Journal of
Plant Pathology & Microbiology
ISSN: 2157-7471

Gajera et al., J Plant Pathol Microb 2012, 3:7
DOI: 10.4172/2157-7471.1000149

Research Article Open Access

Antagonism of *Trichoderma* spp. against *Macrophomina phaseolina*: Evaluation of Coiling and Cell Wall Degrading Enzymatic Activities

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Abstract

In vitro potentialities of seven species of *Trichoderma* were evaluated against phytopathogen *Macrophomina phaseolina* by dual culture techniques. The maximum growth inhibition of test pathogen was observed by antagonist *T. koningi* MTCC 796 (T4) (74.3%) followed by *T. harzianum* NABII Th 1 (T1) (61.4%) at 7 days after inoculation (DAI). Further, mycoparasitism of antagonists were observed upto 14 DAI. Pattern of growth inhibition of test fungus was continued with maximum 14.7% increases in T4 (85.2%) followed by 6.8% elevation in T1 (65.6%) antagonists during 7 to 14 DAI. Microscopic study showed that these two antagonists were capable of overgrowing and degrading *M. phaseolina* mycelia, coiling around the hyphae with apressoria and hook-like structures. At 14 DAI, *T. koningi* MTCC 796 completely destroyed the host and sporulated. The specific activities of cell wall degrading enzymes- chitinase, β-1, 3 glucanase, protease and cellulase were tested during different incubation period (24, 48, 72 and 96 h) when *Trichoderma* spp. grew in presence of pathogen cell wall in synthetic media. The antagonist *T. koningi* MTCC 796 induced higher chitinase and protease activity at 24 h incubation while β-1, 3 glucanase activities was elevated 1.18 fold during 72 to 96 h. Total phenol was produced significantly higher in culture supernatant of *T. koningi* MTCC 796 antagonist followed by *T. hamatum* NBAII Tha 1 and *T. harzianum* NBAII Th 1 at 48 h incubation. The growth inhibitions of pathogen during antagonism was positively correlated with coiling pattern of antagonists at 14 DAI, and induction of chitinase, β-1, 3 glucanase and total phenol content.

Keywords: *Trichoderma* spp.; *Macrophomina phaseolina*; Antagonism; Coiling pattern; Cell wall degrading enzymes

Introduction

Trichoderma spp. are effective biocontrol agents for several soil-borne fungal plant pathogens including *Macrophomina phaseolina* [1] and some species are also known for their abilities to enhance systemic resistance to plant diseases as well as overall plant growth [2]. The biocontrol exercised by *Trichoderma* can occur by means of several antagonistic mechanisms such as nutrient competition, antibiotic production, and mycoparasitism [3,4]. Mycoparasitism has been proposed as the major antagonistic mechanism displayed by *Trichoderma* spp. [5]. After host recognition, *Trichoderma* spp. attaches to the host hyphae via coiling, and penetrate the cell wall by secreting cell wall-degrading enzymes [6]. Mycoparasites produce cell wall degrading enzymes which allow them to bore holes into its fungi host and extract nutrients for their own growth. Most phytopathogenic fungi have cell wall that contain chitin as a structural backbone arranged in regularly ordered layers and β-1,3-glucan as a filling material arranged in an amorphic manner. Chitinases and β -1,3-glucanases have been found to be directly involved in the mycoparasitism interaction between *Trichoderma* spp. and its hosts [5].

A considerable amount of research has been aimed at elucidating the chitinase and β-1,3-glucanase systems of *Trichoderma* spp. during growth on different carbon sources. Major advances have been made in chitinase and β -1,3-glucanase expression by various *Trichoderma* spp. but little is known regarding factors affecting production of these enzymes and the nature of the inducers and repressors [5,7]. The *Trichoderma* mechanism of inducing chitinolytic and glucanolytic enzymes and their regulation is still a matter of speculation, despite many studies. During mycoparasitism, the synthesized cell wall degrading enzymes act synergistically. Thus, understanding the induction process from these enzymes is necessary in order to select the most efficient *Trichoderma* spp. for biocontrol.

The main objective of this study was to evaluate the interaction between the antagonists *Trichoderma* spp. and fungal pathogen *M. phaseolina*, and the possibility of mycoparasitism through (1) examination of the coiling pattern during antagonism using light microscopy, and (2) the efficiency of *Trichoderma* spp. for the induction of four cell-wall degrading enzymes using cell wall of *M. phaseolina* as carbon source.

Materials and Methods

Sources of *Trichoderma* spp

Slants of six species of *Trichoderma* (*T. harzianum* NBAII Th 1; *T. viride* NBAII Tv 23; *T. virens* NBAII Tvs 12; *T. koningi* MTCC 796; *T. pseudokoningi* MTCC 2048; *T. hamatum* NBAII Tha-1.) were obtained either from IARI, New delhi or from MTCC, Chandigarh. One local isolate of *T. harzianum* was also collected from culture collection of Department of Plant Pathology, Junagadh Agricultural University, Junagadh.

Isolation of phytopathogenic fungi

Macrophomina phaseolina (Tassi) Goid was used as pathogen. The castor plants showing typical symptoms of root rot were collected

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Received October 23, 2012; **Accepted** November 27, 2012; **Published** Dwecember 01, 2012

Citation: Gajera HP, Bambharolia RP, Patel SV, Khatrani TJ, Goalkiya BA (2012) Antagonism of *Trichoderma* spp. against *Macrophomina phaseolina:* Evaluation of Coiling and Cell Wall Degrading Enzymatic Activities. J Plant Pathol Microb 3:149. doi:10.4172/2157-7471.1000149

from university fields. The fresh collected diseased plants of castor showing stem blight and shredded bark on the roots were thoroughly washed under running tap water to remove surface soil, dust and other contaminants. Infected root tissues were cut out from the leading edge of lesion, and placed in 1% sodium hypochlorite for five minutes, washed briefly in sterile distilled water and dried on sterile filter paper. The dried pieces were cut into smaller pieces, plated onto PDA and incubated at 28°C. The cultures were purified by hyphal tip method [8] and maintained on PDA by storing it under refrigeration (4°C). Phytopathogen isolated from infected castor root was sent for identification at Indian type culture collection (ITCC), Division of plant pathology, IARI, New Delhi and they identified the pathogen as *M. phaseolina* (identity number 8160.11) based on morphology and microscopy characters.

Dual culture tests and light microscopy

Interactions between antagonistic fungi and pathogenic fungi were determined by the method described by Dennis and Webster [9]. A 5 mm diameter mycelial disc from the margin of the *Trichoderma* one week-old culture and the pathogen *M. phaseolina* were placed on the opposite of the plate at equal distance from the periphery. The experimental design used was a completely randomized with four petri dishes for each antagonist. In control plates (without *Trichoderma*), a sterile agar disc was placed at opposite side of the pathogen *M. phaseolina* inoculated disc. The plates were incubated at 28 ± 2°C and observed after 7 and 14 days for growth of antagonist and test fungus. Index of antagonism as percent growth inhibition of *M. phaseolina* was determined by following the method of Watanabe [10]. Mycelial samples, cut from the interaction region (*Trichoderma*–pathogen hyphae) in dual-culture tests (14 days), were fixed on slide and observed under an inverted binocular light microscope (Laica brand, 40x) for the presence of coiling structures for wall disintegration.

Plant pathogen cell wall preparations

Pathogen *M. phaseolina* were inoculated into 250 ml flasks with 50 ml of potato dextrose broth and incubated at 28 ± 2°C for 7 days. The mycelia were then collected by filtration. The mycelia were thoroughly washed with distilled autoclaved water and homogenized on ice, with a homogenizer, for 5 min at the highest speed. The mycelial suspension was centrifuged at 30,000×g for 20 min at 4°C. The pellet was resuspended in distilled water and sonicated on ice 4 times for 5 min using a sonicator (Equitron-4.2L100H) [11]. The suspension was centrifuged at 800×g for 10 min at 4°C. The pellet was collected and lyophilized. The dry cell wall of pathogen was used as substrate for the production of cell wall degrading enzymes.

Cell wall degrading enzyme productions

Trichoderma spp. and *M. phaseolina* were cultured at 30°C on a Synthetic Medium (SM) containing (grams per liter of distilled water); glucose, 15; $MgSO_4.7H_2O$, 0.2; KH_2PO_4, 0.9; KCl, 0.2; NH_4NO_3, 1.0; Fe^{2+}, 0.002; and Zn^{2+}, 0.002. Flasks containing 50 ml of liquid SM were inoculated with a spore suspension (107 spores. ml^{-1}) of both microbes. Suitable concentration of spore suspension was prepared using serial dilution technique. The glucose in the medium was substituted with pathogen cell wall preparations (2 mg. ml^{-1}) as a carbon source for *Trichoderma* spp. [11]. For enzyme induction in pathogen *M. Phaseolina* (Control), 1% of either chitin, laminarin, casein or carboxymethyl cellulose were added into liquid culture (SM) and pH adjusted to 5.5 [12]. Cultures were then incubated at 30°C in a rotary shaker at 120 rpm for 96 h. After 24, 48, 72 and 96 h time interval, 5 ml of mycelium was harvested and then centrifuged at 15,000×g at 4°C for 10 min. The supernatant was dialyzed against distilled water. The dialyzate was used for enzymic activity of chitinase, β-1,3-glucanase, protease and cellulase and also for total phenol content. The method of Folin-Lowry [13] was used to estimate the protein content in culture supernatant and used for calculating specific activity of the enzyme.

Enzyme and total phenol assays

Chitinase (EC 3.2.1.14) and β-1,3 glucanase (EC 3.2.1.39) activity were assayed by the colorimetric method using colloidal chitin and laminarin as substrates, respectively. The level of reducing sugar released was determined by the DNSA method, using N-acetylglucosamine and glucose as a standard [14]. Cellulase activity (EC 3.2.1.21) was determined by measuring the amount of glucose formed from carboxy methyl cellulose [15]. Reactions were conducted for 30 min at 55°C [16]. Specific activity of chitinase, β-1, 3 glucanase and cellulase were expressed as 1 µmol of reducing sugars released.h^{-1}.mg^{-1} protein. Non enzymatic controls were also performed using boiled enzymes and were subtracted from the enzymatic values.

Protease activity (EC 3.4.21.4) was measured using casein as a substrate. The reaction was carried out according to Charney and Tomarelli [17]. Blank was treated as zero time incubation. The amount of released total free amino acids was estimated by Ninhydrin method [18]. Proteolytic activity was corresponded to the amount of enzyme required to cause an increase of µg free amino acids .h^{-1}.mg^{-1}protein in culture supernatant. Total phenol content was estimated as method described by Malik and Singh [19] and calculated as µg.ml^{-1} culture supernatant using pyrocatechol as a standard.

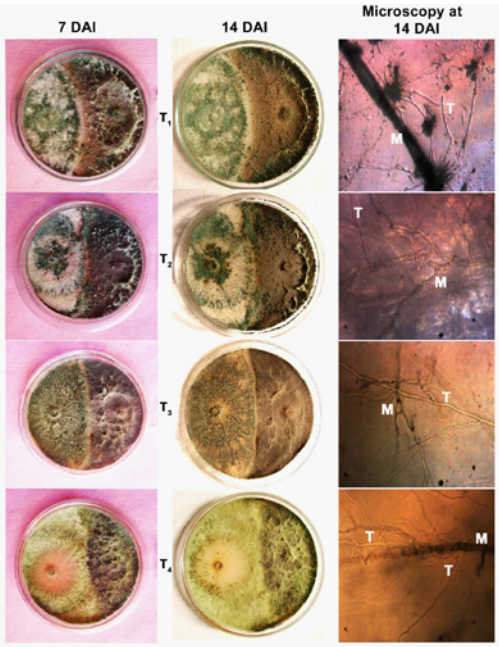

Figure 1a: Antagonism between *Trichoderma* spp. and *Macrophomina phaseolina* at 7 and 14 DAI, and microscopy of interaction between them (Antagonists petri dish (Tl to T4) have Trichoderma spp. at the left and pahtogen M. phaseolina at the right side). (T_1=T. harzianum NABII Th 1×MP; T_2=*T. viride* NBAII Tv 23×MP; T_3=*T. virens* NAB II Tvs 12×MP; T_4=*T. koningii* MTCC 796×MP; DAI=Days After Inoculation; T=*Trichoderma* spp.; M=*Macrophomina phaseolina*).

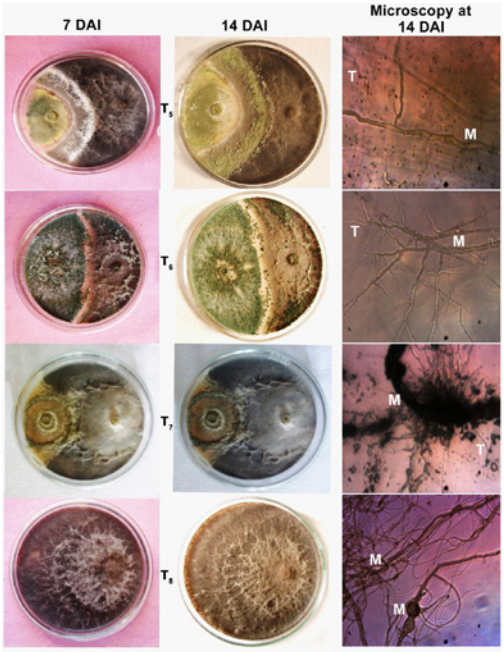

Figure 1b: Antagonism between *Trichoderma* spp. and *Macrophomina phaseolina* at 7 and 14 DAI, and microscopy of interaction between them (Antagonists petri dish (T5 to T8) have Trichoderma spp. at the left and pahtogen *M. phaseolina* at the right side). (T_5=T. pseudokoningii MTCC 2048×MP; T,=*T. hamatum* NBAII Tha 1×MP; T_7=*T. harzianum* Local×MP; T_8= Control-Macrophomina phaseolina (MP); DAI =Days After Inoculation, T=*Trichoderma* spp.; M=*Macrophomina phaseolina*).

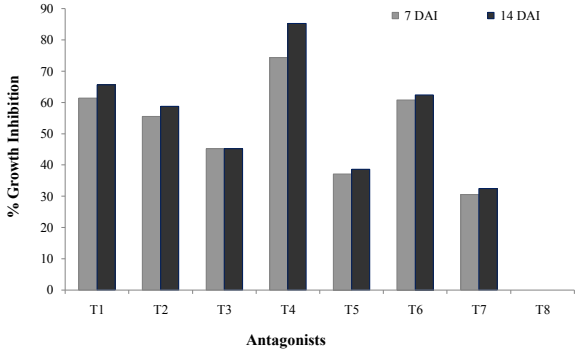

Figure 2: Percent growth inhibition of *Macrophomina phaseolina* during *in-vitro* antagonism with Trichoderma spp. at 7 and 14 DAI

T1=*T. harzianum* NBAII Th 1×*M. phaseolina*; T2=*T. viride* NBAII Tv 23×*M. phaseolina*; T3 =*T. virens* NBAII Tvs 12×*M. phaseolina*; T4=*T. koningii* MTCC 796×*M. phaseolina;* T5=*T. pseudokoningii* MTCC 2048×*M.phaseolina*; T6 =*T. hamatum* NBAII Tha-1×*M. phaseolina*; T7=*T. harzianum* (Local)×*M. phaseolina*; T8=*M.phaseolina*–Control, DAI=Days After Inoculation.

Results

In vitro percent growth inhibition of pathogen - *M. phaseolina*

The first apparent physical contact between *Trichoderma* spp. and its host, *M. phaseolina*, occurred within 2-3 days after inoculation (DAI), followed by growth inhibition. Growth inhibition of *M. phaseolina* (Tassi) Goid during *in vitro* interaction with bio-control agents *Trichoderma* at 7 and 14 DAI was depicted in (Figure 1A and 1B). Percent growth inhibition of pathogen (*M. phaseolina*) was higher in T4 (74.3%) antagonist followed by T1 (61.4%), T6 (60.8%), T2 (55.5%), and T3 (45.2%) at 7 DAI (Figure 2). Further, mycoparasitism of antagonists were observed upto 14 DAI (Figure 1A and 1B). Pattern of growth inhibition of test fungus was continued with maximum 14.7% increases in T4 (85.2%) followed by 6.8% elevation in T1 (65.6%) antagonists during 7 to 14 DAI. The antagonists T5 and T7 were recorded below 40% growth inhibition of test fungus at both intervals. Thus, it was observed that T4 antagonist (i.e. interaction between *T. koningi* MTCC 796 and pathogen *M. phaseolina*) have a better growth inhibition of test fungus followed by T1 (*T. harzianum* NABII Th 1×*M. phaseolina*) compared to other *Trichoderma* spp.

Evaluation of coiling pattern at 14 DAI

Trichoderma spp. tested in this work had a marked significant variation in inhibition of pathogen growth. Maximum pathogen growth inhibition occurred in interacting with *T. koningi* MTCC 796 (T4 antagonist). At 14 DAI, *T. koningi* MTCC 796 completely destroyed the host and sporulated (Figure 1A). This process occurred at different intensities depending on the *Trichoderma* spp., suggesting that various antagonists have different mechanisms of host recognition [20]. Microscopic study showed that *T. koningi* MTCC 796 was capable of overgrowing and degrading *M. phaseolina* mycelia, coiling around the hyphae with apressoria and hook-like structures (Figure 1A). Formation of apressoria-like structures enabled the hyphae of *Trichoderma* spp. to firmly attach to the surface of its host mycelium. However, some antagonists (*T. viride* NABII Tv 23, *T. hamatum* NABII Tha 1) used different mechanism against *M. phaseolina* just touched the hyphae without coiling (Figure 1A and 1B). While, *T. pseudokoningi* showed spore around pathogen not attached to hyphae (Figure 1B).

Production of cell wall degrading enzymes

Seven *Trichoderma* spp., tested for antagonism with fungal pathogen, produced and secreted on induction substantial amounts of various cell wall degrading enzymes when grown in the synthetic media containing pathogen cell wall as the carbon source. There was differing patterns in cell wall degrading enzyme production by various *Trichoderma* spp. during mycoparasitsm at 24, 48, 72 and 96 h incubation with pathogen cell wall.

The chitinolytic activity induced in some antagonists comprises growth of that species. Mean antagonists (T) differences on specific activity of chitinase found to be significant (Table 1). The significantly

***Trichoderma* spp. / Accession No.**	**Chitinase (µmol GlcNAc. h^{-1}.mg^{-1}protein)**				
	24 h	**48 h**	**72 h**	**96 h**	**Mean (T)**
T. harzianum (NBAII Th 1)	17.50	28.71	51.33	45.81	**35.84**
T. viride (NBAII Tv 23)	22.92	21.87	26.10	39.61	**27.63**
T. virens (NBAII Tvs 12)	23.64	22.07	40.31	37.12	**30.78**
T. koningii (MTCC 796)	35.63	37.04	37.94	41.37	**38.00**
T. pseudokoningii (MTCC 2048)	15.10	22.69	28.54	26.39	**2318**
T. hamatum (NBAII Tha-1)	29.60	26.14	36.19	37.40	**32.33**
T. harzianum (Local)	17.69	17.94	25.02	21.60	**20.56**
M. phaseolina - Control	1.36	1.07	2.18	2.32	**1.73**
Mean (H)	**20.43**	**22.19**	**30.95**	**31.45**	
	S.Em.	**C.D. at 5%**	**C.V.%**		
T	0.444	1.264	2.91		
H	0.314	0.894			
T×H	0.888	2.528			

Values represent mean of three experiments, each performed in duplicate.
Table 1: Specific activity of chitinase produced by *Trichoderma* spp. incubated with cell wall of pathogen *Macrophomina phaseolina*.

highest chitinase activity was recorded by *T. koningi* MTCC 796 (38.00 U.mg^{-1} protein) followed by *T. harzianum* NABII Th 1 (35.84 U.mg^{-1} protein) antagonist. Irrespective of antagonists, mean time interval (H) showed continuous rise in enzyme activity during 24 to 96 h when *Trichoderma* spp. grew in the presence of pathogen cell wall. The interaction effect of T×H were significantly differed for chitinase activity. The highest specific activity was recorded by *T. harzianum* NBAII Th 1 at 72 h and it was 1.93 fold higher than 24 h followed by significantly declined at 96 h. The antagonist *T. koningi* MTCC 796 induced higher chitinase activity at 24 h incubation and it was 25 fold higher than control (pathogen alone). Probably, early induction of chitinase by antagonist *T. koningi* MTCC 796 caused high coiling capacity during mycoparasitism at 14 DAI and greater percent growth inhibition of pathogen-*M. phaseolina.*

Mean antagonists (T) differences on specific activity of β-1, 3 glucanase was found to be significant (Table 2). The significantly highest β-1, 3 glucanase activity was recorded by *T. koningi* MTCC 796 (9.97 U.mg^{-1} protein) followed by *T. hamatum* NABII Tha 1 (9.67 U.mg^{-1} protein) antagonist. Irrespective of antagonists, mean time interval (H) showed rise in enzyme activity during 24 to 72 h when *Trichoderma* spp. grew in the presence of pathogen cell wall. The interaction effect of T x H for β-1, 3 glucanase revealed significant differences. The highest specific activity was recorded by *T. hamatum* NABII Tha 1 (14.37 U.mg^{-1} protein) at 72 h and it was 0.85 fold increased than 24 h incubation followed by significantly declined at 96 h. The antagonist *T. koningi* MTCC 796 elevated 1.18 fold specific activities during 24 to 96 h incubation. Overall, the activity of β-1, 3 glucanase was higher in *T. koningi* MTCC 796 followed by *T. hamatum* NABII Tha 1 when grown with pathogen cell wall. These were corresponded to the maximum growth inhibition of pathogen *M. phaseolina* by *T. koningi* MTCC (85.2%) antagonist followed by *T. hamatum* NABII Tha 1 (62.4%) at 14 DAI antagonism. It has been shown that β-1, 3 glucanase inhibited the growth of pathogens in synergistic cooperation with chitinase.

When expressing specific activity of protease (Table 3), antagonist mean (T) activity was found highest in *T. hamatum* NABII Tha 1 (48.17 U.mg^{-1} protein). Protease activity during different time intervals (Mean H) showed about 3 fold rise in activity during 24 to 96 h incubation. Combined effect of T×H indicated that the specific activity of protease attained higher in *T. koningi* MTCC 796 at 24 h.

***Trichoderma* spp. / Accession No.**	**β-1, 3- Glucanase (µmol glucose.h^{-1}.mg^{-1}protein)**				
	24 h	**48 h**	**72 h**	**96 h**	**Mean (T)**
T. harzianum (NBAII Th 1)	4.93	6.09	11.39	9.95	**8.09**
T. viride (NBAII Tv 23)	5.60	5.67	8.46	7.86	**6.90**
T. virens (NBAII Tvs 12)	8.89	4.74	9.97	8.58	**8.05**
T. koningii (MTCC 796)	6.35	6.51	13.14	13.87	**9.97**
T. pseudokoningii (MTCC 2048)	6.44	4.74	7.73	5.38	**6.07**
T. hamatum (NBAII Tha-1)	7.76	6.22	14.37	10.32	**9.67**
T. harzianum (Local)	7.09	6.29	5.99	4.75	**6.03**
M. phaseolina-Control	6.28	2.33	2.13	4.31	**3.76**
Mean (H)	**6.67**	**5.32**	**9.15**	**8.13**	
	S.Em.	**C.D. at 5%**	**C.V.%**		
T	**0.029**	**0.081**	1.35		
H	0.020	0.058			
T×H	0.057	0.163			

Values represent mean of three experiments, each performed in duplicate.
Table 2: Specific activity of β-1, 3 glucanase produced by *Trichoderma* spp. incubated with cell wall of pathogen *Macrophomina phaseolina.*

***Trichoderma* spp. / Accession No.**	**Protease (µg free amino acids.h^{-1}.mg^{-1}protein)**				
	24 h	**48 h**	**72 h**	**96 h**	**Mean (T)**
T. harzianum (NBAII Th 1)	3.75	19.26	46.08	71.25	**35.09**
T. viride (NBAII Tv 23)	0.82	7.03	16.54	5.71	**7.53**
T. virens (NBAII Tvs 12)	38.60	29.52	54.26	60.22	**45.65**
T. koningii (MTCC 796)	39.94	20.15	38.84	25.86	**31.20**
T. pseudokoningii (MTCC 2048)	1.86	13.96	72.86	87.82	**44.13**
T. hamatum (NBAII Tha-1)	11.09	28.85	57.44	95.29	**48.17**
T. harzianum (Local)	4.83	33.27	40.91	62.96	**35.49**
M. phaseolina-Control	2.99	3.77	2.52	1.45	**2.68**
Mean (H)	**12.98**	**19.48**	**41.18**	**51.32**	
	S.Em.	**C.D. at 5%**	**C.V.%**		
T	0.038	0.109	0.43		
H	0.027	0.077			
T×H	0.077	0.218			

Values represent mean of three experiments, each performed in duplicate.
Table 3: Specific activity of protease produced by *Trichoderma* spp. incubated with cell wall of pathogen *Macrophomina phaseolina.*

***Trichoderma* spp.**	**Cellulase (µmol glucose.h^{-1}.mg^{-1} protein)**				
	24 h	**48 h**	**72 h**	**96 h**	**Mean (T)**
T. harzianum (NBAII Th 1)	3.19	25.01	19.1	18.76	**16.52**
T. viride (NBAII Tv 23)	4.16	35.85	18.36	12.23	**17.65**
T. virens (NBAII Tvs 12)	5.72	54.26	22.13	37.67	**29.95**
T. koningii (MTCC 796)	4.34	27.28	12.13	14.82	**14.64**
T. pseudokoningii (MTCC 2048)	3.41	70.21	64.01	60.41	**49.51**
T. hamatum (NBAII Tha-1)	5.99	39.21	23.49	31.93	**25.16**
T. harzianum (Local)	7.37	35.38	15.00	11.03	**17.20**
M. phaseolina-Control	5.43	52.71	63.57	76.35	**49.52**
Mean (H)	**4.95**	**42.49**	**29.72**	**32.90**	
	S.Em.	**C.D. at 5%**	**C.V.%**		
T	0.107	0.304	1.34		
H	0.075	0.215			
T×H	0.213	0.607			

Values represent mean of three experiments, each performed in duplicate.
Table 4: Specific activity of cellulase produced by *Trichoderma* spp. incubated with cell wall of pathogen *Macrophomina phaseolina.*

The highest specific activity was recorded in T. pseudokoningii MTCC 2048 at 96 h incubation which exceptionally did not inhibit the growth of pathogen effectively. The enzyme activity was more or less correlated with growth inhibition of test fungus except *T. viride* NABII Tv 23 and *T. pseudokoningii* MTCC 2048 antagonists.

Considering mean antagonist (T) data, specific activity of cellulase was significantly higher in test fungi control (49.52 U.mg^{-1} protein) followed by *T. pseudokoningii* MTCC 2048 antagonist (Table 4). The significantly minimum cellulase activity (14.64 U.mg^{-1} protein) was found in culture medium of *T. koningi* MTCC 796. Mean time interval data (H) showed maximum 7.5 fold rise in activity during 24 to 48 h incubation. Interaction effect of T×H indicated 13 fold increase in the activity during 24 to 96 h incubation in control treatment (Pathogen-*M. phaseolina*). The activity in the culture medium was negatively correlated to the growth inhibition of pathogen during in vitro antagonism study. Total phenol was produced significantly higher in culture supernatant of *T. koningi* MTCC 796 antagonist followed by *T. hamatum* NBAII Tha 1 and *T. harzianum* NBAII Th 1 at 48 h incubation (Table 5). Total phenol content was raised at early duration of incubation (24 to 48 h) in the antagonists who inhibited growth of test fungus effectively.

***Trichoderma* spp.**	**Total phenol (µg/ml cultural filtrate)**				
	24 h	**48 h**	**72 h**	**96 h**	**Mean (T)**
T. harzianum (NBAII Th 1)	10.49	6.79	4.57	4.70	**6.64**
T. viride (NBAII Tv 23)	8.68	8.49	9.58	7.09	**8.46**
T. virens (NBAII Tvs 12)	11.28	11.33	9.89	6.30	**9.70**
T. koningii (MTCC 796)	11.77	13.62	9.74	9.77	**11.23**
T. pseudokoningii (MTCC 2048)	5.74	6.96	6.43	6.40	**6.38**
T. hamatum (NBAII Tha-1)	11.13	11.06	7.17	5.47	**8.71**
T. harzianum (Local)	6.32	8.21	8.85	9.30	**8.17**
M. phaseolina-Control	5.19	5.26	5.43	8.68	**6.14**
Mean (H)	**8.83**	**8.97**	**7.71**	**7.21**	
	S.Em.	**C.D. at 5%**	**C.V.%**		
T	0.026	0.075	1.11		
H	0.019	0.053			
T×H	0.053	0.149			

Values represent mean of three experiments, each performed in duplicate.
Table 5: Total phenol produced by *Trichoderma* spp. incubated with cell wall of pathogen *Macrophomina phaseolina.*

	% Growth inhibition	**Chitinase**	**β-1,3 Glucanase**	**Protease**	**Cellulase**	**Total phenol**
% Growth inhibition	1.0000					
Chitinase	0.9553**	1.0000				
Glucanase	0.9191**	0.9255**	1.0000			
Protease	0.3855	0.5804	0.5770	1.0000		
Cellulase	- 0.7350*	- 0.7021*	- 0.6441*	- 0.1155	1.0000	
Total phenol	0.6526*	0.6124	0.7329*	0.2508	- 0.5982	1.0000

n-24, critical value *(p=0.05) = 0.632; **(p=0.01) = 0.765
Table 6: Correlation matrix between percent growth inhibition of test fungi at 14 DAI and production of hydrolytic enzymes by *Trichoderma* spp. using cell wall of test fungi as carbon source.

Correlation between antagonism study and release of cell wall degrading enzymes by *Trichoderma* spp. using pathogen cell wall as substrate

A highly significant positive correlation (p=0.01) was observed between percent growth inhibition of pathogen *M. phaseolina*, and chitinase and β-1, 3-glucanase enzymes (Table 6) which established a relationship to inhibit the growth of fungal pathogen by increasing the levels of these cell wall degrading enzymes during antagonism. Protease showed positive but no significant correlation among percent growth inhibition, chitinase and β-1, 3-glucanase enzymes. The significant negative correlation (p=0.05) was established between growth inhibitions of test fungus and cellulase, suggested that percent growth inhibition of pathogen decreased with increasing concentration of cellulase during antagonism. Cellulase was significantly negatively correlated with pathogenesis related enzymes mainly chitinase and β-1, 3-glucanase. Among chitinase and β-1,3-glucanase, the correlation was highly positive (0.9255) indicated that these enzymes worked together in synergistic cooperation to inhibit the growth of pathogen. Total phenol content was positively correlated (p=0.05) with β-1, 3-glucanase and percent inhibition of pathogen suggesting their role in growth inhibition of pathogen during antagonism.

So, it can be summarized that among seven *Trichoderma* spp., *T. koningi* MTCC 796 was the best agent to inhibit the growth of pathogen *M .phaseolina* during antagonism study on PDA media. Microscopic study also showed that *T. koningi* MTCC 796 started the parasitism earlier and was capable of overgrowing and degrading *M. phaseolina* mycelia, coiling around the hyphae with apressoria and hook-like structures. Percent growth inhibition of pathogen and production of cell wall degrading enzymes-chitinase and β-1,3 glucanase correlated positively suggesting that these enzymes were released during antagonism and inhibited the growth of fungal pathogen *M. phaseolina*. Thus, *T. koningi* MTCC 796 is most suitable strain to be used in biological control of plant pathogen *M. phaseolina*, causing charcoal rot disease in castor.

Discussion

Trichoderma spp. are widely used in agriculture as biocontrol agents because of their ability to reduce the incidence of disease caused by plant pathogenic fungi, particularly many common soil borne pathogens [21-29]. In present work, in vitro antagonism of 7 species of *Trichoderma* with *M. phaseolina*, causing charcoal rot in castor, were studied. The results of dual culture revealed the maximum (74.3%) growth inhibition of test pathogen with *T. koningi* MTCC 796 (T4) followed by *T. harzianum* NABII Th 1 (T1) (61.4%) at 7 DAI (Figure 2). Further, mycoparasitism of antagonists were observed upto 14 DAI (Figure 1A and 1B). Pattern of growth inhibition of test fungus was continued with maximum 14.7% increase in T4 (85.2%) followed by 6.8% elevation in T1 (65.6%) antagonists during 7 to 14 DAI.

These results was in confirmation with the finding of Melo and Faull [30], who reported that the *T. koningi* and *T. harzianum* were found to be effective in reducing the radial growth of *R. solani*, *T. koningi* strains produced toxic metabolites with strong activity against *R. solani,* inhibiting the mycelial growth. Ramezani [31] also documented that *T. harzianum* significantly inhibited the growth of *M. phaseolina. T. viride* and *T. harzianum* had a greater inhibition on *M. phaseolina* than *T. hamatum*. Our study showed *T. koningi* MTCC 796 and *T. harzianum* NABII Th 1 have a better growth inhibition of *M. phaseolina* compared to *T. viride*. *Trichoderma* spp. is known to act through several mechanisms such as hyperparasitism, inhibition and antibiosis. Shalini and Kotasthane [32] screened seventeen *Trichoderma* strains against *R. solani in vitro*. All strains including *T. harzianum*, *T. viride* and *T. aureoviride* were inhibited the growth of *R. solani.*

Mycoparasitism involves morphological changes, such as coiling and formation of appressorium- like structures, which serve to penetrate the host [33]. The first contact between *Trichoderma* spp. and pathogen *M. phaseolina* occurred after 2 to 3 days of inoculations, followed by growth inhibition. Differential antagonistic activity has even been observed for various *Trichoderma* spp. which demonstrates semi-specificity in the interaction of *Trichoderma* with its host [4]. Our results on light microscopic study revealed that *T. koningi* MTCC 796 and *T. harzianum* NABII Th 1 showed effectively coiling on pathogen *M. phaseolina* at 14DAI, and may start the parasitism earlier during antagonism compared to other *Trichoderma* spp. Similar results have been reported for *T. harzianum* against *Crinipellis perniciosa* [34], *Sclerotium rolfsii* [35] and *Rhizoctonia cerealis* [36].

Monteiro et al. [37] reported that *T. harzianum* ALL42 were capable of overgrowing and degrading *Rhizoctonia solani* and *M. phaseolina* mycelia, coiling around the hyphae with formation of apressoria and hook-like structures. The interaction between fifteen isolates of *T. harzianum* and the soil-borne plant pathogen, *Rhizoctonia solani*, was studied using light microscopy and Transmission Electron Microscopy (TEM) which showed efficient coiling process followed by a substantial production of hydrolytic enzymes [14]. Howell [1] examined *Trichoderma* that attaches to the pathogen with cell-wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is

the pathogen and forms the appresoria. The following step consisted of the production of pathogenesis related enzymes and peptaibols.

Many reports suggested the involvement of signal transduction pathways components such as G proteins, cAMP and MAP kinase, which control extracellular enzyme and coiling around host hyphae [20,38]. In *Trichoderma*, there is a biochemical evidence for participation of G-α in coiling since an increase in coiling around nylon fibers was detected after addition of activators of G-protein [39].

The specific activities of the four enzymes of the *Trichoderma* spp. were tested using cell wall of the pathogen *M. phaseolina* as substrate during different incubation period ranged from 24 to 96 h (Tables 1-4). The species were cultured on cell walls of *M. phaseolina* as the sole carbon source. All tested species showed significant variation in chitinase, β-1, 3-glucanase and protease activities when grown in the *M. phaseolina* cell wall except cellulase. The release of chitinase and β-1, 3-glucanase was higher in *T. koningi* MTCC 796 followed by *T. harzianum* NBAII Th 1 and *T. hamatum* NABII Tha 1 than other *Trichoderma* spp. The antagonist *T. koningi* MTCC 796 induced highest chitinase activity at 24 h incubation and it was 25 fold higher than control (pathogen alone). Early induction of chitinase by antagonist *T. koningi* MTCC 796 compared to other species caused high coiling capacity during mycoparasitism at 14 DAI and greater percent growth inhibition of pathogen *M. phaseolina*.

It has been shown that *T. koningi* MTCC 796 elevated 1.18 fold specific activities of β-1, 3 glucanase during 72 to 96 h compared to 24 h incubation. It indicated that β-1, 3 glucanase inhibited the growth of pathogen in synergistic cooperation with chitinase [35,40]. Similar to present study, Marco et al. [12] noted that two isolates of *T. harzianum* (39.1 and 1051) produced and secreted on induction of substantial amounts of chitinolytic enzymes and it increased within 72 h, while β-1, 3 glucanase activity was found to be maximum during 72 to 120 h growth in presence of specific substrate.

The cell wall degrading enzymes-chitinase and β-1,3-glucanase were produced in the presence of phytopathogen cell walls as the carbon source, suggesting that these substrates can also act as inducers of lytic enzyme synthesis. This result is similar to that by reported Sivan and Chet [41] in which *T. harzianum* produced high levels of chitinase and glucanase when grown on *Rhizoctonia solani* mycelia. Considerable variation has been reported with respect to biocontrol activity among the *Trichoderma* spp. analyzed. The production of hydrolytic enzymes has been shown to be affected by culture conditions and by the host [4]. Some other studies also demonstrated that *T. harzianum* revealed chitinase and gluconase activity when their cultures were supplemented with cell walls from *Sclerotium rolfsii* [11], *Fusarium oxysporum*, *Rhizoctonia solani* [22] and *Botrytis cinerea* [4]. However, our earlier study [42] confirmed the relationship between the RAPD polymorphism showed by three species of twelve *Trichoderma* isolates and their hardness to *Aspergillus niger*, in terms of *in vitro* production of cell wall degrading enzymes–chitinase, β-1, 3 glucanase and protease during antagonism.

Correlation between antagonism study and release of cell wall degrading enzymes by *Trichoderma* spp. in the presence of pathogen cell wall indicated a highly significant positive correlation (p=0.01) between percent growth inhibition of pathogen *M. phaseolina*, chitinase and β-1, 3-glucanase enzymes which established a relationship to inhibit the growth of fungal pathogen by increasing the levels of these cell wall degrading enzymes during antagonism (Table 6). Protease showed positive but non significant correlation while cellulase confirmed negative correlation, among percent growth inhibition, chitinase and β-1,3-glucanase enzymes. Total phenol content was positively correlated (p=0.05) with β-1, 3 glucanase and percent inhibition of pathogen suggesting their role in growth inhibition of test fungus during antagonism.

There are several mechanisms involved in *Trichoderma* antagonism namely antibiosis whereby the antagonic fungus shows production of antibiotics, competition for nutrients. In case of mycoparasitism, *Trichoderma* directly attacks the plant pathogen by excreting lytic enzymes such as chitinases, β-1,3 glucanases and proteases [43]. Because of the skeleton of pathogenic fungi cell walls 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 [44,45]. Filamentous fungal cell wall also contains lipids and proteins [46]. It therefore, was expected that antagonistic fungi synthesized proteases which may act on the host cell-wall [1].

Most of the bio-control agents are known to produce chitinase and β-1, 3-glucanases enzymes which could degrade the cell wall leading to the lysis of hyphae of the pathogen [47]. The pathogen cell-wall and chitin induce *nag1* gene, but it is only triggered when there is contact with the pathogen [1,2,45]. Present findings showed higher specific activity of enzymes-chitinase and β-1, 3 glucanase in *Trichoderma* spp. incubated with pathogen cell wall. Activity of these enzymes varied with various *Trichoderma* species. This may be due to the expression of certain gene in *Trichoderma* spp. during incubation as *Chit33* is expressed only during the contact phase and not before overgrowing *R. solani* [48]. However, *chit36Y* does not need the direct contact of the pathogen to be expressed. *Chit33*, *chit42* and *chit36* have been over expressed in *Trichoderma* spp. in order to test the role of these chitinases in mycoparasitism, and the 42-kDa chitinase is believed to be a key enzyme [1]. The production of β-1, 3 glucanase was also reported as an important enzymatic activity in bio-control of pathogen because β-1, 3 glucan is a structural component of fungal cell walls. Many β-1, 3-glucanases have been isolated, but only a few genes have been cloned, e.g. *bgn13.1* [40].

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