

Secondary metabolites produced *Trichoderma* spp. against *Fusarium oxysporum* in *Solanum lycopersicum* L.

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Abstract

Trichoderma species are well-known biological control agents. In this study, secondary metabolites from *Trichoderma* spp. (*Trichoderma harzianum* - Tr01-333257, *T. viride*- Tr02-333256) were extracted from liquid culture using ethyl acetate and tested for their activities against fungal pathogen *Fusarium oxysporum* (Fu04 - KY977424). The fungal pathogen *Fusarium* responsible for wilt disease observed on *Solanum lycopersicum* L. (Tomato) treated with *Trichoderma* extract. This experiment was conducted by different treatments such as Control, Tr01, Tr02, Fu04, Tr01+ Fu04, Tr02+ Fu04. *Trichoderma harzianum* (Tr01) showed strongest inhibitory activities against *Fusarium oxysporum*. The expression of two pathogenesis-related gene SIWRKY31, SIWRKY4, which are responsible for production of glucanase and chitinase contributing to pathogen inhibition. In this results showed the potential for use of *Trichoderma* metabolites as biological control agents against *Fusarium* wilt disease.

Keywords: antagonistic effects, *Fusarium*, biological control, *Trichoderma*

Introduction

Secondary metabolites are an heterogeneous group of natural compounds that are considered to aid the producing organisms in survival and basic functions such as competition, symbiosis, metal transport, differentiation, etc... (Demain and Fang, 2000) [24]. Chemical and biological properties of such compounds, they are widely used for medical, pharmaceutical, (or) agricultural purposes (Calvo *et al.*, 2002) [23]. Fungi of the genus *Trichoderma* are biocontrol agents that are successfully used as bio pesticides worldwide, and many species are well known producers (Ghisalberti and Sivasithambaram 1991, Reino *et al.*, 2008, Sivasithambaram and Ghisalberti, 1998) [27, 25, 28] of secondary metabolites in *Trichoderma* spp. is strain – dependent and includes volatile and non-volatile antifungal substances, such as 6-n-penty-6H-pyrone-2-one, gliotoxin, viridian, harzianopyridine and peptaibols (Reino *et al.*, 2008, Sivasithambaram and Ghisalberti, 1998, Vey *et al.*, 2001) [25, 28, 30]. The biocontrol effects of *Trichoderma* spp. was significantly enhanced by the stimulation of plant defense responses to pathogen attack. In addition the inoculation of the living fungal antagonist produced a growth promotion effect in plants. In this study, we isolated the major secondary metabolites produced in liquid culture by *Trichoderma harzianum* and *Trichoderma viride* biocontrol strains (Tr01-333257, Tr02-333256). There are four compounds were extracted, isolated and characterized from fungal culture filtrate (Tetradecane, Tetracontane, Octadecanoic acid, 2- furancarboxaldehyde).

Materials and Methods

Trichoderma and *Fusarium* isolates

Soil sample (50g each) collected from different ecological habitat of Salem, Tamil Nadu India for the isolation of *Trichoderma* spp. and collected from top 10-15 cm depth of root rhizospheric soli. The sample were labeled in Polythene

paper bag separately and brought to laboratory stored at 4 °C until used. Five-fold serial dilutions of each soil samples were prepared in sterilized distilled water and 0.5ml 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 and used during the course of study. The fungal pathogen *Fusarium oxysporum*, causing wilt and rot diseases in cowpea, was isolated from naturally infected cucumber plants using standard isolation techniques (Riker and Riker, 1936). The infected plant materials were collected and brought to the laboratory. The sterilized bits were then placed in sterile petridishes containing oatmeal agar medium and incubated at 28 ± 2 °C. 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 pathogens were maintained for further studies.

Identification of *Trichoderma* spp. and fungal pathogen *Fusarium oxysporum*

Morphological Anamorphic characterization of *Trichoderma* isolate were done by growing them in potato dextrose agar medium in order to unequivocally verify taxa and species, Morphological characteristics viz., conidiophores length and shape, phialophes and chlamydo spores shape, length and 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. All conidiophores, phialides, conidia and chlamydo spores were measured the morphological characteristics of *Trichoderma* spp. The pathogen associated with cowpea wilt and rot diseases was identified as 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 lacto phenol cotton blue staining.

DNA isolation and 16s rRNA amplification

The isolates was inoculated in 50 ml of culture medium and incubated until it reached the 600nm. The cells were harvested by centrifuging at 1250rpm at ambient temperature. The cell pellet resuspend in 467 μ l TE buffer and further incubated with 33 μ l of lysing buffer (30 μ l of 10% SDS and 3 μ l of 20 mg/ml proteinase K) for 1 hrs at 37 °C. The lysate extracted with an equal volume of 25:24:1 phenol: chloroform: isoamyl alcohol. The aqueous phase transferred to a 1.5 micro centrifuge tube and added with 1/10 volume of 3M sodium acetate and incubated at -20 °C for 30 min. after the incubation 0.6 volumes of isopropanol added and mixed gently and centrifuged at 14000rpm for 20 min at 4 °C. DNA pellet washed with 1 ml of 70 % ethanol twice by spinning at 14000rpm for 20 min at 4 °C. The DNA pellet was resuspended in 100 μ l TE buffer and stored at 4 °C, overnight. The quality and quantity of the DNA was estimated by OD ratio of 260/280,260/230 nm through 8 port nanodrop (Thermo Fisher Scientifics, USA) and 1% agarose gel respectively. The 16S rRNA gene sequence of bacteria was amplified with the extracted DNA using universal primers 8 F 5'-AGA GTT TGA TCC TGG CTC A and 1492 R 5'-GGT TAC CTT GTT ACG ACG ACT T (Edwards *et al.*, 1989).

Antagonistic characteristics of *Trichoderma* against pathogenic fungi in dual culture plate technique

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 inoculated aseptically. The petridishes containing PDA and incubated at 28 \pm 2 °C for 24 hours. After this *Trichoderma* isolates were inoculated in the same petridishes, away from the pathogen and incubated for 120 hours. Three replications were maintained for each isolate. Pathogen grown in monoculture served as control. Growth measurements were taken at regular intervals after 24 h of inoculation of antagonistic for 96 hours. Natures of reaction of the antagonist on the pathogen were recorded.

Assay for Extra Cellular Enzyme Activity (Ressing and Strominger 1995)

β -1, 3-Glucanase Activity

For assay of β -1, 3-glucanase enzyme, 0.5 ml laminar in, 1.0 ml of 0.05 M citrate buffer (pH 4.8) and 0.5 ml culture filtrate was mixed and incubated at 4 °C for 60 min. An equal volume of dinitrosali cyclic acid reagent was added to the reaction mixture and warmed in boiling water for 15 min. The absorbance of reaction mixture 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 used in the assay and activity of β -1, 3-glucanase was expressed in ml. One corresponds to the release of 1in mol glucose equivalent per second.

Chitinase Activity

A mixture of 0.5 ml culture filtrate, 0.5 ml suspension of colloidal chitin and 1.0 ml of McIlvaines buffer (pH 4.0) was mixed and incubated at 37 °C for 2 h in a water bath with constant shaking. The reaction was stopped by boiling 3 min in heated water bath. 3 ml potassium ferricyanide reagent was added and warmed in boiling water for 15 min. The amount of N-acetyl glucosamine released was estimated following the methods (Reissing *et al.*, 1955) [28]. The absorbance of reaction mixture was measured in a spectrophotometer at 420 nm. The amount of reducing sugar released was calculated from standard curves and chitinase enzyme activity was expressed in (pmol/s) per millilitre.

Extraction of *Trichoderma* spp. metabolites using Ethyl acetate

After 14-days of growth, sterile water (8ml) was poured into the plates, and mycelia were harvested by scratching using a glass stick. The amount of harvested mycelia was dependent on isolates; however, the same concentration of extracts was used for treatment. The harvested mycelia were 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 150rpm (Bae *et al.*, 2011) [10]. Half of the volume of ethyl acetate (EtOAc, 250 ml) was added into the liquid culture and shaken at 150 rpm for 10min. After 1h of incubation without shaking, the top layer was transferred into a flask, concentrated, and dried using a rotary vacuum evaporator at 36 °C (Rouini *et al.*, 2006) [12]. The dried extracts were weighted and dissolved in acetone-water (1:9, V/V).

Analysis of secondary metabolites of strain of *Trichoderma harzianum* and *Trichoderma viride* in GCMS

To determine the presence/absence of metabolites, each sample was subjected to thin layer chromatography (TLC) with a mobile phase of hexane: acetone (50:50) (Analytika®) and as a stationary phase with a silica gel matrix in aluminium plate (Whatman®). 200 μ l of non-inoculated culture medium (by several successive applications of 20 μ l at the same point, allowing drying between each application) was added to each plate, which was used as a reference, and at another three points, 200 μ l of the culture of each isolate was added by successive application. The plate was then air dried for 20 min. The development was performed by exposure of the plate to iodine vapors for 15 min. The procedure was repeated in triplicate for each sample. The qualitative analysis of the *Trichoderma* spp. extracts was performed from 3 weeks old samples by gas chromatography-mass spectrometry (GC-MS) on an Agilent® 6890 chromatograph, with an Agilent® 5973N mass detector using a 5% phenylmethylpolysiloxane column with helium mobile phase. Analysis of each sample was performed in triplicate. (Yuef *et al.*, 2018) [21].

FTIR analysis of crude extract of Tr01 and Tr02

The extract was totally dried and prepared to FTIR examination (Periyar University, in Salem) utilizing assimilation method of 4000-400 cm⁻¹ territory. The IR range frequencies were resolved dependent on its useful gatherings (Vinale *et al.*, 2006) [5].

Analysis of plant hormone

Plant hormones were analyzed using a high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) system to monitor changes in response to the EtOAc extract of KACC 40557 in the detached pepper leaves with or without *P. capsici* (Pan *et al.*, 2010) [11]. Five hormones were analyzed: auxin (IAA), gibberellin (GA3), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA). Briefly, the leaf tissues were ground into fine powder, after which 50 mg of the ground samples were used for hormone analysis. Internal standard (IS) solution (50 L L) was also added to the samples to give a final concentration of 1 mg/mL. Next, extraction solvent (500L L of 2-propanol/H₂O/concentrated HCL, 2:1:0.002, v/v/v) was added to the sample, after which the samples were placed in a shaker at 100 rpm and 4 °C for 30 min. After adding 1 mL of dichloromethane, samples were returned to the shaker for 30 min at 4 °C. This step was followed by centrifugation of samples at 14,000 rpm and 4 °C for 5 min. The supernatant was then removed and concentrated in a nitrogen evaporator. Finally, samples were dissolved in 0.1 mL methanol and stored at 20 °C until further analysis. HPLC for hormone analysis was conducted using an Agilent 1100 series HPLC system equipped with a degasser, pump, auto-sampler and column oven. Chromatographic separations were performed using a SunFireC18 column (2.1 ×10 mm) (Waters, Milford, MA, USA). The isocratic mobile phase of 15:85 v/v, 0.1% formic acid in water and 0.1% formic acid in methanol was applied at a flow rate of 300 L/min, column temperature of 30 °C and an injection volume of 10 L in all experiments. Measurements for MS analysis were made using a 500 °C source temperature, 5.5 kV (positive) and 4.5 kV (negative) ion spray voltages, 3 collision gas (CAD), 15 curtain gas (CUR), 45 ion source gas and dwell times of 1, 2 and 150 ms. Calibration curves of the standards were prepared using 0.25, 0.5, 1.0, 2.5, 5.0, 50 and 500 ng/mL ABA, JA and SA, 0.5, 1.0, 2.0, 5.0, 10, 100 and 1000 ng/mL GA3, and 12.5, 25, 50, 125, 250 and 2500 ng/mL IAA. Calibration curves were estimated using the ratio of ABA, GA3, IAA, JA, and SA area/IS area versus the ratio of ABA, GA3, IAA, JA and SA/IS concentration. Each calibration curve was assayed individually using a 1/x weighted linear regression.

Histochemical Staining for Assessment of Lignification

The histochemical analysis for detection of the lignified tissue was done following the protocol as suggested by Guo *et al.*, (2001) [8]. The transverse section (TS) of stem tissues from the second internode region was cut with blade used for cutting the thin sections collected from all the treated tissues, and further, mounted in 1% phloroglucinol solution made with 95% alcohol. The mounted samples were covered with 0.1 mL concentrated HCl and was placed on a clean glass slide covered with the coverslip (Guo *et al.*, 2001) [8]. The stained samples were visualized under a compound light microscope (Nikon, Japan). The lignified tissues were identified as intense pink coloration of deposited lignified material.

Phylogenetic analysis

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987) [1]. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next

to the branches (Felsenstein, 1985) [2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.*, 2004) [3]. And are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013) [4].

Results

Isolation of *Trichoderma* spp. and *Fusarium oxysporum*

Fungal was isolated from the different agricultural field. Soil sample collected from Salem District. Serial diluted samples were placed on Potato dextrose Agar medium colony that produced on light green in colour and dark green in colour. The sample was named as (Tr01, Tr02) maintained in potato dextrose agar (PDA) medium. Fungal isolates was carried out using morphological characterization as observed the result was depicted in (figure-1). Plant pathogen isolated from infected plant sample for wilt, leaf spot, and blight of tomato. A specific fungus was successfully isolated from tomato leaf. The infected plant sample pure cultured on PDA medium. Identified through morphological Characterization of *Fusarium oxysporum* produced white color or pink color pigmentation, for naked eyes. Observe under the light microscope (10x×40x) and the result was defected (Plate.1, 2, 3).

Antagonistic activity test

Trichoderma harzianum isolate was test their antagonistic activity against fungal pathogen *Fusarium oxysporum* using dual culture techniques. A Biocontrol agent is highly inhibiting the plant pathogen. The isolate tested positive for antifungal activity will be further exploded in lab experiments to plant growth, yield, and their ability on *Solanum lycopersicum* L. (Plate. 4, 5).

Extra cellular enzymes activity of *Trichoderma* spp.

All isolates showed significantly of chitinase and β-1,3-glucanase activities with supplement of different carbon sources as substrates in the basal media. The highest chitinase enzyme activity was recorded with Tro1 (34. 88) compared with β-1-3-glucanase enzyme activity was recorded with (24.93). The production of chitinase and β-1, 3-glucanase are summarized in (Figure -1).

PCR analysis

The microbial isolate (Tr01) were confirmed through genetic DNA isolation & PCR analysis.

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

The secondary metabolites were observed strain selected according to thin layer chromatography (TLC) analysis. The presence of more metabolites extracts were evaluated by GC-MS showed that the strains of *Trichoderma* produced secondary metabolites. Analysis of the spectra showed that the ten number of Compounds were analyzed. The *Trichoderma harzianum* (Tr01) produced molecules of 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,

which act in an antibiotic way against plant pathogenic fungi and can promote the growth of the same, as well as grant them systemic resistance. In this study, secondary metabolites of n-hydrocarbons type *T. harzianum* produced secondary metabolites, the compounds analyzed are volatile in nature, these molecules are related to the interaction between the fungi and the environment, it was observed that the number of compounds varies between species, intraspecific variation as a possible result of the different environmental conditions to which they were submitted at their respective collecting sites however, *Trichoderma harzianum* (Tr01) showed the highest antibiotic resistance (Fig.2, 3 Table.1-4).

IR spectrum sample of Tr01 and Tr02

IR spectra show that there are Stretching vibration of C-H bond in - CH₃ noticeable at 3427.32cm⁻¹, 2935.15cm⁻¹, 2355.92cm⁻¹, Carbonyl (- C=O) signals obvious at 1743.35cm⁻¹, 1508.79cm⁻¹. Twisting vibration of C-H bond in - CH₃ at 1384.32cm⁻¹, 1151.39cm⁻¹. Extending vibration of C-H bond in - CH₃ noticeable at 3599.63cm⁻¹, 3014.84cm⁻¹, 2882.07cm⁻¹, 2356.28cm⁻¹. Carbonyl (- C=O) signals obvious at 1742.35cm⁻¹, 1509.26cm⁻¹. Twisting vibration of C-H bond in - CH₃ at 1149.80cm⁻¹, 1102.95cm⁻¹, 960.97cm⁻¹ (Fig.4, 5 and Table. 5,6).

FTIR range of *Trichoderma* extract demonstrated development of carboxylic acids, aldehydes, aromatics, alcohols, esters, ethers, and alkyl halides bunches at various frequencies. The most grounded stretch at 1384.32 in *Trichoderma harzianum* and at 1149.80 in *Trichoderma viride* found as sulphonyl antizyme stretch having the best effect on plant protection system.

Hormone analysis

Plant hormone comprise a collection of structurally unrelated signaling molecules derived from different metabolic pathways involved in diverse arrays of biotic/abiotic stress responses (Hiomi *et al.*, 2013; Robert-Seilaniantz *et al.*, 2011)^[13, 15]. In this study, *Solanum lycopersicum* L. plant hormones were analyzed in *Fusarium oxysporum* and the extract.

The IAA levels were 0.94, 0.98, 0.97, 1.01 and 1.18 µg/g in the leaves of Tr01, Tr01+Fuo4, Tr02, Tr02+Fuo4, Fuo4, and control samples at 96h, respectively. The IAA level of Tr02 was significantly higher than that of the other five samples. A synergistic effect on IAA was observed in responses to combined treatment with Fuo4 and the extract (Tr02, Tr01) at 96h, while the IAA levels was low at 96h in Tr01+Fuo4 and Tr02+ Fuo4. The combination of *Fusarium* and extract Tr02+Fuo4 could induce the IAA, which might be important to protection of plants from pathogens (Table. 7).

At 96h, The GA3 levels were 1.15, 1.89, 1.18, 1.93 and 3.58 µg/g in Tr01, Tr01+Fuo4, Tr02, Tr02+Fuo4, Fuo4, and control respectively, with similar values being observation in Fuo4 and Tr01+Fuo4 samples, but significantly lower values in Tr01, Tr02, significant induction of the Fuo4 sample was observed at 96h. These findings indicate GA3 production, while the extract inhibited Fuo4 growth and led to minor GA3 induction in Tr01+Fuo4.

ABA was present at 0.58, 0.92, 0.61, 0.32, and 0.23 µg/g in Tr01, Tr01+Fuo4, Tr02, Tr02+Fuo4, Fuo4, and control at 96h respectively, with a significantly higher level occurring in Fuo4 than that in Tr01+Fuo4. The absence of extract in

Fuo4 sample allowed plants to undergo *Fusarium* wilt growth, leading to the induction of ABA. This induction is a well-known defense mechanism against pathogens that occurs through the ROS scavenging system (Fath *et al.*, 2001)^[14]. Enhanced aba has also been reported to lead to pathogen susceptibility (Ton *et al.*, 2009)^[16]. While the extract in Tr01, Tr02 and Tr02+Fuo4 led to significant up regulation of ABA, which might be important for plant production.

Plant responses to different biotic/ abiotic stresses are locally (or) systematically orchestrated by signaling molecules such as JA, which inhibits plant growth, induces plant defense, and regulates fungal pathogen attack (Kunkel and Brooks, 2002)^[20]. In this study JA levels were 1.06, 0.44, 1.1, 0.5, and 1.44 µg/g in Tr01, Tr01+Fuo4, Tr02, Tr02+Fuo4, Fuo4, and control at 96h respectively, The JA levels were significantly up-regulated in Fuo4 and Tr01+Fuo4 samples during treatment, while no significant changes were observed in Tr01+Fuo4. In plants, enhanced JA levels led to pathogen susceptibility (Thaler *et al.*, 2012)^[18]. In this present study *Trichoderma* inoculated plants to suppressed *Fusarium* wilt growth (Tr01+Fuo4): however, pathogen infection induced wilt in leaf tissues (Fuo4), which led to development of systemic acquired resistance (SAR) via up- regulation of JA. Arabidopsis triple mutant fad3 (fatty acid desaturase 3), fad 7 and fad 8 with impaired JA production (or) jar (jasmonic acid resistant 1) exhibited enhanced susceptibility to various pathogens, including *Alternaria brassiciola*, *Botrytis cinera* and *Pythium* species (Stintzi *et al.*, 2001)^[19].

SA is also an important signaling molecule that plays a central role in plant defense against pathogens (Kunkel and Brooks, 2002)^[20]. We detected SA at 1.23, 1.08, 1.27, 1.11 and 1.05 µg/g in Tr01, Tr01+Fuo4, Tr02, Tr02+Fuo4, Fuo4, and control at 96h respectively, Similar levels of SA were detected in Fuo4 and Tr02+Fuo4, while significantly higher levels were found in Tr01 and Tr02. SA was significantly up-regulated at later, points for each treatment without significant differences among treatments. SA levels were also significantly up-regulated after pathogen infection and SA treatment of plants resulted in enhanced resistance to a broad range of pathogens (Vlot *et al.*, 2009)^[17]. In the present study, no significant differences were observed between Fuo4 and Tr02+Fuo4. However, the Tr02, Tr02 alone significantly up- regulated SA. Overall, these findings confirm that the *Trichoderma* highly up-regulated SA in plants, which might prevent pathogen infection of the host plant.

Histochemical Analysis for Lignin Assessment

The amount of lignified tissues was found to be greater in *T.harzianum* plants co-inoculated with Fuo4 (Tr01+ Fuo4, Tr02+ Fuo4) followed by *T.harzianum* followed by Tr01 samples. We have measured the deposited lignified tissues in the following treatments are Tr01, Fuo4, Tr02, Tr01+Fuo4, Tr02+Fuo4 and control plants. The pink colored staining of phloroglucinol -Hcl stained lignified tissues was more pronounced and was shown to have intense coloration. The Fuo4 challenged sample had a comparatively higher amount of lignified tissues than uninoculated control as visualized under the compound microscope. The lignified tissues in Fuo4 challenged stem (Fig-6B, C) showed less lignified tissues observed on (Fig-6D) whereas the control and Tr01+ Fuo4, Tr02+ Fuo4 plant

observed (Fig-6E, G) and (Fig-6A) was found to have a comparatively lesser amount of lignified tissues. However, in all the histochemical tissues sections analyzed the deposited lignin was quantitatively more in Tr01 tissues sections. The histochemical tissues sections from the Tr01 plants (Fig-6E) and (Fig-6F) respectively, The Tr01+ Fuo4 and Tr01+ Fuo4 tissues were found to have comparatively more lignified tissues on respectively (Fig – 6E, F) which

confirmed that the defense programming of the host was more pronounced in the presence of *T. harzianum* induced the *Fusarium oxysporum*. Moreover, Tr01+ Fuo4 had the maximum amount of deposited lignified tissues which confirmed that the defense responses in the presence of beneficial microbe *T. harzianum*, become more robust when plants further encounter the pathogen.

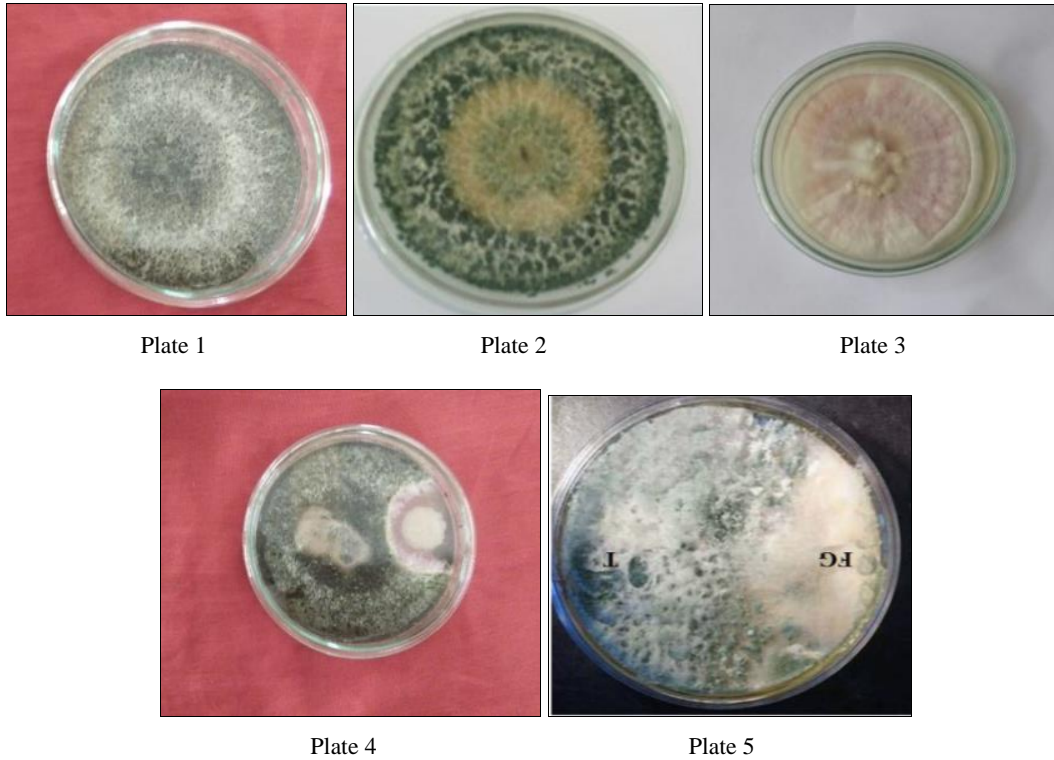


Fig 1: Extra cellular enzyme produced by *Trichoderma harzianum*

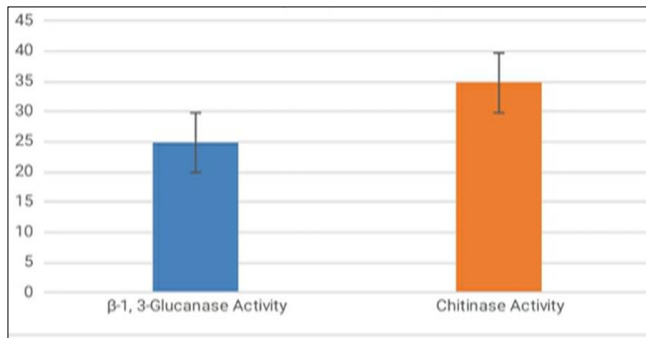


Fig 2: GCMS-Tr01-*Trichoderma harzianum*

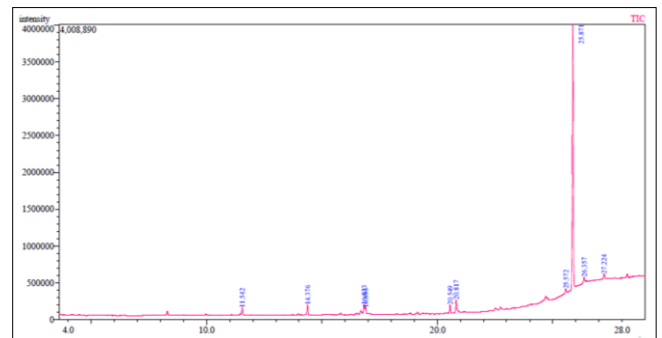


Fig 3

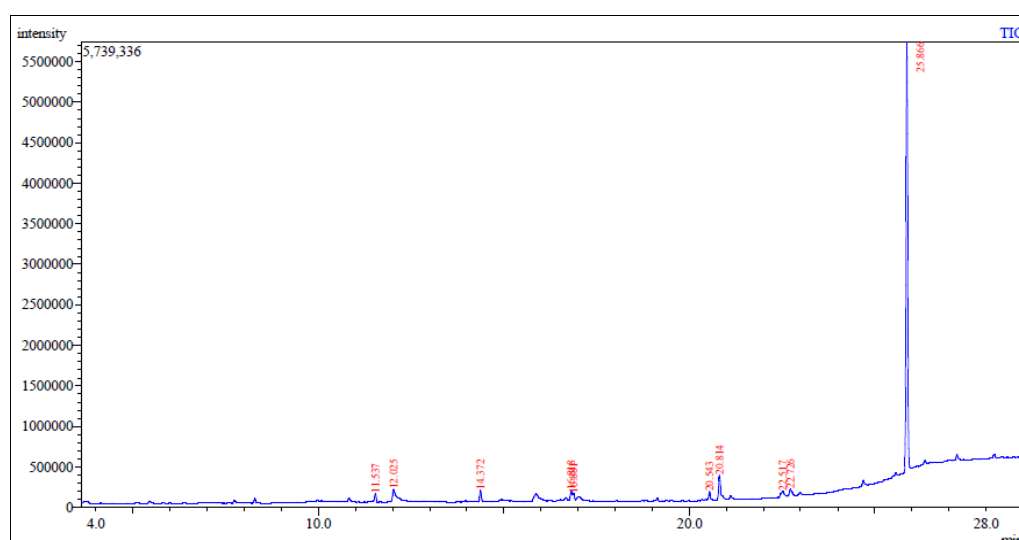
Secondary metabolites identified from *Trichoderma harzianum* in GC-MS analysis using Ethyl acetate solvent and their respective retention times (RT)

Table 1: GCMS –Tr01–*Trichoderma harzianum*

Peak	R.Time	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	10725399 8	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: GCMS–Tr01–*Trichoderma harzianum*

S.no	Compound Name	Biological Activity
1	Dodecane	Enhance Antifungal activity
2	Tetradecane	Antifungal, Antibacterial
3	Diethyl Phthalate	Plasticizer
4	Hexadecane	Antifungal, Antibacterial, Antioxidant.
5	Benzenepropanoic acid, 3,5- bis (1,1-dimethylethyl)-4-hydroxy-, methylester	Antifungal and Antioxidant property.
6	n-Hexadecanoic acid	Antioxidant activity, Pesticide, Nematicide.
7	Tetracontane	Anti-inflammatory, Analgesic activity.
8	Bis(2-ethylhexyl) phthalate	Plasticizer
9	Eicosane	Antifungal, Antibacterial, Antitumor, Cytotoxic.
10	Tetracontane	Anti-inflammatory, Analgesic activity.

**Fig 3:** GCMS – Tr02 – *Trichoderma viride*

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

Table 3: GCMS– Tr02 – *Trichoderma viride*

Peak	R.Time	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 4: GCMS – Tr02 – *Trichoderma viride*

S.no	Compound Name	Biological Activity
1	Dodecane	Enhance Antifungal activity
2	2-Furancarboxaldehyde, 5-(hydroxymethyl)	Antimicrobial activity
3	Tetradecane	Antifungal, Antibacterial
4	Diethyl Phthalate	Plasticizer
5	Hexadecane	Antifungal, Antibacterial, Antioxidant.
6	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl	Antifungal and Antioxidant property.
7	n-Hexadecanoic acid	Antioxidant activity, Pesticide, Nematicide.
8	Oleic Acid	Anemiagenic, Allergenic, Antiandrogenic
9	Octadecanoic acid	Antibacterial, Cosmetic, Flavour, Hypocholesterolemic, Lubricant, Perfumery, Propepic, Suppository.
10	Tetracontane	Anti-inflammatory, Analgesic activity.

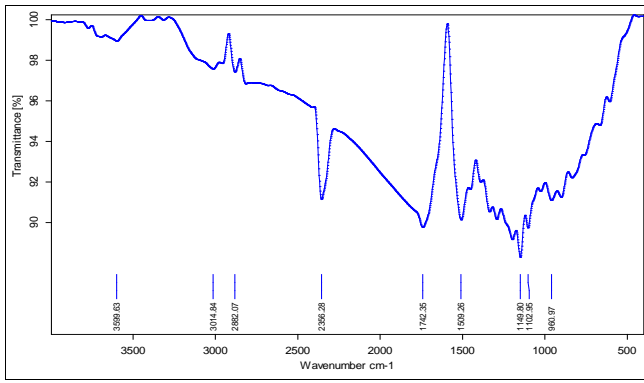


Fig 4: FTIR sample of Tr01

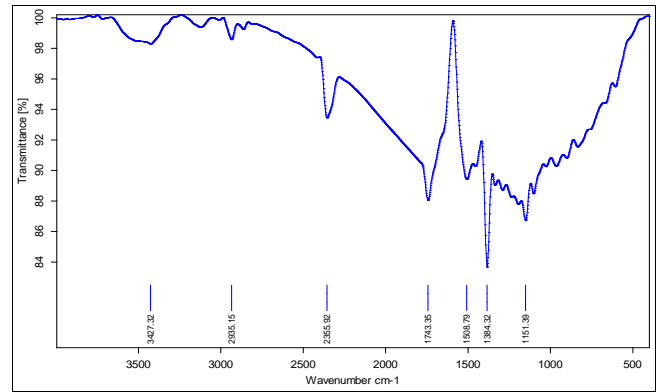


Fig 5: FTIR sample of Tr02

Table 5: Functional groups of compounds found in extract in *Trichoderma viride*

Extract	Peak value	Functional group	Groups
Methanol	960.97	CH=CH- in trans desubstituted alkenes	C=C bending
	1102.95	C-O-C aromatic compounds	C-O stretching
	1149.80	Sulfonic acid	S=O stretching
	1509.26	Nitro compounds	N-O stretching
	1742.35	Esters	C=O stretching
	2356.28	Carboxylic acid	O-H stretching
	2882.07	Alkane	C-H stretching
	3014.84	Alkene	C-H stretching
	3599.63	Alcohol	O-H stretching

Table 6: Functional groups of compounds found in extract in *Trichoderma harzianum*

Extract	Peak value	Functional groups	Groups
Methanol	3427.32	NH2 in aromatic amines	NH stretch
	2935.15	C-H in alkane	Alkane stretch
	2355.92	_NH3 in amine hydrohalides	NH stretching mode
	1743.36	C=O in esters	C=O stretch
	1508.79	NO2 in aromatic nitro compound	NO2 antisym stretch
	1384.32	SO2 sulphonyl chloride	SO2 antisym stretch
	1151.39	C-O-C in aliphatic ethers	C-O-C Antisym stretch

Table 7: Hormone responses of *Solanum lycopersicum* L.

Treatments	IAA	GA	ABA	JA	SA
Control	0.23	0.23	0.23	0.23	0.23
Tr01	0.97	1.18	0.61	1.1	1.27
Tr02	0.13	0.32	0.12	0.24	0.26
Fuo4	1.18	3.58	0.23	1.44	1.05
Tr01+ Fuo4	1.01	1.93	0.32	0.5	1.11
Tr02+ Fuo4	0.86	0.68	0.10	0.34	0.10



Fig 6: Evaluation of the plant protection reaction as lignification. The figure shows the cross over segments of stem tissues with lignin statement in the dividers of interfascicular strands and xylem cells gathered from four diverse cross over part of control, T. harzianum, Fuo4 and PP2 were gathered. The stem tissue areas from second internode locale were analyzed through Vibratome, recolored with Phloroglucinol-HCl (pink or fuchsia tone). The power of the pink tone speaks to higher statement of lignin. Vascular groups with lignified essential xylem: sclerenchyma beginning to separate with no lignified cell dividers. (A) Microscopic perspective on the histochemical segment (cross over) of the control stem tissue plant (control) B) Tr01, (C) Tr02, (D) Fuo4, (E) Tr01+ Fuo4, (F)Tr02+ Fuo4.

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