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Abstract: *Fusarium oxysporum* f. sp. *lycopersici* causing tropical wilt of tomato is a destructive phytopathogen. To study bio efficacy of fungal bio agents, fifteen isolates of *Trichoderma* species were isolated from rhizosphere soil of healthy tomato plants. Among the isolates, TA12 showed higher antagonistic efficacy against the pathogen. Upon analysis of the rDNA of internal transcribed spacers (ITS) and molecular data, the isolate was identified as *Trichoderma atroviride*. The *in vitro* antagonistic assessment indicated that the *T. atroviride* isolate caused significant inhibition of *F. oxysporum* f. sp. *lycopersici*. *Trichoderma atroviride* (TA12) showed antagonistic activity against *F. oxysporum* with mycelial inhibition of 71.25%. The culture filtrates of *Trichoderma atroviride* exhibited its antifungal activity against *F. oxysporum* with a suppression of 77.77%. Moreover, the ethyl acetate extracts of *T. atroviride* TA12 showed the highest antifungal potency against *F. oxysporum* f. sp. *lycopersici*. The main bioactive constituents of *T. atroviride* were 6-pentyl - 2H-pyran-2-one, quinoline, phenol, 2-(6-hydrazino-3-pyridazinyl) and heptadecane. In conclusion, the isolate TA12 could be exploited to develop an effective biocontrol management practice for combating Fusarium wilt disease of *Fusarium oxysporum* f.sp. *lycopersici* in tomato.

Keywords: Anti-fungal activity, *Fusarium oxysporum* f. sp. *lycopersici*, tomato and *Trichoderma* and GC-MS.

INTRODUCTION

Tomato (*Lycopersicon esculentum*) belonging to solanaceae family, ranks first and second in processing crops and vegetables respectively in India (Fatima and Anjum, 2017). It is commercially cultivated globally in both indoor and outdoor conditions. It contains lycopene, a rich source of anti-oxidant property (Miller *et al.*, 2002). Cultivation of tomato becomes limited due to invasion of wide pests *viz.*, insects, diseases, weeds and nematodes which accounts for major yield loss. *Fusarium oxysporum* f. sp. *lycopersici* (Fol) infecting tomato is a destructive pathogen, causing severe

economic losses all over the world. Major symptoms include yellowing of lower leaves, stunted growth, wilting of leaves and finally death of plant (Prihatna *et al.*, 2018). Chemical control of plant disease management is commonly employed approach (Hirooka and Ishii, 2013). The efficiency of fungicides chiefly depends on the timing of application, method of application, disease intensity, the efficiency of disease forecasting systems and the rate of emergence of fungicide resistant strains (Skamnioti and Gurr, 2009). Since, plant disease management using fungicides have constraints on environment and paves for evolution of resistant in pathogen, biological control using potential antagonists play a key approach in managing tomato wilt disease (Horinouchi *et al.*, 2010, Zhao *et al.*, 2011). *Trichoderma* spp. (*Hypocrea*) have found to be the most effective antagonists as they have mechanisms like mycoparasitism, antibiosis, competition and induced systemic resistance in host plants (Rodriguez *et al.*, 2020). Numerous *Trichoderma* isolates secreted many volatile and non-volatile substances that one anti-fungal in nature against soil borne pathogens (Nagamani *et al.*, 2017). Besides disease control, *Trichoderma harzianum* also associated with enhancing soil fertility (Liton *et al.*, 2019). This study exploits the anti-fungal efficiency of *Trichoderma atroviride* against wilt disease of tomato.

MATERIALS AND METHODS

Isolation and identification of pathogen

The various isolates of the pathogen tomato wilt were collected from infected tomato plants in different places of Madurai district. The isolate FO (Maa)-5 was found highly virulent. This isolate was identified as *Fusarium oxysporum* f.sp. *lycopersici* based on sequencing of ITS region (Accession number: MZ043720). The pathogen was maintained on PDA slants and used for further studies.

Isolation of Trichoderma

Soil samples from rhizosphere region (3 cm) of healthy tomato plants were collected from 15 different locations of Madurai district, Tamil Nadu. The collected samples were dried and subjected to serial dilution (up to 10^{-4}). The biocontrol agent was isolated using the selective medium of *Trichoderma* (TSM) and incubated for 7 days at $25 \pm 3^\circ\text{C}$ (Awad *et al.*, 2018). Later the putative colonies were purified by single hyphal tip method. General biochemical tests were done to confirm the biocontrol agent. Later these cultures were preserved in PDA slants for further studies.

Antifungal assay using Trichoderma spp. against pathogen

Antifungal assay was carried out to evaluate the antimicrobial efficacy of the potential isolates of *Trichoderma* spp. against the pathogen.

i. **Dual culture assay**

The dual culture described by Yassin et al. (2021) was followed to test the antagonistic ability of *Trichoderma* species against the pathogen. Small block (5 mm disc) of *Fusarium* cut from the periphery was placed at one cm away from the periphery of the Petri dish previously poured with PDA. Similarly, the *Trichoderma* isolate was placed one cm away from the edge of the same Petri plate aseptically on the opposite end and plates were incubated at room temperature for 5 days. The experiment was replicated thrice and per cent growth inhibition was calculated by using the following formula,

$$I = \frac{(A - B)}{A} \times 100$$

Where A is mycelial growth of pathogen in control plate, B is mycelial growth of pathogen in treatment plate and I is the percent inhibition of mycelial growth.

ii. **Effect of culture filtrates on inhibition of pathogen**

Mycelial plugs were taken from the freshly grown *Trichoderma* cultures and inoculated into conical flask containing fresh 100 ml potato dextrose broth and incubated for 7 days at 150 rpm at 28°C (You et al., 2016). Supernatant of the cultures were collected and centrifuged at 9000 rpm for 10 min. Then the cell free filtrates were sterilized through a 0.22 µm millipore filters and mixed with unsolidified PDA medium at 10% (v/v) concentration. Uninoculated PDB was added to PDA with same ratio for control. Mycelial disc of the pathogen was placed in all PDA plates and kept for incubation at 28°C for 5 days. Reduction in mycelial growth of the pathogen was measured and per cent inhibition over control was arrived by the formula of Sreedevi et al. (2011),

$$I = \frac{(C - R)}{C} \times 100$$

Where,

C - Mean linear growth of pathogen in control , R - Mean linear growth of pathogen in treatments

Extraction of Trichoderma DNA and PCR amplification

The potent cultures were inoculated in conical flask containing 100 ml of potato dextrose broth and incubated in shaker 150 rpm for 7 days. The mycelial mat was sieved and pierced into powder using liquid nitrogen (Liu et al., 2020). DNA extraction of virulent isolates was done by using the procedure of Zhang et

al. (2010). Genomic DNA was isolated by using CTAB method. PCR amplification was carried out using universal primers – internal transcribed spacer ITS1 (5'TCCGTTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Polymerase chain reaction was performed in a reaction mixture of 50 µl with 35 cycles including 63 ng of genomic DNA, 50 pmol of each primer, 500 µM concentrations of dNTPs and 1.25 units of *Taq* DNA polymerase in an Eppendorf thermal Cycler. The PCR programme was performed with initial denaturation (95oC for 2 mins), followed by the repeated cycles of denaturation (94oC 1min), annealing (56oC for 30 sec) and extension (72oC for 1 min), and final extension of 72oC for 10 min. Amplicons were detected by 2% (w/v) agarose gel electrophoresis. Sequencing of purified PCR product was done at Eurofins Genomics India Pvt. Ltd. Bangalore.

Identification of the Trichoderma sp. and phylogenetic relationships

ITS region of potential isolate was sequenced and BLAST searched with sequences in the NCBI, GenBank. Phylogenetic dendrogram was constructed by the neighbor-joining method in MEGA 10.0 software depending over multiple sequence alignment with an evolutionary distance of 0.05. The tree topologies were evaluated by performing analysis of 1000 data sets. The sequence was submitted to GenBank for obtaining accession number.

Preparation of crude extracts

Mycelial disc from an actively growing colony of *Trichoderma* isolate was inoculated into fresh potato dextrose broth and incubated for seven days. The culture filtrates were collected by filtering using Whatmann no.1 filter paper followed by centrifugation at 9000 rpm for 15 min and finally the metabolites were extracted using ethyl acetate as solvent (Jantarach and Thanaboripat, 2010). Further concentration of extracts and elimination of solvents were done using rotary evaporator (Sharma *et al.*, 2016).

Gas chromatography mass spectrometry (GCMS)

The extract possessing high antimicrobial property has been subjected to GCMS analysis. The antibiotics, volatiles and secondary metabolites present in the sample were detected by injecting one microlitre of sample in Capillary Standard Non – Polar Column of GC - MS in which Helium was used as carrier gas. The analytical conditions were adjusted by following the procedures given by Yassin *et al.*, (2020). The m/ z peaks representing mass to charge ratio, characteristic of the antimicrobial fractions were compared with those of the corresponding organic compounds in the NIST library (Manigundan *et al.*, 2020).

Thin layer chromatography (TLC)

Thin Layer Chromatography was performed to identify the presence of antifungal compounds in crude extract of *Trichoderma* isolate. TLC tank was filled with acetone and chloroform solvents in the ratio of 3:1 and sealed the tank immediately (Vivek *et al.*, 2013). Desired size of TLC plate (60 F254, Merck, India) was taken and marked 0.5 cm above the bottom corner of plate. Samples were spotted at 1 cm distance and labelled. Spotted TLC plate was allowed to run in TLC tank. Then the plate was removed and visualized in laminar under UV fluorescence light (254 nm) and marked the dark purple fluorescence with pencil. The R_f value was calculated based on the distance covered (Fried and Sherma, 1982), R_f= Distance travelled by substance / Distance travelled by solvent

Statistical analysis

Statistical analysis were performed using analysis of variance (ANOVA) by SPSS software version 16 (SPSS.Chicago). The data were tabulated as mean of triplicates $\pm$ standard error and will be considered significant when the P < 0.05 and the means were compared by Duncan's Multiple Range Test (DMRT).

RESULTS

Antifungal assay

The results of antifungal assay revealed that all the *Trichoderma* isolates possessed certain amount of antifungal activity both in dual and culture filtrate assays.

a) **Dual culture assay**

A total of fifteen isolates of *Trichoderma* spp. were isolated from the rhizosphere soil of healthy tomato plants. Among the isolates, *Trichoderma* isolate TA 12 was found superior against *Fusarium oxysporum* with 71% mycelial inhibition over control (Fig 1). The next best isolate was TA 2 with mycelial inhibition of 68.75%. Isolate TA 5 recorded minimum inhibition percentage of 46.22 (Table 1)

b) ***Trichoderma* culture filtrate assay against Fol**

The experimental results revealed that all the isolates inhibited the mycelial growth of pathogen at significant level.. Among the isolates tested, TA12 showed the maximum mycelial inhibition of 77.77% (Fig2). This was followed by the isolate TA 2 (75.65%). The least

Table 1
Antifungal assay of *Trichoderma* isolates against *Fusarium oxysporum* fsp *lycopersici*

S.No.	Treatments	Mycelial growth (cm)*	Per cent mycelial inhibition
1.	TA - 1	4.2 ^{ij}	47.50(43.57)
2.	TA - 2	2.5 ^b	68.75(56.01)
3.	TA - 3	3.9 ^h	51.23(45.70)
4.	TA - 4	3.3 ^e	58.75(50.04)
5.	TA - 5	4.3 ^j	46.22(42.83)
6.	TA - 6	3.5 ^f	56.23(48.58)
7.	TA - 7	2.7 ^c	66.25(54.48)
8.	TA - 8	3.2 ^e	60.00(50.77)
9.	TA - 9	4.1 ⁱ	48.77(44.30)
10.	TA - 10	3.5 ^f	56.21(48.57)
11.	TA - 11	3.0 ^d	62.50(52.24)
12.	TA - 12	2.3 ^a	71.25(57.58)
13.	TA - 13	4.1 ⁱ	48.74(44.28)
14.	TA - 14	2.9 ^d	63.76(52.99)
15.	TA - 15	3.7 ^g	53.73(47.14)
Control		8.0	0.00(0.00)
CD (P=0.05)		1.37	

* Mean of three replications

Values with different superscripts are significantly differ from each other at $p < 0.05$ Values in the parenthesis are arc sine transformed values



Fig. 1
In vitro dual confrontation assay of antagonistic *Trichoderma* isolates against *Fusarium oxysporum* f.sp. *lycopersici*



Fig. 2
Anti-mycotic potency of culture filtrate of *Trichoderma* spp (TA12) against *Fusarium oxysporum* f.sp. *lycopersici*

mycelial growth was observed in TA 5 at the rate of 52.20% (Table 2).

Molecular confirmation of potential Trichoderma isolate TA12

PCR of *Trichoderma* isolate with ITS-1 and ITS-4 primer pairs resulted in amplification of a fragment of size 636 bp (Fig 3).

Table 2
Antifungal assay of culture filtrates of *Trichoderma*
isolates against *Fusarium oxysporum* f.sp. *lycopersici*

S.No.	Treatments	Mycelial growth (cm)*	Per cent mycelial inhibition
1.	TA - 1	4.2 ^j	53.33(46.91)
2.	TA - 2	2.2 ^b	75.65(60.43)
3.	TA - 3	3.7 ^{hi}	58.88(50.11)
4.	TA - 4	3.0 ^{de}	66.67(54.74)
5.	TA - 5	4.3 ^j	52.20(46.26)
6.	TA - 6	3.1 ^{ef}	65.55(54.06)
7.	TA - 7	2.3 ^{bc}	74.45(59.64)
8.	TA - 8	2.8 ^d	68.88(56.09)
9.	TA - 9	4.0 ^{ij}	55.54(48.18)
10.	TA - 10	3.3 ^{fg}	63.33(52.73)
11.	TA - 11	2.5 ^c	72.22(58.19)
12.	TA - 12	2.0 ^a	77.77(61.87)
13.	TA - 13	3.8 ⁱ	57.76(49.46)
14.	TA - 14	2.5 ^c	72.22(58.19)
15.	TA - 15	3.4 ^{gh}	62.20(52.06)
Control		9.0	0.00(00.00)
CD (P=0.05)		1.97	

* Mean of three replications

Values with different superscripts are significantly differ from each other at $p < 0.05$ Values in the parenthesis are arc sine transformed values

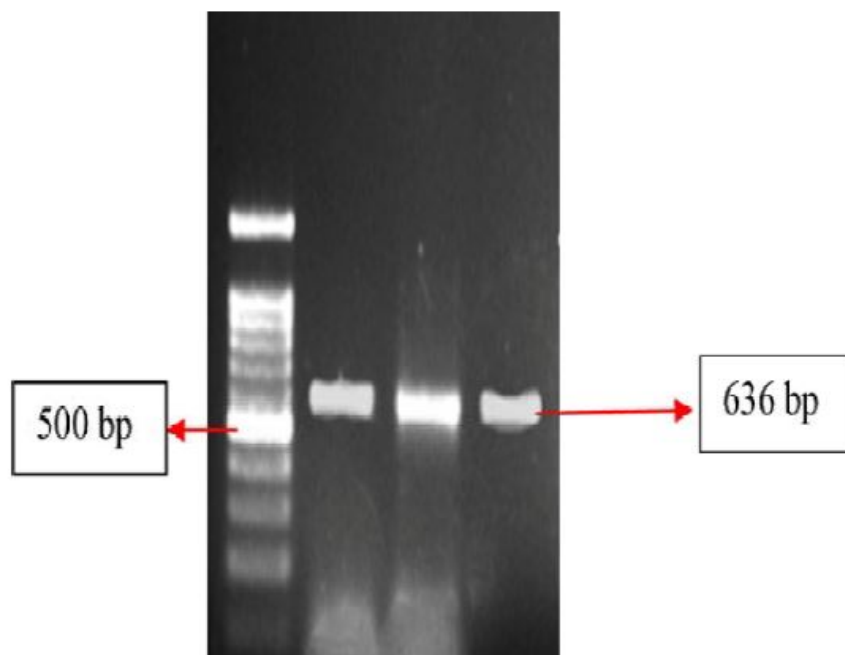


Fig. 3
The results of ITS amplification in potent

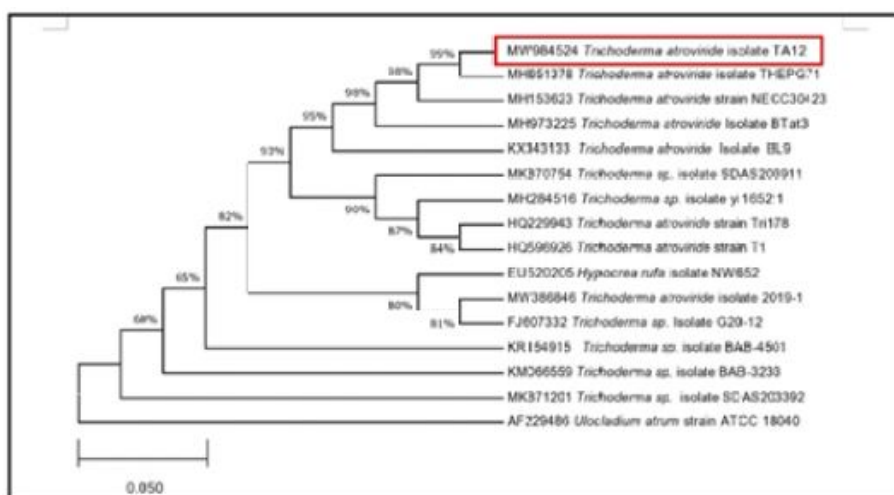


Fig. 4
Phylogenetic analysis of *Trichoderma atroviride* using neighbor joining method. The numbers over branches indicates bootstrap coefficient.

Trichoderma isolates

Phylogenetic analysis of the sequence (TA 12) with existing sequences in the NCBI database showed 99% sequence similarity with *Trichoderma atroviride* (Fig 4). The sequence was deposited in Genbank and obtained accession number (MW984524; Fig 5).

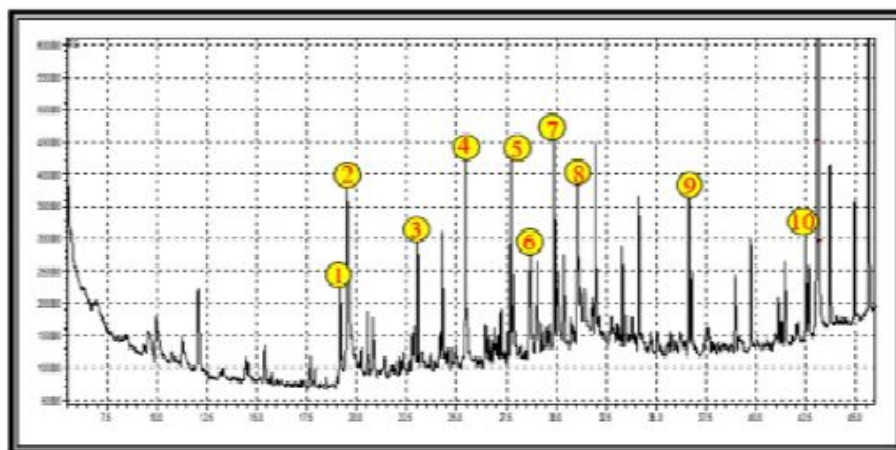


Fig. 5
GCMS chromatogram for detection of secondary metabolites
in *Trichoderma atroviride* using ethyl acetate as solvent.

GC- MS analysis of extracts of Trichoderma

The extracts of *T. atroviride* were analyzed to determine its active chemical constituents. Active constituents of *T. atroviride* extract were demonstrated in Fig 5 and Table 3. The results showed that a numerous compounds produced by *Trichoderma atroviride*, possessing high antimycotic property.

TLC of Trichoderma spp.

The TLC plate with the sample was observed under UV laminar fluorescence. The spot was resolved without any smear or streak pattern in TLC plate. In case of chitinase (developed in acetone:chloroform (3:1)) distinct spots were visualized under UV light (254 nm) with Rf value of 0.84 The distance travelled by the substance was 5.1 cm.

Table 3
Identification of secondary metabolites from *Trichoderma atroviride* through GCMS analysis

Peak	Retention Time	Compound Name	Structure	Molecular Formula	Molecular Weight (g/mol)	Peak area %
1	19.162	Quinoline	Importar imagen	C9H7N	129.16	1.92
2	19.518	2H-Pyran-2-one, 6-pentyl	Importar imagen	C10H14O2	166.22	6.25
3	23.056	Nonadecane	Importar imagen	C19H40	268.5	1.81
4	25.450	Heptadecane	Importar imagen	C17H36	240.5	3.20
5	27.725	Heneicosane	Importar imagen	C21H44	296.57	3.11
6	27.866	Eicosane	Importar imagen	C20H42	282.54	1.68
7	28.690	Phenol	Importar imagen	C6H5OH	94.11	1.77
8	31.062	Dibutyl phthalate	Importar imagen	C16H22O4	278.34	2.79
9	36.633	Hexadecane, 1-iodo	Importar imagen	C16H33I	352.34	3.54
10	42.653	Benzene propanoic acid	Importar imagen	C9H10O4	182.17	1.78

DISCUSSION

Trichoderma species have a global range of distribution and live in a variety of ecological niches, including decaying bark and wood, other fungus, soil, and healthy plant roots, stems, and leaves (Du Plessis *et al.*, 2018; Mukherjee *et al.*, 2013). The number of *Trichoderma* species used in biocontrol has drastically increased in modern era. Up to date, more than 290 *Trichoderma* species have been discovered (Bissett *et al.*, 2015; Du Plessis *et al.*, 2018; Zhu *et al.*, 2017).

In this study, a survey was conducted and obtained fifteen isolates of *Trichoderma* after isolation. Among the isolates, *Trichoderma* isolate TA12 showed greater inhibition against the *Fusarium* strain than the other *Trichoderma* isolates. TA12 suppressed the mycelial growth of the pathogen (*Fusarium oxysporum* f. sp. *lycopersici*) by 71%. The results were in accordance with Schoffen *et al.* (2020), who reported that *T. atroviride* strain suppressed the mycelial growth of *F. oxysporum* in the range of 52.37% – 70.56%. *Trichoderma virens* exhibited a mycelial inhibition percentage of 80 against *Fusarium* wilt (Banerjee *et al.*, 2020). Sallam *et al.* (2019) confirmed the antagonistic potency of *T. atroviride* strain against *Fusarium* wilt of tomato with a mycelial inhibition rate of 66.80%.

The metabolites produced by *Trichoderma* spp. inhibited *Fusarium* isolates. Among the 15 isolates tested, 12 were able to inhibit the growth of *Fusarium oxysporum* (>50%) within which six isolates showed relatively strong inhibitory effect (>60%). Further in vitro assay of *Trichoderma* culture filtrates against Fol confirmed the similar trend as

the TA 12 isolate recorded the highest inhibition of the pathogen (77%). Our findings are consistent with those of Rudresh *et al.* (2005), who reported the antimicrobial efficiency of culture filtrates of *T. harzianum* against *F. oxysporum* strain, recording mycelial inhibition rates of 78.5%. Alvarez-Garcia *et al.* (2020) also reported the suppression of mycelial growth of *Fusarium* spp. by the culture filtrates of *T. harzianum* and recorded the inhibition rate of 76.27%. Findings of Tomah *et al.* (2020) proved that *Trichoderma citrinoviride* retarded the growth of fungal pathogen at 77.8%.

The potent antagonist (TA 12) was subjected to sequencing of ITS regions and phylogenetic analysis. The phylogenetic analyses indicated that the isolate shown 99% similarity with other *T. atroviride* isolates thus TA12 confirmed as *T. atroviride*.

The antifungal ability of *T. atroviride* was confirmed by performing GCMS. Previous studies indicated that these compounds inhibited the mycelial growth of different pathogenic fungal strains (Keszler *et al.*, 2000, Jelen *et al.*, 2014, Mallaiah *et al.*, 2016). The main constituents alone do not attribute to the antifungal activity but also the presence of other bioactive substances attributed to antifungal potency. The antifusarial potency of *T. atroviride* extract may be attributable to the presence of many bioactive compounds such as 6-pentyl-2H-pyran-2-one, quinoline, phenol, 2-(6-hydrazino-3-pyridazinyl), heptadecane, 17-methoxy-4-methyl-*d*-homo-18-norandrosta, nonadecane, heneicosane, eicosane, dibutyl phthalate, hexadecane and benzene propionic acid. The antifungal efficacy of the extract may also be referred to the synergistic effect among the bioactive components (Khan *et al.*, 2020).

Thin layer chromatography was done to separate and identify antifungal compound of *T. atroviride*.

The Rf value calculated was similar to the values obtained with separation of enzymes from *Trichoderma* isolates (Rabinal and Bhat, 2017).

Vinale *et al.* (2008) also revealed the same range of values when isolated from *Trichoderma*. The Rf value of 0.86 was identified in TLC separation of *T. harzianum* isolates (Kiss *et al.*, 2000).

Many studies indicated that *Trichoderma* spp. possess the multiple mechanisms, including mycoparasitism, extracellular enzymes such as cellulase, amylase, pectinase, protease and chitinase, antagonistic compounds and induced resistance, to inhibit pathogens and reduce diseases (Cherkupally *et al.*, 2017). Thus, the *Trichoderma atroviride* TA12 possibly uses multiple mode of action to inhibit pathogen, while antifungal compounds secreted by it could have played a major role in inhibiting pathogen and controlling fusarium wilt incidence of tomato.

The potent antagonist, *Trichoderma atroviride* isolate exhibited excellent antimycotic activity against Fusarial phytopathogen of tomato. Hence its antimicrobial potency of culture filtrates and organic solvent extracts against fusarial pathogen of tomato highlights the ability to employ novel and safe biofungicide in order to neglect the hazards of chemical fungicides on the human health and environment.

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