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CONTENTS

In this Issue

i-ii

Review

- Phytoremediation of indoor air pollutants: Harnessing the potential of plants beyond aesthetics** 131-143
Shalini Jhanji and U.K.Dhatt

Research Articles

- Response of fruit yield and quality to foliar application of micro-nutrients in lemon [*Citrus limon* (L.) Burm.] cv. Assam lemon** 144-151
Sheikh K.H.A., Singh B., Haokip S.W., Shankar K., Debbarma R.
- Studies on high density planting and nutrient requirement of banana in different states of India** 152-163
Debnath Sanjit Bauri F.K., Swain S., Patel A.N., Patel A.R., Shaikh N.B., Bhalerao V.P., Baruah K., Manju P.R., Suma A., Menon R., Gutam S. and P. Patil
- Mineral nutrient composition in leaf and root tissues of fifteen polyembryonic mango genotypes grown under varying levels of salinity** 164-176
Nimbolkar P.K., Kurian R.M., Varalakshmi L.R., Upreti K.K., Laxman R.H. and D. Kalaivanan
- Optimization of GA3 concentration for improved bunch and berry quality in grape cv. Crimson Seedless (*Vitis vinifera* L)** 177-184
Satisha J., Kumar Sampath P. and Upreti K.K.
- RGAP molecular marker for resistance against yellow mosaic disease in ridge gourd [*Luffa acutangula* (L.) Roxb.]** 185-192
Kaur M., Varalakshmi B., Kumar M., Lakshmana Reddy D.C., Mahesha B. and Pitchaimuthu M.
- Genetic divergence study in bitter melon (*Momordica charantia* L.)** 193-198
Nithinkumar K.R., Kumar J.S.A., Varalakshmi B, Mushrif S.K., Ramachandra R.K. , Prashanth S.J.
- Combining ability studies to develop superior hybrids in bell pepper (*Capsicum annuum* var. *grossum* L.)** 199-205
Varsha V., Smaranika Mishra, Lingaiah H.B., Venugopalan R., Rao K.V. Kattedgoudar J. and Madhavi Reddy K.
- SSR marker development in *Abelmoschus esculentus* (L.) Moench using transcriptome sequencing and genetic diversity studies** 206-214
Gayathri M., Pitchaimuthu M. and K.V. Ravishankar



Generation mean analysis of important yield traits in Bitter gourd (<i>Momordica charantia</i>)	215-221
Swamini Bhoi, Varalakshmi B., Rao E.S., Pitchaimuthu M. and Hima Bindu K.	
Influence of phenophase based irrigation and fertigation schedule on vegetative performance of chrysanthemum (<i>Dendranthema grandiflora</i> Tzelev.) var. Marigold	222-233
Vijayakumar S., Sujatha A. Nair, Nair A.K., Laxman R.H. and Kalaivanan D.	
Performance evaluation of double type tuberose IHR-4 (IC-0633777) for flower yield, quality and biotic stress response	234-240
Bharathi T.U., Meenakshi Srinivas, Umamaheswari R. and Sonavane, P.	
Anti-fungal activity of <i>Trichoderma atroviride</i> against <i>Fusarium oxysporum</i> f. sp. <i>Lycopersici</i> causing wilt disease of tomato	241-250
Yogalakshmi S., Thiruvudainambi S., Kalpana K., Thamizh Vendan R. and Oviya R.	
Seed transmission of bean common mosaic virus-blackeye cowpea mosaic strain (BCMV-BICM) threaten cowpea seed health in the Ashanti and Brong-Ahafo regions of Ghana	251-260
Adams F.K., Kumar P.L., Kwoseh C., Ogunsanya P., Akromah R. and Tetteh R.	
Effect of container size and types on the root phenotypic characters of <i>Capsicum</i>	261-270
Raviteja M.S.V., Laxman R.H., Rashmi K., Kannan S., Namratha M.R. and Madhavi Reddy K.	
Physio-morphological and mechanical properties of chillies for mechanical harvesting	271-279
Yella Swami C., Senthil Kumaran G., Naik R.K., Reddy B.S. and Rathina Kumari A.C.	
Assessment of soil and water quality status of rose growing areas of Rajasthan and Uttar Pradesh in India	280-286
Varalakshmi LR., Tejaswini P., Rajendiran S. and K.K. Upreti	
Qualitative and organoleptic evaluation of immature cashew kernels under storage	287-291
Sharon Jacob and Sobhana A.	
Physical quality of coffee bean (<i>Coffea arabica</i> L.) as affected by harvesting and drying methods	292-300
Chala T., Lamessa K. and Jalata Z	
Vegetative vigour, yield and field tolerance to leaf rust in four F1 hybrids of coffee (<i>Coffea arabica</i> L.) in India	301-308
Divya K. Das, Shivanna M.B. and Prakash N.S.	
Limonene extraction from the zest of <i>Citrus sinensis</i>, <i>Citrus limon</i>, <i>Vitis vinifera</i> and evaluation of its antimicrobial activity	309-314
Wani A.K., Singh R., Mir T.G. and Akhtar N.	
Event Report	315-318
National Horticultural Fair 2021 - A Success Story	
Dhananjaya M.V., Upreti K.K. and Dinesh M.R.	
Subject index	319-321
Author index	322-323

Original Research Paper

Anti-fungal activity of *Trichoderma atroviride* against *Fusarium oxysporum* f. sp. *lycopersici* causing wilt disease of tomato

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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 $0.22 \mu\text{m}$ 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'TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990). Polymerase chain reaction was performed in a reaction mixture of $50 \mu\text{l}$ with 35 cycles including 63 ng of genomic DNA, 50 pmol of each primer, $500 \mu\text{M}$ concentrations of dNTPs and 1.25 units of *Taq* DNA polymerase in an eppendorf

thermal Cycler. The PCR programme was performed with initial denaturation (95°C for 2 mins), followed by the repeated cycles of denaturation (94°C 1min), annealing (56°C for 30 sec) and extension (72°C for 1 min), and final extension of 72°C 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),

$$R_f = \frac{\text{Distance travelled by substance}}{\text{Distance travelled by solvent}}$$

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% (Fig 2). This was followed by the isolate TA 2 (75.65%). The least

Table 1. Antifungal assay of *Trichoderma* isolates against *Fusarium oxysporum* f.sp. *lycopersici* in dual culture assay

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

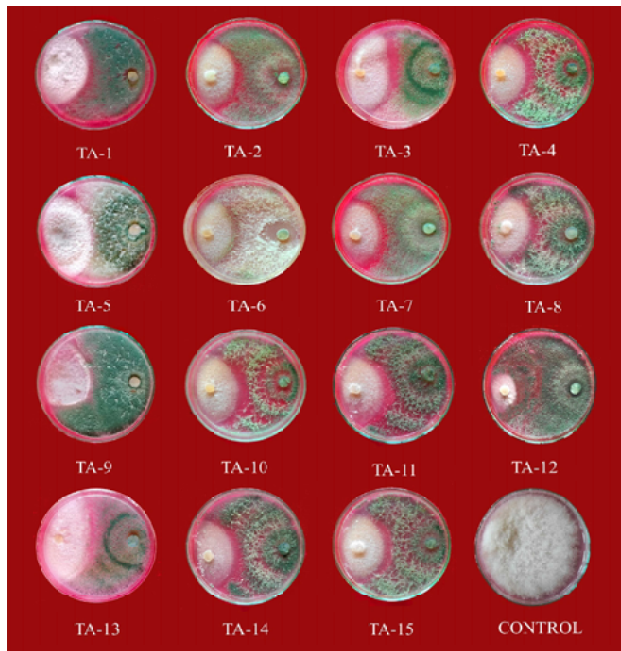


Fig. 1. In vitro dual confrontation assay of antagonistic *Trichoderma* isolates against *Fusarium oxysporum* f.sp. *lycopersici*



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 ^{dc}	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

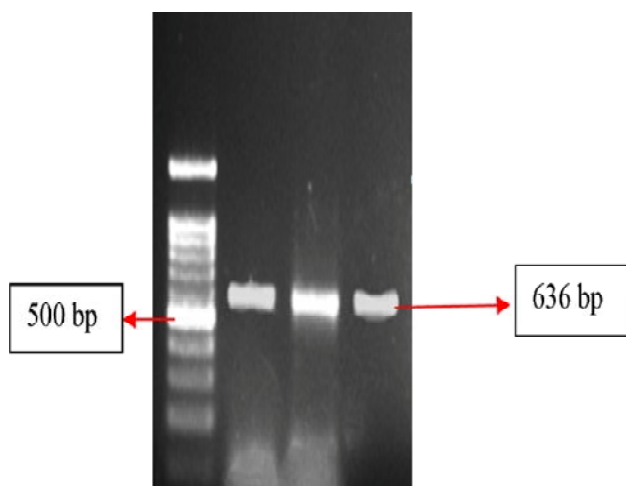


Fig. 3. The results of ITS amplification in potent *Trichoderma* isolates

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).

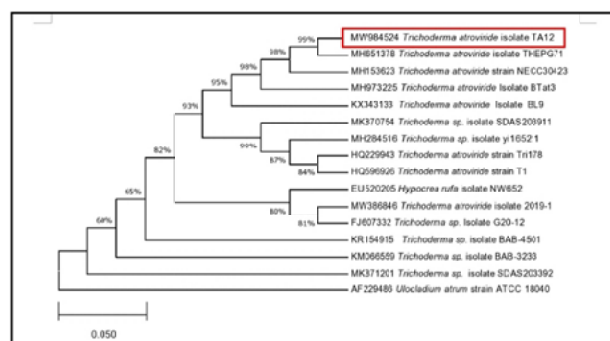


Fig. 4. Phylogenetic analysis of *Trichoderma atroviride* using neighbor joining method. The numbers over branches indicates bootstrap coefficient.

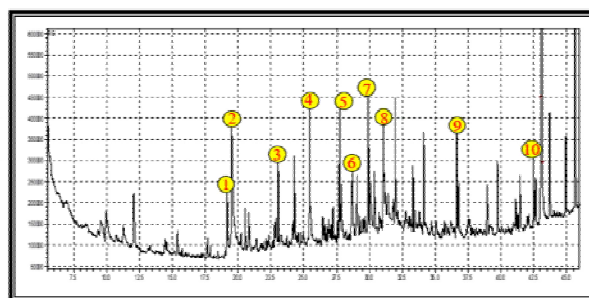


Fig. 5. GCMS chromatogram for detection of secondary metabolites in *Trichoderma atroviride* using ethyl acetate as solvent.

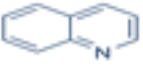
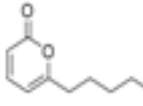




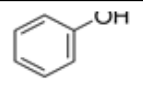
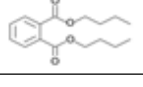


GC- MS analysis of extracts of *Trichoderma*

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 R_f 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		C_9H_7N	129.16	1.92
2	19.518	2H-Pyran-2-one, 6-pentyl		$C_{10}H_{14}O_2$	166.22	6.25
3	23.056	Nonadecane		$C_{19}H_{40}$	268.5	1.81
4	25.450	Heptadecane		$C_{17}H_{36}$	240.5	3.20
5	27.725	Heneicosane		$C_{21}H_{44}$	296.57	3.11
6	27.866	Eicosane		$C_{20}H_{42}$	282.54	1.68
7	28.690	Phenol		C_6H_5OH	94.11	1.77
8	31.062	Dibutyl phthalate		$C_{16}H_{22}O_4$	278.34	2.79
9	36.633	Hexadecane,1-iodo		$C_{16}H_{33}I$	352.34	3.54
10	42.653	Benzene propanoic acid		$C_9H_{10}O_4$	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, Mallaiiah *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 anti-fusarial 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-3pyridazinyl), heptadecane, 17-methoxy-4-methyl-d-homo-18-norandrostane, 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 R_f 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 R_f 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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