cepa* L. seedlings 10 days after inoculation: Mean of three replicates per treatment.

Treatment	Disease incidence (%) in treated seedlings
sterile distilled water 1ml (control)	5 (12.92)[b]
Fusarium sp.	60 (50.85)[a]
Alternaria sp.	5 (12.92)[b]
Curvularia sp. 1	6 (11.44)[b]
Curvularia sp. 2	5 (12.92)[b]
P value	<0.001

Note: Means in column followed by different letters indicate significant difference at $P<0.05$

The number of seedlings with damping-off symptoms was recorded and disease incidence (%) caused by each fungal genus was assessed (Table 5). Compartments with soil that were inoculated with spore suspension of the *Fusarium* sp. showed the highest disease incidence *i.e.* 60% while the soils that were inoculated with spore suspensions of *Alternaria* sp. and two *Curvularia* sp. showed very low disease incidence *i.e.* 5–6 %. 1×10^5 spores ml^{-1} concentration of the *Fusarium* sp. spore suspension was effective in developing the seedling damping-off disease.

Re-isolation of the pathogens

Fusarium sp. was isolated from the inoculated seedlings that showed symptoms when cultured on PDA+Tet. Hyphae of the reisolated *Fusarium* sp. were observed to be branched, septate and there were two types of conidia; microspores and curved macrospores similar to the initially isolated *Fusarium* sp.

PCR amplification of ITS region of *Fusarium* isolates

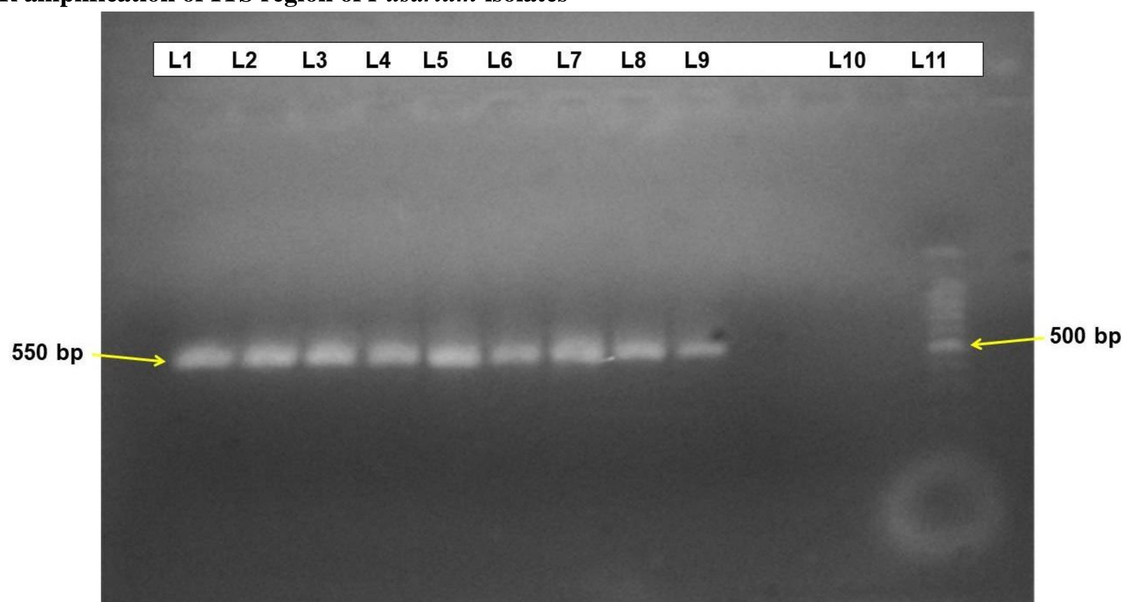

Figure 3. Lane 1-9: ITS PCR amplified DNA for *Fusarium* spp. with ITS 1/ ITS 4 primers (Lane 1-3: *Fusarium* sp. isolated from Matale District: Lane 4-6: *Fusarium* sp. isolated from Anuradhapura: Lane 7-9: *Fusarium* sp. isolated from Mahaweli H region) Lane 10: PCR negative control, Lane 11: 100 bp DNA ladder, respectively.

Agarose gel electrophoresis of PCR products obtained from PCR amplification of DNA isolated from morphologically similar *Fusarium* spp. isolated from Matale and Anuradhapura districts using primers ITS 1 and ITS 4 are shown in figure 3. DNA fragments with 550 bp were observed.

Table 6. Antagonistic activity of the tested *Trichoderma* spp. against *Fusarium* sp.

***Trichoderma* spp.**	**% Growth inhibition of *Fusarium* sp. ± SE**
Trichoderma virens (Tr.1)	31.92 ± 4.63[b]
Tr.2	38.61 ± 4.67[b]
Trichoderma asperellum(Tr.3)	55.74 ± 1.65[a]
Tr.4	33.61 ± 3.64[b]
Tr.5	37.40 ± 1.99[b]
Tr.6	42.42 ± 1.75[ab]
Tr.7	36.36 ± 1.24[b]
P value	0.010

Note: Each value represents the mean of four replicates (±) SE; Means followed by different letters within column are significantly different ($p \leq 0.05$) according to ANOVA.

Table 7. Average colony diameter of *Trichoderma* spp. after two days of inoculation.

Fungal isolate	**Average colony diameter (cm) ± SE**
Trichoderma virens (Tr.1)	8.60 ± 0.06 [b]
Tr.2	8.40 ± 0.06 [b]
Trichoderma asperellum (Tr.3)	9.00 ± 0.00 [a]
Tr.4	8.50 ± 0.20 [b]
Tr.5	3.90 ± 0.10 [e]
Tr.6	4.30 ± 0.10 [d]
Tr.7	6.70 ± 0.17 [c]
P value	<0.001

Note: Data are means of three replications; *Means in column followed by different letters indicate significant difference at $P < 0.05$

Table 8. Identification of fungal isolates.

Sample	**Fungal Isolate**	**GenBank accession numbers**
Sample 11 (*Fusarium* sp.)	*Fusarium solani*	MF685335
D4_Tri (*Trichoderma* sp.3-Tr.3)	*Trichoderma asperellum*	MG198706
Cul_2 (*Trichoderma* sp.1-Tr.1)	*Trichoderma virens*	MG199587

Molecular identification of pathogenic *Fusarium* isolate

DNA sequence analysis using BLAST revealed that *Fusarium* isolates showed 99 % identity to the previously isolated *Fusarium solani* (ex: accession numbers KF999012.1, HG 798753.1, HQ 833835.1) Hence, based on its 18 S ribosomal RNA gene, internal transcribed spacer 1, partial sequence; 5.8 S ribosomal RNA gene, internal transcribed spacer 2 and 28 S ribosomal RNA gene, partial sequence the pathogenic *Fusarium* sp. isolated in the current study was assigned as *Fusarium solani.* The NCBI GenBank accession number is indicated in table 8.

Isolation and identification of fungal species from soil samples

Fungi isolated from soil samples collected from big onion fields were identified to be *Trichoderma* spp. *Aspergillus, Penicillium, Rhizopus, Mucor.*

In vitro tests for antagonistic activity of *Trichoderma* spp.

Trichoderma spp. isolated from the soils of Matale and Anuradhapura districts were tested using dual culture assays for their ability to control the growth of the *Fusarium solani* identified as the causative agent of onion damping-off disease.

Dual culture assays

After 6 days of incubation, *Trichoderma* colonies showed dominant growth while the growth of *Fusarium* colonies was restricted in both dual culture plates and PDA coated slides.

All tested *Trichoderma* spp. suppressed the mycelial growth of *Fusarium solani* in different degrees. Results of antagonistic capability revealed that Tr. 3 caused the highest reduction of the growth of *Fusarium solani* compared to other species *i.e.* 55.74%. While isolate Tr. 1 showed percentage inhibition equaling 31.92% (Table 6).

Determination of the average colony diameter of *Trichoderma* spp. after two days of inoculation

Tr. 3 has the highest colony diameter after two days of incubation while Tr.1 has the second-highest colony diameter (Table 7).

PCR amplification of DNA fragment in ITS region of rDNA gene in *Trichoderma* spp.

Agarose gel electrophoresis of PCR products obtained from PCR amplification of DNA isolated from *Trichoderma* spp. using primers ITS 1 and ITS 4 are shown in figure 4. DNA fragments with 600 bp were observed.

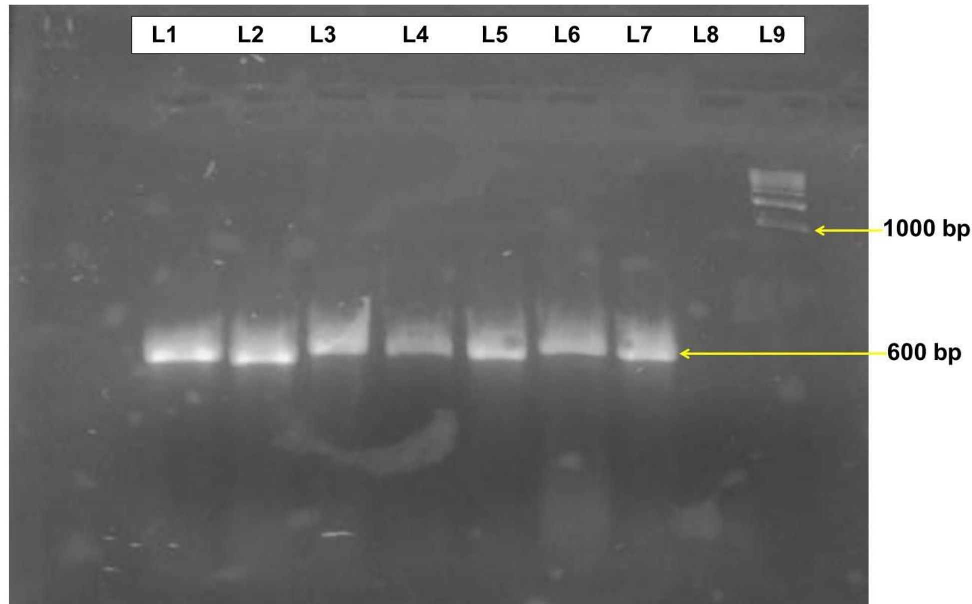

Figure 4. PCR amplified DNA for amplification ITS region of *Trichoderma* spp. Lane 1-9: Tr.2, *Trichoderma asperellum*, Tr.4, Tr.5, *Trichoderma virens*, Tr.7, Tr.6, PCR negative control, 1kb DNA marker respectively.

Molecular identification of promising biocontrol agents

Two *Trichoderma* spp. (Tr. 3 and Tr. 1) were selected as promising biocontrol agents based on the antagonistic activity, growth rate, sporulation capacity and tentative microscopic identification. Their amplified DNA produced using ITS 1 and ITS 4 primers were sequenced and compared to the GenBank database through BLAST search. This demonstrated 100 % identity with *Trichoderma asperellum* (ex. accession numbers KU 145462.1, KP 059114.1, KP 784424.1) for Tr. 3. Whilst the Tr. 1 presented a 100 % similarity with the accession numbers KT278905.1, KT 278873.1, KT 278870.1 for *Trichoderma virens.*

Hence, based on its 18 S ribosomal RNA gene, partial sequence; the Tr.3 and Tr.1 isolated in the current study were assigned as *Trichoderma asperellum* and *Trichoderma virens* respectively. The NCBI GenBank accession number are indicated in table 8.

PCR amplification of *Trichoderma* spp. 18S rRNA genes with the NS 1 / FR 1 primer set

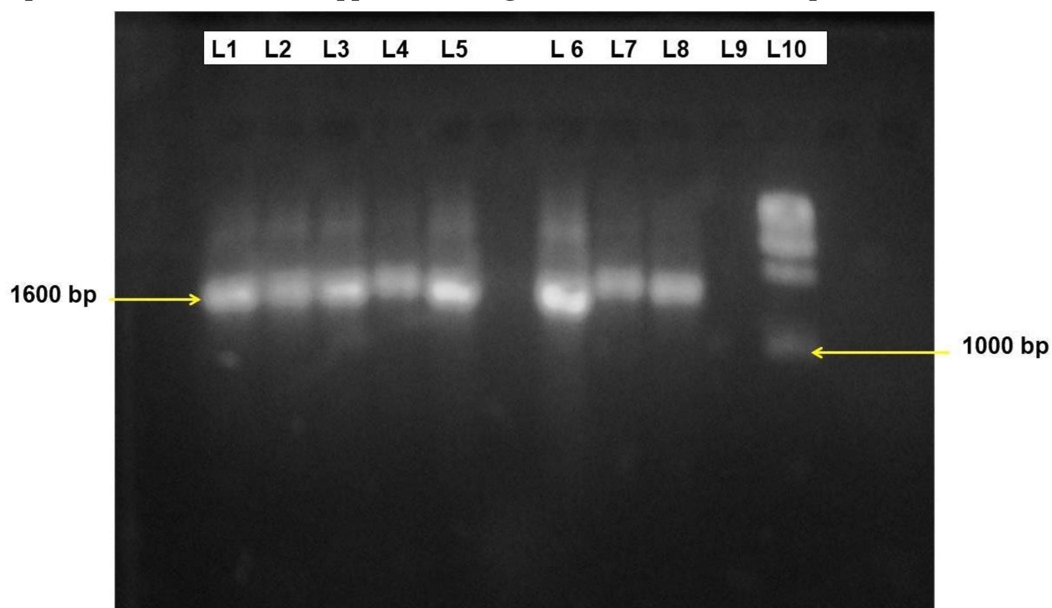

Figure 5. PCR amplified DNA of *Trichoderma* spp. using NS 1 and FR 1 primers Lane 1-10: Tr.2, *Trichoderma asperellum*, Tr.4, Tr.5, *Trichoderma virens*, Tr.7, Tr.6, *Ceratocystis paradoxa* (Positive control), PCR negative control, 1 kb DNA marker respectively.

Agarose gel electrophoresis of PCR products obtained for PCR amplification of DNA extracted from *Trichoderma* spp. using primers NS 1 and FR 1 are shown in figure 5. DNA fragments 1600 bp in size were observed.

RAPD-PCR analysis of *Trichoderma* spp.

RAPD-PCR amplification of genomic DNA extracted from Trichoderma spp. using two primers OPD 6 / A-4

The oligonucleotide primers i.e. OPD 6 and A 4 produced multiple bright bands (Fig. 6) and revealed potentially intra-specific polymorphisms among the *Trichoderma* isolates.

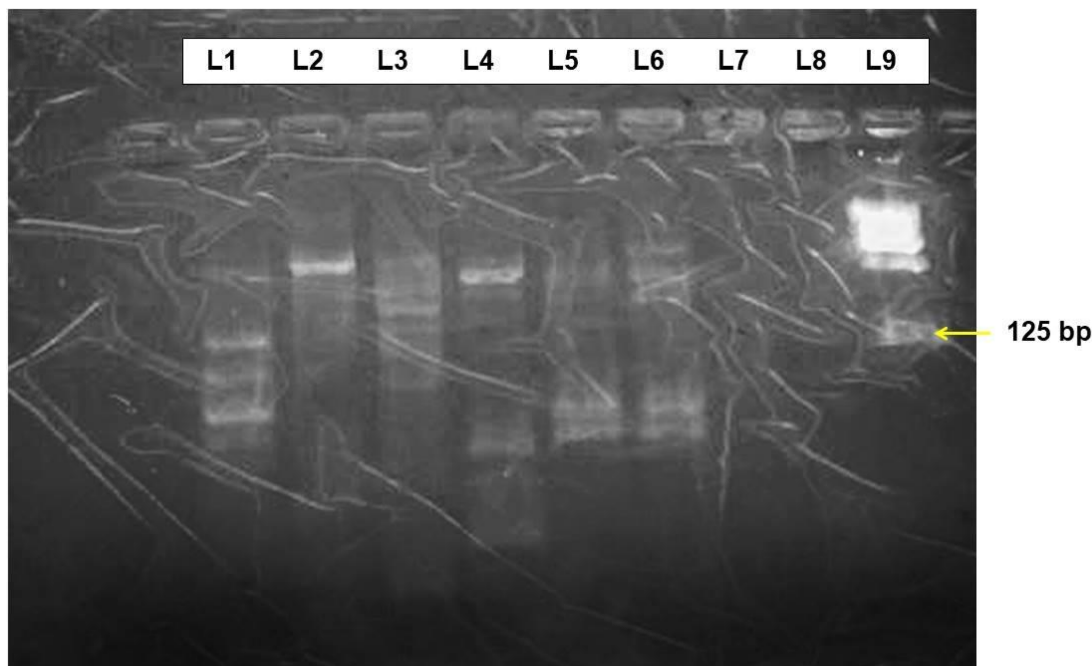

Figure 6. Representative random amplified polymorphic DNA patterns of the seven *Trichoderma* spp. amplified with the primers OPD 6 and A 4. Lane 1-9: Tr.2, *Trichoderma asperellum*, Tr.4, Tr.5, *Trichoderma virens*, Tr.7, Tr.6, PCR negative control, Lambda *Hind* III DNA Ladder[1]

[[1]The artifacts within the Fig.6 is due to cellophane paper laid on the UV trans illuminator]

RAPD-PCR amplification of genomic DNA extracted from Trichoderma spp. using primers OPD 6, A-4, A-5 and AA-11

The oligonucleotide primers *i.e.* OPD 6, A-4, A-5, AA-11 produced amplified DNA of multiple bright bands (Fig. 7) and revealed potentially intra-specific polymorphisms among the *Trichoderma* isolates.

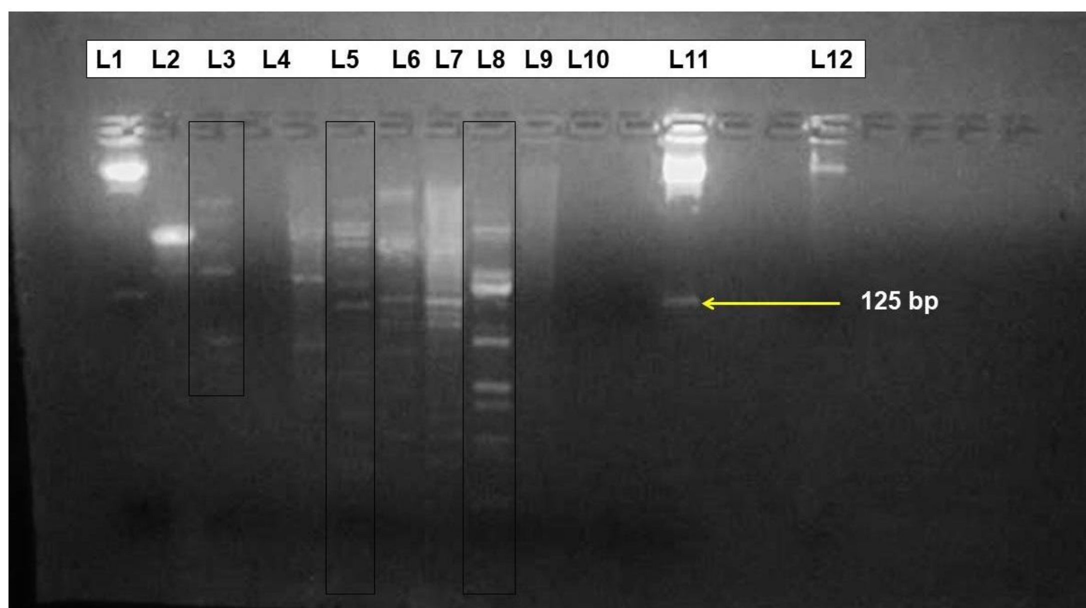

Figure 7. Representative random amplified polymorphic DNA patterns of the seven *Trichoderma* spp. amplified with primers OPD 6, A-4, A-5, AA-11, Lane 1-11: Lambda *Hind* III DNA Ladder Tr.2, *Trichoderma asperellum*, Tr.4, Tr.5, *Trichoderma virens*, Tr.7, Tr.6, *Fusarium solani*, *Alternaria* sp. PCR negative control, Lambda *Hind* III DNA Ladder

Growth rates of *Fusarium* sp. and *Trichoderma* spp. (*Trichoderma virens* (Tr.1) and *Trichoderma asperellum* (Tr.3))

1 cm diameter discs of *Fusarium* sp. and *Trichoderma* spp. were transferred on to PDA plates (3 replicates for each fungus) and incubated at room temperature. Colony diameters were measured and growth rates of *Fusarium* sp. and *Trichoderma* spp. (*Trichoderma virens* (Tr.1) and *Trichoderma asperellum* (Tr.3)) were calculated after 6 days and 3 days of incubation respectively. The growth rates of *Trichoderma* spp. were faster than that of *Fusarium solani* at room temperature (Table 9).

Table 9. Growth rates of *Fusarium* sp. and *Trichoderma* spp.

Fungal isolate	Growth rate (mm/day) ± SE
Fusarium solani	8.167 ± 0.095^{c}
Trichoderma virens (Tr.1)	21.223 ± 0.640^{b}
Trichoderma asperellum (Tr.3)	29.833 ± 0.167^{a}
P value	<0.001

Note: Data are means of three replications.
*Means in column followed by different letters indicate significant difference at P < 0.05

Determination of the mechanisms of antagonism of *Trichoderma* spp.

Formation of special structures

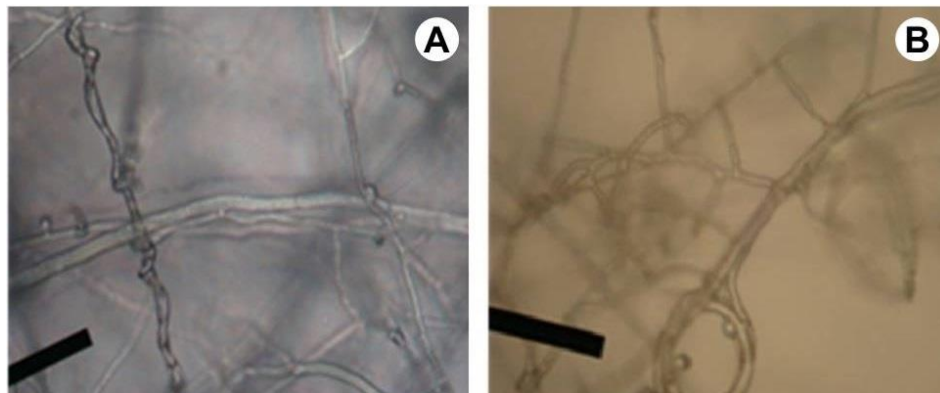

Figure 8. Antagonistic activity of *Trichoderma asperellum*.: **A**,Thin *Trichoderma* hyphae coiling around the thicker hyphae of *Fusarium solani* and Attachment of the *Trichoderma asperellum* hyphae by tips on the large hyphae of *Fusarium solani* (10×40×3); **B**, Loops formed by *Trichoderma asperellum* to trap *Fusarium solani* hyphae (10×40×3).

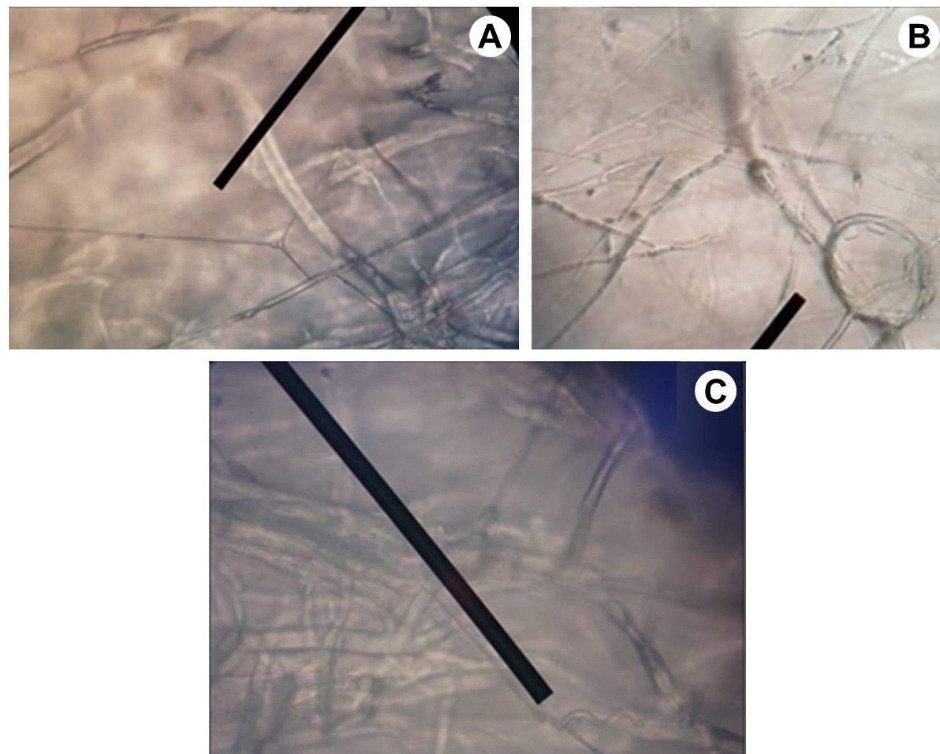

Figure 9. Antagonistic activity of *Trichoderma virens* against *Fusarium solani*: **A**, Attachment of the *Trichoderma virens* hyphae on the hyphae of *Fusarium solani* (10×40×3); **B**, Loops formed by *Trichoderma virens* to trap *Fusarium solani* hyphae. (10×40×3); **C**, thin *Trichoderma virens* hyphae coiling around the thicker hyphae of *Fusarium solani* (10×40×3)

Interaction between the two fungi when observed through the light microscope indicated that *Trichoderma asperellum* and *T. virens* formed special structures such as coils, loops and also attached to the larger hyphae of *Fusarium solani* with hyphal tips that seemed to restrict the colony growth of *Fusarium solani* (Figs. 8 & 9).

Chitinase activity of Trichoderma asperellum, Trichoderma virens

Chitinase detection medium supplemented with colloidal chitin as sole C source and bromocresol purple (BCP) as an indicator (pH 4.7) was used to test for chitinase production by *Trichoderma asperellum* and *Trichoderma virens*. *Trichoderma* spp. tested produced a yellow to purple zone around the colonies indicating chitin utilization by producing chitinase. (Figs. 10 & 11).

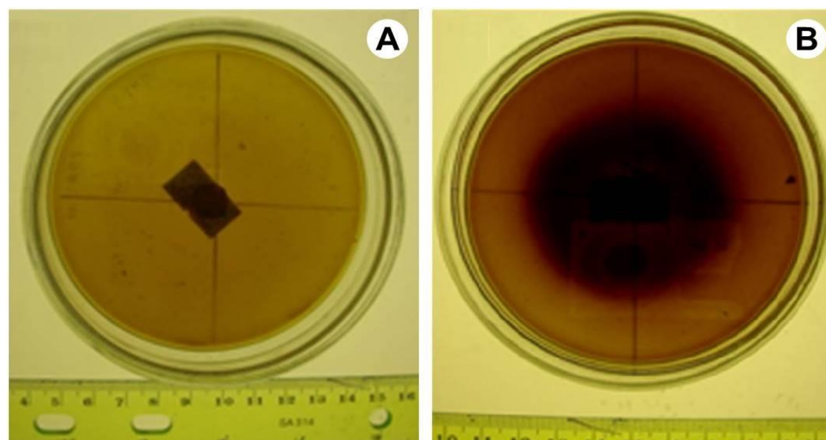

Figure 10. Screening of *Trichoderma asperellum* for chitinase activity on medium supplemented with colloidal chitin: **A**, Uninoculated control; **B**, (3 days) after inoculation.

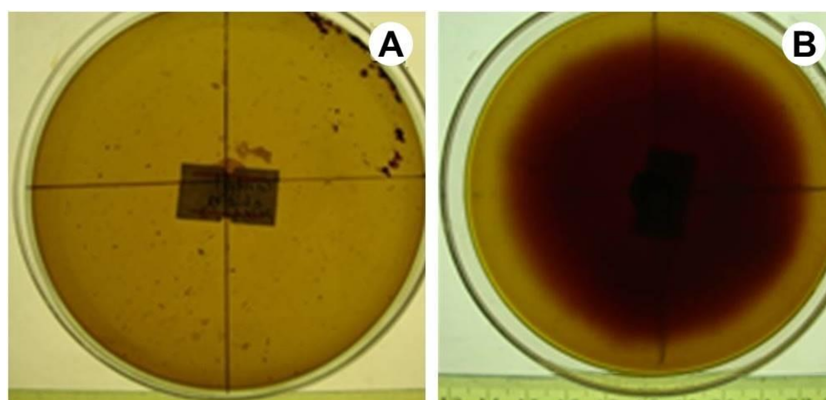

Figure 11. Screening of *Trichoderma virens* for chitinase activity on medium supplemented with colloidal chitin: **A**, Uninoculated control; **B**, (3 days) after inoculation.

Determination of effect of volatile metabolites

The volatile metabolites produced from *Trichoderma virens* and *Trichoderma asperellum* showed 15 % and 25 % inhibition of mycelial growth of *Fusarium solani* (Table 10).

Table 10. Effect of volatile metabolites from *Trichoderma* spp. on radial growth of *Fusarium solani.*

	Percentage growth inhibition (%) of *Fusarium solani* ± SE
Trichoderma asperellum	24.767 ± 0.45
Trichoderma virens	15.133 ± 0.13

Note: Data are means of three replications.

Nucleotide sequence accession numbers

Sequences obtained in this study have been submitted to NCBI GenBank and the Internal Transcribed Spacer gene sequence accession numbers are indicated in table 8.

DISCUSSION

Big onion cultivations in Sri Lanka is mostly concentrated in Matale and Anuradhapura districts and Mahaweli H region yielding about 75,776 MT annually (AESD 2016). However, the amount harvested is insufficient even to meet the local demand and one factor that contributes significantly towards reduced yields is diseases that affect onion cultivations deleteriously at different stages of growth. A survey carried out in a previous study (Gunaratna & Deshappriya 2014) revealed that the more common diseases that affected the

plants in all areas of cultivation were seedling damping-off at the nursery stage and basal rot, leaf and flower stalk anthracnose (twister) under field conditions. The causative agents of these diseases were identified as *Fusarium* sp., *Colletotrichum gloeosporioides*, *Alternaria* sp. and *Sclerotium* sp. (Gunaratna & Deshappriya 2014). Amongst these diseases, damping-off and basal rot were the more important production constraints. The pathogenicity of *Fusarium* sp. as the causative agent of damping-off disease of *Allium cepa* was confirmed by following Koch's postulates. *Fusarium* sp. has been mentioned as the causative agent of damping-off disease in onion at the nursery stage (Mishra *et al.* 2014). *Fusarium* spp. have been confirmed to be the causative agents of many diseases of economic importance *i.e. F. solani* caused root rot and pre-emergence damping-off at the seedling stage of soybean (Killebrew *et al.* 1988); *Fusarium solani* and *Fusarium oxysporum* wilt incidence, chlorosis of leaves and vascular discoloration in roots in cucumber Akrami (2015); *Fusarium solani* and *Rhizoctonia solani* damping-off and root rot diseases in tomatoes Karima & Nadia (2012) .

According to the bi-directional sequencing results of the ITS region, of rDNA *Fusarium* sp. the causative agent of damping-off disease of *A. cepa* was identified as *Fusarium solani*. As damping-off disease caused by *Fusarium solani* causes substantial losses to onion seedlings at the nursery stage, it is important that effective control measures are in place. The current practices used by farmers to control damping-off disease of onions is the application of fungicides such as Mancozeb, Homai, Thiram, Carbendazim, Captan as soil and seed treatments, use cultural practices such as farm sanitation, weeding and crop rotation. Although fungicide applications are effective, their use can pose many problems such as high costs incurred, the variability of results, deleterious effects on soil organisms, and most importantly the adverse effects on the environment and human health. Such deleterious effects have renewed the interest in using alternative agricultural practices in Sri Lanka. One such practice for disease management is the use of biological control agents.

Biological control agents have been used successfully to control a number of diseases in numerous crops and could be the best alternative especially against soil-borne pathogens such as *Fusarium solani* that causes damping-off of onions. *Trichoderma* spp. that are common saprophytic fungi found in almost all environments, have been investigated as potential biocontrol agents because of their ability to reduce the incidence of disease caused by plant pathogenic fungi by means of numerous mechanisms (Hanson 2000, Harman *et al.* 2003).

After confirmation of pathogenicity of *Fusarium solani*, the possibility of using the isolated *Trichoderma* spp. to control the pathogenic *Fusarium solani* was investigated under laboratory. The dual culture assay used for the preliminary evaluations on the effect of the *Trichoderma* isolates showed that all *Trichoderma* spp. isolated could control the mycelial growth of the pathogenic *Fusarium solani* significantly after six days of incubation indicating the possibility of using any one of them for the control of the pathogen. However, two of the *Trichoderma* isolates *i.e.* Tr. 3 and Tr. 1 showed higher control ability, growth rate and sporulation capacity were thus selected for further tests under greenhouse and field conditions. According to the sequencing results of the ITS region, Tr.1 and Tr.3 were identified as *Trichoderma virens* and *Trichoderma asperellum* respectively.

In the present study, the preliminary identification of fungal isolates was carried out using their morphological features and by observing their sporulating structures. Their identity was further confirmed through PCR analysis of rDNA gene ITS region using ITS 1 and ITS 4 primers. The amplified ITS fragment of rDNA gene of *Fusarium* spp. isolated from *A. cepa* seedlings from different regions were approximately 550 bp in size which was similar to those reported by Elsalam *et al.* (2003).

ITS region of rDNA gene of the *Trichoderma* spp. was amplified using ITS-1 and ITS-4 primers. The size of the amplified DNA products was 600 bp. These results are in accordance with several workers who observed that the amplified rDNA fragment to be approximately 500 to 600 bp by PCR in *Trichoderma* (Cornea *et al.* 2008, Chakraborty *et al.* 2010).

PCR-amplified rDNA ITS sequences have been used for the characterization, identification and detection of *Verticillium albo-atrum*, *V. dahlia, Trichoderma* spp. *Rhizoctonia solani*, *R. bataticola*, *Ramularia areola* and *Alternaria macrospora* (Nazar *et al.* 1991, Chakrabarty *et al.* 2007, Cornea *et al.* 2008, Wijesinghe *et al.* 2010). Accordingly, the PCR-amplified rDNA gene ITS sequences showed polymorphism at ITS region among the *Trichoderma* spp. But no distinct amplified product size variation in the ITS region of rDNA of the seven *Trichoderma* isolates could be observed after agarose gel electrophoresis and the migration of amplified products of each *Trichoderma* isolate was similar in size *i.e.* 600 bp suggesting that there was little variation between the amplified products. It is also possible that amplified product size variation present between *Trichoderma* isolates was not properly resolved by amplification profile of rDNA ITS sequences of the fungal genome with specific primers *i.e.* ITS-1 and ITS-4 but there may be a sequence level difference among these

amplified products.

Hoshino & Morimoto (2008) reported that NS 1 and FR 1 primers can be used to estimate fungal diversity in agricultural soils. Fagbola *et al.* (2004) have also used NS 1 and FR 1 primers to distinguish *Colletotrichum circinans* and *Colletotrichum coccodes.* PCR amplification of the 18S ribosomal DNA of both species produced 1.65 kb long fragments that covered almost the entire 18S rDNA molecule. Similarly, DNA fragments of 1600 bp in size were observed for *Trichoderma* spp.

Random amplification of polymorphic DNA (RAPD) or arbitrarily primed polymerase chain reaction (AP-PCR) is a simple and rapid method for detecting genetic diversity. Small changes in ITS sequence, *e.g.* single point mutations or deletions are often accompanied by RAPD patterns (Bridge *et al.* 2000). Accordingly, RAPD-PCR amplification of genomic DNA extracted from *Trichoderma* spp. using primers OPD 6, A-4, A-5, AA-11 produced multiple bright bands and revealed inter-specific polymorphisms among the *Trichoderma* isolates. Accordingly, the close morphological resemblance that exists among the species of *Trichoderma* can be resolved clearly without any controversy using RAPD as similar observations have been reported by several workers (Shalini *et al.* 2006, Cornea *et al.* 2008, Chakraborty *et al.* 2010). Also, it appears that RAPD is a promising molecular marker for the identification of *Fusarium solani i.e.* damping-off pathogen of *A. cepa* and ideal method for the identification and monitoring *Trichoderma asperellum* and *Trichoderma virens* after introduced into the soil to control -damping-off disease through antagonistic activity. This RAPD marker system should be further optimized to obtain sharp reproducible banding pattern to differentiate effective *Trichoderma* spp. *i.e. Trichoderma asperellum* and *Trichoderma virens* against *Fusarium solani.*

The ability of *Trichoderma* spp. to control plant diseases are attributed to a number of mechanisms such as competition, mycoparasitism, formation of restrictive structures, antibiosis, and production of secondary metabolites and even induction of resistance in the plant (Harman 2000, Howell 2003, Shalini *et al.* 2006).

As the dual plate assays carried out in the present study showed that the *Trichoderma* spp. tested possess a clear ability to control the growth of the pathogen, the mechanisms through which the control is achieved was investigated. Interaction between the two fungi when observed through the light microscope indicated that a mechanism adopted by two *Trichoderma* spp. to restrict *Fusarium solani* was formation of loops and coils around the pathogen hyphae and attachment of the hyphal tips to the hyphae of *Fusarium solani.* Barakat *et al.* (2006) have reported the control of *Sclerotium rolfsii* by *Trichoderma* spp. through the formation of similar structures.

The possibility of the more effective *Trichoderma* isolates (*Trichoderma virens* and *Trichoderma asperellum*) showing additional mechanisms of control was also investigated. As enzymes have been reported to be associated with mycoparasitic activities of *Trichoderma* spp. Chitinase production by the two *Trichoderma* spp. was tested using plate assays. The results showed that both isolates produced Chitinase enzymes and although an accurate quantitative assessment is difficult in such plate assays, a variation in the level of enzyme production was observed. These results indicate that chitinase activity may be contributing to the control of the pathogenic *Fusarium* by acting on the chitin component of the cell walls. These results were in agreement with laboratory experiments carried out by Abo-Elyousr *et al.* (2014) who reported that mycoparasitism in *Trichoderma* was associated with its capacity to produce extracellular chitinase. Chitinases and/or glucanases function by breaking down the polysaccharides, chitin, and β-glucans that are responsible for the rigidity of pathogen cell walls (Howell 2003). Additionally, the results of the present study showed that both *Trichoderma* spp. also produced volatile compounds which reduced the hyphal growth of *F. solani* under *in vitro* conditions.

CONCLUSIONS

The present evaluation thus gave clear indication that *Trichoderma virens* and *Trichoderma asperellum* isolated from soils of *Allium cepa* growing areas possess a diverse array of mechanisms to control the damping-off pathogen, *Fusarium solani* of big onion and thus have the potential to be effectively used in the management of *A. cepa* damping-off disease in the field.

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