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Screening of *Trichoderma* Isolates as a Biological Control Agent against *Ceratocystis paradoxa* Causing Pineapple Disease of Sugarcane

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In this study, dual culture, poison agar, and direct methods were used to assess the ability of *Trichoderma virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433, and *T. harzianum* IMI-392434 to control *Ceratocystis paradoxa*, which causes the pineapple disease of sugarcane. The highest percentage inhibition of radial growth (PIRG) values were observed with *T. harzianum* IMI-392432 using two dual culture methods, 63.80% in Method I and 80.82% in Method II. The minimum colony overgrowth time was observed with *T. harzianum* IMI-392432 and the maximum was observed with *T. pseudokoningii* IMI-392431. Different concentrations of different day-old metabolites of *Trichoderma* isolates were tested against mycelial growth of *C. paradoxa*. The highest PIRG (84.685%) exhibited at 80% concentration of 30-day-old metabolites of *T. harzianum* IMI-392432 using the modified bilayer poison agar method. In the direct assay method the maximum mycelial growth weight (PIGW) was observed at the same concentration and the same day-old metabolites of *T. harzianum* IMI-392432. This study showed that *Trichoderma* isolates have a good antagonistic effect on *C. paradoxa* mycelial growth and *T. harzianum* IMI-392432 has the most potential to control the pineapple disease pathogen.

KEYWORDS : *Ceratocystis paradoxa*, PIGW, PIRG, Secondary metabolites, *Trichoderma*

Pineapple disease caused by *Ceratocystis paradoxa* results in a considerable loss in sett germination and can reduce cane yield by 31~35% (Anonymous, 2000). The disease is severe in heavy textured soils and poorly drained fields, and it can reduce germination by up to 47% (Anonymous, 1999). The affected setts emit a smell resembling that of the mature pineapple fruit (Went, 1896), which is due to ethyl acetate formed by metabolic activity of the pathogen. The ethyl acetate content in the infected tissue may rise to 1%, which is sufficient to inhibit germination of buds (Kuo *et al*., 1969). The fungus is essentially soil-borne and is transmitted to cane setts via two types of spores: thin-walled cylindrical conidia (6~24 μm × 2~5.5 μm) and thick-walled oval chlamydospores (10~25 μm × 7.5~20 μm). The latter ensures the long-term survival of the pathogen in the soil. Infection occurs mainly through cut ends but also through wounds caused by insects and through cracks in cuttings. The fungus spreads rapidly through the parenchyma, which becomes red and breaks down leading to a hollow and blackened interior. The economic importance of the disease is also significant.

When shoot population is reduced, yield is directly reduced. Furthermore, grapy stands necessitate costly replanting or recruiting, and weed problems may also result if leaf canopy development is retarded. Fungicide is most commonly used as a treatment to manage, but there is a need for non-chemical methods of control to reduce the adverse effects of toxic chemicals on the environment, particularly the sugarcane ecosystem. Biological control of plant pathogens by microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods (Baker and Paulitz, 1996). The antagonistic activity of *Trichoderma* species against plant pathogens has been studied extensively (Burgess and Hepworth, 1996; Burns and Benson, 2000; Etabarian, 2006; Hjeljord *et al.*, 2001). Knowledge about the behavior of these fungi as antagonists is essential for their effective use because they can act against pathogens in several ways like produce lytic enzymes, antibiotics etc (KüçüK and Kivanç, 2003). A number of commercial formulations, based on *T. harzianum* and *T. virens*, are available for the control of soil borne and foliar disease in a range of horticulture crops (Etabarian, 2006; Samuels, 1996). *T. harzianum* isolate, T39, is the active ingredient of Trichodex, which is reported to control botrytis grey mold on a range of crops (Elad, 1994). *T. harzianum* has been evaluated for the control of black seed rot disease of oil palm sprouted seeds in Nigeria (Eziashi *et al.*, 2007), but there is little information on the efficacy of *T. virens* and other isolates of *T. harzianum* against *Ceratosystis paradoxa*, the pathogen that causes pineapple disease in sugarcane. Therefore, the focus of this investigation was to evaluate the potential of select *Trichoderma* isolates to biologically control *C. paradoxa*. The physical mode of antagonism and the effect of secondary metabolites produced by selective *Trichoderma* strains on the growth and development of *C. paradoxa* were also investigated.

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Materials and Methods

Source of *Trichoderma* isolates. Five *Trichoderma* isolates, *T. virens* (Miller) IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* (Rifai) IMI-392432, *T. harzianum* (Rifai) IMI-392433, and *T. harzianum* (Rifai) IMI-392434, were collected from the Biotechnology and Microbiology Laboratory, Department of Botany, Rajshahi University, Bangladesh. These isolate were previously verified by CABI Bioscience, Surrey, U.K. (Rahman, 2009).

Isolation and identification of *C. paradoxa*. Pineapple disease infected setts with typical symptoms were collected (Fig. 1a) from experimental fields of the Bangladesh Sugarcane Research Institute, Ishurdi, Pabna, Bangladesh. The samples were washed with tap water to remove sand and soil particles, split open longitudinally

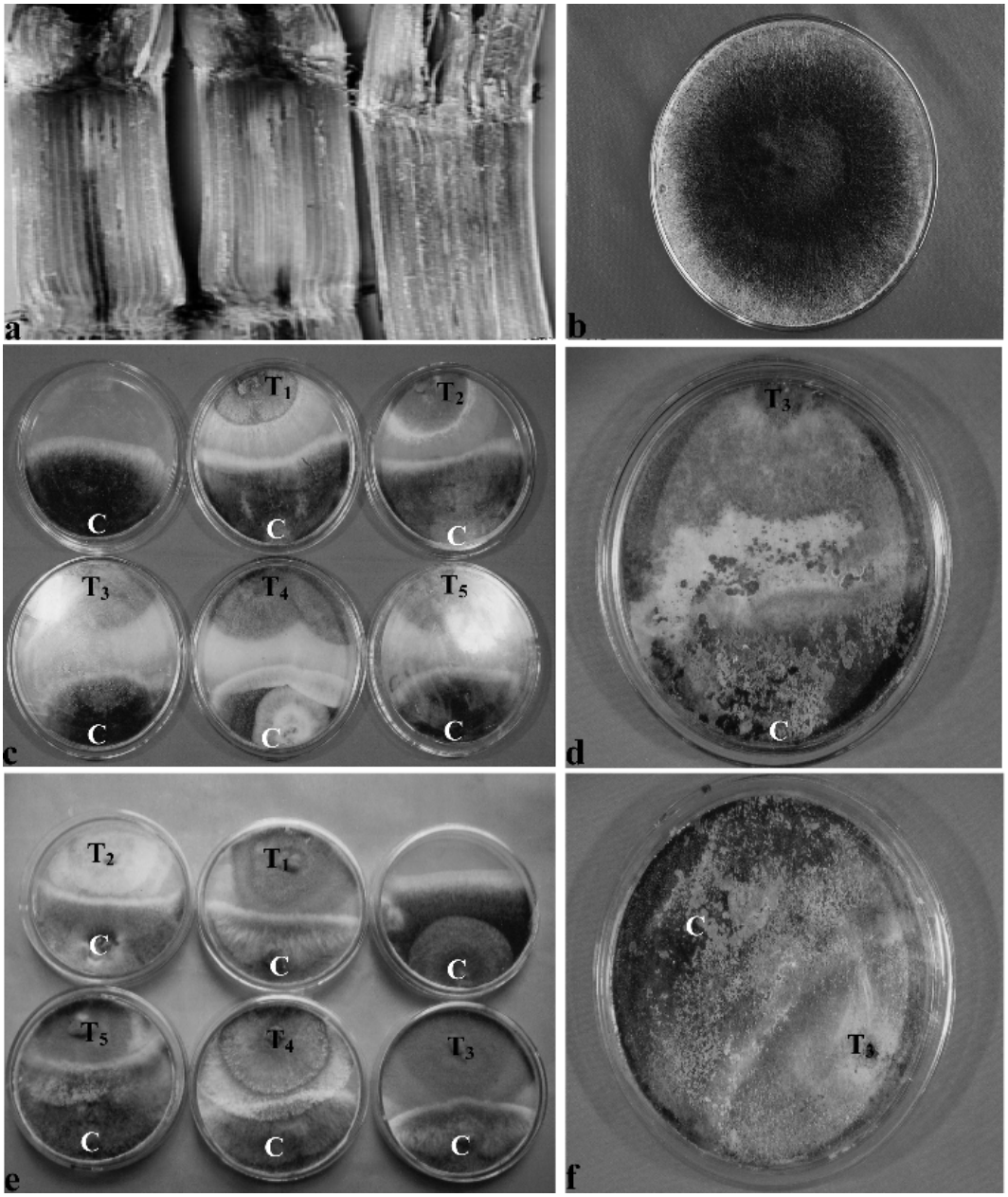

Fig. 1. a & b. Symptoms of pineapple disease of sugarcane and *C. paradoxa* colonies on PDA. **c & e**, Antagonistic effects of *Trichoderma* isolates against *C. paradoxa* in dual culture Method I and Method II, respectively; **d & f**, Shows overgrowth of *Trichoderma* covering the *C. paradoxa* colony after 7 and 6 days of inoculation in dual culture (Method I and Method II). C, T_1, T_2, T_3, T_4, and T_5 indicate *C. paradoxa*, *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum*, IMI-392432, *T. harzianum*, IMI-392433, and *T. harzianum* IMI-392434, respectively.

with a sharp knife, and surface sterilized with a 1:1000 mercuric chloride solution for 1 minute. The specimens were then washed three times with sterile distilled water. The blackish spores of *C. paradoxa* were collected with a sterile needle, transferred to Potato Dextrose Agar (PDA) medium, incubated at room temperature (28 ± 1°C), and observed regularly to monitor fungi growth. The fungi that grew on PDA (Fig. 1) were isolated and purified by the hyphal tip culture method. The fungi were identified following the key outline by Edgerton (1959). Pure culture of *C. paradoxa* was preserved on a PDA slant at 4°C. The pathogenicity of the *C. paradoxa* isolate was confirmed on a local sugarcane cultivar. All the cultures were stored at 4ºC until further study.

Screening by dual culture method. Two methods were followed for dual culture technique. In the first method (Method-I), an agar disc (6 mm) was taken from 4-day-old PDA culture plates of each *Trichoderma* isolate and placed at the periphery of the PDA plates (9 mm). Another agar disc of the same size of *C. paradoxa* was also placed at the periphery but on the opposing end of the same Petri dish (Fig. 2). In the second method (Method-II), an agar disc (6-mm) of the antagonist, *Trichoderma* (T), was placed 2 cm away from the periphery of the Petri dish, and a same sized agar disc of the test fungus, *C. paradoxa* (C), was similarly placed 2 cm away from the edge of the Petri plate but on the end opposite of *Trichoderma* sample. As a control, *C. paradoxa* was placed in a similar manner on a fresh PDA plate (Fig. 2). All pairings were carried out in quadruplicate and incubated at 28°C. Antagonistic activity was tested 4 days after incubation by measuring the radius of the *C. paradoxa* colony in the direction of the antagonist colony (R_2) and the radius of the *C. paradoxa* colony in the control plate (R_1). The two readings were transformed into percentage inhibition of radial growth (PIRG) using the formula developed by Skidmore and Dickinson (1976),

$$\text{Where, } PORG = \frac{R_1 - R_2}{R_1} \times 100$$

Observations were continued on the dual culture plates after 4 days of incubation and PIRG was calculated. The number of days required for the antagonist to overgrow the whole colony of *C. paradoxa* was recorded.

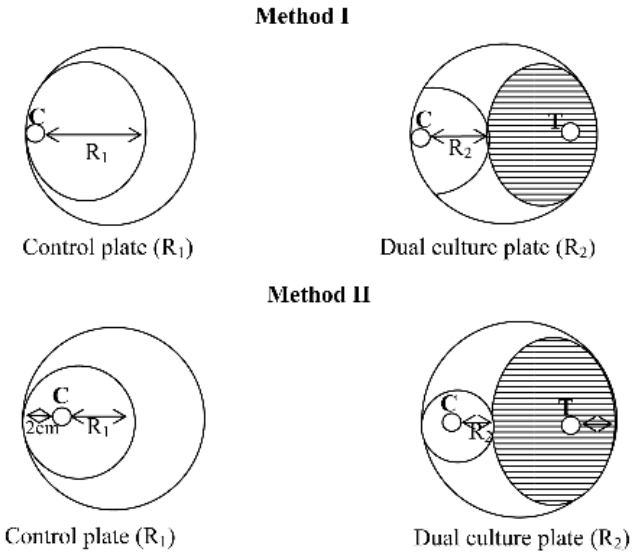

Fig. 2. Measurement of radial growth of *Ceratocystis paradoxa* mycelia by Method I where culture plug placement was at the margin and Method II where placement was 2 cm away from the margin. Note: R_1, Radius of *C. paradoxa* colony in control plate; R_2, Radius of *C. paradoxa* colony in dual culture plate; C, *Ceratocystis paradoxa* isolate; T, *Trichoderma* isolate.

Screening by Poison Agar Technique Using Crude Metabolites

Preparation of culture filtrates of *Trichoderma*. Two hundred milliliters of Richard's solution (KNO_3: 1.0 g, KH_2PO_4: 0.5 g, $MgSO_4 \cdot 7H_2O$: 0.25 g, glucose: 34 g, and trace amounts of $FeCl_3$ in 1 *l* distilled water, pH 6.5) was prepared and poured into 500 *ml* conical flasks and autoclaved for 15 minute at 121°C/1.05 kg/cm^2 pressure. Six pieces of agar discs (6 mm) were kept in a flask (with media) for each strain of *Trichoderma* with four replicates. The flasks were incubated on a Gallenkamp orbital incubator at 100 rpm at 28°C (Dennis and Webster, 1971). The culture filtrates were collected after 10, 20, and 30 days of incubation. These were then concentrated to about 50% using a vacuum evaporator at 38~40°C and filtered by sterilized membrane filter.

Preparation of poison agar plate. Initially, 20, 40, 60, and 80% of PDA medium was prepared and kept in 250 ml conical flask for each medium and autoclaved for 15 minutes. The sterilized metabolites were then added to this PDA medium respectively. The molten PDA, with different concentrations of metabolites, was poured into Petri plates and allowed to solidify. As a control, Richard's solution was mixed with PDA in the same concentrations as used for *Trichoderma* metabolites.

Screening technique. For normal poison agar method, seven-day-old culture discs (6 mm) of *C. paradoxa* were inoculated at the centre of previously prepared poison agar plates and incubated at room temperature (28 ± 2°C) for 10 days. For the modified bilayer poison agar method, an agar disc (6 mm) of *C. paradoxa* was inoculated on the center of a normal PDA plate for 4 days. Afterwards a second layer of molten PDA, incorporated with ascending concentrations of sterilized metabolites of *Tricho-*

derma, was poured over the *C. paradoxa* colony. As a control, a second layer of molten PDA, incorporated with only sterilized Richard's solution instead of *Trichoderma* metabolites, was poured over the *C. paradoxa* colony. Observation was made on radial extension of the mycelia on culture plates for both the experimental treatment and control. Data were recorded on the mycelial extension of colony diameter after 4 to 10 days of inoculation. The readings were calculated for the percentage inhibition of radial growth (PIRG) based on the formula by Skidmore and Dickinson (1976), the same as for the dual culture experiment.

Direct Assay of *Trichoderma* Metabolites

Two methods were carried out to assess the inhibition of mycelial growth of *C. paradoxa.* In the first method, the PDB was prepared in quadruplicate at concentrations of 20, 40, 60, and 80%. Previously prepared sterilized *Trichoderma* metabolites were added proportionally into each conical flask. Then *C. paradoxa* mycelial discs were placed in each flask and incubated at room temperature (28°C) for 7 days. As a control, Richard's solution without the *Trichoderma* culture filtrates was added to the same concentrations of PDB media. In the second method, *C. paradoxa* mycelial discs were cultured in PDB as described in first method. On the 7th day of culture, *Trichoderma* filtrates at 20, 40, 60, and 80% were incorporated respectively into particular *C. paradoxa* cultures and incubated for another 7 days. As a control, Richard's solution without *Trichoderma* metabolites was added as earlier described. On the 7th day of mycelial growth, *C. paradoxa* mycelia was harvested from the flask, gently washed with distilled water, and oven dried at 60°C. The mean mycelial weight of the treatments was compared to the dry mean weight of *C. paradoxa* mycelia from the control flask. Data on mycelial weight for treatment flask at different concentrations and control flasks were recorded. The differences between the two readings multiplied by 100 were taken as the percentages of inhibition of mycelial growth weight (PIWG) following the modified method of Skidmore and Dickinson (1976).

$PIWG = (A_1 - A_2)/A_1 \times 100$, where A_1 = mycelial weight of *C. paradoxa* in control flasks and A_2 = mycelial weight of *C. paradoxa* mycelia in treatment flasks.

Results

Screening by dual culture technique. Each *Trichoderma* isolate inhibited the radial mycelial growth of *C. paradoxa*. The percentage inhibition of radial growth (PIRG) values ranged from 40.47 to 63.80% for first method and 58.12 to 80.82% for second method (Table 1 and Fig. 1c & e). The highest PIRG values (63.80% and 80.82%) were observed with *T. harzianum* IMI-392432 and the lowest recorded (40.47% and 58.12%) were observed with *T. pseudokoningii* IMI-392431 for first and second methods, respectively. With both methods, the highest PIRG values recorded were for *T. harzianum* IMI-392432, which was significantly different ($P \leq 0.05$) from the others. Colony overgrowth times varied from 7 to 12 days for the first method and 6 to 10 days for the second method (Table 1 and Fig. 1d & f). For both methods, the minimum colony overgrowth time recorded was for *T. harzianum* IMI-392432 and the maximum colony overgrowth time recorded was for *T. pseudokoningii* IMI-392431 for both methods.

Screening by poison agar technique. The PIRG values by metabolites of *Trichoderma* strains varied significantly ($p \leq 0.05$) at different concentrations and different days. With the normal poison agar method, the highest PIRG values (82.63%) were achieved at 80% concentration on the 4th day by 30-day-old metabolites of *T. harzianum* IMI-392432 using the normal poison agar method (Table 2). However, with the modified bilayer poison agar method, the highest PIRG values (84.68%) were achieved at 80% concentration of 30-day-old metabolites of *T. har-*

Table 1. Mean PIRG values and colony overgrowth time of *Trichoderma* isolates against *C. paradoxa* by dual culture method

Methods	*Trichoderma* isolates	Mean % inhibition of radial growth (PIRG)	No. of days to over growth *C. paradoxa* colony
Method-I	*T. virens*, IMI-392430	46.16 c	10
	T. pseudokoningii, IMI-392431	40.47 d	12
	T. harzianum, IMI-392432	63.80 a	7
	T. harzianum, IMI-392433	56.7 b	8
	T. harzianum, IMI-392434	54.24 b	9
Method-II	*T. virens*, IMI-392430	62.83 d	8
	T. pseudokoningii, IMI-392431	58.12 e	10
	T. harzianum , IMI-392432	80.82 a	6
	T. harzianum, IMI-392433	75.93 b	7
	T. harzianum, IMI-392434	70.33 c	7

In a column the same letters are not significantly different by DMRT at 5% level.

Table 2. Mean PIRG values by normal poison agar method using *Trichoderma* metabolites

Isolates of *Trichoderma*	No of days	10-day-old metabolites				20-day-old metabolites				30-day-old metabolites			
		20%	40%	60%	80%	20%	40%	60%	80%	20%	40%	60%	80%
T. virens IMI-392430	4	24.54 a	26.82 e	42.38 ef	50.29 de	34.26 cd	49.69 ef	55.82 ef	58.38 hi	57.98 ab	59.78 ef	61.84 hi	68.98 ij
	5	21.01 b	24.97 fg	39.16 hi	48.31 fg	32.18 e	46.38 hi	54.38 fg	55.87 jk	57.23 cd	57.21 ghi	58.71j	64.59 j
	6	14.93 fghi	22.18 ij	36.31 j	42.63 ij	28.34 hi	42.81 k	52.84 gh	55.29 l	55.82 cd	56.69 efg	57.26 jk	60.72 k
	7	12.21 klm	19.32 lm	34.25 kl	40.28 kl	26.51 jk	40.26 1l	50.28 i	51.47 lm	52.39 hi	52.76 kl	55.98 kl	58.91 l
	8	9.9 no	16.1 o	30.19 n	39.58 klm	20.26 n	38.31 m	47.37 jkl	48.32 op	48.18 k	49.84 mn	53.81 mn	57.85 lm
	9	7.98 p	13.28 p	26.12 p	36.98 no	16.45 o	36.48 n	40.32 op	45.31 r	44.28 no	47.31 o	50.39 o	54.72 n
	10	5.89 q	11.18 q	19.28 rs	34.58 pq	14.23 p	34.84 nop	39.41 pq	42.81 s	40.23 r	45.72 op	48.92 op	51.48 o
T. pseudokoningii IMI-392431	4	18.91 c	22.38 i	34.81 jk	39.82 klm	29.38 ghi	42.92 k	46.36 kl	49.48 nop	46.38 klm	51.49 lm	56.96 jk	56.51 m
	5	15.81 efgh	20.13 kl	29.16 no	36.12 op	26.19 jkl	40.34 l	45.68 lm	47.81 pq	44.81 mno	50.51 mn	54.48 lm	54.32 n
	6	12.83 jkl	19.36 lm	27.65 op	34.26 q	24.26 m	38.45 m	43.32 n	46.34 qr	42.39 pq	48.96 n	52.72 n	53.48 n
	7	10.77 mn	16.19 o	23.29 q	30.19 r	20.48 n	37.14 op	40.98 op	42.71 s	40.88 qr	46.68 o	50.98 o	51.62 o
	8	8.12 p	14.38 p	20.46 r	28.89 r	14.21 p	35.19 no	38.46 qr	40.28 t	38.26 s	44.54 p	48.45 pq	50.86 o
	9	7.04 pq	10.96 q	18.38 s	26.78 s	12.68 p	33.14 pq	36.92 r	38.98 tu	36.19 t	42.28 q	46.92 q	49.12 p
	10	4.02 r	8.16 r	14.98 t	24.63 t	8.95 q	31.98 q	34.45 s	37.48 u	35.35 b	39.81 r	44.84 r	47.24 q
T. harzianum IMI-392432	4	24.54 a	34.87 a	49.62 a	58.68 a	39.82 a	59.18 a	62.86 a	71.27 a	60.36 a	69.71 a	78.45 a	82.63 a
	5	18.41 cd	32.38 b	46.19 bc	56.16 b	35.96 bc	56.36 b	60.48 b	69.41 b	59.12ab	68.34 a	77.21 a	80.48 b
	6	16.21 efg	31.12 bc	44.26 d	52.86 c	32.46 e	54.82 b	57.82 cd	66.97 cd	56.28 cde	67.42 b	74.38 b	78.82 b
	7	15.68 efgh	26.29 ef	42.1 ef	50.31de	31.78 ef	51.96 cd	55.61 ef	64.58 e	54.75 ef	64.3 c	71.96 c	76.26 c
	8	14.56 ghij	23.17 hi	38.45 i	46.91 g	29.91 gh	50.45 def	51.92 h	62.29 f	52.64 hi	62.15 d	69.84 d	73.14 de
	9	13.82 ijk	21.51 ijk	35.18 jk	43.86 hi	26.37 ljk	48.92 fg	49.12 ij	58.81 gh	50.18 j	60.48 e	67.36 f	70.28 ef
	10	12.14 klm	18.31 mn	32.24 m	41.29 jk	24.83 klm	46.15 hi	47.82 jk	56.77 ij	57.16 k	58.36 fgh	65.48 g	69.28 fgh
T. harzianum IMI-392433	4	23.64 a	31.27 bc	47.63 b	53.92 c	36.74 b	52.92 c	60.31 b	68.42 bc	59.21 ab	66.58 b	74.38 b	79.86 b
	5	16.61 ef	30.19 cd	43.19 de	49.28 1ef	34.19 d	50.87 de	58.82 bc	66.37 d	57.63 abc	64.3 c	71.92 c	76.34 c
	6	14.9 fghi	28.67 d	40.32 gh	46.68 g	30.28 fg	49.94 ef	54.92 f	62.81 f	54.36 fg	62.85 cd	68.45 def	73.21 de
	7	14.1 hij	26.12 ef	38.98 hi	44.82 h	28.36 hi	47.37 gh	52.12 h	59.93 gh	52.81 gh	60.12 ef	64.98 g	70.81 fg
	8	13.56 ijk	24.37 gh	36.12 j	42.62 ij	27.81 ij	45.98 hij	49.81 i	58.18 hi	50.92 ij	58.93 efg	62.84 h	68.72 h
	9	12.8 jkl	18.32 mn	32.92 lm	41.23 jk	24.29 m	44.31 jk	46.37 kl	54.36 k	47.38 k	56.68 hi	61.92 hi	66.88 i
	10	11.32 lmn	16.72 no	28.26 o	38.95 lm	20.41 n	42.81 k	44.42 mn	51.42 lm	45.24 lmn	54.84 j	60.86 i	64.51 j
T. harzianum IMI-392434	4	21.85 b	31.32 bc	44.68 cd	51.13 d	34.82 cd	50.38 def	56.81 de	66.32 d	57.76 ab	64.37 c	69.37 de	76.91 c
	5	19.19 c	29.81 cd	41.26 fg	48.93 ef	32.39 e	49.86 ef	54.26 fg	63.92 ef	56.82 bc	62.56 d	67.82 ef	74.24 d
	6	16.94 de	26.17 ef	38.89 hi	44.68 h	28.46 hi	47.38 gh	52.88 gh	60.26 g	54.98 ef	59.92 ef	65.56 g	71.58 ef
	7	13.49 ijk	23.29 ghi	35.18 jk	42.88 ij	26.14 jkl	44.82 ij	50.12 i	54.83 k	52.39 hi	58.51 fg	62.46 g	69.93 fgh
	8	10.15 mn	20.49 jkl	33.92 klm	40.12 kl	24.48 lm	42.91 k	48.92 ij	52.39 l	48.14 k	55.86 ij	60.78 hi	68.81 h
	9	9.49 mn	16.23 o	29.16 no	38.19 mn	20.68 n	40.45 l	44.29 mn	50.46 mn	46.86 kl	54.45 jk	58.61 j	65.54 ij
	10	8.52 op	13.18 p	25.98 p	36.18 op	16.46 o	39.34 lm	41.28 o	49.73 mno	43.34 op	52.95 kl	57.92 j	61.58 k

In a column the same letters are not significantly different by DMRT at 5% level.

Table 3. Mean PIRG values by modified bilayer poison agar method using *Trichoderma* metabolites

Isolates of *Trichoderma*	No. of days	10-day-old metabolites				20-day-old metabolites				30-day-old metabolites			
		20%	40%	60%	80%	20%	40%	60%	80%	20%	40%	60%	80%
T. virens IMI-392430	4	26.98 bcd	26.98 a	42.96 fgh	51.34 cd	34.81 ef	49.36 lm	55.92 ghij	58.92 jkl	58.16 bc	60.87 ghij	62.21 h	66.13 l
	5	24.12 cde	24.12 def	41.48 hij	50.92 cde	32.96 gh	48.19 m	55.36 hijk	57.23 lm	57.34 cd	59.26 jkl	60.36 i	65.28 lm
	6	20.16 ghijk	22.18 ghi	38.31 kl	47.32 hij	30.18 i	46.32 n	54.28 jklm	56.31 mn	56.29 defg	57.58 lmn	59.33 i	64.36 mn
	7	18.34 ijkl	20.37 ijk	36.18 m	42.16 m	28.41 ij	44.21 pq	53.81 klm	54.86 no	55.34 efgh	56.19 n	58.87 ij	62.84 n
	8	16.92 klm	18.48 lm	32.26 o	40.36 n	26.34 k	42.48 qr	52.47 mn	53.92 op	52.28 j	52.28 o	57.26 j	60.31 o
	9	13.54mn	16.73 mn	28.18 q	38.58 n	22.19 m	39.21 st	51.34 n	52.49 p	50.17 kl	51.16 o	55.16 k	59.98 o
	10	12.26 no	14.89 op	22.34 t	36.19 o	18.93 n	37.12 u	49.48 op	49.27 qr	48.93 l	49.32 p	52.51 l	57.48 p
T. pseudokoningii IMI-392431	4	16.32 lm	20.92 ij	38.62 k	40.24 n	32.86 gh	43.26 pq	48.82 p	50.61 q	47.19 m	52.29 o	57.87 j	57.48 p
	5	14.24 mn	18.31 lm	36.16 m	38.81 n	28.98 i	42.48 qr	46.32 q	49.24 qr	45.45 n	51.73 o	55.28 k	56.98 p
	6	13.16 no	16.46 no	34.26 n	36.56 o	26.34 k	41.29 rs	44.94 q	47.84 r	43.81 o	50.98 o	54.12 k	55.16 q
	7	12.38 no	14.38 pq	31.83 o	32.91 p	22.18 m	40.14 st	42.58 r	45.26 s	42.26 op	48.94 p	52.22 l	54.16 q
	8	10.64 op	12.83 q	29.9 p	30.34 q	20.29 n	38.62 u	40.36 s	43.98 s	41.48 p	47.18 q	50.16 m	53.92 q
	9	8.32 p	10.36 r	26.18 q	28.81 q	18.86 n	37.21 u	38.51 t	40.14 t	40.67 pq	46.87 q	49.43 mn	51.18 r
	10	7.81 p	8.48 s	24.14 s	26.61 r	12.61 o	35.92 u	36.26 u	38.62 t	39.26 q	45.14 r	48.17 n	49.46 s
T. harzianum IMI-392432	4	30.46 a	28.28 a	51.39 a	60.14 a	40.41 a	60.62 a	63.52 a	72.16 a	61.54 a	70.47 a	79.63 a	84.68 a
	5	29.32 ab	26.46 ab	50.34 b	59.41 a	39.64 ab	59.31 ab	61.28 ab	70.84 ab	60.92 a	69.37 ab	78.41 a	83.26 ab
	6	28.16 ab	24.98 bcde	48.92 bc	56.68 b	36.52 de	58.72 bc	60.58 bcd	68.26 cd	58.38 bc	67.91 b	76.52 b	82.14 bc
	7	27.46 abc	22.96 fgh	46.42 d	52.91 c	33.98 fgh	57.26 cd	58.91 def	67.29 cd	57.14 cde	65.28 c	74.88 bc	80.38 de
	8	24.17 cdef	21.34 hij	42.91 fgh	50.84 cde	30.19 i	55.48 ef	57.63 efg	65.78 ef	56.82 cdef	62.29 fgh	72.29 d	79.23 e
	9	20.96 fghi	20.92 ij	40.48 ij	48.82 ghi	28.46 ij	54.91 efg	56.88 gh	63.65 g	55.21 fgh	60.48 hijk	70.16 ef	77.37 f
	10	18.84 hijkl	19.48 jkl	39.81 jk	46.48 j	26.82 jk	51.19 jk	54.94 ijk	60.18 hij	54.64 ghi	60.24 ijk	69.28 ef	76.64 fg
T. harzianum IMI-392433	4	27.31 abc	27.21 a	49.21 b	56.92 b	38.48 bc	56.36 de	62.26 bc	69.36 bc	59.91 ab	66.19 c	76.26 b	81.32 cd
	5	26.86 bc	26.14 abc	47.34 cd	54.28 bc	37.91 cd	54.59 fgh	60.39 cd	68.24 cd	58.63 bc	64.47 cde	75.89 bc	80.18 de
	6	23.28 defg	24.38 cdef	45.92 de	52.54 c	34.54 fg	54.48 fgh	59.12 de	67.28 de	58.48 bc	63.16 ef	74.43 c	79.28 e
	7	21.22 efghi	22.19 ghi	43.84 fg	49.46 efg	32.96 gh	53.28 ghi	56.64 ghi	65.49 ef	56.82 cdef	62.94 fg	72.22 d	77.54 f
	8	20.36 ghijk	20.54 ijk	42.45 gh	46.71 j	29.28 i	52.91 hi	54.93 ijk	61.26 hi	54.26 hi	60.87 ghij	70.19 ef	76.89 f
	9	18.45 ijkl	18.82 kl	39.92 jk	44.12 kl	26.46 k	50.14 kl	52.81 lmn	59.51 ijk	52.14 j	59.64 ijk	69.34 ef	75.12 gh
	10	17.93 ijkl	17.84 lmn	36.81 lm	42.38 m	24.28 l	48.26 m	51.28 n	58.28 kl	51.87 jk	58.86 klm	68.96 f	73.26 i
T. harzianum IMI-392434	4	26.38 bcd	26.45 ab	46.31 d	52.31 c	35.54 ef	52.71 ij	58.81 def	67.82 de	58.63 bc	64.97 cd	72.11 d	74.48 hi
	5	24.41cde	25.82 abcd	44.38 ef	50.42 def	34.31 fgh	50.56 kl	57.28 fg	66.46 def	56.21 defg	63.44 def	71.98 de	73.28 i
	6	22.16 efgh	23.29 efg	42.36 gh	48.91 fgh	32.49 h	48.34 m	56.12 fghi	64.94 fg	55.81 defgh	61.27 ghi	70.33 ef	71.17 j
	7	20.46 ghij	20.92 ij	41.92 hi	46.68 j	30.16 i	46.21 n	54.48 jkl	61.41 h	54.26 hi	60.18 ijk	69.64 ef	70.87 j
	8	18.38ijkl	18.45 lm	40.48 ij	44.94 k	28.82 i	45.98 no	52.68 lmn	60.28 hij	53.34 ij	59.88 ijk	67.26 g	69.97 j
	9	17.16 jklm	17.81 lmn	38.16 kl	42.71 lm	26.36 k	44.36 op	51.19 no	59.58 ijk	52.16 j	57.21 mn	67.14 g	67.88 k
	10	16.38 lm	16.38 no	35.31 mn	39.86 n	23.12 lm	44.12 pq	49.48 op	57.92 klm	50.26 kl	56.91 n	66.21 g	66.82 kl

In a column the same letters are not significantly different by DMRT at 5% level.

Table 4. Mean PIGW values in direct assay method using *Trichoderma* metabolites

Isolates of *Trichoderma*	10-day-old metabolites				20-day-old metabolites				30-day-old metabolites			
First method	20%	40%	60%	80%	20%	40%	60%	80%	20%	40%	60%	80%
T. virens, IMI-392430	58.28 d	62.12 c	66.86 c	69.58 c	60.92 d	64.96 c	68.74 c	72.54 c	63.91 d	66.57 d	69.19 c	74.86 c
T. pseudokoningii, IMI-392431	48.27 e	51.29 d	55.86 d	58.26 d	52.37 e	57.81 d	59.97 d	60.87 d	56.28 e	59.34 e	60.24 d	62.48 d
T. harzianum, IMI-392432	67.14 a	72.46 a	74.92 a	77.18 a	69.71 a	75.92 a	76.58 a	81.93 a	72.45 a	78.15 a	79.63 a	82.73 a
T. harzianum, IMI-392433	65.32 b	68.21 b	70.47 b	73.59 b	67.54 b	71.64 b	72.87 b	76.18 b	69.81 b	74.53 b	74.51 b	79.21 b
T. harzianum, IMI-392434	62.81 c	66.48 b	69.54 b	70.87 c	64.84 c	69.87 b	71.31 b	72.91 c	66.52 c	71.91 c	72.87 b	74.69 c
Second method												
T. virens, IMI-392430	55.18 d	58.92 c	61.18 d	63.91 c	57.19 d	58.96 c	59.18 c	63.29 c	59.54 d	62.24 c	63.68 c	68.31 d
T. pseudokoningii, IMI-392431	44.38 e	46.46 d	48.92 e	51.56 d	49.26 e	52.12 d	54.27 d	57.81 d	52.21 e	56.89 d	58.75 d	59.58 e
T. harzianum, IMI-392432	64.82 a	66.59 a	68.64 a	70.89 a	67.54 a	70.29 a	71.48 a	74.38 a	69.81 a	73.18 a	73.21 a	78.92 a
T. harzianum, IMI-392433	62.19 b	64.87 a	66.82 b	68.18 b	64.73 b	68.79 a	69.97 a	71.19 b	66.75 b	71.63 a	71.14 b	74.49 b
T. harzianum, IMI-392434	59.78 c	62.18 b	64.58 c	66.42 b	62.49 c	66.14 b	67.54 b	69.71 b	64.68 c	69.59 b	69.49 b	71.12 c

In a column the same letters are not significantly different by DMRT at 5% level.

zianum IMI-392432 (Table 3). The lowest PIRG values, 4.02 and 7.81%, were recored at 20% concentration on the 10th day of 10-day-old metabolites of *T. pseudokoningii* IMI-392431 using the normal poison agar and modified bilayer poison agar methods, respectively. The observed PIRG values of each strain were significant ($P \leq 0.05$) among different concentrations and different days of metabolites in both methods.

Direct assay of *Trichoderma* metabolites on growth of *C. paradoxa*. *Trichoderma* metabolites significantly ($P \leq 0.05$) inhibited mycelial growth of *C. paradoxa* at different concentrations and on different days. The highest percentage of inhibition of mycelial growth weight (PIGW) recorded were 82.73 and 78.92% with *T. harzianum* IMI-392432 at 80% metabolite concentration for the first and second method, respectively. The lowest PIGW values, 48.27 and 44.38%, were recorded at 20% metabolites on 10-day-old metabolites of *T. pseudokoningii* IMI-392431 by the first and second method, respectively (Table 4). It was also observed that PIGW values were statistically significant ($P \leq 0.05$) for each isolate of *Trichoderma* at different concentrations for both methods.

Discussion

Mycelial interaction is a basic method to assess antagonistic properties of microorganisms. These results revealed that all strains of *Trichoderma* antagonized *C. paradoxa* growth to various degrees and that different isolates within the same species also showed different degrees of inhibition. Jinantara (1995) reported that all nine isolates of *T. harzianum* possessed different abilities to attack *Sclerotium rofsii*, which was also in agreement with Henis *et al.* (1983) who found that different isolates of *T. harzianum* parasitized sclerotia of *S. rolfsii* with varying percentages of inhibition. Two comparative methods were used to test for variation in screening results in the placement of fungal inocula on discs. Results showed that although the percentage of inhibition values varied but the ranking of antagonisticity remained in the same order.

However, the first method is recommended for an accurate radius measurement of the test fungi within the dual culture plate because when test fungi are placed on the margin of plate it is easy to take measurements from margin towards the centre. Based on two criteria, the highest percentage inhibition of radial growth (PIRG values) and minimum colony overgrowth time, the *T. harzianum* IMI-392432 isolate was the best antagonist. Dharmaputra *et al.* (1994) tested two isolates of *T. harzianum* and one isolate of *T. viride* against *Ganoderma* and found that all isolates inhibited the mycelial growth of the pathogen, but *T. harzianum* (isolate B10-1) showed the best performance. Etabarian (2006) reported that *T. viridie* (MO) reduced the colony area of *Macrophomina phaseoli* by 19.2 and 34.9% using the dual culture and cellophane methods, respectively. Other than mycelial interaction and hyperparasitism by the *Trichoderma* species, scientists have also considered the action use of antibiotic metabolites as a contributing mechanism in the biocontrol of plant pathogens (Ghisalberti and Rowland, 1993). This study showed that secondary metabolites produced by *Trichoderma* strains were effective inhibitors of growth of *C. paradoxa*. The ability of *Trichoderma* species to produce inhibitory substances against microorganisms has been described by Dennis and Webster (1971) and Jinantara (1995). In this study, *T. harzianum* IMI-392432 displayed the best performance using the poison agar method at different concentrations of metabolites and on different days. To know whether the antibiotic action of secondary metabolites of *Trichoderma* were diffusible as well as antifungal, the modified bilayer agar experiment was carried out. The inhibition of radial growth of *C. paradoxa* was very pronounced compared to the growth of the uninoculated control bilayers. It is clear that the presence or absence of *Trichoderma* metabolites can have a significant role on the outcome of *C. paradoxa* mycelia. This experiment confirmed that the metabolites produced by *T. harzianum* are diffusible and can prevent, inhibit, or suppress the growth of *C. paradoxa* in culture. Therefore, *Trichoderma* has a large potential as a biocontrol agent against *C. paradoxa*. In previous studies, Schoeman *et al.* (1996) reported that metabolites of *T. harzianum* could influence the outcome of the decay caused by Basidiomycetes in freshly-felled pine. Eziashi *et al.* (2007) reported *T. polysporum* significantly reduced the growth of *C. paradoxa* followed by *T. viridie, T. hamatum* and *T. aureoviride.* The actual effect and mechanism involved is not known, but *Trichoderma* spp. are known to produce a range of metabolites that may affect the growth of microorganisms and plants (Ghisalberti and Rowland, 1993).

The antifungal properties of *Trichoderma* strains against *C. paradoxa* were confirmed where cultures filtrates of *Trichoderma* were used to control *C. paradoxa* in both experiments.The high PIGW value recorded at 80% metabolite concentration indicates that a high percentage of culture filtrate makes inhibition more effective. Eziashi *et al.* (2007) also reported that *C. paradoxa* was inhibited at high concentrations of 100% and 70% of metabolites by *T. polysporum* and *T. viride*, respectively. Based on the PIGW values, *T. harzianum* IMI-392432 showed the best inhibitory effect on C. *paradoxa* growth. Filtrates from *Trichoderma* species have been reported to exhibit antifungal activities (Calistru *et al*., 1997). Doi and Mori (1994) successfully found antifungal potential of culture filtrates of two *Trichoderma* species on wood decay fungi. Papavizas (1982) demonstrated that the culture filtrates of various *T. harzianum* strains suppressed growth of the

white rot pathogen, *Sclerotium cepivorum*. Our results that culture filtrates of *Trichoderma* inhibited mycelial growth of *C. paradoxa* were very similar to above findings. This result also suggests that, secondary metabolites of *T. harzianum* IMI-392432 produced antifungal compounds and such compounds may play an active role in the inhibitory effects on *C. paradoxa* colony growth.

Due to variable antagonistic potential of individual *Trichoderma* isolates, it is important that they be screened first to select for the most active antagonist against a particular pathogen thus a particular *Trichoderma* species can be considered as a biocontrol agent.

In vitro results obtained using different techniques suggest that *T. harzianum* IMI-392432 was the best at inhibiting the mycelial growth of *C. paradoxa*. So, it might be use as a potential biocontrol agent in future.

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