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Hydrolytic Enzyme Activity in Relation to Mycoparasitic Action of Trichoderma Species against Fusarium Oxysporum

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Abstract Mycoparasitic action of *Trichoderma* spp. on the pathogen through the production of lytic enzymes *viz* β-1, 3 glucanase (laminarinase) and chitinase was studied. Hyphal interaction between the antagonist and the pathogen was investigated through scanning electron microscope. Coiling around, penetration and overgrowth of the antagonistic mycelium on the pathogenic mycelium were the result of the death of the pathogen mycelium through lysis. Studies on the production of hydrolytic enzymes viz. β-1, 3 glucanase and chitinase by the five *Trichoderma* spp. have reflected their inducible nature. On the other hand, various carbon sources influenced differently the production of β-1, 3 glucanase and chitinase enzyme by the antagonists, as there exists a parallel relationship between carbon source in the medium and *Trichoderma* species. Different species of *Trichoderma* also showed variable degrees of enzyme production and *T. harzianum* was recorded to be the most efficient producer. The variation in enzyme activity clearly reflects the varying degree of substrate utilization by the different species of *Trichoderma* indicating thereby their varying degree of biocontrol potential as mycoparasitic fungi.

Keywords Fusarium oxysporum, hyphal interaction, lytic enzymes, mycoparasitism, Trichoderma species

Introduction

Coleus forskohlii (Wild.) Briq. is a potentially important medicinal plant of the future, being immensely valued for its pharmacoproperties that have been discovered only recently. Its tuberous roots are found to be rich source of forskolin, which is being developed as a remedy for hypertension, glaucoma, asthma, congestive heart failures, and certain types of cancers. The plant suffers tremendously from a wilt pathogen, *Fusarium oxysporum*, which results in great economic loss to our country during its cultivation [1]. Mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential events, including recognition, attack and subsequent penetration and killing of the host. This is followed by the growth of the mycoparasite on the host fungal content [2, 3]. Mycoparasitism of plant pathogenic fungi by *Trichoderma* isolates has been well researched and is widely considered to be a major contributing factor to the biocontrol of *Trichoderma* spp. of a range of commercially important plant diseases [4]. Mycoparasitism involves morphological changes, such as coiling and formation of appressorium like structures, which serve to penetrate the host and contain concentrations of osmotic solutes such as glycerol [5]. *Trichoderma* attaches to the pathogen with cell wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms appressoria. The following step consists of the production of cell wall degrading enzymes and peptaibols [6], that facilitate both entry of *Trichoderma* hypha in the lumen of the parasitized fungus and assimilation of the cell-wall content. Dubey and Suresh [7] observed the principle mechanism of mycoparasitism of *Trichoderma* strains as coiling around the *Fusarium oxysporum* hyphae and lysis. Mycoparasitism, one of the main mechanisms involved in the antagonistic activity of *Trichoderma* strains, depends on the secretion of complex mixtures of hydrolytic enzymes able to degrade the host cell wall [8]. Interstrain and interspecific variations exist among the *Trichoderma* isolates with regard to their ability to produce

both laminarinase and chitinase [9]. It has also been reported that *Trichoderma* species produced chitinase and β-1, 3 glucanase in liquid culture containing cell wall of *Fusarium* spp. as sole carbon source. The article deals with the activities of hydrolytic enzymes, viz., β-1, 3 glucanase (laminarinase) and chitinase that may contribute to their mycoparasitic action [10].

Materials and Methods

Selection of Antagonistic Fungi

The mycoflora isolated from the rhizosphere of *Coleus forskohlii* were tested against the pathogen *Fusarium oxysporum* in order to select the potential antagonists. Five species of *Trichoderma* had been found to reduce the growth of the pathogen drastically of which three species *viz. T. viride, T. lignorum and T. reesei* were isolated from the rhizosphere soil of *Coleus* plants in Burdwan and Hooghly, and the other two species *viz. T. harzianum* and *T. hamatum* had been procured from Indian Agricultural Research Institute (I.A.R.I), New Delhi. The pathogen as well as *Trichoderma* strains were grown on potato dextrose agar (PDA) plates for a week at at 28°± 1°C.

Lytic Enzyme Production by the Antagonists

Preparation of Fungal Cell Wall Material

The pathogen was grown in potato dextrose medium at 28±1 °C. After 20 days, the mycelia were removed by filtration and dried at 60^0C. Cell wall material was then prepared following the method of Bruce et al. [11]. Mycelia were ground in a mortar and pestle before being exposed to ultrasonic disintegration (Braun-sonic 1510) for 3 minutes at 150 W. Then the resulting mycelia pastes were washed thrice (with repeated centrifugation at 5000 rpm) with 0.1 M NaCl in 0.5 M acetate buffer at pH 5.5 (0.5 M anhydrous sodium acetate and 0.5 M acetic acid mixed at 6.2:1 respectively) followed by three washes with deionized distilled water. The samples then lyophilized overnight before being ground to a fine powder in a mortar and pestle.

Preparation of Enzyme Source

Enzyme source was prepared following the methods of Bruce et al. [11], with slight modifications [12]. The *Trichoderma* isolates *viz. T. hazianum, T. viride* and *T. hamatum* were cultured separately in 50 ml amounts of each of the three different media like Czapek's Dox Broth (CDB) as basal medium, CDB + *Fusarium oxysporum* cell wall material (0.1 g) [CDB +FOC] and CDB without sucrose + *F. oxysporum* cell wall material (0.1 g) [CDB-S+FOC]. The flasks were incubated at 25°C for 10 days. After incubation, mycelial mat was removed by filtration and culture filtrates were sterilized by passing them through 0.45 µm membrane filters . The filtrates were then dialysed overnight (to remove residual sugars) in a continuous flow of cold water at 10-12°C using 2.4 nm pore size dialysis bag prior to assay for β -1, 3-glucanase (laminarinase) and chitinase activity.

Assay of β-1, 3 Glucanase

Assay of β-1, 3 glucanase or laminarinase *i.e.* enzymatic hydrolysis of laminarin (Sigma) was measured according to dinitrosalicylic acid method of Miller [13]. The reaction mixture contains 0.5 ml of enzyme source, 0.2 ml of citrate buffer (pH 4.8) and 1.6 mg of soluble laminarin. The reaction mixture contains 0.5 ml of enzyme source, 0.2 ml of citrate buffer (pH 4.8) and 1.6 mg of soluble laminarin. The reaction was stopped by boiling and the amount of reducing sugar was determined. The enzyme activity was expressed as release of µmol glucose/ ml of fitrate/ hour. The results are presented in Table 1.

Table 1: Levels of production of lytic enzymes by *Trichoderma* spp. on CDB, CDB+FOC, CDB-S+FOC

Antagonists (*Trichoderma* spp.)	**β-1,3 glucanase (1 unit=1µ mole glucose realeased / ml. of filtrate / min / mg protein)***			**Chitinase (1 µ mole glucose released / ml. of filtrate / min / mg protein)***		
	CDB	**CDB+FOC**	**CDB-S+FOC**	**CDB**	**CDB+FOC**	**CDBS+FOC**
T. hamatum	870.23±2.81	638.66±2.33	1128.63±3.15	129.22±4.90	72.43±0.02	130.73±0.87
T. lignorum	666.03±1.84	505.55±1.99	981.83±0.89	121.04±0.47	67.16±0.06	124.83±0.56
T. reesei	520.25±6.13	445.42±2.48	791.28±1.36	103.04±0.02	52.23±0.08	115.43±0.88
T. harzianum	1193.43±41.07	979.19±4.79	1903.47±46.68	153.41±2.72	111.19±1.06	181.11±2.33
T. viride	1014.01±3.19	873.83±11.53	1423.16±2.90	142.45±0.21	98.24±0.56	155.37±0.83

(CDB= Czapek's Dox Broth, FOC= Cell wall material of *F. oxysporum*, S=Sucrose)

*Data are the mean values of five replicates ($P < 0.05$)

Data are the mean ± standard deviation.

Assay of Chitinase

The assay of chitinase is based on the estimation of reducing sugars released during the hydrolysis of swollen chitin . The reaction mixture, containing 1 ml of 0.5% swollen chitin (suspended in 50 mM sodium acetate buffer at pH 5.21 containing 0.02% sodium azide) and 1 ml of enzyme source , was incubated at 40 °C for 60 minutes with shaking,

and then centrifuged at 4000 rpm for 15 minutes. The amount of reducing sugar released in the supernatant was determined by the method described by Miller [13], using N-acetyl glucosamine as standard. One unit (u) of activity was defined as the amount of enzyme which catalyses the release of 1 µmol reducing sugar in 60 min at 40°C. The results are presented in Table 1.

Hyphal Interaction between the Antagonists and the Pathogen

Lysis of the Hypha of the Pathogen

The hyphal mats were gently lifted with a needle from the zone of interactions between *Trichoderma* spp. and the pathogen in dual culture plates. The mat was placed on a microscopic slide, spread with needle, stained with cotton blue and observed under microscope for hyphal interaction.

Lysis of pathogenic hyphae by the antagonist is one of the key mechanisms involved during mycoparasitism. Lysis of pathogenic hyphae by *Trichoderma* spp. was studied following the method of Ko and Lock Wood [14] and Mumpuni et al. [15]. Strands of hyphae of the test fungi were suspended in the respective culture filtrates of the antagonists and incubated at 25°C for 24 hours and then stained with 1% Rose Bengal and observed under a bright field microscope (Lieca DFC295, version V3, Germany). Lysed areas of hyphae under microscope revealed as hyaline and unstained zones, while unlysed hyphae appeared deep red (Fig.1).

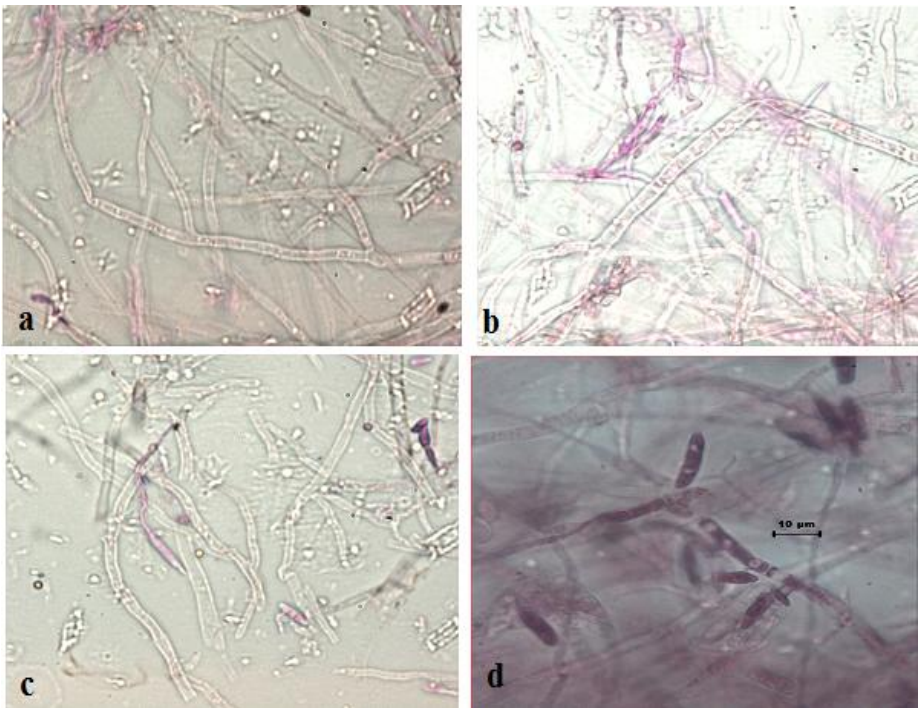

Figure 1: Photomicrographs showing the lysis of hyphae of F. oxysporum (a) lysis by T. harzianum, (b) lysis by T. viride, (c) lysis by T. hamatum, (d) Control (lysed hyphae appeared unstained and hyaline; unlysed hyphae remain deep red) (Bar=10 µm).

Hyphal Interactions through Scanning Electron Microscope

Study of hyphal interaction between *F. oxysporum* and most potent antagonist *T. harzianum* was also studied by scanning electron microscope (SEM). Mycelial samples from the interaction zone of 15 days old dual culture plate were cut by cork borer and were processed for SEM [16] (Fig.2).

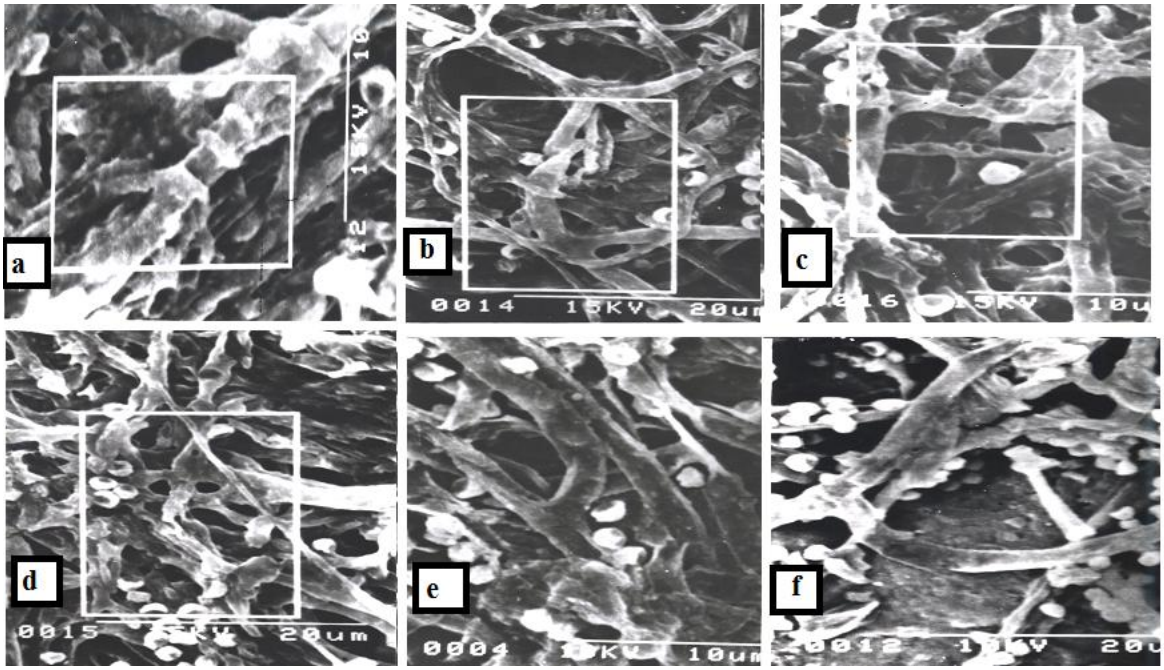

Figure 2: Scanning electron micrographs of hyphal interaction between the pathogen, F. oxysporum and the antagonist, T. harzianum (a) showing coiling (b) showing penetration, (c) showing overgrowth, (d) formation of pore on mycelium of the pathogen, and (e) & (f) showing cell wall degradation and lysis of mycelium of the pathogen.

Results

Mycoparasitic Action of *Trichoderma* spp.

Lytic Enzyme Production

Studies on the production of hydrolytic enzymes viz. β-1, 3 glucanase and chitinase by the five *Trichoderma* spp. (Table 1) have reflected their inducible nature. Wide variations existed in respect of the production of β-1, 3 glucanase (laminarinase) among the different species but similar pattern of enzyme production was recorded in the media types used. In Czapek's Dox Broth (basal medium) supplemented with cell wall material of *F. oxysporum*, lower quantity of enzyme was excreted by all *Trichoderma* spp. but when cell wall material of the pathogen alone was considered as the sole carbon souce (i.e. in CDB-S+FOC), higher β-1, 3 glucanase activities of the antagonists were recorded where *T. harzianum* comparatively produced better β-1, 3 glucanase (1903.47 units) than *T. viride* (1423.16 units), *T. hamatum* (1128.63 untis), *T. lignorum* (981.83 units) and *T. reesei* (791.28). Statistically it is evident that addition of cell wall material of the pathogen significantly increased β-1, 3 glucanase activity which was significantly more in *T. harzianum.*

On the other hand, various carbon sources influenced differently the production of chitinase enzyme by the antagonists, as there exists a parallel relationship between carbon source in the medium and *Trichoderma* species. All the *Trichoderma* spp. showed highest enzyme activity in medium containing Czapek's Dox Broth (CDB) omitting sucrose but substituted with cell wall material of the pathogen (CDB-S+FOC) and lowest in CDB with cell wall material of the pathogen (CDB+FOC). Different species of *Trichoderma* also showed variable degrees of chitinase production and *T. harzianum* was recorded to be the most efficient producer. Statistically all the treatments with different carbon sources were found to be significant ($P<0.05$) and the chitinase activity of different *Trichoderma* spp. varied significantly and the enzyme production remained highest in CDB-S+FOC medium.

Hyphal Interactions Between *Trichoderma* sp. and the Pathogen

The lysed areas of the mycelium of *F. oxysporum* were recorded as hyaline and unstained zones and the unlysed hyphal areas appeared deep red. *T. harzianum* was responsible for the highest degree of lysis of pathogen hyphae which is evident from the occurrence of more clear areas in *F. oxysporum* hyphae (Fig. 1a). *T. viride* also showed lysis of *F. oxysporum* when the strands of hyphae suspended in culture filtrate of the antagonist (Fig. 1b). So it is clear from the results that both the antagonists were able to secrete hydrolytic enzymes that act directly on the pathogen during mycoparasitism.

Scanning electron microscopic (SEM) studies of hyphal interaction between *T. harzianum* and the pathogen revealed that the hyphae of the antagonist coiled around the hyphae of the pathogen (Fig. 2a) followed subsequently by their penetration (Fig. 2b) resulting in the formation of the pores on the hyphae of the pathogen (Fig. 2d). All these activities ultimately lead to the overgrowth of the antagonistic mycelia (Fig. 2c) followed by distortion of the pathogenic mycelia (Fig. 2e & 2f).

Discussion

Mycoparasitic Action of *Trichoderma* spp.

Lytic Enzyme Production

Trichoderma spp. parasitizes a range of other fungi. Mycoparasitism, one of the most important mechanisms of antagonism by *Trichoderma* spp., depends on the secretion of complex mixtures of hydrolytic enzymes able to degrade the host cell wall [17].

It is observed (Table 1) that all the *Trichoderma* species tested were found to excrete hydrolytic enzymes viz. β-1, 3-glucanase (laminarinase) and chitinase. Among the isolates, *T. harzianum* was found to be most significant ($P < 0.05$) producer of β-1, 3- glucanase and chitinase (Table 1). The chitinolytic enzyme from *T. harzianum* has been reported to be more effective against wide range of fungi [18] and that corresponds to the present findings. Culture filtrates of *T.harzianum* inhibiting spore germination of phytopathogenic fungi has been reported [19]. β-1, 3-glucanase inhibits spore germination or the growth of pathogen in synergistic co-operation with chitinases [20]. *T. hamatum* was able to produce extracellular lytic enzymes such as β-1, 3-glucanase and chitinase [21]. Chitinase and β-1, 3-glucanase, produced by *T. viride*, were efficient in the management of root pathogens [22].

Mycoparasitism is a behavioral process involving a number of sequential stages including target location, chemotrophic growth, recognition, attachment, lysis and nutrient acquisition [11]. Lectins were shown to be involved in the recognition of *Trichoderma* spp. and their host fungus, while chitinase involved in the degradation of host cell wall [23]. Thus it is quite natural that induction of the production of lytic enzymes largely depends on the composition of the culture media on which the antagonists grow. Persson and Baath [24], were of the opinion that the mycoparasitic behavioral pattern of *Arthrobotrys oligospora* was influenced by the type and the amount of carbon sources influenced β-1, 3 glucanase and chitinase activities in all the *Trichoderma* spp. remain much higher in the Czapek's Dox Broth (CDB) omitting sucrose but get it substituted with cell wall material of the pathogen, *F. oysporum* (CDB-S+FOC) than medium with (CDB+FOC). The level of both the enzymes was lower in the CDB+cell wall material than their activities in the CDB which may possibly be due to the binding of the enzyme to the cell wall substrate prior to extraction of the filtrate for assay [25]. It is implied that the nutrient content of the medium may have direct influence on the production of the enzymes.

Production of hydrolytic enzymes appeared to be higher by *Trichoderma* spp. when the growth medium was supplemented with the cell wall of host fungi than in the medium supplemented with cell wall of some other fungi which are not being parasitized by *Trichoderma* [26]. This observation suggested that the cell wall of host fungi certainly provides some biochemical stimulus for enzyme induction [26]. Excellular enzymes corresponding to the main chemical constituents of the fungal cell wall, i.e. chitin, glucans, proteins have been detected when *T. harzianum* is grown on *Rhizoctonia solani* mycelia or cell wall as the sole carbon source [27]. The difference in enzyme activity may be due to varying degree of substrate utilization by the *Trichoderma* spp. indicating thereby their varying degree of biocontrol efficacy as mycoparasitic fungi where *T. harzianum* was proved to be most effective antagonist which is in line similar to that reported by Sivan and Chet [26]. Because of the skeleton of filamentous fungal cell wall contains chitin, glucan and proteins, enzymes that hydrolyze these components have to be present in a successful antagonist in order to play a significant role in cell wall lysis of the pathogen [28]. This fact strongly suggests that hydrolytic enzyme over-producing fungi may show superior biocontrol capacity [17].

Chitinase and β-1, 3-glucanase also are known to release fungal cell wall fragments which elicit other defense responses. The induction of these two enzymes in chilli plants in response to *T. harzianum* may therefore, trigger multipronged defense responses contributing to both local and systemic resistance to the pathogen [29]. Production of lytic enzymes and the factors which influence their production are therefore, the aspects which will determine the potential of any *Trichoderma* isolate selected for the biological control of plant pathogenic fungi [30].

Hyphal Interactions Between *Trichoderma* sp. and the Pathogen

Interacting mycelia regions of *T. harzianum* and the pathogen in dual culture were scanned through SEM which clearly showed coiling of the mycelium of *Trichoderma* around the hyphae of the pathogen (Fig. 2a). The mycelia of the antagonist directly penetrated and overgrew around the mycelium of the host pathogen (Fig. 2b and 2c).

It is interest that when the strands of hyphae of the pathogen were suspended in the respective culture filtrates of the antagonistic fungi, lysis of the mycelium of the pathogen took place due to secretion of lytic enzymes by *Trichoderma* spp. (Fig.1). *T. harzianum* showed higher degree of lysis compared to *T. viride* due to the production of higher lytic enzymes. Microscopic examination of *F. oxysporum* mycelia after staining with 1% Rose Bengal revealed unstained hyaline portion in the hyphae that may possibly be due to lysis of cell wall and cell constituents. Similar observations on lysis of fungal mycelia by *Trichoderma* species were made [31, 32]. Mycoparasitism of *Trichoderma* spp. has been reported on the fungi causing soil borne diseases of plants [33, 34].

Different strains can follow different patterns of induction, but the fungi apparently always produce low levels of an extracellular exochitinase. Diffusion of this enzyme catalyses the release of cell-wall oligomers from target fungi, and this in turn induces the expression of fungitoxic endochitinases [35], which also diffuse and begin the attack on the target fungus before contact is actually made [36]. Once the fungi come into contact, *Trichoderma* spp. attach to the host and can coil around it and form appressoria on the host surface. Attachment is mediated by the binding of carbohydrates in the *Trichoderma* cell wall to lectins on the target fungus [37]. Once in contact, the *Trichoderma* produce several fungitoxic cell-wall-degrading enzymes [38]. The combined activities of these compounds result in parasitism of the target fungus and dissolution of the cell walls. Mycoparasitic fungi recognize the cell wall components of the host fungi, hydrolyze, solubilize and finally utilize them as substrates. The variation in enzyme activity clearly reflects the varying degree of substrate utilization by the different species of *Trichoderma* indicating thereby their varying degree of biocontrol potential as mycoparasitic fungi.

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References

1. Khatun S, Chatterjee NC. *Glomus fasciculatum* in defense responses to fusarial wilt of *Coleus forskohlii*. *Acta Agriculturae Scandinavica, Section B - Soil and Plant Science*, 2011, 61: 136-142.
2. Chet I, Harman GE, Baker R. *Trichoderma hamatum*: its hyphal interactions with *Rhizoctonia solani* and *Pythium* spp. *Microbiology Ecology*, 1981, **7**: 29-38.
3. Chakraborty MR. Studies on *Fusarium* wilt of brinjal (*Solanum malongena*) and its integrated management. Ph.D. Thesis. The University of Burdwan, West Bengal, India. 2005.
4. Harman GE, Chet I, Baker R. Factors affecting *T. hamatum* applied to seeds as a biocontrol agent. *Phytopathology*, 1981, 71: 569-572.
5. Mclntyre M, Nielsen J, Arnau J, van der Brink, H, Hansen K, Madrid S. Proceedings of the 7th European Conference on Fungal Genetics. Copenhagen, Denmark, 2004.
6. Howell CR. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Disease,* 2003, 87: 4-10.
7. Dubey SC, Suresh M. Randomly amplified polymorphic DNA markers of *Trichoderma* species and antagonism against *Fusarium oxysporum* f. sp. *ciceris* causing chickpea wilt. *Journal of Phytopatholo*gy, 2006, 154: 663-669.
8. Sanz L, Montero M, Redondo J, Llobell A, Monte E. Expression of a α-1, 3-glucanase during mycoparasitic interaction of *Trichoderma asperellum*. *FEBS. Journal,* 2005, 272: 493-499.
9. Viterbo A, Ramot O, Chemin LY, Chet I. Significance of lytic enzymes from *Trichoderma* spp. in the biocontrol of fungal plant pathogens. *Antonie van Leeuwenhoek Journal of Microbiology,* 2002, 81: 549-556.
10. Shoulkamy MA, Guebitz GM, El-Katatny MHA, Shaban GM. Abdelzaher, H.M.A., Antagonism of *Trichoderma* or *Gliocladium* species on two phytopathogenic Species of *Fusarium. Journal of Natural Fibers,* 2006, 3: 1-17.
11. Bruce A, Srinivasan U, Staines HJ, Highley TL. Chitinase and laminarinase production in liquid culture by *Trichoderma* spp. and their role in biocontrol of wood decay fungi. *Int. Biodeterio Biodegrad,* 1995, 95: 337-353.
12. Dutta S, Chatterjee NC. Raising of carbendazim tolerant mutants of *Trichoderma* and variation in their hydrolytic enzyme activity in relation to mycoparasitic action against *Rhizopus stolonifer. Journal* of *Plant Diseases* and *Protection,* 2004, 111: 557-565.

13. Miller GL. Use of dinitrosalicylic acid reagent for detection of reducing sugar. *Analytical Chemistry*, 1959, 31: 426-438.
14. Ko KH, Lockwood JL. Mechanism of lysis of fungal mycelia in soil. *Phytopathology*, 1970, 60: 148-154.
15. Mumpuni A, Sharma HSS, Brown AE. A possible role of lytic enzymes produced by *Trichoderma harzianum* biotypes during interaction with *Agaricus bisporus.* In: *Advances in Mushroom Biology and Production*, pp. 223-239, Eds. Rai, R.D., Dhar, B.L., Verma, R.N., India. 1996.
16. Pham GM, Srivastava A, Saxena AK, Pareek A, Varma A. Protocol to understand the interaction between rhizobacteria and symbiotic fungus. In: *Basic Research and Applications of Mycorrhizae*, I.K. International Private Ltd., Eds. Podila, G.K., A. Verma, A., New Delhi. 2005.
17. Marco JL, Felix CR. Characterization of protease produced by a *Trichoderma harzianum* isolate which controls cocoa plant witches broom disease. *BMC Biochemistry*, 2002, 3:3.
18. Lorito M, Harman GE, Hayes CK, Brodway RM, Troncoso A, Woo SL, di Pierto A. Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiase. *Phytopathology*, 1993, 83:302-307.
19. Viterbo A, Haran S, Friesem D, Ramot O, Chet I. Antifungal activity of a novel endochitinase gene (chit 36) from *Trichoderma harzianum* Rifai TM. *FEMS. Microbiology Letters*, 2001, 200: 169-174.
20. El-Katatny MH, Gudelj M, Robra KH, Elnaghy MA, Gubitz GM. Characterization of a chitinase and an endo-β-1, 3-glucanase from *Trichoderma harzianum* Rifai T24 involved in control of the phytopathogen *Sclerotium rolfsii*. *Applied Microbiology* and *Biotechnology*, 2001, 56: 137-143.
21. Maj A, Witkowska D, Robak M. Biosysthesis and glucanases of *Trichoderma hamatum. Electronic Journal* of *Polish Agricultural Universities*, 2002, 2.
22. Nakkeeran S, Renukadevi P, Marimuthu T. Antagonistic potentiality of *Trichoderma viride* and assessment of its efficacy for the management of cotton root rot. *Archives of Phytopathology and Plant Protection*, 2005, 38: 209-225.
23. Chet I, Inbar J, Hadar I. Fungal antagonists and mycoparasites. In: The Mycota IV, *Environment and Microbial relationships*, pp. 165-184, Eds. Wicklow, D.T., Soderstrom, B., Springer-Verlag, Berlin. 1997.
24. Persson Y, Baath E. Quantification of mycoparasitism by the nematode trapping fungus *Arthrobotrys oligospora* on *Rhizoctonia solani* and the influence of nutrient levels. *FEMS Microbiology Ecology*, 1992, 101: 11-16.
25. Chakraborty MR, Chatterjee NC. Hydrolytic enzymes in relation to the fusarial wilt of Brinjal. *International Journal Plant Science*, 2008, 1: 112-115.
26. Sivan A, Chet I. Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum. Journal of General and Applied Microbiology*, 1989, 135: 675-682.
27. Geremia RA, Goldman GH, Jacobs D, Ardiles W, Vila SB, van Montagu M, Herrera-Estrella A. prb1, a gene specifically expressed during simulated mycoparasitism by *Trichoderma harzianum. Molecular Microbiology*, 1993, 8: 603-613.
28. Carsolio C, Benhamou N, Haran S, Cortes C, Gutierrrez A, Chet I. Herrera-Estrella A. Role of *Trichoderma harzianum* endochitinase Gene ech 2 in mycoparasitism. *Applied and Environmental Microbiology*, 1999, 65: 929-935.
29. Jangid PK, Mathur PN, Mathur K. Role of chitinase and β-1, 3-glucanase elicitation in the *Trichoerma harzianum* induced resistance in capsicum. *Indian Phytopathology*, 2004, 57: 217-221.
30. Karasuda S, Tanaka S, Yamamoto Y, Koga D. Plant chitinase as a possible biocontrol agent for use instead of chemical fungicides. *Bioscience Biotechnology and Biochemistry*, 2003, 67: 221-224.
31. Goodwin-Egein MI, Arinzae AE. Antagonism between *Trichoderma harzianum* Rifai and *Fusarium oxysporum* Schlecht Emend Sny and Hans. *Journal of Mycology and Plant Pathology*, 2001, 31: 22-30.
32. Ojha S. Studies in wilt of tomato (*Lycopersicon esculentum*) caused by *Fusarium oxysporum* and its integrated control. Ph.D. Thesis. The University of Burdwan, West Bengal, India. 2008.
33. Ozbay N, Newman SE. Biological control with *Trichoderma* spp. with emphasis on *T. harzianum. Pakistan Journal* of *Biological Sciences*, 2004, 7: 478-484.
34. Ojha S, Chatterjee NC. Mycoparasitism of *Trichoderma spp.* in biocontrol of Fusarial wilt of tomato. *Archives of Phytopathology and Plant Protection*, 2011, 44: 771-782.
35. Brunner K, et al. The Nag1 *N*-acetylglucosaminidase of *Trichoderma atroviride* is essential for chitinase induction by chitin and of major relevance to biocontrol. *Current Genetics*, 2003, 43: 289-295.
36. Zeilinger S. et al. Chitinase gene expression during mycoparasitic interaction of *Trichoderma harzianum* with its host. *Fungal Genetics* and *Biology*, 1999, 26: 131-140.

37. Inbar J, Menendez A, Chet I. Hyphal interaction between *Trichoderma harzianum* and *Sclerotinia sclerotiorum* and its role in biological control. *Soil Biology* and *Biochemistry*, 1996, 28: 757-763.
38. Chet I, Benhamou N, Haran S. In: *Trichoderma* and *Gliocladium*, pp. 153-172, Eds. Harman, G.E., Kubicek, C.P., Taylor and Francis, London, 1998.