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Tetradecane producing biocontrol agent, *Trichoderma* spp. against *Fusarium oxysporum* in tomato (*Solanum lycopersicum* L.)

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Abstract The secondary metabolites from *Trichoderma harzianum* - Tr01 and *Trichoderma viride*- Tr02 were extracted from liquid culture using ethyl acetate and tested for their activities against fungal pathogen, *Fusarium oxysporum (*Fuo4*)* causing wilt disease in tomato (*Solanum lycopersicum* L.). Two isolates of *Trichoderma harzianum* (Tr01), *Trichoderma viride* (Tr02) were antagonized *F. oxysporum* causing tomato wilt. *T. harzianum* Tr01 showed the highest chitinase activity of β-1-3-glucanase. The treated inoculated tomato with *Trichoderma* extract was investigated. *Trichoderma harzianum (*Tr01) showed strongest inhibitory activities against *Fusarium oxysporum*. *T. harzianum* (Tr01) found to produce Dodecane, tetradecane, Diethyl Phthalate, Hexadecane, Benzenepropanoic acid, 3,5- bis (1,1-dimethylethyl)-4-hydroxy-, methylester, n-Hexadecanoic acid, Tetracontane, Bis(2-ethylhexyl) phthalate, Tetracontane. It concluded that *Trichoderma harzianum* (Tr01), *Trichoderma viride* (Tr02) can be produced active metabolites to control *Fusarium* wilt of tomato. The bioinoculants of *T. harzianum* Tr01 and *Trichoderma viride* Tr02 was highly inhibited the tested plant pathogen and enhanced plant growth.

Keywords: Antagonistic effects, *Fusarium*, Biological control, *Trichoderma* spp., Tetradecane.

Introduction

Tomato (*Solanum lycopersicum* L.) belongs to *Solanaceae*, and is an important nutritive and nursery-based vegetable crop cultivated for its fleshy fruit in throughout the world. Tomato fruits can be eaten raw or cooked and also used to preparae soup, juice, ketchup, puree, paste and powder. It is a good source of vitamin A and C, β-carotene pigment, and also contains minerals like iron, phosphorus etc. (Abed *et al.*, 2013). But the tomato plant is infected by distinct diseases that significantly affect its growth and yield. *Fusarium* wilt is very harmful disease affecting crop loss which is caused by *Fusarium oxysporum, Fusarium lycopersici* (Sacc.) (Enespa and Dwivedi, 2014). *Fusarium oxysporum* is a soil borne fungal pathogen that infects plants through

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roots at all stages of plant growth which causing major economic losses by inducing necrosis and wilt in many crops. The disease causes great losses, especially on the susceptible varieties of tomato when soil and air temperature are high during the warm season (Mandal *et al.*, 2009). *Trichoderma* species are biocontrol agents which successfully used as biofungicide worldwide (Ghisalberti and Sivasithambaram, 1991; Reino *et al.*, 2008; Sivasithembaram and Ghisalberti, 1998) and produced secondary metabolites as antifungal substances such as 6-n- pentyi-6H-pyrone-2-one, gliotoxin, viridian, harzianopyridine and peptaibols (Reino *et al.*, 2008, Sivasithembaram and Ghisalberti, 1998; Vey *et al.*, 2001). Volatile organic compounds **(**VOCs) are diverse group of natural compounds that is produced by organisms in survival during the basic functions of competition, symbiosis, metal transport, differentiation (Demain and Fang, 2000). Chemical and biological properties of compounds used for medical, pharmaceutical, and agricultural purposes (Calvo *et al.*, 2002).

The objectives were to isolate the major secondary metabolites produced in liquid culture of *Trichoderma harzianum* and *Trichoderma viride* as biocontrol strains (Tr01-333257, Tr02-333256), and elucidated tetradecane, tetracontane, octadecanoic acid, and 2- furancarboxaldehyde from culture filtrates.

Materials and methods

Trichoderma and Fusarium isolates

Soil samples (50g each) were collected from distant habitation in Salem, Tamil Nadu, India. It collected from top 10-15 cm depth of root rhizosphere soil. The samples were labeled in polythene paper bag separately and brought to laboratory, then stored at 4° C until used. Five-fold serial dilutions of each soil sample were prepared in sterilized distilled water, and 0.5 ml diluted sample was poured on the surface of Potato Dextrose Agar (PDA) (Himedia, India). Plates were incubated at 28 ± 2° C for 8 days. Morphologically different colonies appearing on the plates were purified and preserved at 4° C. The fungal pathogen, *Fusarium oxysporum,* causing wilt and rot disease in tomato using standard isolation technique (Riker, 1936). The infected plant parts were collected and brought to the laboratory. The disinfect bits were then placed in steriled petri dishes containing oatmeal agar medium and incubated at 28 ± 20C. Mycelia bits were purified by hyphal tip method, and transferred to potato sucrose agar (PSA) and potato dextrose agar (PDA) slant and pure cultures of the pathogen were maintained for further studies.

Identification of Trichoderma spp. and fungal pathogen, Fusarium oxysporum

Morphological observation of *Trichoderma* isolates were done by extending them in PDA medium in order to unequivocally verify taxa and species, structure viz., conidiophores length and shape, philosophores, philospores and chlamydospore shape, length including coloration. For morphological characterization, slide culture technique was used and incubated at 25° C with alternating 12 hour dark and 12 hour cool white fluorescent light. Morphological characteristics of *Trichoderma* were measured. The pathogen associated with wilt disease was identified by cultural and morphological characters. Cultural characters of pathogen such as rate of growth, growth pattern etc. in the potato dextrose media were studied. Morphological characters of the pathogen like length of sporangia, L/B ratio, stalk length etc. were studied by slide culture technique using lactophenol cotton blue staining.

Antagonistic effects of Trichoderma spp. against pathogenic fungus in dual plate method

The antagonistic effect of *Trichoderma* against *Fusarium oxysporum* was tested by dual culture plate technique outlined by Skidmore and Dickinson (1976). Plant pathogen from 84 hours old culture grown on PDA was aseptically transfered. The petri dishes containing PDA were incubated at 28 ± 2°C for 24 hours. After *Trichoderma* isolates were transfered in the same petri dishes away from the pathogen, and incubated for 120 hours. Three replications were maintained for each isolate. Pathogen grew in monoculture served as control. Growth measurements were taken at regular intervals after 24 h subcultured antagonists for 96 hours. The reaction of antagonists on the pathogen were recorded.

Assay for extracellular enzymatic activity of β-1, 3-glucanase activity

According to the method of Reissig *et al.* (1995), the assay of β-1,3-glucanase enzyme was performed using 0.5 ml laminar in, 1.0 ml of 0.05 M citrate buffer (pH 4.8), and 0.5 ml mixed together incubated at 40 C for 60 min. Same amount of dinitro salicyclic acid added to the mixture into the water both for 15 min. The absorbance was measured at 575 nm in a spectrophotometer, and compared with standard graph drawn by following the same procedure, but using different concentrations of glucose instead of culture filtrate. The quantity

of reducing sugar was calculated from the glucose standards. One corresponds to the release of 1 in mol glucose equivalent per second.

Chitinase activity

The chitinase enzyme activity was followed by the method of Reissig *et al.* (1955). The amount of released reducing sugar was calculated from standard curves, and chitinase enzyme activity was expressed in pmol/s) per millitre.

Extraction and detection of secondary metabolites from Trichoderma spp. using ethyl acetate solvent

After 14-days old mycelia were harvested by scratching using glass rod. The harvested mycelia and extracts was taken in same concentration. Then, it was poured into 500 mL of minimal salts broth (MIN) media in 1,000 mL Erlenmeyer flasks, and grown for 14 days at 25°C at 150 rpm (Hanhong, 2011). Ethyl acetate (EtOAc, 250 Ml) added into the liquid culture and shaken at 150 rpm for 10min. After 1 h without shaking mycelia layer was transferred into flask, and dried using a rotary vacuum evaporator at 36°C (Rouini *et al.*, 2006). Finally, the extracts were weighted and dissolved in acetone-water (1:9, V/V). Then the secondary metabolites were identified by GC-MS device (Martínez-Padrón *et al.*, 2018).

Collection and seed selection

The seeds of tomato (*Solanum lycoprescum* L.) variety PKM-2 were collected from collected from agricultural office (seelanayakanpatty) in Salem. The seeds are sterilized with 0.5% sodium hypochlorite for 3 min and repeated washing in distilled water.

Experimental design

The experiment was designed with the following combinations as *Trichoderma harzianum* (Tr01), *Trichoderma viride* (Tr02), *Fusarium oxysporum* (Fuo4), Tr01 + Fuo4, Tr02 + Fu04 and control. The pots were maintained in appropriate distances. Water was supplied after intervals of 24 to 48 hours as required. The plant sample data were subjected to analyze at 60 days after inoculation from pot culture.

Growth characteristics of plant

Plants were harvested at 60 DAI (day after inoculation) and separated into leaves, shoot and roots. The plant shoot, root length (cm), plant fresh, dry weight (g) and chlorophyll content were calculated in bioinoculants treated plants and non-inoculants plants. Three plants were taken each to measure the mean values in all the treated and control plants.

Extraction

Two hundred mg of fresh young leaves were ground with 10 ml of 18% acetone with pestle mortar, and spun at 2500 gm for 10 min at 200°C in centrifuge. The homogenate was re-extracted with 80 percent acetone until the green color was disappears in the residue, and the extract was pooled, then made up of 20 ml with 80% acetone.

Estimation of photosynthetic pigments (mg/g/f. wt)

Chlorophyll a and b contents were extracted from respective amount of leaves and estimated according to the method of Arnon (1949) and the carotenoids content was determined by Kirk and Allen method (1965).

Soil nitrogen content

The soil nitrogen content was estimated by Subbish and Asija (1956) Method. Soil phosphorus was estimated by Olsen, 1954. Potassium content of the soil was estimated by the method of (Jackson, 1973).

Estimation of total nitrogen

The dried plant material was ground in a mortar and pestle, and the total nitrogen content was estimated by the conventional micro-Kjeldahl method (Umbriet, 1972).

Soil microbial population

Microbial population was determined by the dilution plate's techniques. The soil sample (1g) was serially diluted water to get 10-3 dilutions. An aliquot of the sample was plated either in potato dextrose, and rose bengal agar medium for total fungal population. After 2 to 5 days of incubation at 30^{o} C

plates were observed colony formation. The total number of colonies in each pate was scored. The results were expressed as colony forming unit per gram soil.

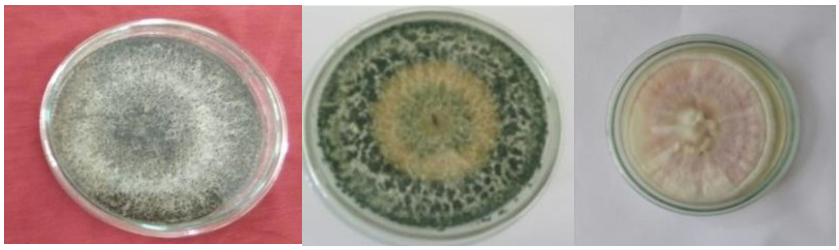
Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa was clustered together in the bootstrap test (100 replicates) and expressed the next to the branches (Felsenstein, 1985). Evolutionary analyses were conducted in MEGA6 (Tamura *et al.,* 2013).

Results

Isolation and Identification of Trichoderma spp.

Trichoderma spp. were isolated from different agricultural fields. Soil samples were collected from Salem District. The samples were serially diluted and placed on PDA medium. The colony showed in light and dark green in color. The samples were named as Tr01, and Tr02 which maintained in PDA medium. Fungal isolates were observed morphological characterization which shown in Figures 1 and 2. *Trichoderma* spp. isolate Tr01 showed light green colony, chlamydospores, conidia and conidiophores. Isolate Tr 02 showed morphological characterization of dark green colony colour, conidial shape. These isolates were observed under light microscope (10×40X) as seen in Figures 1 and 2.



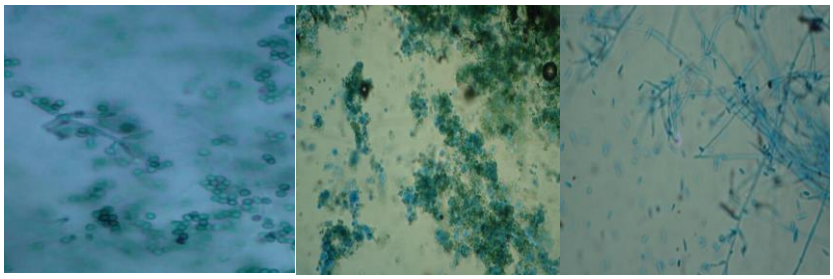
A **B** **C**

Figure 1. Morphological observation of *Trichoderma* spp. and *Fusarium oxysporum,* A= *Trichoderma harizanum* Tr01, B= *Trichoderma viride* Tr02, *C = Fusarium oxysporum*

Isolation and identification of plant pathogen

The pathogen was isolated from wilt disease of tomato. The infected plant sample pure culture was cultured on PDA medium, and identified through

morphological characterization. It is confirmed to be *Fusarium oxysporum* which produced white or pink color pigmentation and observe under the light microscope (10 ×40X) as seen in Figures 1 and 2.



A **B** **C**

Figure 2. Microscopic view of the isolates (10x × 40x) A=*Trichoderma harzianum* Tr01, B=*Trichoderma viride* Tr02, C = *Fusarium oxysporum*

Antagonistic activity test

Two isolates of *Trichoderma harzianum* (Tr01), *Trichoderma viride* (Tr02) were proved to be antagonize *F. oxysporum* causing tomato wilt using dual culture technique. These isolates were positively tested for antifungal activity (Figure 3).

a ***b***

Figure 3. Antagonistic activity of *Trichoderma* spp. against *Fusarium oxysporum*, A = *Trichoderma harzianum* Tr01, b = *Trichoderma viride* Tr02

Extracellular enzymatic activity of Trichoderma spp.

Result showed that the highest chitinase activity was recorded with *T. harzianum* Tro1. β-1-3-glucanase activity was also found as seen in Figure 4.

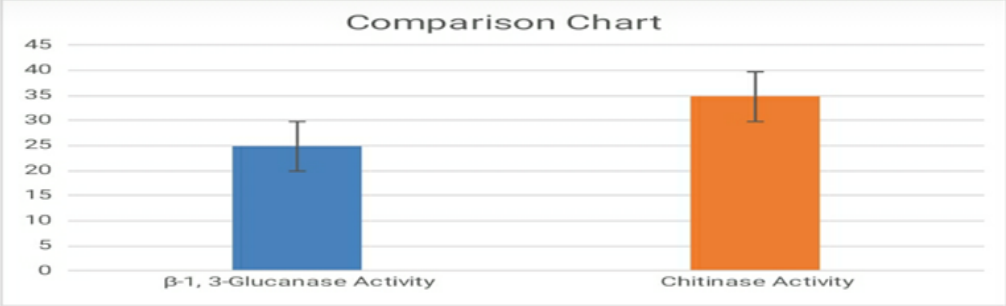
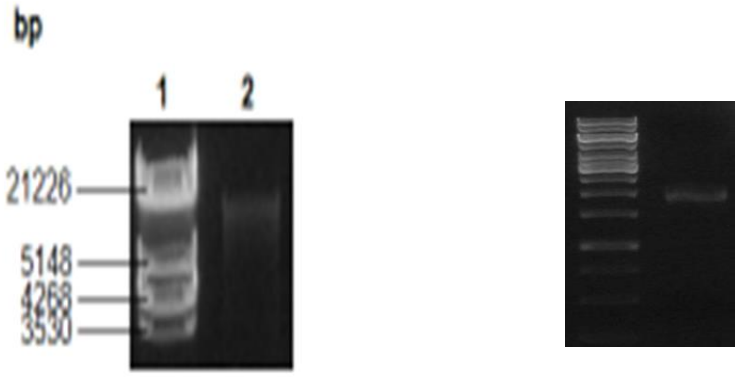


Figure 4. Enzyme activity of chitinase and β-1,3 –glucanase activity

PCR analysis

T. harzianum Tr01, *T. viride* Tr02 and *Fusarium oxysporum* Fuo4 were confirmed identification through genetic DNA isolation and PCR analysis (Figure 5).



a **b**

Figure 5. PCR analysis of 1 Kb16s rRNA amplification of *Trichoderma harzianum* Tr01 (a) and *Trichoderma viride* Tr02(b) a) *Trichoderma harzianum*

Production and extraction of secondary metabolites by Trichoderma strains in GCMS Analysis

The secondary metabolites were observed according to thin layer chromatography (TLC) analysis. *Trichoderma* was proved to produce secondary metabolites by GC-MS. Analysis of the spectra showed that the ten number of compounds which were analyzed from *T. harzianum* (Tr01) to produce Dodecane, tetradecane, Diethyl Phthalate, Hexadecane,

Benzenepropanoic acid, 3,5- bis (1,1-dimethylethyl)-4-hydroxy-, methylester, n-Hexadecanoic acid, Tetracontane, Bis(2-ethylhexyl) phthalate, Tetracontane. All compounds showed antibiotic activity against the tested plant pathogen and promoted the growth of tomato. In this study, the metabolites of n-hydrocarbons type of *T. harzianum* Tr01 which produced secondary metabolites, the compounds analyzed as volatile. *T. harzianum* (Tr01) showed the highest antibiotic resistance as shown in Figure 6, 7 and 8, Table 1, 2 and 3).

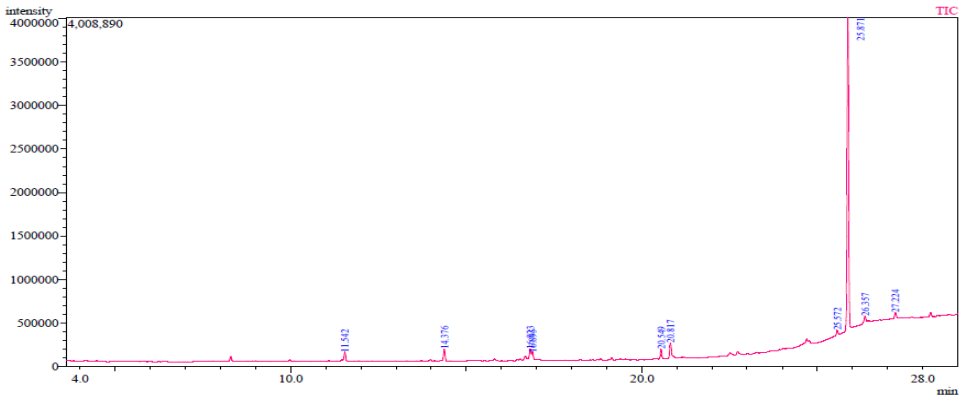


Figure 6. Identification of secondary metabolites from *Trichoderma harzianum* Tr01 in GC-MS analysis using ethyl acetate solvent and their respective retention times (RT)

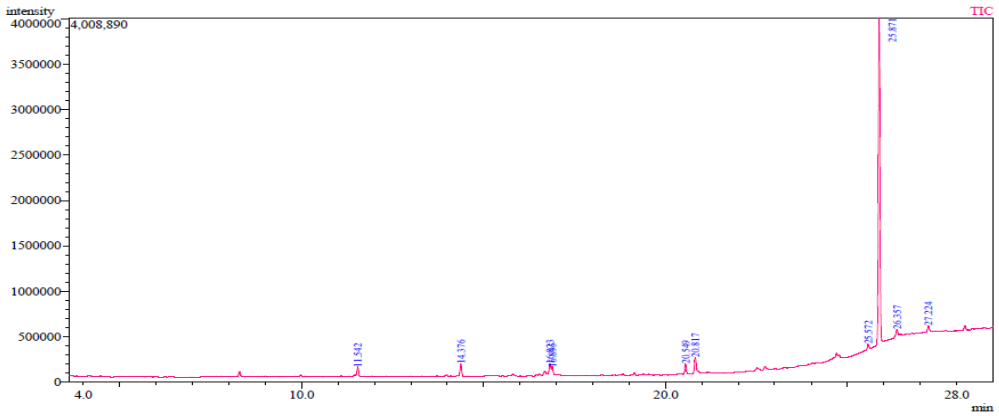


Figure 7. Identification of secondary metabolites from *Trichoderma viride* Tr02 in GC-MS analysis using ethyl acetate solvent and their respective retention times (RT)

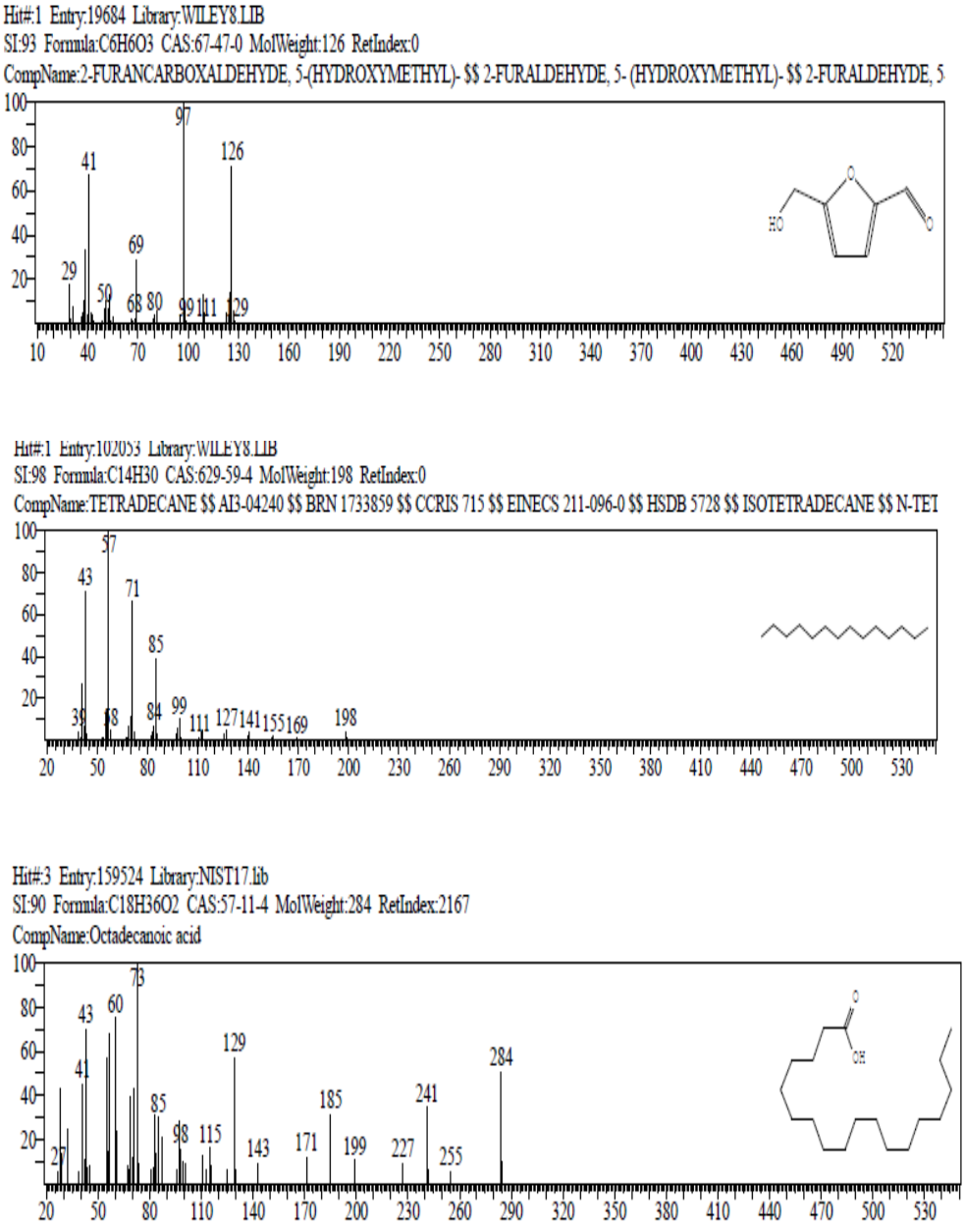


Figure 8. GCMS - compound structures of *Trichoderma* spp.

Table 1. Identification of secondary metabolites from *Trichoderma harzianum* Tr01in GC-MS analysis using ethyl acetate solvent and their respective retention times (RT)

Peak	RT	Area	Area %	Height	Height %	Name
1	11.542	268012	2.01	96328	2.16	Dodecane
2	14.376	373920	2.80	131351	2.95	Tetradecane
3	16.823	373368	2.80	117182	2.63	Diethyl Phthalate
4	16.895	221163	1.66	75059	1.68	Hexadecane
5	20.549	310649	2.33	107603	2.41	Benzenepropanoic acid, 3,5- bis (1,1-dimethylethyl)-4-hydroxy-, methyl ester
6	20.817	515776	3.87	164492	3.69	n-Hexadecanoic acid
7	25.572	146217	1.10	52243	1.17	Tetracontane
8	25.871	107253998	0.45	3577137 8	0.28	Bis(2-ethylhexyl) phthalate
9	26.357	192222	1.44	67381	1.51	Eicosane
10	27.224	205058	1.54	67250	1.51	Tetracontane
		13331784	100.00	4456026	100.00	

Table 2. Secondary metabolites identified from *Trichoderma viride* Tr02 in GC-MS analysis using Ethyl acetate solvent and their respective retention times (RT)

Peak	RT	Area	Area%	Height	Height%	Name
1	11.537	294528	1.46	102588	1.59	Dodecane
2	12.025	740157	3.67	140511	2.18	2-Furancarboxaldehyde, 5-(hydroxymethyl)
3	14.372	423445	2.10	145217	2.26	Tetradecane
4	16.818	389306	1.93	119822	1.86	Diethyl Phthalate
5	16.881	234772	1.16	87793	1.36	Hexadecane
6	20.543	288706	1.43	101027	1.57	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl
7	20.814	1076631	5.33	297258	4.62	n-Hexadecanoic acid
8	22.517	420847	2.08	72298	1.12	Oleic Acid
9	22.726	388131	1.92	86040	1.34	Octadecanoic acid
10	25.866	15934615	78.92	5282770	82.09	Bis(2-ethylhexyl) phthalate
		20191138	100.00	6435324	100.00	

Table 3. Effect of bioinoculants on total nitrogen content in tomato at 60 DAI (mg N/g dry plant)

S.no	Treatments	Total nitrogen content (mg N/g dry plant)
1	Control	1.12
2	Tr01	2.38
3	Tr02	1.57
4	Fuo4	0.84
5	Tr01+Fuo4	1.86
6	Tr02+Fuo4	1.24

Pot experiment

The result obtained from the study on the effect of two different treatments, Control, *Trichoderma harzianum* (Tr01), *Fusarium* (Fu04), Tr01 + Fu04, Control, *Trichoderma viride* (Tr02) *Fusarium* (Fu04), Tr02 + Fu04 management of tomato seedling.

Determination of plant length in (cm)

The inoculated tomato plants with *T. harzianum* - Tr01 and *T. viride*- Tr02 inhibited *F. oxysporum* Fu04 after 60 days. The tomato plants had higher plant height in *T. harzianum* Tr01 ((27.8 cm) and *T. viride* Tr02 which the plant height was 23.8 cm than the control (18.2cm) as shown in Figure 9.

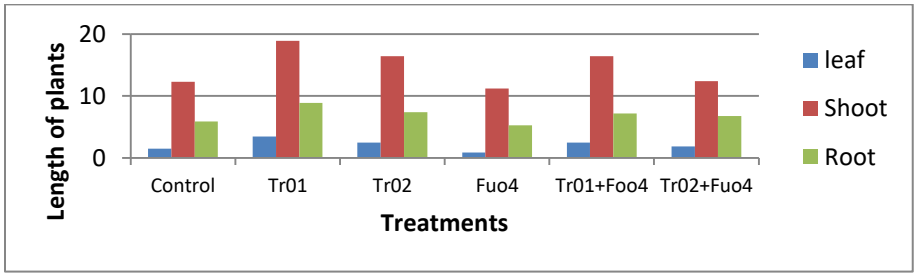


Figure 9. Growth characters of tomato at 60 DAI of leaves, shoots and root length

Determination of chlorophyll

Chlorophyll a, b and carotenoids content ultimately affected and its accumulation was significantly reduced to compare with control. The result showed that there was high chlorophyll accumulation in *T. harzianum* - Tr01 and *T. viride*- Tr02 treated pots. Bioinoculants were either *T. harzianum* - Tr01

or *T. viride*- Tr02 showed the highest enhancing plant chlorophyll content (Figure 10).

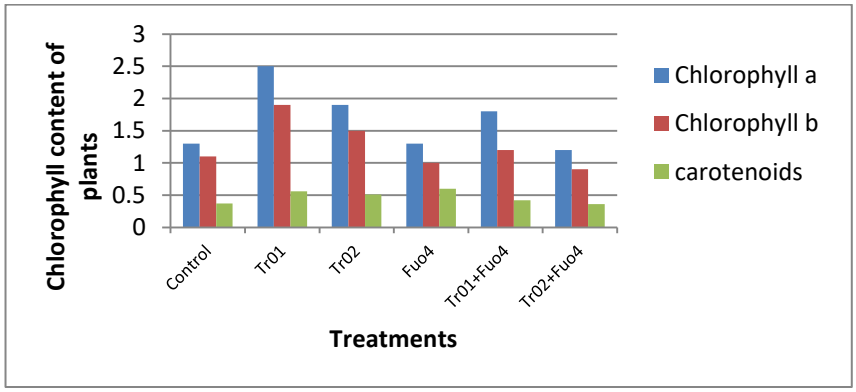


Figure 10. Analysis of chlorophyll a, b and carotenoids content in tomato at 60 DAI (mg/g/fr.wt) of *Trichoderma viride* Tr02

Fresh and dry weight of plants

Result showed that bio- inoculants agent (*Trichoderma viride* Tr02) inoculated to tomato plant resuleading to get the fresh weight was 11.2g when compared to the control (8.6 g). Bio-inoculant was highly enhanced the plant fresh weight. Moreover, *T. harzianum* Tr01 applied to inoculated plant with pathogen leading to get the fresh weight of 13.4g. The bioinoculants control agent of *Trichoderma viride* Tr02 applied to inoculated plants with pathogen got the dry weight of 5.4g when compared to the control (3.6g) and Fu04(2.3g) plant pathogen. The bioinoculant-*Trichoderma viride* Tr02 was highly inhibited the tested plant pathogen. Bioinoculants were also highly enhanced the plant dry weight (Figure 11).

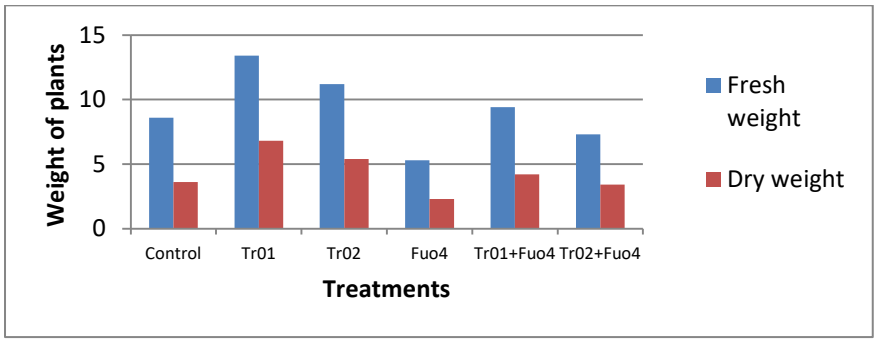


Figure 11. Growth characters of tomato fresh weight at 60 DAI (g/plant)

Total nitrogen content

There was a statistically significant effect showed in total nitrogen content (g/plant) of tomato plants. The highest total nitrogen content was recorded as 2.38 mg N/g dry plant in bioinoculant of *T. harzianum* Tr01 applied to inoculated plants. In contrast, less nitrogen content was shown to be 0.84 mg N/g dry plant as seen in Table 3.

Soil analysis

Soil NPK contents were more pronounced in dual inoculated soils and as well as in field trials. In uninoculated soil had less NPK content, and Bioinoculants inoculated soil contain high level of nutrients (Table 4).

Table 4. Characterization of soils

S.no	Treatments	PH	EC	Macro nutrient			Micro nutrient			
				N	P	K	Fe	Mn	Zn	Cu
1	**Control**	7.5	0.23	87.2	4.3	454.2	6.43	11.26	0.42	2.01
2	**Tr01**	4.5	0.52	232	10.3	354.4	4.45	13.25	2.65	3.65
3	**Tr02**	6.2	0.67	193	7.6	342.3	4.16	09.41	2.12	2.94
4	**Fu04**	5.4	0.52	130	5.4	207.1	2.18	02.37	0.54	1.94
5	**Tr01+ Fu04**	8.1	0.44	205	7.3	323.0	4.27	12.03	2.04	2.16
6	T**r02+ Fu04**	7.2	0.48	198	6.1	316.0	3.45	7.43	1.75	1.38

Note: EC = Electrical Conductivity, N = Available Nitrogen, P = Available Phosphorus, K = Available Potassium

Nitrogen	Phosphorus	Potassium
0 to 46 mg/Kg soil-low	0 to 113 mg/kg soil-low	0.0 to 4.5 mg/kg soil – low
47 to 113 mg/kg soil-medium	113 to 181mg/kg soil-medium	4.6 to 9.0 mg/kg soil – medium
113 above mg/kg soil-High	181 above mg/kg soil-high	9.0 above mg/kg soil – high

Soil microbial population

Analysis of microbial population in soil sample from control and bio inoculants inoculated plants sown in the pots. Count the microbial colonies in naked eye. Respect to all the two parameters, namely, fungal population was analyzed. After the inoculation, the effects significantly higher in soil sample inoculation with bio inoculants for the entire soil sample studied, as compared with inoculated control. The soil had a fungal population of 12 X107 CFU/g soil and pathogen showed soil microbial population was 2.6 X107 CFU /g soil. (Figure 12, Table 5).

Table 5. Impact of after inoculated soil counting of microbial population at 60 DAI (CFU/g soil)

S.no	Treatments	Total fungal population in soil (CFU/ g soil)
1	Control	1.2 X 10^5
2	Tr01	12 X 10^5
3	Tr02	10 X 10^5
4	Fu04	2.6 X 10^5
5	Tr01+ Fu04	5.4 X 10^5
6	Tr02+ Fu04	6 X 10^5

Note: CFU = Colony forming unit

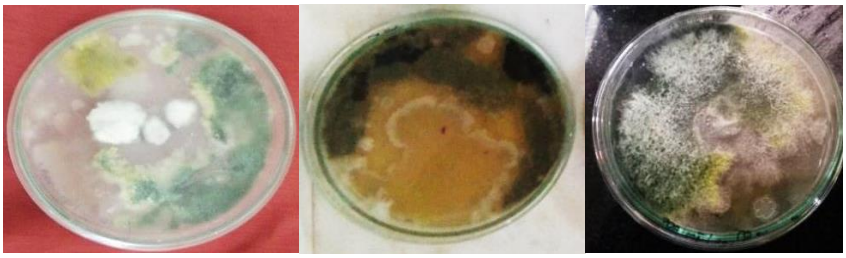


Figure 12. Isolation of microbial population

Discussion

The samples were collected from Salem district. *Fusraium oxysporum* is proved to be pathogenic isolate and caused wilt disease of tomato. It is also reported by Baysal *et al.* (2013) who stated that the tested pathogen, *F. oxysporum* leaded to severe crop loss and it is a major constraint in the production of tomato under greenhouse and fields. Similar experiments for the identification of microbes through morphological and microscopic observation were enumerated by Singh *et al.* (2006). The research finding of *T. harzianum* Tr01 and *T. viride* Tr02 were confirmed identification by molecular phylogenetic. The isolates showed maximum similarity with *Trichoderma* spp. based on 16S r RNA analysis (Lau *et al.*, 2006).

Results showed *T. harzianum* Tr01 and *T. viride* Tr02 actively antifungal activity against *F. oxysporum* causing tomato wilt. *Trichoderma* spp. are an important biocontrol agent to control pathogen growth and to protect plant. *Trichoderma* spp. have been investigated as potential biocontrol agents because of their ability to reduce the incidence of diseases caused by plant pathogenic fungi, particularly many common soilborne pathogens.

T. harzianum Tr01 was proved to produce β-1-3-glucanase activity. It has been reported that several fungal cell wall degrading enzymes, amongst them chitinase and gluganase play an important role in the antagonistic action

against a wide range of fungal plant pathogens (Kubicek *et al.*, 2008). Two isolates of *T. harzianum* Tr01 and *T. viride* Tr02 were to be antagonize *F. oxysporum* causing tomato wilt using dual culture technique. With this, Angélica *et al.* (2001) stated that the rapid growth of *Trichoderma* revealed an important advantage in the competition for space and nutrients with plant pathogenic fungi. It concluded that *Trichoderma* spp. (Tr01, Tr02) actively controlled the tested plant pathogen, *F. oxysporum* (Fu04), and improved yield and quality of crop. Bioinoculant-*Trichoderma viride* Tr02 significantly enhanced plant biomass, chlorophyll content, nitrogen content, NPK content and soil microbial population. Bioinoculant-*Trichoderma harzianum* Tr01 showed highly inhibition *F. oxysporum* (Fu04), and increased in plant growth.

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