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Characterization of secondary metabolites and enzymes produced by *Trichoderma* species and their efficacy against plant pathogenic fungi

S C DUBEY[1], ARADHIKA TRIPATHI[2], P DUREJA[3] and ANITA GROVER[4]

Indian Agricultural Research Institute, New Delhi 110 012

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ABSTRACT

The secondary metabolites from culture filtrate and mycelial mass of potential isolates of *Trichoderma viride* (IARI P1 and IARI P2), *T. virens* (IARI P3) and *T. harzianum* (IARI P4) were extracted by solvent extraction and soxhlet water bath distillation methods and evaluated at different concentrations against *Rhizoctonia bataticola* (dry root rot of chickpea) and *Fusarium oxysporum* f. sp. *ciceris* (wilt of chickpea). All concentrations (500, 250, 125 and 75 ppm) were found to be inhibitory to these pathogens. Higher concentrations were found to be inhibitorier than the lower ones. 47.8% to 78.0% inhibition of mycelial growth was recorded even at 75ppm concentration. Some of the compounds, namely, 6-nonylene alcohol, massoilactone, methyl-cyclopentane, methyl cyclohexane, N-methyl pyrollidine, dermadin, ketotriol, koningin-A, 3-methyl-heptadecanol, 2-methyl heptadecanol, palmitic acid, 3-(2'-hydroxypropyl)-4-(hexa-2'-4-dineyl)-2-(5H)-furanone and 3-(propenone)-4-(hexa-2'-4'-dineyl)-2-(5H)-furanone from these metabolites were chemically characterized by GC-MS/MS. Growing culture in shaker incubator at 25°C and 6.5 *p*H was found to be the optimum conditions for production of maximum proteins. The activities of defense related enzymes, namely, chitinase and ß-1, 3-glucanase produced by the above mentioned *Trichoderma* species were determined. All *Trichoderma* species showed the activity of these enzymes. Moreover, chitinase activity was maximum in *T. virens*, whereas, ß-1, 3- glucanase activity was maximum in *T. viride* among the tested cultures.

Key words: Bioefficacy, ß-1, 3- glucanase, Chitinase, Secondary metabolites, *Trichoderma* spp

Trichoderma species have been described as biological control agents against fungal plant pathogens (Vinale *et al*. 2008). The mycoparasitism has been reported as a major mechanism accounting for the antagonistic activity against phytopathogenic fungi by *Trichoderma*. *Trichoderma* species are known to excrete the hydrolytic enzymes such as chitinase, ß-glucanase and proteases. The anti-fungal mechanism of *Trichoderma* involves fungal cell wall degrading enzymes (Harman *et al*. 2004). Various volatile and non-volatile metabolites are produced by *Trichoderma* species and they are playing a major role in antibiosis. Earlier studies reported effectiveness of four isolates of *Trichoderma* spp. namely, *T. viride* (IARI P1 and IARI P2), *T. virens* (IARI P3) and *T. harzianum* (IARI P4) as seed dressing and soil application formulation (Dubey *et al*. 2007; Dubey *et al*. 2009). The present investigation was undertaken with the objectives of isolation and characterization of secondary compounds from the above mentioned *Trichoderma* spp and determination of enzymatic activity responsible in plant defense mechanism.

[1]Principal Scientist, (e mail: scdube2002@yahoo.co.in), Division of Plant Pathology, [2]Research Associate, Division of Plant Pathology, (e mail: tripathi.aradhika@gmail.com), [3]Emiritous Scientist, (e mail: pd_dureja@yahoo.com), Division of Agricultural Chemicals, [4]Principal Scientist, (e mail: anitagrover@hotmail.com), National Centre for Plant Biotechnology

MATERIALS AND METHODS

The potential isolates of *T. viride* (IARI P1; MTCC No. 5369 and IARI P2), *T. virens* (IARI P3; MTCC No. 5370) and *T. harzianum* (IARI P4; MTCC No.5371) (based on earlier report) were selected for the present study.

Extraction and evaluation of secondary metabolites

The *Trichoderma* spp were grown on potato dextrose broth (PDB) in 4 litre haffkine flat bottom flasks containing 2 litre medium at 25±1°C for 25 days. The culture filtrate was obtained by straining through the muslin cloth. Metabolites were extracted by solvent extraction method into hexane at the ratio of 1: 1 (v/v). The upper layer of solvent, which may contain antifungal compounds, was collected through separating funnel into conical flasks. The solvent (hexane) was evaporated from the solution using rotary evaporator. The residues were re-suspended in solvent (acetone) for

further use.

The fungal mass of the bioagents grown on PDB at 25±1°C for 25 days were air-dried (5–7-days) and cut into small pieces for the extraction of metabolites by Soxhlet water bath distillation method. The fungal mats were wrapped in blotting paper and kept inside the Soxhlet. The extractor was connected with the flask, which contain 400 ml methanol for distillation. Distillation was carried out at 62°C temperature using hot water bath for 3 successive days and methanol was removed using rotary evaporator.

The metabolites obtained from the isolates of *Trichoderma* species were evaluated against *Rhizoctonia bataticola* (dry root rot of chickpea) and *Fusarium oxysporum* f. sp. *ciceris* (wilt of chickpea). The potato dextrose agar medium containing metabolites at different concentrations (500, 250, 125 and 75 ppm) was poured in 90 mm Petri-plates and allowed to solidify. The plates were inoculated by placing 3 mm discs of 7-day old culture of *R. bataticola* and *F. oxysporum* f. sp. *ciceri*s in the centre. For each treatment three replications were used. Controls were maintained without metabolites and acetone separately. The plates were incubated at 25±1°C for 3-days for *R. bataticola* and 6 days for *F. oxysporum* f. sp. *ciceri*s. The colony diameter was recorded and percent growth inhibition was calculated.

Purification of secondary metabolites

Aliquots of the solvents containing metabolites were spotted on thin layer chromatography (TLC) plates having 0.25 mm thickness of silica gel layer. The plates were developed in hexane and benzene mixture in ratio of 1: 1(v/v), air dried for 5 min and visualized by iodine. The compounds were separated using a glass column (75 cm × 2 cm id) containing 50 g of 60 × 120 mesh pre-activated silica gel in hexane. The column was successively eluted with hexane-ethyl acetate, collected 25 ml fractions, distilled on a water bath and the purity was checked on TLC plates. Hexane fractions after concentration were analyzed by gas liquid chromatography (GLC) using flame ionization detector (FID) and OV-I column (10 m×0.3 mm id). Nitrogen was used as a carrier gas with a flow rate of 24 ml/min. Injection port and detector temperature was at 250°C and column temperature programmed from 130°C-250°C at 10°C/min. Further, for characterization of specific compounds/secondary metabolites from each *Trichoderma* isolate, the compounds were extracted in large quantity using 30 L of PDB and stored for later use. Hexane/methanol fractions were analyzed by Varian GC/MS/MS using DB 5 columns (30 m × 0.25 mμ). Injection port and detector were 250°C and column temperature programmed from 100°C-250°C at 10°C/min.

Estimation and characterization of extra cellular proteins and enzymes

The stock solution of bovine serum albumin (BSA) was prepared by dissolving 25 mg BSA in 100 ml of double distilled water. The samples for different concentrations (25, 50, 100, 150, 200 and 250 μg/100μl) of protein were prepared by adding double distilled water and OD values were recorded with the help of spectrophotometer of each concentration separately at 660 nm absorbance and standard curve was plotted**.**

Effect of environmental conditions and pH on production of proteins

To determine the effect of different incubation conditions on production of proteins, three sets of experiments were conducted using minimal synthetic medium (in g/L: chitin 5.0, corn steep solid 5.0, $MgSO_4 7H_2O$ 0.2, K_2HPO_4 0.9, KCl 0.2, NH_4NO_3 1.0, $FeSO_4 7H_2O$ 0.002, $MnSO_4$ 0.002 and $ZnSO_4$ 0.002) in four replications. The flasks of the first set of experiment for four isolates of *Trichoderma* were kept in open condition with shaking at room temperature (25±4°C) with 120 rpm for 7 days. The flasks of second set were kept in BOD with shaking (25±1°C) at 120 rpm for 7 days. The flasks of third set of experiment were placed in BOD without shaking at 25±1°C for 7 days. All the flasks (50 ml medium in each) were inoculated with 7 days old culture of *Trichoderma* species. After incubation, the mycelium was filtered through Whatman No. 1 filter paper in sterilized conical flask under aseptic condition. The culture filtrates were either used immediately for protein estimation or stored at 4°C for further use. Four pH levels (4.5, 5.5, 6.5 and 7.0) were maintained in minimal synthetic medium for each isolate of *Trichoderma* species in three replications. After inoculation with 7 days old culture, the flasks were incubated at 25±1°C for 7 days. The culture filtrates were either used immediately for protein estimation or stored at 4°C until further use.

For estimation of protein the culture filtrate (5 to10 ml) of each bioagent was centrifuged at 1000 rpm for 10 min. and supernatant was transferred into a centrifuge tube. Supernatant of each isolate was pipetted out in 0.1, 0.2, 0.4, 0.6, 0.8 and 1 ml separately into tubes and added 5 ml solution C (carbonate-Cu^{++} soln.) in each tube. Mixed well by vortex and kept for 5 min. Folin – Ciocalteu reagent (1N) was added (0.5 ml) in each tube and vortex well. These tubes were kept 30 min at room temperature for colour development. OD values were recorded for each isolate which developed darker colour with the help of spectrophotometer for estimation of proteins. Protein was estimated by computing the absorbance value as 'y' in equation (y = mx + c) obtained from BSA standard curve.

Estimation of enzyme activity

The minimal synthetic medium [Chitin (from crab shells, practical grade), N-acetyl ß-D-glucosamine (GlcNAc), p-dimethyl aminobenzaldehyde (DMAB), ß-glucosidase from almonds, laminarin from *Laminaria digitata,* p-

hydroxybenzoic acid hydrazide, corn steep solids were obtained from Sigma-Aldrich Company Ltd, USA] was used for the cultivation of *Trichoderma* isolates. The pH was maintained at 6.5 (with 50 mM phosphate buffer). The 7 days old cultures were inoculated with 100 ml of medium and incubated at 25±1°C for 72 hr on a rotary shaker at 120 rpm. The culture filtrate was obtained through Whatman No.1 filter paper and concentrated by lyophilization and used as a source of ß-1, 3-glucanase and chitinase.

Laminarin was used as the substrate to estimate the specific activity of ß-1, 3 glucanase. The degradation of laminarin by the ß-1, 3 glucanase was assayed by determining the reducing sugar (glucose) released by the enzyme. For ß -1, 3-glucanase activity assay standard curve of different concentrations of glucose was made following the method of Lever (1972). The assay was performed as described by Keen and Yoshikawa (1983) with certain modifications where p-hydroxybenzoic acid hydrazide was used as colour reagent to determine reducing sugars. 1 ml reaction mixture contained 50 µl enzyme extract and 1 mg laminarin in assay buffer. 0.1 M potassium acetate (pH 5.2) used as assay buffer. The reaction mixture was incubated in water bath shaker at 37°C for 30 min then the reaction was terminated by heating the mixture in boiling water bath for 3 min. 10 ml samples were taken out and added 3 ml of 0.5% p-hydroxybenzoic acid hydrazide in 0.5 M NaOH then the mixture was boiled in water bath for 5 min. The tubes were then cooled in tap water and absorbance recorded at 410 nm in a spectrophotometer (EC Spectronic) and the amount of glucose released was calculated from the glucose standard curve. One unit of ß -1, 3-glucanase activity was defined as the amount of enzyme that produced 1µmol of glucose released/min/mg of protein under the given conditions. The protein content in the enzyme solution was determined by the method of Bradford (1976) using bovine serum albumin as standard protein.

Hydrolysis of colloidal chitin by three *Trichoderma* species produced by a GlcNAc was determined colorimetrically. For chitinase activity assay the colloidal chitin was prepared according to the method described by Ungappa and Lockwood (1962). A standard curve relating the amount (n moles) of GlcNAc with the OD at 585 nm was prepared following the protocol given by Reissig *et al*. (1955). Chitinase activity was measured calorimetrically by the method described by Pegg and Young (1982). 0.5 ml reaction mixture contained 0.01 ml enzyme extract, 1 mg colloidal chitin and 0.003 mg ß-glucosidase from almonds in 0.1 M sodium acetate buffer (*p*H 5.2) was incubated in water bath shaker at 37°C for 30 min. After incubation to this 0.1 ml of 0.8 M potassium tetra borate buffer *p*H 9.1 was added then the mixture was boiled in water bath exactly for 3 min and cooled in tap water. From this mixture 0.5 ml was taken in tube to which 2.5 ml p-dimethyl aminobenzaldehyde solution was added, incubated at 37°C for 20 min and cooled in tap water. A reaction mixture with all the ingredients was heat killed immediately after addition of enzyme extract to serve as reference or blank. The absorbance recorded at 585 nm in a spectrophotometer and the amount of GlcNAc released was calculated from the GlcNAc standard curve. One unit of chitinase activity was defined as the amount of enzyme that produced 1 nmol GlcNAc released min^{-1} mg^{-1} of protein under the given conditions. The protein content in the enzyme solution was determined by the method of Bradford (1976) using bovine serum albumin as standard protein.

RESULTS AND DISCUSSION

Evaluation of secondary metabolites

Amongst the secondary metabolites present in culture filtrate and that in mycelial mass, the metabolites of *T. harzianum* caused maximum growth inhibition followed by

Table 1 Effect of secondary metabolites isolated from culture filtrates and mycelial mass of various species of *Trichoderma* on *Rhizoctonia bataticola*

Trichoderma specis	Radial growth inhibition (%) of *R. bataticola* at different concentrations (ppm) of compounds from culture filtrate and mycelial mass									
	75 ppm		125 ppm		250 ppm		500 ppm		Mean	
	Filtrate	Mycelia	Filtrate	Mycelia	Filtrate	Mycelia	Filtrate	Mycelia	Filtrate	Mycelia
T. viride (IARIP 1)	72.8(58.3)	72.6(54.3)	84.6(66.9)	76(60.6)	89.7(71.2)	87(68.9)	100(90)	100(90)	86.8(71.6)	83.9(68.5)
T. viride (IARIP 2)	78.7(62.2)	67.6(55.3)	87.9(69.7)	74.3(59.5)	94.8(77.3)	80.4(63.7)	100(90)	100(90)	90.3(74.8)	80.6(67.1)
T. virens (IARIP 3)	78.9(62.7)	70.4(57.1)	84.6(66.6)	80.8(64.1)	88.4(70.1)	80.4(63.8)	100(90)	100(90)	88(72.4)	82.9(68.7)
T. harzianum (IARIP 4)	81.7(64.7)	82.1(65.1)	87.4(67.2)	83.9(66.3)	95.4(77.6)	88.2(70)	100(90)	100(90)	91.1(74.9)	88.6(72.8)
Mean	78.0(62.0)	73.2(58)	86.1(67.6)	78.7(62.7)	92.1(74.0)	84(66.6)	100(90)	100(90)		
LSD (*P*= 0.05) for *Trichoderma* species									(1.0)	(2.1)
LSD (*P*= 0.05) for concentrations									(1.0)	(2.1)
LSD (*P*= 0.05) for *Trichoderma* × concentrations									(2.0)	(4.26)

Figures in parentheses are transformed angular values

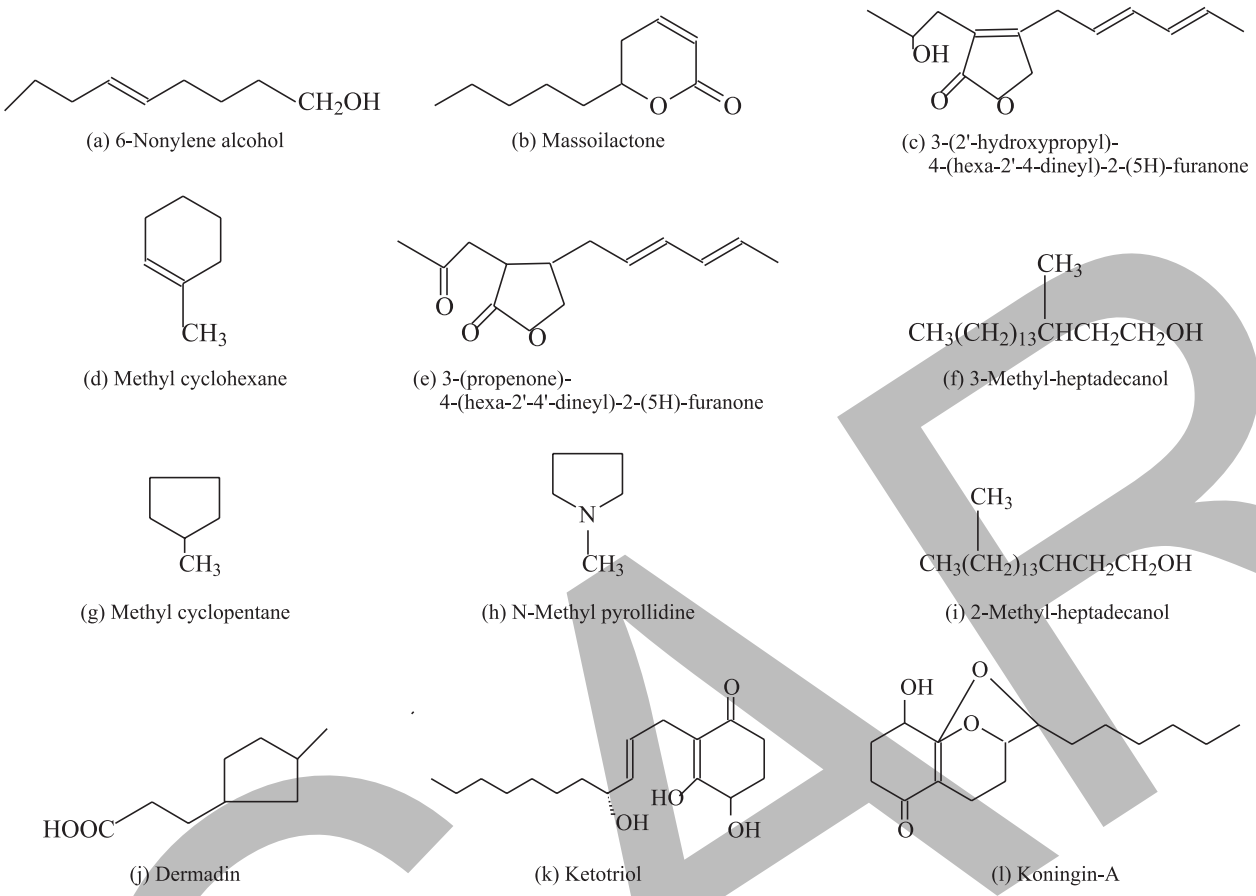

Fig 1 Secondary metabolites characterized from culture filtrate and mycelial extract of *Trichoderma* species

T. viride and *T. virens* against *R. bataticola*. There was gradual increase in the inhibition of fungal growth as the concentrations of the metabolites increased and 100% growth inhibition was observed at 500 ppm concentartion. Even at 75 ppm concentration, metabolites obtained from culture filtrate and mycelial mass caused 78% and 73.2% growth inhibition, respectively (Table 1). Of the metabolites obtained from culture filtrate, *T. virens* caused maximum growth inhibition of *Fusarium oxysporum* f. sp. *ciceris*, followed by *T. viride* and *T. harzianum*. Whereas, in case of metabolites of mycelial mass, maximum inhibition was recorded in *T. viride* followed by *T. harzianum* and *T. virens* (Table 2). There was linear relationship in between concentration and growth inhibition. The metabolites obtained from culture filtrates of *Trichoderma* species caused 100% growth inhibitions at 500 ppm, whereas in case of mycelial mass, more than 60% growth inhibition at 500 ppm was observed. The lowest concentration in this study (75 ppm) caused more than 47% growth inhibition.

Thus, the secondary metabolites extracted from culture as well as mycelial mass of *Trichoderma* species caused variable inhibition to the plant pathogenic fungi *R. bataticola* and *F. oxysporum* f. sp. *ciceris*. Higher the concentration greater was the inhibition. Moreover, the growth inhibition of these fungi at 75 ppm concentration was significantly superior over the control. *Trichoderma* species are free-living fungi that are highly interactive in root, soil and foliar environments and have been used successfully in field trials to control many crop pathogens. Structural and biological studies of the metabolites isolated from *Trichoderma* species are reviewed other authors (Reino *et al*. 2008). Siddiquee *et al*. (2009) demonstrated inhibitory properties from both volatile and non-volatile antifungal compounds produced by *T. harzianum* (against *Ganoderma boninense*) and *Trichothecium roseum* (against *R. solani*). The culture filtrate of *T. roseum* strongly inhibited the mycelial growth and sclerotial formation, its germination and viability, which proved that the biocontrol activity is antibiosis-mediated. The extracellular crude antifungal metabolites of *T. roseum* were thermo-and photo-stable and effectively reduced the sheath blight disease in rice up to 47.7% (Jayaprakashvel *et al*. 2010). Vinale *et al*. (2009) also evaluated *in vitro* production and antibiotic activities of the major compounds synthesized by *T. harzianum* strains T22 and T39 against several plant pathogenic fungi. The secondary metabolites showed different levels of antibiotic activity. The inhibitory property of the metabolites produced by *Trichoderma* species evaluated in the present study clearly indicated the antibiosis

Table 2 Effect of secondary metabolites isolated from culture filtrates and mycelial mass of various species of *Trichoderma* on *Fusarium oxysporum* f. sp. *ciceris* (FOC)

Trichoderma specis	Radial growth inhibition (%) of FOC at different concentrations (ppm) of compounds from culture filtrate and mycelial mass									
	75 ppm		125 ppm		250 ppm		500 ppm		Mean	
	Filtrate	Mycelia	Filtrate	Mycelia	Filtrate	Mycelia	Filtrate	Mycelia	Filtrate	Mycelia
T. viride (IARIP 1)	25.3(30.2)	52.3(46.3)	70.2(56.9)	56.2(48.6)	72.5(58.3)	63.6(52.9)	100(90)	67.2(55.0)	67(58.8)	59.8(50.7)
T. viride (IARIP 2)	36.7(37.3)	56(48.4)	69.6(56.5)	59.1(50.3)	80.9(64.1)	63.6(52.9)	100(90)	70.1(56.7)	71.8(62)	62.2(52.1)
T. virens (IARIP 3)	60.1(50.9)	52.8(46.6)	61.9(51.9)	55.7(48.3)	83.9(66.3)	58.7(50.1)	100(90)	62.3(52.2)	76.5(64.8)	57.4(49.3)
T. harzianum (IARIP 4)	36.6(36.8)	56.4(48.7)	45(42.1)	57.5(49.3)	73.1(58.8)	61.5(50.9)	100(90)	63.3(52.7)	63.7(56.9)	59.7(50.4)
Mean	47.8(38.4)	54.4(47.5)	61.7(51.9)	57.1(49.1)	69.9(61.9)	61.8(51.7)	100(90)	65.7(54.2)		
LSD (P= 0.05) for *Trichoderma* species									(0.6)	(1.5)
LSD (P= 0.05) for concentrations									(0.6)	(1.5)
LSD (P= 0.05) for *Trichoderma* × concentrations									(1.3)	(N.S.)

Figures in parentheses are transformed angular values

activity of the isolates which could be utilized further for development of bio-formulation for disease management.

Characterization of secondary metabolites

The TLC and GLC analysis showed the presence of several secondary metabolites/compounds. Further, these metabolites were characterized using varian GC/MS/MS (Table 3). The mass spectrum of metabolites obtained from culture filtrate of *T. viride* identified as 6-nonylene alcohol (Fig 1a), massoilactone (Fig 1b), 3-(2'-hydroxypropyl)-4-(hexa-2'-4'-denyl)-2-(5H)-furanone (Fig 1c). The mycelial extract of *T. viride* also showed the presence of several secondary metabolites. The compounds were identified as methyl cyclohexane (Fig 1d), palmitic acid [CH_3 $(CH_2)_{14}COOH$], 3 (- propenone)-4-(hexa-2'-4-dineyl)-2-(5H)-furanone (Fig 1e) and 3-methyl heptadecanol (Fig 1f). Ion chromatogram of metabolites of culture filtrate of *T. virens* showed several secondary compounds. Some of them were identified as methyl-cyclopentane (Fig 1 g), methyl cyclohexane (Fig 1d), N-methyl pyrollidine (Fig 1 h), 2-methyl heptadecanol (Fig 1i), dermadin (Fig 1j) and massoilactone (Fig 1b). Similarly from mycelial extract the compound was identified as Ketotriol (Fig 1 k). The metabolites of culture filtrate of *T. harzianum* showed the presence of several compounds and some of them were characterized. They were methyl cyclopentane (Fig 1 g), cyclonerodiol, koningin-A (Fig 1l), 2-methyl heptadecanol (Fig 1i) and methyl cyclopentane (Fig 1 g). Total ion chromatogram generated from mycelial extract of *T. harzianum* also showed the presence of several compounds. Only palmitic acid and ketotriol (Fig 1 k) were characterized. *Trichoderma* species secrete diverse secondary metabolites with antibiotic properties and antifungal activities (Singh *et al*. 2005). The secondary metabolites were isolated and characterized from culture filtrates of two commercial *T. harzianum* strains T22 and T39 and their production during the antagonistic interaction with *R. solani* was also investigated (Vinale *et al*. 2006).

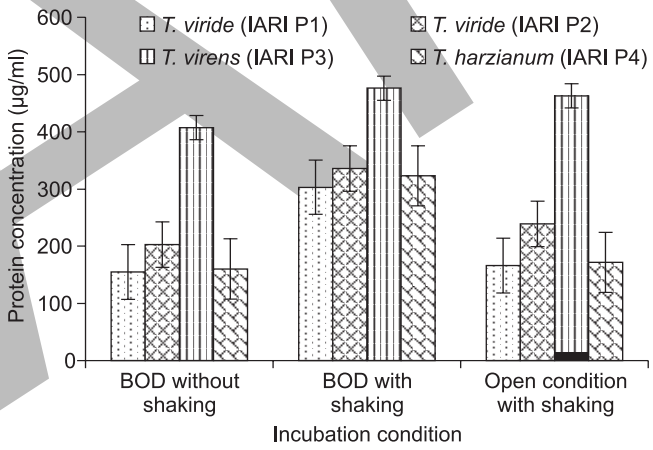

Fig 2 Concentration of protein in culture filtrates of *Trichoderma* species under different incubation conditions

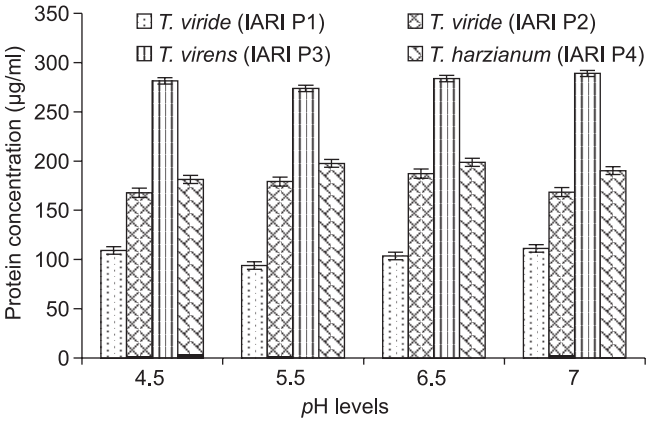

Fig 3 Effect of different pH levels on production of proteins in culture filtrates of *Trichoderma* species

Table 3 The molecular ion peak and identified compounds from the metabolites obtained from culture filtrate and mycelial extract of *Trichoderma* species

Trichoderma spp	Molecular ion peak (m/z) with fragmented ion	Compounds identified
T. viride (culture filtrate)	128, 127	6-Nonylene alcohol
	168, 152, 124	Massoilactone
	226	3-(2'-hydroxypropyl)-4-(hexa-2'-4-dineyl)-2-(5H)-furanone
T. viride (mycelial extract)	98, 55	Methyl cyclohexane
	256	Palmitic acid
	220, 80	3-(propenone)-4-(hexa-2'-4'-dineyl)-2-(5H)-furanone
	270, 90	3-Methyl-heptadecanol
T. virens (culture filtrate)	84, 57	Methyl-cyclopentane
	98, 57	Methyl cyclohexane
	95, 70	N-Methyl pyrollidine
	270, 149, 135, 105, 57	2-Methyl-heptadecanol
	147, 129, 111	Dermadin
	168	Massoilactone
T. virens (mycelial extract)	282, 264, 95, 54	Ketotriol
T. harzianum (culture filtrate)	84	Methyl cyclopentane
	240, 215, 164,	Cyclonerodiol
	284, 263, 237, 55	Koningin-A
	270, 256, 242, 158, 92,78	2-Methyl heptadecanol
	84	Methyl cyclopentane
T. harzianum (mycelial extract)	256	Palmitic acid
	282	Ketotriol

Table 4 Productivity of ß -1, 3-glucanases and chitinases by three potential isolates of *Trichoderma* species

Trichoderma species	Activity (units)		Protein content (mg/ml)		Specific activity (m units/mg of protein)	
	ß -1, 3-glucanases*	Chitinases**	ß -1, 3-glucanases	Chitinases	ß -1, 3-glucanases	Chitinases
T. viride (lARI P l)	0.70±0.04	1.0±0.04	44.0±0.91	44.0±0.41	15.91±0.54	22.72±0.64
T. virens (lARI P 3)	0.52±0.05	2.2±0.11	34.0±0.41	34.0±0.46	15.29±0.34	64.71±0.38
T. harzianum (IARI P 4)	0.37±0.03	0.87±0.08	45.0±0.41	45.0±0.46	8.22±0.26	19.33±0.35

* µmoles of glucose released min^{-I}
** nmoles GlcNAc released min^{-I}

Estimation and characterization of extra cellular proteins and enzymes

The absorbance was recorded with help of spectrophotometer for each concentration of protein (25, 50, 100, 150, 200 and 250 □g/100□l) separately at 660 nm. The curve was plotted on the basis of standard peek appeared for preparation of Bradford standard (BSA) curve. The graph drew on the basis of recorded absorbance value of BSA standard at different concentrations and it was found suitable and accurate with linear equation y = 0.0021x + 0.0117 and R^2 coefficient value 0.9938 (<1). Out of 3 different conditions of incubation, incubation in BOD with shaking was the best for maximum production of proteins (359.5 □g/ml) followed by shaking in open condition (Fig 2) for *Trichoderma* species. Out of *p*H levels evaluated, *p*H 6.5 was the best for maximum production of proteins (282.03 □g/ml) for *Trichoderma* species (Fig 3). Subsequently these optimized conditions were used for production of extra cellular enzymes, because the optimum conditions also favours maximum production and activity of the enzymes.

In addition to secondary metabolites, *Trichoderma* species spp also produces different classes of fungal cell wall degrading hydrolytic enzymes such as chitinases, ß-1, 3-glucanases and proteases, which play an important role in mycoparasitism (Harman *et al.* 2004). Production of cell wall degrading enzymes by *Trichoderma* species were monitored after 72 hr of growth in minimal synthetic medium containing chitin as a carbon source.

ß- 1, 3 glucanase and chitinase activity

The enzyme was active only towards glucan containing ß-1, 3 linkages such as laminarin. All the isolates showed different levels of the activity of ß-1, 3 glucanase. It was highest in *T. viride*, followed by *T. virens* and *T. harzianum*.

The highest specific activity of chitinase was found in *T. virens* followed by *T. viride* and *T. harzianum* (Table 4). Thus, the isolates were found variable in respect of production of chitinases.

The antifungal arsenal of *Trichoderma* spp. includes a great variety of lytic enzymes, most of which play a great role in biocontrol (Kubicek *et al*. 2001). A number of *Trichoderma* isolates are able to excrete hydrolytic enzymes such as chitinases, proteases and ß-glucanases into the medium in the presence of laminarin, chitin or cell walls of phytopathogenic fungi. Extracellular chitinase and ß-1, 3-glucanase of the newly isolated *T. harzianum* T24 inhibited growth of *Sclerotium rolfsii*. A combination of these enzymes which exhibited reasonable thermostability and low ED_{50} values against the pathogen, seems to offer a primary means of controlling the pathogen (El-Katatny *et al*. 2001). Enzymes and antibiotic substance are produced that kill and/or degrade the target hyphae and permitte penetration of the *Trichoderma* strains. Both the enzymes and antibiotics are strongly antifungal and are synergistic in their action (Shoresh *et al*. 2010). The application of metabolites for crop protection, such as host defense inducers and antibiotics, may become a reality in the near future as they can be produced cheaply in large quantities on an industrial scale, easily separated from the fungal biomass, dried and formulated for spray or drench applications (Vinale *et al*. 2008). The isolates of *Trichoderma* included in the present study showed the activity of enzymes and production of metabolites (antibiotics) may be the reason for their effectiveness against *R. bataticola* and *F. oxysporum* f. sp. *ciceris*.

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