



Chitinolytic efficacy and secretion of cell wall-degrading enzymes from *Trichoderma* spp. in response to phytopathological fungi

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ABSTRACT

Chitinolytic activity and major antifungal enzyme secretion from *Trichoderma* spp. were studied. Soil samples were collected from different environmental niche of North Gujarat Region, India, and 12 different species of *Trichoderma* were obtained and identified. Among 12 isolates, 4 isolates were identified as *Trichoderma harzianum*, 5 isolates were identified as *Trichoderma viride*, and remaining 3 isolates were as of *Trichoderma hamatum*. These isolates were identified using species-specific primers amplification by polymerase chain reaction. All identified isolates were screened for chitinase activity using colloidal chitin derived from commercial chitin on the media supplemented with bromocresol purple. According to the results of chitinase activity screening assay, *T. viride* was found to be more potential isolate for chitinase production. From biocontrol assay using dual culture method, *T. viride* was found to be more potent antagonist against fungal plant pathogens such as *Aspergillus niger*, *Fusarium oxysporum*, and *Sclerotium rolfsii*. *T. viride* was selected for further study of biocontrol potential and production of cell wall-degrading enzymes. *T. viride* was inoculated in media containing basal media and mycelia of fungal pathogens for cell wall-degrading enzyme production. It was found that *T. viride* secretes three major cell wall-degrading enzymes, i.e., chitinase, protease, and β -glucanase. Optimum production of all three enzymes was found at 96 h incubation. Details of antifungal protein secretion are mentioned in this paper.

1. INTRODUCTION

Genus *Trichoderma* and efficient biocontrol strain are being industrialized as effective biological fungicides, and their biocontrol mechanism of biological control involves the role of secondary metabolites with prospective applications as new antibiotics [1,2]. They produce many antifungal enzymes including chitin-degrading enzymes which used economically as a basis of these kind of proteins. Many reports suggested that chitinolytic enzymes producing species of *Trichoderma* are prove most effective agents as different biological control of plant infections or diseases [3-8]. Chitinase enzymes are chitinolytic proteins which hydrolyze the β -1, 4-glycosidic linkages within the N-acetyl glucosamine monomers in the structure of chitin and are extensively distributed in nature [9].

The biocontrol mechanisms of *Trichoderma* involve several events such as competition for nutrition, production of antibiotic, and mycoparasitism [10,11]. Mycoparasitism has been proposed as most effective and very important incompatible machinery exhibited by

different *Trichoderma* spp. [12]. After recognition of host, different events with *Trichoderma* spp. are occurred like coiling around hyphae and enter the cell wall by cell wall digestive enzymes action [13]. Cell wall digestive enzymes produced by mycoparasites allow them to make holes into its respective host and consumed nutrients for their own development. Chitin is major structural components having regularly arranged β -1, 3-glucan material in most phytopathogenic fungi cell wall. Two enzymes, chitinases and β -1, 3-glucanases, play a significant role in the mycoparasitism competitor interaction between *Trichoderma* spp. and its respective hosts [12].

A extensive reviews of research have been aimed at explaining the mechanism of secretion of chitinase and β -1,3-glucanase by different *Trichoderma* spp. on variety of carbon sources media. Most information has been known for expression of chitinase and β -1,3-glucanase enzymes by different *Trichoderma* spp., but the factors which are affecting secretion of these enzymes and the characters of the inducers and repressors are not well understood yet [5-12]. The antagonist mechanism of *Trichoderma* spp. inducing secretion of chitinolytic, proteolytic, and glucoytic enzymes and their controlling mechanism is still a hypothesis, inspite of numerous studies and reports [14]. In time course of mycoparasitism, the cell wall-degrading enzymes act synergistically and exerted their action on pathogenic fungi. Thus, considerate the induction process of these enzymes is essential for selection of most significant *Trichoderma* spp. for biocontrol purpose.

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The aim of this study was to isolate the screening of chitinolytic *Trichoderma* spp. and estimate the communication between the antagonists *Trichoderma* spp. and three fungal pathogens *Aspergillus niger*, *Fusarium oxysporum*, and *Sclerotium rolfsii* and the possibility of mycoparasitism through the efficacy of different *Trichoderma* spp. for the stimulation of three cell wall digestive enzymes using mycelia of three pathogenic fungus as source of carbon.

2. MATERIALS AND METHODS

2.1. Purification and Identification of Different *Trichoderma* spp.

Soil was sampled from agricultural field and rhizosphere of plantation crops of three districts of North Gujarat. *Trichoderma* selective medium supplemented with Captan (TSMC) was used as specific selective medium for various *Trichoderma* species and it contained (g/L) MgSO₄·7H₂O - 0.2, K₂HPO₄ - 0.9, KCl - 0.15, NH₄NO₃ - 1.0, glucose - 3.0, chloramphenicol - 0.25, fenaminosulf - 0.3, pentachloronitrobenzene - 0.2, rose bengal - 0.15, captan - 0.02 (post autoclaving), agar - 20 as well as in modified TSM (Smith and Goodman 1990): Containing (g/L): Ca(NO₃)₂ - 1.0, KNO₃ - 0.26, MgSO₄·7H₂O - 0.26, KH₂PO₄ - 0.12, CaCl₂·2H₂O - 1.0, citric acid - 0.05, sucrose - 2.0, agar - 20.0, chlortetracycline - 0.05, and captan (50% wettable powder) - 0.04 [15]. The identification of *Trichoderma* isolates was confirmed by amplification of species-specific primer polymerase chain reaction (PCR). The sequence of primer was adopted from method reported by Meena Surya in his Ph.D. thesis [16]. One primer was OLIGO 361 (-5'-ATCCGTACGC -3') specific to *T. viride*, and other one was OPA-5 (-5'-TGCCTGACTC -3') specific to *Trichoderma harzianum* and *Trichoderma hamatum*.

2.2 Chitinolytic Activity Screening

2.2.1. Preparation of colloidal chitin

Colloidal chitin was prepared with the help of commercial chitin (Himedia) by the method of Roberts and Selitrennifoff [17] with a little modifications and supplemented in the chitinase assay medium for the source of carbon. Chitin was digested by concentrated HCl by constant stirring using a magnetic stirrer in lower temperature at 4°C (refrigerator) overnight followed by extraction of colloidal chitin in 2000 ml of ice-cold ethanol (95%) for neutralization and kept at 26°C for overnight. Centrifugation was performed at 3000 rpm for 20 min at 4°C, and pellet was washed with sterile distilled water at 4°C till the alcohol residues was absolutely removed. The soft, pasty consistency of colloidal chitin obtained having 90-95% moisture, and it was stored at 4°C until further use in chitinase assay.

2.2.2 Solid medium for screening of chitinase positive *Trichoderma* spp.

Chitinase secretion detection medium consists of 0.3 g of MgSO₄·7H₂O, 3.0 g of (NH₄)₂SO₄, 2.0 g of KH₂PO₄, 1.0 g of citric acid monohydrate, 15 g of agar, 200µl of Tween-80, 4.5 g of colloidal chitin, and 0.15 g of bromocresol purple (BCP) (pH: 4.7) per liter and then autoclaved at 121°C for 15 min. Petri plates were poured with Lukewarm medium, and culture plugs were inoculated for testing chitinase activity. Plates were incubated at 25°C ± 2°C for colored zone formation.

2.3. Antagonism against Fungal Pathogen

In vitro test for biocontrol *in vitro* antifungal activity of selected *Trichoderma* spp. against *F. oxysporum*, *S. rolfsii*, and *A. niger* was tested on dual culturing method. *F. oxysporum*, *S. rolfsii*, and *A. niger* were taken from Shri A. N. Patel Postgraduate Institute, Sardar Patel

University, Vallabh Vidyanagar, Anand. 5 mm discs of above fungal pathogen strains and *Trichoderma* disc cut from 7-day old culture were placed 5 cm distance from each other and kept at 30°C. Inhibition of fungi and infringement over pathogens by *Trichoderma* were measured and matched with the control.

2.4. Profiling of Secretary Antifungal Enzymes from Culture Filtrate of *Trichoderma*

2.4.1. Culture preparation

F. oxysporum, *S. rolfsii*, and *A. niger* were maintained on potato dextrose agar (PDA) and grown in potato dextrose broth at 28°C followed by incubation on shaker at 150 rpm in a 500 ml Erlenmeyer flask for 7 days. Mycelia were separated by centrifugation for 10 min at 5000 rpm at room temperature. Washing of mycelia was done using 50 ml of double distilled water followed by boiling to obtain deactivated mycelia, which were then stored at -20°C until further use. Deactivated mycelia were also incubated on PDA and observed for a week to determine whether the deactivated mycelia were completely deactivated or not.

Selected *Trichoderma* was grown and maintained on PDA plates. To simulate the biocontrol environment, glucose was separately added to a basic liquid medium. The basic liquid medium contains 1.4 g of (NH₄)₂SO₄, 0.2 g of KH₂PO₄, 6.9 g of NaH₂PO₄·H₂O, 0.3 g of MgSO₄·7H₂O, 1.0 g of peptone, and 0.3 g of urea. The additions were 5 g of deactivated fungal (plant pathogen) mycelia and 5 g of glucose [18]. The conidia were grown in a 250 ml liquid medium at 22°C with shaking, and centrifugation at 5000 rpm at 4°C for 10 min was performed at every 24-120 h.

2.4.2 Enzymatic assay

Ammonium sulfate (Qualigen, Mumbai) was added to selected *Trichoderma* culture supernatant kept on magnetic stirrer at 4°C for overnight (approximate for 12 h) for 80% saturation. Supernatant than centrifuged at 15000 rpm for 30 min at 4°C to obtain precipitated protein pellets, which then resuspended in 3 ml of deionized water. Total protein concentration was measured by the method of Oswald Lowry [19].

The activity of Chitinase (EC 3.2.1.14) was performed colorimetrically using colloidal chitin as substrate and β-1,3 glucanase (EC 3.2.1.39) using laminarin as substrate. Reducing sugar was released and determined by a method involving DNSA, N-acetyl glucosamine, and glucose as a standard [20]. The specific activity of chitinase and β-1,3 glucanase was expressed as 1 µmol of reducing sugars released h⁻¹ mg⁻¹ of protein.

Protease activity (EC 3.4.21.4) was measured using casein, as a substrate. The method reported by Charney and Tomarelli was carried out for protease assay [21]. Total free amino acids were released and estimated using ninhydrin method [22]. Proteolytic activity was expressed as the amount of enzyme required to increase of µg free amino acids h⁻¹ mg⁻¹ protein. Blank was treated as zero time incubation.

3. RESULT

3.1. Identification of Different *Trichoderma* spp.

Twelve different cultures of *Trichoderma* spp. were obtained from the selective media plates. Identification was made by PCR amplification of DNA using three species-specific primers. PCR amplification of 12 isolates was shown in Figs. 1 and 2, and specific bands were amplified from each fungus. Among 12 isolates, 4 isolates showed 1.6 kb amplified polymorphic band, which is specific to *T. harzianum* using

OLIGO 631 primer. 5 isolates showed 600 bp amplified polymorphic band with OPA 5 primer, which is specific to *T. viride*, and 3 isolates gave amplified polymorphic band of 400 bp with OPA 5 primer, specific for *T. hamatum*.

3.2. Effect of Antagonists against Mycelial Growth of Plant Fungal Pathogens

Potential biocontrol isolates *Trichoderma viride*, *T. harzianum*, and *T. hamatum* were screened against *F. oxysporum*, *S. rolfsii*, and *A. niger*. The effectiveness of the biocontrol agents against mycelial growth of *F. oxysporum*, *S. rolfsii*, and *A. niger* was studied *in vitro* (Table 1). *T. viride* strain was the most effective, suppressing 88.7%, 86.4%, and 87.34% of *F. oxysporum*, *S. rolfsii*, and *A. niger* mycelia growth, respectively. *T. harzianum* strain was the next most effective, suppressing 82.3%, 80.21%, and 79.34% of *F. oxysporum*, *S. rolfsii*, and *A. niger* mycelia growth, respectively. None of the *T. hamatum* isolates was significantly effective against this pathogen suppressing 58.32%, 56.12%, and 55.89% of *F. oxysporum*, *S. rolfsii*, and *A. niger*, respectively. Suppression of fungal plant pathogens against *Trichoderma* spp. was showed in Fig. 3.

Trichoderma grew on possibly surrounds of the pathogenic fungus (*F. oxysporum*, *S. rolfsii*, and *A. niger*) in plate to overwhelm additional growth of pathogenic fungi. No additional growth of pathogenic fungi was observed in *Trichoderma* controlled plates. All of the pathogenic fungi were fully grown on control plates after continued incubation.

These preliminary results showed that present strain of *Trichoderma* can be used as a biological controlling agent against the verified fungal organisms.

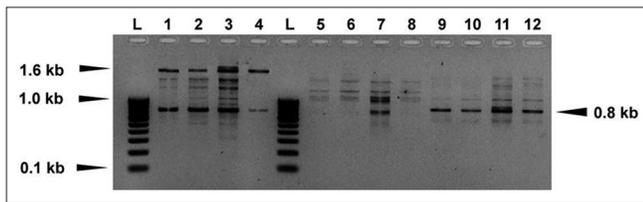


Fig. 1: Polymerase chain reaction amplification of different species of *Trichoderma* using OLIGO-631 RAPD primer showing 1.6 kb species-specific band in *Trichoderma harzianum*.

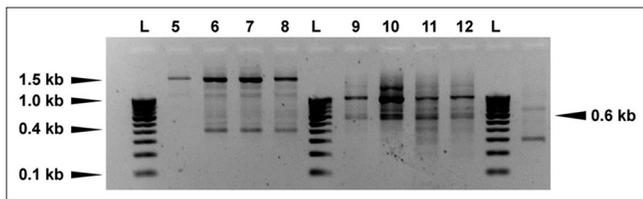


Fig. 2: Polymerase chain reaction amplification of different species of *Trichoderma* using OPA-5 RAPD primer showing 600 bp specific band in *Trichoderma viride* and 400 bp in *Trichoderma hamatum*.



Fig. 3: Antagonism between *Trichoderma* spp. and three pathogenic fungi, *Aspergillus niger*, *Fusarium oxysporum*, and *Sclerotium rolfsii* at 7 and 14 DAI *Trichoderma* spp. at the left and pathogen at right side. (a and b) Dual culture of *Trichoderma* spp. and *A. niger* at 7 days and 14 days after inoculation, respectively. (c and d) Dual culture of *Trichoderma* spp. and *S. rolfsii* at 7 days and 14 days after inoculation, respectively. (e and f) Dual culture of *Trichoderma* spp. and *F. oxysporum* at 7 days and 14 days after inoculation, respectively.

Table 1: Effect of three different *Trichoderma* spp. on the growth of three different plant fungal pathogens through mycoparasitism.

Treatment	<i>A. niger</i> (cm)		<i>F. oxysporum</i> (cm)		<i>S. rolfsii</i> (cm)	
	Radial growth of pathogen	Radial growth of antagonist	Radial growth of pathogen	Radial growth of antagonist	Radial growth of pathogen	Radial growth of antagonist
<i>T. harzianum</i>	0.57	7.51	0.63	7.23	0.62	7.22
<i>T. viride</i>	0.53	7.42	0.60	7.12	0.57	7.09
<i>T. hamatum</i>	2.91	6.23	2.80	6.10	2.94	6.03
Control	5.00	8.00	5.00	8.00	5.00	8.00

A. niger: *Aspergillus niger*, *F. oxysporum*: *Fusarium oxysporum*, *S. rolfsii*: *Sclerotium rolfsii*, *T. harzianum*: *Trichoderma harzianum*, *T. viride*: *Trichoderma viride*, *T. hamatum*: *Trichoderma hamatum*

3.3. Dyeing of Chitinase Detection Medium

BCP (0.15 g/l) and colloidal chitin (4.5 g/l) were added to chitinase detection medium. Forming substrate had a sunny yellow color, and adequate BCP was recollected after adjustment of pH to 4.7 followed by 15 min sterilization at 121°C. Colored zones diameter (mm) was shown in Table 2. No complex protocols for staining of the chitinous material and mordant for fixing of colors were necessary as per the previous reports [23].

3.4. Qualitative Determination of Chitinase activity of *Trichoderma* Isolates on Medium Supplemented with Colloidal Chitin

Trichoderma isolate, which has chitin degradation capacity, was inoculated on colloidal chitin media containing BCP (pH 4.7) showed degradation of chitin to N-acetyl glucosamine causing a parallel shift in pH toward alkalinity and change of color of BCP from yellow to purple zone surrounding the inoculated fresh culture plugs where the chitin was utilized. Activity of chitinase qualitatively exhibited by 12 isolates of *Trichoderma* was measured by the width of the purple-colored zone after 3 days of incubation in the agar medium supplemented with colloidal chitin and was classified into different groups (1 = no chitinase activity; 2 = low chitinase activity; 3 = medium chitinase activity; and 4 = high chitinase activity) (Table 2). Four isolates of *Trichoderma* (# 4, 9, 10, 11, and 12) presented rapid and maximum response in colloidal chitin (derived from commercial chitin) and represented efficient chitinase activity groups. One isolate (# 6) exhibits very low chitinase activity, and remaining six isolates (# 1, 2, 3, 5, 7, and 8) showed the medium activity of chitinase.

3.5. Extracellular Cell Wall-Degrading Enzymes of *T. viride* in the Presence of Pathogens

Isolate identified as *T. viride* proved high chitinase producing strain and also significantly inhibits the growth of pathogenic fungus. Hence, *T. viride* was selected for the further enzymatic study. From culture grown in media containing deactivated fungal pathogen, mycelia and glucose secrete extracellular proteins which was harvested and saturated using 80% ammonium sulfate. Quantitative measurement of total protein was determined by the method of Oswald Lowry [19].

Significant activities of chitinase, β -glucanase, and protease were found when *T. viride* grown in the presence of deactivated mycelia of plant pathogenic fungi. The higher activity of this cell wall-degrading *T. viride* enzymes demonstrates that these enzymes

are stimulated in the presence of deactivated mycelia of fungal pathogens. The phenomenon was observed in the daily decrease of the fungal mycelia growth when grew along with *T. viride* in dual culture PDA plate.

Three fungal pathogens were tested in this experiment for evaluating antagonist effect of *T. viride*, and it produces a significant level of various cell wall-degrading enzymes when grew along with pathogen mycelia. There were paradoxical arrays in cell wall-degrading enzymes secretion by *T. viride* against all tested fungal pathogens during mycoparasitism at different time intervals, namely, 24, 48, 72, 96, and 120 h incubation with the pathogen. The chitin-degrading activity induced in some antagonists inhibits the growth of that species. Mean differences in antagonists (T) on chitinase-specific activity found to be important (Table 3). The considerably maximum chitinase activity was observed by *T. viride* (38.00 U/mg protein) in the presence of *A. niger*, followed by 35.84 U/mg protein in the presence of *F. oxysporum* and 34.35 U/mg protein in the presence of *S. rolfisii*. Opposite of antagonists, mean time interval (H) indicates unbroken increase in enzyme activity during 24-96 h.

The interaction effect of T \times H was notably varied for stimulation of chitinase. The significant level of chitinase activity was exerted by *T. viride* at 96 hrs, and it was 21.96, 20.71, and 19.85 fold higher compared to control in the presence of *A. niger*, *F. oxysporum*, and *S. rolfisii*, respectively, followed by considerably decreased at 120 h. Possibly, this rise in chitinase by antagonist *T. viride* caused more coiling capacity during mycoparasitism at 7 DAI and bigger percent growth retardation of different fungal pathogens.

The protease activity showed by *T. viride* inhibiting the growth of that species. Mean differences of antagonists (T) on protease activity found to be very important (Table 4). The considerably higher level of protease activity was exerted by *T. viride* (48.17 U/mg protein) in the presence of *A. niger*, followed by 35.49 U/mg protein in the presence of *F. oxysporum* and 07.53 U/mg protein against *S. rolfisii* antagonist. Opposite of antagonists, mean time interval (H) showed uninterrupted increase in activity of enzyme during 24-96 h when *T. viride* grew along with pathogen mycelia.

The β -1, 3 Glucanase activity induced in *T. viride* comprises the growth of that species. Mean antagonists (T) differences on the specific activity of glucanase found to be significant (Table 5). The significantly highest glucanase activity was recorded by *T. viride* (10.17 U/mg protein) in the presence of *A. niger*, followed by 09.49 U/mg protein in the presence of *F. oxysporum* and 07.45 U/mg protein in the presence

Table 2: Colored zone diameter generated by *Trichoderma* isolates for chitinase activity on medium supplemented with colloidal chitin.

<i>Trichoderma</i> spp.	Chitinase activity shown by isolates belonging to three <i>Trichoderma</i> spp.			
	Color Zone (mm)			
	1-5 (nil)	10-15 (low)	20-25 (medium)	>30 (high)
<i>T. harzianum</i>	-		Isolate 1, 2 and 3	Isolate 4
<i>T. viride</i>	-		Isolate 5	Isolate 9, 10, 11 and 12
<i>T. hamatum</i>	-	Isolate 6	Isolate 7 and 8	

T. harzianum: *Trichoderma harzianum*, *T. viride*: *Trichoderma viride*, *T. hamatum*: *Trichoderma hamatum*

Table 3: Specific activity of chitinase produced by *T. viride* incubated with mycelia of three different fungal pathogens, *A. niger*, *F. oxysporum*, and *S. rolfisii*.

Organism	Chitinase (μ mol GlcNAc/h/mg protein)					Mean (T)
	24	48	72	96	120	
<i>A. niger</i>	35.63	37.04	37.94	41.37	37.86	38.00
<i>F. oxysporum</i>	17.5	28.71	41.33	45.81	33.24	35.84
<i>S. rolfisii</i>	30.6	27.14	38.19	37.4	26.47	34.35
<i>T. viride</i> (control)	1.36	1.07	2.18	2.32	1.40	01.53
Mean (H)	21.27	23.49	26.91	31.72	22.24	

Values represent mean of three experiments, each performed in duplicate.

A. niger: *Aspergillus niger*, *F. oxysporum*: *Fusarium oxysporum*, *S. rolfisii*: *Sclerotium rolfisii*, *T. viride*: *Trichoderma viride*

Table 4: Specific activity of protease produced by *T. viride* incubated with mycelia of three different fungal pathogens, *A. niger*, *F. oxysporum*, and *S. rolfisii*.

Organism	Protease (µg free amino acids/h/mg protein)					Mean (T)
	24	48	72	96	120	
<i>A. niger</i>	11.09	28.85	57.44	95.29	45.21	48.17
<i>F. oxysporum</i>	4.83	33.27	40.91	62.96	34.21	35.49
<i>S. rolfisii</i>	0.82	7.03	16.54	5.71	7.42	07.53
<i>T. viride</i> (control)	2.99	3.77	2.52	1.45	1.78	02.68
Mean (H)	19.73	72.92	117.41	165.41	88.62	

Values represent mean of three experiments, each performed in duplicate.

A. niger: *Aspergillus niger*, *F. oxysporum*: *Fusarium oxysporum*, *S. rolfisii*: *Sclerotium rolfisii*, *T. viride*: *Trichoderma viride*

Table 5: Specific activity of β-1, 3 glucanase produced by *T. viride* incubated with mycelia of three different fungal pathogens, *A. niger*, *F. oxysporum*, and *S. rolfisii*.

Organism	β-1, 3 glucanase (µmol glucose/h/mg protein)					Mean (T)
	24	48	72	96	120	
<i>A. niger</i>	6.35	6.51	13.14	13.87	10.64	10.17
<i>F. oxysporum</i>	7.76	6.22	10.37	14.32	9.43	09.49
<i>S. rolfisii</i>	5.60	5.67	7.86	8.46	7.21	07.45
<i>T. viride</i> (control)	2.99	3.77	2.52	1.45	1.98	02.76
Mean (H)	22.7	22.17	33.89	38.10	29.26	

Values represent mean of three experiments, each performed in duplicate.

A. niger: *Aspergillus niger*, *F. oxysporum*: *Fusarium oxysporum*, *S. rolfisii*: *Sclerotium rolfisii*, *T. viride*: *Trichoderma viride*

of *S. rolfisii* antagonist. Irrespective of antagonists, mean time interval (H) showed a continuous rise in enzyme activity during 24-96 h when *T. viride* grew in the presence of pathogen mycelia.

The specific activities of the three enzymes of the *Trichoderma* spp. were tested using mycelia of the fungal pathogen as a substrate during different incubation period ranged from 24 to 96 h (Tables 3-5). The species were cultured on mycelia of fungal pathogen. All confirmed species indicated a significant difference in three enzymes activities when grown in the fungal pathogen mycelia. The release of chitinase and β-glucosidase was higher in *T. viride* against *A. niger*, *F. oxysporum*, and *S. rolfisii*. *T. viride* induced highest chitinase activity at 96 h incubation, and it was 21.96, 20.71, and 19.85 fold higher than control (each pathogens). Early induction of chitinase by antagonist *T. viride* compared to other species caused high coiling capacity during mycoparasitism at 07 DAI and greater percent growth inhibition of pathogen fungal pathogens.

4. DISCUSSION

Many reports worldwide stated that, because of their ability to reduce the incidence of disease caused by plant pathogenic fungi, *Trichoderma* spp. is widely used in agriculture as biocontrol agents [24-31].

In this manuscript, antagonistic effect of 12 different *Trichoderma* spp. on three pathogenic fungi *A. niger*, *F. oxysporum*, and *S. rolfisii* was observed *in vitro*. Dual culture experiment results showed the maximum growth inhibition of *A. niger* (89.4%), *F. oxysporum* (88%), and *S. rolfisii* (88.6%), respectively, with *Trichoderma* spp.

In 2013, Bhale et al. [32] reported about inhibition of growth of *A. niger* by antagonist action of *T. viride*, *T. harzianum*, and *S. rolfisii* [33,34], which were very similar to our findings. Alwathnani et al. [35] also reported that *T. viride* and *T. harzianum* considerably repressed the growth of *F. oxysporum*. *T. viride* and *T. harzianum* had a greater inhibition on *F. oxysporum* than *T. hamatum*. Our study showed that *T. harzianum* and *T. viride* have a superior growth retardation efficiency to all three pathogens compared to *T. hamatum*. *Trichoderma* spp. is known to act using several machineries like hyperparasitism, antibiosis, and inhibition. In 2007, Shalini and Kotasthane [36] confirmed screening of 17 *Trichoderma* spp. against *F. oxysporum* and *S. rolfisii* *in vitro*. All strains including *T. harzianum*, *T. viride*, and *T. hamatum* were able to retardation the development of those plant pathogens.

Mechanism of mycoparasitism involves some important events in morphological behavior such as coiling around hyphae and establishment of appressorium-like structures (appressoria), which help to penetrate the fungus inside host [37]. After 2 to 3 days of inoculations, the first physical growth contact was observed between antagonist strain of *Trichoderma* spp. and plant pathogenic fungus in dual culture Petri plate followed by growth inhibition of pathogenic fungus. Various *Trichoderma* spp. showed differential antagonistic behavior with their hosts, and this interaction is semi-specific against a variety of hosts [11]. Analogous findings have been reported against *S. rolfisii* using *T. harzianum* in 2001 [38]. It was reported that *S. rolfisii* and mycelia of other fungus were inhibited by *T. harzianum* ALL42 strain which found to overgrow in Petri plate in dual culture method [39]. It also forms coiling around hyphae resulting in generation of appressoria. Proficient coiling process followed by secretion of hydrolytic enzymes was observed during the interaction of 15 *Trichoderma* isolates with *Rhizoctonia solani* which were reported by Almedia et al. [20].

In the present work, selection of a potent chitinolytic strain of *Trichoderma* was studied. Chitinolytic potential of all isolated *Trichoderma* spp. was identified using Basal chitinase detection medium supplemented with colloidal chitin. Chitin is a polymer of chitobiose, which consists of two NAGA residues bound to each other by β-1, 4 glycosidic linkage. The active groups present in NAGA molecules are hydroxyl, carbonyl, and imine groups at which anionic dye like BCP is bind and generates color-bound complex (yellow) [2] (pH 4.7) when *Trichoderma* inoculated in colloidal chitin media having BCP resulted in the formation of chitin into N-acetylglucosamine causing change in pH to more alkalinity and also change in color of indicator dye (BCP) from yellow to purple zone where the chitin got utilized.

BCP (BCP or full form: 5',5''-dibromo-o-cresolsulfophthalein [pKa 6.3]) is a pH depending halo chromic dye which used for indicating change in pH. Hydronium ions (H₃O⁺) or hydrogen ions (H⁺) are detected by BCP in the Arrhenius model. Initially, for chitinolytic microorganisms, plate with chitin agar has been used for isolation through clear zone formation [40-42]. There was a use of remazol brilliant blue as coloring dye for chitinolytic screening of microorganisms, but this method has low sensitivity, and it is affected by particle size and concentration of chitin, media thickness, and inoculum size [43,44].

Vaidya et al. reported the use of glycol chitin and Calcofluor White M2R, fluorescein isothiocyanate, and Rhodamine B in the screening of hyperchitinase secretory bacteria and fungus [45]. Expensive substrate required for more sensitive techniques, which are more appropriate to learn specificity of chitinases [46-49]. Many reports suggested

the involvement of G proteins, cAMP, and MAP kinase which are signal transduction pathways components, which is responsible for controlling the secretion of extracellular enzyme and coiling surround host hyphae [50,51]. In *Trichoderma*, there is a biochemical confirmation for a significant role of G-protein activator responsible for coiling around nylon fibers [52].

Different antifungal enzymes from *Trichoderma* spp. were studied by growing it with plant pathogenic fungi mycelia incubated for 24-96 h (Tables 3-5). The different *Trichoderma* spp. were cultured on basal medium having mycelia of pathogens as the only source of carbon. All species of *Trichoderma* showed considerable difference found in antifungal proteins when grew in basal medium. The secretion of enzymes chitinase and β -1, 3-glucanase was higher in *T. viride* than *T. harzianum* and *T. hamatum*. *T. viride* was found to induce 18 fold more secretion of chitinase after 96 h of incubation compared to control (only pathogen). Early and quick initiation of chitinase by *T. viride* showed more coiling capability during mycoparasitism on 14th DAI and more percent growth retardation of fungal pathogens compared to other species.

After 72 h of incubation, *T. viride* showed more secretion of β -1, 3 glucanase, and it reached 2.18 fold higher within 96 h. It indicated that like chitinase, β -1, 3 glucanase also plays a role in retardation of pathogens growth in synergistic manner [5,38]. Marco *et al.* [53] also noted similar results that two different *T. harzianum* (39.1 and 1051) induce a remarkable level of chitinolytic enzymes during 72 h, whereas β -1, 3 glucanase activity induced during 72-120 h growth with substrate.

Chitinase and β -1, 3-glucanase were found to secret by *Trichoderma* spp. when grew with mycelia of pathogen as sole carbon source, indicating the role of mycelia of pathogen as a stimulator of these enzyme syntheses. This finding is much related to Sivan and Chet [54] observations in which *T. harzianum* secreted elevated levels of glucanase and chitinase when grown in medium having on *R. solani* mycelia. Different environmental factors affect the host and culture, resulting in differentially secretion of antifungal enzymes [11]. Some related observations also indicating that *T. harzianum* showed higher chitinase and glucanase activity when it grows on media fortified with cell walls of *S. rolfisii* [55], *F. oxysporum*, *R. solani* [25], and *Botrytis cinerea* [11]. However, observations by Gajera and Vakharia [56] established the association between the RAPD analysis of 12 different *Trichoderma* isolates and their biocontrol efficacy against *A. niger*, in context of secretion of cell wall-degrading enzymes including chitinase, β -1, 3 glucanase during mycoparasitism.

Various mechanism of antagonism exerts by *Trichoderma* spp. are known which include competition for nutrition and antibiosis. In the event of mycoparasitism, *Trichoderma* spp. produces cell wall-degrading enzymes such as proteases, chitinases, and β -1,3 glucanase and restrains the development of plant pathogens [57]. It is said that, to play an important role in lysis of pathogen cell wall, successful antagonists must have secretion of enzymes that hydrolyzed a fungi cell wall skeleton which mainly consists of chitin, glucan, and proteins [58,59]. Some fungi also contain lipids in its skeleton.

It is well known that the hyphae of pathogenic fungi are degraded by lytic enzymes produced by biocontrol strains, resulting in retardation of growth of pathogenic fungi [60]. When antagonist came in contact with pathogen, some cell wall components and chitin induce a *nag 1* gene in antagonist [57,59,61]. In our results, it was showed that *Trichoderma* spp. secretes more amount of cell wall lytic enzymes

in the presence of mycelia of pathogenic fungi. The differential producing ability of these enzymes varied with different *Trichoderma* species, possibly because of the gene expression of certain enzymes in *Trichoderma* spp. during mycoparasitism only but not before pathogen overgrowing [62]. However, some enzymes such as chit36Y does not required the through contact with the pathogen for gene expression. During the event of mycoparasitism, *Trichoderma* spp. found to overexpress the chit36, chit42, and Chit33 genes confirm the role of chitinases in mycoparasitism. It is well known that fungal cell wall contains β -1, 3 glucan as principal structural component and β -1, 3 glucanase enzyme is responsible for degradation of glucan present in cell wall of pathogenic fungi. In the recent years, many β -1, 3-glucanases have been identified, but very few genes have been cloned yet such as *bgn13.1* gene [63].

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