

## Antagonistic Effect of *Trichoderma* species against *Alternaria tenuis* a Fruit Rot Pathogen of Chili

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### ABSTRACT

Five native strains of *Trichoderma*, viz., *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433 and *T. harzianum* IMI-392434 were evaluated for antagonist potential against chili's fruit rot pathogen (*A. tenuis*) using dual culture, poison agar and direct assay methods. Two dual culture methods were applied and the highest percent inhibition of radial growth (PIRG) values occurred at  $60.81 \pm 0.85$  and  $77.59 \pm 2.14\%$  with *T. harzianum* IMI-392432 for first and second method, respectively. The minimum colony overgrowth time was recorded in *T. harzianum* IMI-392432 and maximum was exhibited in *T. pseudokoningii* IMI-392431. The PIRG values of *Trichoderma* strains against *A. tenuis* were significantly ( $P < 0.05$ ) varied at different concentrations of metabolites of different days. The highest PIRG values ( $84.64 \pm 1.25\%$ ) were achieved at 80% concentration on the 4<sup>th</sup> day, with 30-day-old metabolites of *T. harzianum* IMI-392432 in normal poison agar. But in modified bilayer poison agar, the highest PIRG values ( $85.9 \pm 0.44\%$ ) were recorded at the same concentration and the same 30-day-old-metabolites of *T. harzianum* IMI-392432. In direct assay method, maximum percentage of inhibition of mycelial growth weight (PIGW) was achieved at the same concentration and the same days old metabolites of *T. harzianum* IMI-392432. Present study showed that the *Trichoderma* has a good antagonistic effect on mycelial growth of *A. tenuis*, and *T. harzianum* IMI-392432 has the highest potential to be applied in order to control fruit rot pathogen of chili.

**Keywords:** *Trichoderma*, Secondary metabolites, PIRG, PIWG, *Alternaria tenuis*

### INTRODUCTION

Chili (*Capsicum annum* L.) is one of the most important spice crops of Bangladesh with average yield of 0.042 t/ha which is very low as compared to that of other chili-growing countries of the world (BBS, 2003). Fungal diseases play a vital role in reducing yield and among them; fruit rot is an important disease. Several species of *Trichoderma* have been extensively studied for their biological control effects against fungal plant pathogens (Ozbay and Newman, 2004). The genus is known to produce various secondary metabolites that have a

wide-spectrum of effects on various fungal groups (Islam et al., 2008). It has been commercially produced as a means of preventing the development of several soil pathogenic fungi. Strains of *T. harzianum* are marketed in a number of products; such as Plantshield7/ Root shield7 from the U.S., Trichodex7 from Israel, Binab T7 from Sweden and Supresivit7 from the Czech Republic. The antagonistic activity has often been associated with production of secondary metabolites (Silva et al., 2001). It was reported that the production of metabolites from different *Trichoderma* strains depends on ecological factors and the strains show varying effects on pathogens. Some of these metabolites have been isolated from sporulating or mycelial cultures but subcultivation decreased the production of the peptide antibiotics produced by *Trichoderma* isolates (Ghisalberti and Sivasithamparam, 1991). Different *Trichoderma* species showed different inhibitory results towards test fungi (Roiger and Jeffers, 1991). Research concerning the behavior of these fungi as antagonists demonstrated that they can act against target organisms in several ways (Chet, 1987). Isolates of *T. harzianum* can produce antifungal antibiotics (Ghisalberti and Rowland, 1993) and produce degradative lytic enzyme such as chitinase (Reino et al., 2008). Due to this variability, it is very important to select better isolates of *Trichoderma* as antagonist against particular pathogens. Therefore, the present investigation was aimed to evaluate the potentiality of *Trichoderma* isolates as a biological control agent against *A. tenuis*. The physical mode of antagonism and the effect of secondary metabolites produced by *Trichoderma* strains were also investigated.

## MATERIALS AND METHODS

### Sources of *Trichoderma*

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

### Isolation of *Alternaria tenuis*

*A. tenuis* was isolated from infected fruit parts of chili that were collected after recording the symptoms of the disease. Following standard phytopathological methods (Booth, 1971), the pathogen was isolated from the transitional zone of infected tissues and cultured on PDA medium. The pathogenicity of *A. tenuis* isolate was proved on local chili cultivars. All the cultures were stored at 4°C for further study.

### Screening by dual culture

Two methods were followed for the dual culture technique. In the first method, a mycelial plug (6 mm in diameter) was taken from a 4-day-old PDA culture plate of *Trichoderma* strain and placed at the periphery of the PDA plates (9 cm). Then, another mycelial plug of the same size of *A. tenuis* was similarly

placed at the periphery but on the opposing end of the same Petri dish. In the second method, a mycelial plug (6 mm) of the antagonist (*Trichoderma*) was placed 2 cm away from the periphery of the Petri dish and a plug of the same size of the test fungus (*A. tenuis*) was similarly placed 2 cm away from the edge of the Petri plate at the opposite site to *Trichoderma*. For the control, a mycelial plug of *A. tenuis* was placed alone in a similar manner on fresh PDA. All experiments were carried out in four replicates and incubated at 28°C. Antagonistic activity was assessed at 4 days after incubation by measuring the radius of the *A. tenuis* colony in the direction of the antagonist colony ( $R_2$ ) and the radius of the *A. tenuis* colony in the control plate ( $R_1$ ). The two readings were transformed into percent inhibition of radial growth (PIRG), using the formula of Skidmore and Dickinson (1976),

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

Observation was continued on the dual culture plates after 4 days incubation and followed by calculation of the PIRG. The number of days taken for the antagonist to overgrow the whole colony of *A. tenuis* was recorded.

#### **Screening by poison agar technique using crude metabolites**

**Preparation of culture filtrates of *Trichoderma*:** 200 ml of Richard's solution ( $\text{KNO}_3$ : 1.0g,  $\text{KH}_2\text{PO}_4$ :0.5g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ : 0.25g, glucose: 34g, trace amounts of  $\text{FeCl}_3$  in 1L distilled water, pH6.5) was prepared and poured into 500 ml conical flasks and autoclaved for 15 min at 121°C/1.05kg/cm<sup>2</sup> pressure. Six mycelial plugs (6 mm in diam.) of each strain were inoculated into each flask (with media) with four replications. The flasks were incubated on a orbital incubator (Gallenkamp) at 100 rpm at 28°C (Dennis and Webster, 1971). The culture filtrates were collected after 10, 20 and 30 days incubation. These were then concentrated to about 50%, using a vacuum evaporator at 38-40°C and finally filtered by sterilized membrane filter.

**Preparation of poison agar plate:** Firstly, 20, 40, 60 and 80% PDA media were prepared, and taken per bottle with four replications and sterilized by autoclaving at 121°C/1.05kg/cm<sup>2</sup> pressure for 15 minutes. Then, the sterilized metabolites were incorporated with this PDA media at the concentrations of 20, 40, 60 and 80%( v/v). The molten PDA at different concentrations of metabolites were poured onto the Petri plates and allowed to solidify. For control, only Richard's solution was used and incorporated with PDA in the same concentrations as that for *Trichoderma* metabolites.

**Screening technique:** For the normal poison agar method, on seven-day-old mycelial plug (6 mm) of *A. tenuis* was inoculated at the centre of each of previously-prepared poison agar plate and incubated at 28°C for 10 days. In the modified bilayer poison agar method, 7 days old mycelial plug (6 mm) of *A. tenuis* was inoculated on the centre of a normal PDA plate for 4 days. After that,

a second layer of molten PDA was incorporated with ascending concentrations of sterilized metabolites of *Trichoderma* was poured over the *A. tenuis* colony. In the control, a second layer of molten PDA was incorporated with sterilized Richard's solution instead of *Trichoderma* metabolites was used instead. Observation was made on radial extension of the mycelia on the culture plate for both treatments and control. Data were recorded on the mycelial extension of colony diameter after 4 to 10 days inoculation. The readings were calculated for PIRG, based on the Skidmore and Dickinson (1976) formula.

### Direct assay of *Trichoderma* metabolites

Two techniques were followed to assess the inhibition of mycelial growth of *A. tenuis*. In the first technique, the potato dextrose broth (PDB) was prepared at concentrations of 20, 40, 60 and 80 % with four replications of each. Previously-prepared sterilized *Trichoderma* metabolites with different concentrations were incorporated proportionally into each conical flask. Then 7-day-old *A. tenuis* mycelial plugs were placed in each flask and incubated at 28°C for 7 days. For the control, the same concentrations of Richard's solution without the *Trichoderma* culture filtrates were incorporated into the PDB. In the second technique, *A. tenuis* was cultured in different concentrations of PDB described previously. On the 7<sup>th</sup> day of culture, *Trichoderma* filtrates at 20, 40, 60 and 80 % (v/v) concentrations were incorporated reciprocally into particular *A. tenuis* culture and incubated for another 7 days. For the control, Richard's solution without *Trichoderma* metabolites was incorporated proportionally as earlier described. After that, *A. tenuis* mycelia were harvested from the flask, and washed gently with distilled water and oven-dried at 60°C until the weight was constant. The mean mycelial weight of the treatments was compared to the dry mean weight of *A. tenuis* mycelia from the control flask. Data on mycelial weight for each concentration in treatment and control flasks were recorded. The differences between the two readings multiplied by 100 were taken as the percentage inhibition of mycelial growth weight (PIGW), following the modified method of Skidmore and Dickinson (1976).

$$\text{PIGW} = \frac{A_1 - A_2}{A_1} \times 100, \text{ where } A_1 = \text{mycelial weight of } A. \text{tenuis in control}$$

flasks and  $A_2$  = mycelial weight of *A. tenuis* mycelia in treatment flasks.

## RESULTS

### Screening by dual culture

*Trichoderma* isolates inhibited the radial mycelial growth of *A. tenuis*. The PIRG values ranged from 37.99±0.64 to 60.81±0.85% for the first method and 53.06±1.76 to 77.59±2.14% for the second method, respectively (Table 1). The highest PIRG values recorded were 60.81±0.85 and 77.59±2.14% in *T. harzianum* IMI-392432 and the lowest recorded were 37.99±0.64 and 53.06±1.76% in *T. pseudokoningii* IMI-392431 for first and second method, respectively (Table 1 and Fig 1). In both methods, the highest PIRG values were recorded in

*T. harzianum* IMI-392432, which was significantly ( $P=0.05$ ) different from other strains. *A. tenuis* colony overgrowth times for all *Trichoderma* strains varied from 8 to 13 days for first method and 7 to 12 days for second method (Table 1). The minimum colony overgrowth time was recorded in *T. harzianum* IMI-392432 and the maximum colony overgrowth time was recorded in *T. pseudokoningii* IMI-392431 for both methods.

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

Methods	<i>Trichoderma</i> isolates	Mean % Inhibition of Radial growth (PIRG)	No. of days to overgrowth of <i>A. tenuis</i> colony
Method-1	<i>T. virens</i> , IMI-392430	42.08±0.61 cd	12
	<i>T. pseudokoningii</i> , IMI-392431	37.99±0.64 d	13
	<i>T. harzianum</i> , IMI-392432	60.81±0.85 a	8
	<i>T. harzianum</i> , IMI-392433	54.89±0.46 ab	10
	<i>T. harzianum</i> , IMI-392434	49.10±0.62 bc	10
Method-2	<i>T. virens</i> , IMI-392430	59.99±0.76 c	11
	<i>T. pseudokoningii</i> , IMI-392431	53.06±1.76 d	12
	<i>T. harzianum</i> , IMI-392432	77.59±2.14a	7
	<i>T. harzianum</i> , IMI-392433	72.06±1.04 ab	10
	<i>T. harzianum</i> , IMI-392434	69.71±1.28 b	10

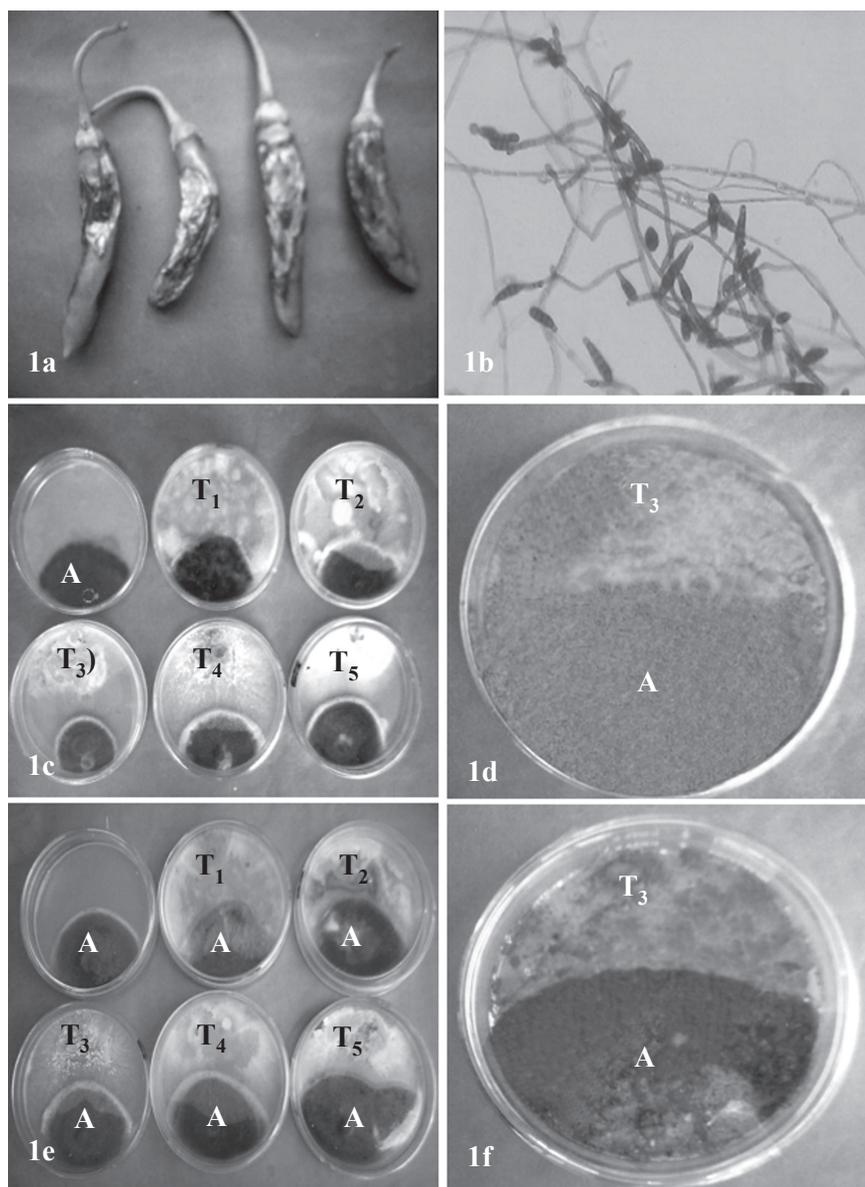
In a column, same letters are not significantly different by DMRT at 5% level.

### Screening by poison agar technique using crude metabolites

The PIRG values of *Trichoderma* strains against *A. tenuis* were significantly ( $P=0.05$ ) varied at different concentrations of metabolites. The highest PIRG values (84.64±1.25%) were achieved at 80% concentration on the 4<sup>th</sup> day with 30-day-old metabolites of *T. harzianum* IMI-392432 in normal poison agar (Table 2). But in modified bilayer poison agar, the highest PIRG values (85.9±0.44%) were recorded at the same concentration and same day-old metabolites of *T. harzianum* IMI-392432 (Table 3). The lowest PIRG values were recorded at 20% concentration on the 10<sup>th</sup> day with 10-day-old metabolites of *T. pseudokoningii* IMI-392431 in both methods. From statistical analysis, it was observed that the PIRG values of each strain were significantly ( $P=0.05$ ) different at different concentrations and different days of metabolites.

### Direct assay of *Trichoderma* metabolites

*Trichoderma* metabolites inhibited mycelial growth of *A. tenuis* significantly ( $P=0.05$ ) at different concentrations of different day-old metabolites. The highest PIGW was recorded at 84.63±0.65 and 75.78±1.18% in *T. harzianum* IMI-392432 at 80% metabolite concentration for first and second method, respectively (Table 4). The lowest PIGW values were recorded at 20% metabolite concentrations on 10-day-old metabolites of *T. pseudokoningii* IMI-392431.



**Figure 1.** 1a & 1b. Photographs show fruit rot symptom of chili and pathogen *A. tenuis*.

1c & 1e. Antagonistic effect of *Trichoderma* isolates against *A. tenuis* in dual culture (method-I & method-II).

1d & 1f. Overgrowth of *Trichoderma* cover the *A. tenuis* colony after 7 days and 6 days of inoculation in dual culture (Method-I and Method-II).

A, T<sub>1</sub>, T<sub>2</sub>, T<sub>3</sub>, T<sub>4</sub>, and T<sub>5</sub> indicate *A. tenuis*, *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431,

*T. harzianum* IMI-392432, *T. harzianum* IMI-392433 and *T. harzianum* IMI-392434, respectively.

**Table 2.** Mean PIRG values of *A. tenuis* by normal poison agar using *Trichoderma* metabolites.

<i>Trichoderma</i> isolates	No of days	10-day-old metabolites			20-day-old metabolites			30-day-old metabolites			80%	60%	80%
		20%	40%	60%	80%	20%	40%	60%	80%	20%			
<i>T. vires</i> IMI-392430	4	20.35±0.89 ef	24.98±1.18 f	40.00±1.13 ab	48.78±0.82 bc	32.51±1.09 d	49.72±0.53 cd	56.84±0.45 g	59.48±0.34 i	58.87±0.43 a	59.71±0.47 hi	62.66±0.37 k	64.02±0.74 lm
	5	18.32±0.96 ghi	22.77±0.88 hi	38.57±1.01 bc	47.73±0.85 cd	29.39±0.65 fg	44.49±0.73 h	52.25±0.91 ij	56.64±0.63 i	53.33±0.69 e	57.12±0.74 j	59.46±0.45 l	59.82±1.14 n
	6	13.05±0.75 mn	21.42±0.25 ij	37.14±0.75 cde	45.56±0.96 e	25.06±1.11 kl	40.95±0.73 i	47.97±0.97 l	53.51±0.71 kl	48.29±0.84 f	43.29±0.87 f	52.86±0.64 l	55.15±1.31 o
	7	11.86±0.72 no	18.23±0.43 k	35.45±0.81 ef	42.8±0.22h i	23.38±1.31 lm	36.88±0.73 o	43.04±1.43 mm	48.87±0.95 nn	43.68±0.97 hij	49.66±1.29 m	50.8±1.41 no	52.31±1.56 pq
	8	10.57±0.35 op	13.25±0.42 n	31.85±0.69 h	38.38±0.51 lm	22.34±1.08 lm	33.97±0.41 p	35.73±0.59 p	46.40±1.12 p	36.88±0.94 m	43.68±0.97 hij	46.53±1.53 n	49.67±0.35 op
	9	8.58±0.35 qr	10.31±0.53 op	23.93±0.73 kl	25.47±0.45 s	19.49±0.38 o	30.88±0.43 q	34.36±0.56 p	41.52±0.59 r	34.47±1.11 n	44.69±1.57 o	46.46±0.33 q	48.98±1.38 st
	10	6.82±0.32 s	8.90±0.54 p	15.18±0.82 n	20.24±0.49 u	12.77±0.45 q	25.46±0.67 s	30.64±0.48 q	36.53±0.86 t	31.25±1.14 o	42.49±1.26 p	45.38±0.63 q	47.48±1.79 t
	4	16.37±0.85 jk	18.67±0.91 k	32.83±1.07 gh	36.70±0.97 mn	28.14±0.69 gh	38.36±1.15 mm	47.41±0.44 l	50.78±0.68 m	45.46±1.05 gh	50.48±0.65 m	51.58±1.01 n	53.28±0.92 p
	5	13.06±0.69 mn	14.79±0.72 mn	31.02±1.24 h	33.69±1.37 pq	25.61±0.61 ijk	36.66±1.07 o	44.56±0.37 m	47.34±0.61 op	42.59±1.15 j	46.23±0.52 no	48.79±0.58 p	50.96±0.93 qr
	6	11.26±0.82 no	13.42±0.41 n	28.08±1.27 ij	32.18±1.18 q	22.78±1.02 mm	34.52±0.83 p	42.12±0.71 ln	44.42±0.85 q	40.33±0.87 l	45.02±0.13 no	46.94±0.59 q	48.58±0.91 st
<i>T. pseudokoningii</i> IMI-392431	7	9.31±0.65 pq	11.44±0.51 o	24.36±0.43 kl	29.91±1.48 r	15.54±0.49 p	32.04±0.69 q	38.34±0.85 o	41.01±1.43 r	38.5±1.12 m	42.57±0.34 p	45.76±0.54 q	45.75±1.61 u
	8	6.99±0.76 rs	8.88±0.71 p	20.61±1.71 m	26.38±0.99 s	12.89±1.09 q	28.66±0.67 r	34.41±0.97 p	39.08±1.33 s	33.28±1.35 n	39.82±0.52 q	41.39±0.45 r	43.81±1.45 v
	9	4.52±0.37 t	7.06±0.81 q	16.44±0.95 n	23.45±0.37 t	10.38±0.93 r	25.27±1.21 s	29.94±1.48 q	35.58±1.52 t	30.02±1.24 o	38.10±0.37 r	39.76±0.53 s	40.27±0.97 w
	10	2.20±0.35 u	4.81±0.85 r	13.21±0.77 o	18.54±1.04 v	7.37±0.43 s	21.70±2.03 t	24.69±1.14 r	28.58±0.69 u	23.31±1.23 p	33.11±1.23 s	37.01±0.19 t	38.55±1.06 x
	4	27.73±0.36 a	32.52±0.84 a	40.58±0.58 a	51.24±0.71 a	37.05±0.72 a	53.1±0.64 a	66.40±1.53 a	70.76±0.91 a	58.34±1.11 ab	73.51±1.06 a	80.67±0.72 a	84.64±1.13 a
	5	24.87±0.24 b	29.79±1.53 c	36.66±0.79 cd	49.19±0.81 b	34.71±0.46 bc	50.92±0.67 b	64.39±1.45 b	68.14±1.28 b	56.09±1.20 cd	71.13±1.57 b	79.08±0.72 a	82.16±0.83 b
	6	22.13±0.79 cd	27.34±1.57 de	34.53±0.69 fg	46.69±0.68 de	32.38±0.73 d	49.06±0.75 cd	62.84±1.85 b	65.84±1.61 d	53.06±0.91 e	68.05±1.35 de	76.85±0.69 b	78.91±0.68 c
	7	19.83±0.63 fgh	24.74±0.67 fg	31.55±0.54 h	45.06±0.53 efg	30.64±0.53 ef	46.46±0.26 fg	60.88±1.48 c	63.66±1.39 ef	48.72±1.08 f	66.36±1.31 ef	74.8±0.58 d	76.21±0.93 d
	8	18.77±0.58 fgh	21.45±0.63 j	27.62±0.58 ij	42.89±0.44 hi	27.81±0.39 gh	44.12±1.07 h	59.37±1.34 cd	61.65±1.51 gh	47.15±1.07 fg	64.23±1.46 g	72.64±0.98 d	72.28±1.24 f
	9	16.70±0.46 ijk	18.14±0.85 k	25.03±0.52 k	39.67±0.34 kl	25.73±0.39 ijk	40.58±0.42 kl	57.58±1.13 fg	59.57±1.58 i	45.02±0.91 hi	61.22±1.35 h	70.26±0.99 f	70.49±1.14 g
<i>T. harzianum</i> IMI-392432	10	15.79±0.56 kl	16.27±0.46 lm	22.79±0.87 l	36.76±0.46 mm	23.05±0.83 mm	39.86±0.55 lm	54.19±1.05 h	56.99±1.79 i	43.46±1.01 i	59.11±1.68 i	68.13±1.27 gh	68.23±0.87 hij
	4	25.33±0.47 b	30.51±0.78 b	36.94±0.45 cde	45.85±0.68 def	35.73±0.61 ab	50.71±0.57 bc	59.08±0.32 def	67.21±0.76 c	57.63±0.48 abc	71.47±1.48 b	76.72±0.33 b	77.91±1.14 c
	5	24.39±0.44 b	28.49±1.09 d	36.12±0.71 def	44.62±0.69 fg	33.34±0.72 cd	48.56±0.59 de	56.95±0.41 g	65.22±0.79 de	55.88±0.49 cd	68.94±1.73 cd	74.77±0.67 c	75.29±1.25 de
	6	22.31±0.88 bc	25.95±0.73 f	34.52±0.62 fg	43.69±0.36 gh	30.28±0.81 ef	46.71±0.38 fh	54.68±0.61 h	62.87±1.13 fg	53.02±0.82 e	65.86±2.12 f	72.04±0.81 de	73.23±1.26 f
	7	19.97±0.65 efg	24.31±0.71 fg	32.06±0.63 h	41.78±0.51 ij	27.21±0.59 hi	43.72±0.96 hi	53.73±0.56 hi	60.4±0.46 hi	48.31±1.67 f	63.71±2.22 g	69.17±0.28 fg	69.11±2.85 ghi
	8	16.87±0.43 ijk	21.82±0.62 j	27.38±0.71 ij	40.34±0.42 jk	25.32±0.82 jk	41.81±1.25 jk	52.15±0.73 i	56.93±0.73 i	45.48±0.62 gh	60.25±2.31 hi	67.24±0.81 hi	67.65±2.93 ij
	9	15.10±0.19 kl	17.91±0.26 kl	23.38±0.69 l	39.31±0.25 kl	23.53±1.31 mm	39.64±1.31 mm	50.96±0.66 jk	52.82±0.72 l	43.4±1.99 ijk	56.79±2.89 j	65.5±0.98 ij	66.53±2.84 jk
	10	12.69±0.21 mn	15.02±0.25 m	20.51±0.25 m	34.77±0.86 op	22.37±1.46 mm	38.32±1.24 mm	49.63±0.72 k	51.76±0.68 lm	42.21±1.81 jk	55.08±2.42 k	64.91±0.85 j	65.62±0.89 kl
	4	24.79±0.41 b	28.89±1.03 cd	35.10±0.62 f	44.84±0.74 fg	34.35±0.44 bc	48.87±0.87 d	64.38±0.74 b	65.27±1.46 d	56.77±0.72 bcd	69.82±0.42 bc	73.69±1.23 cd	75.14±1.21 de
	5	23.79±0.42 bc	27.35±0.73 de	32.15±0.76 h	42.48±1.13 hi	31.90±0.62 ef	47.09±0.62 ef	62.83±0.91 b	63.54±1.35 ef	55.67±1.07 d	67.07±0.74 ef	70.72±0.91 ef	73.73±1.42 ef
<i>T. harzianum</i> IMI-392434	6	21.65±0.48 de	25.69±0.61 ef	29.17±0.83 i	39.55±0.56 kl	29.52±1.05 fg	45.18±0.78 gh	60.36±0.39 cd	60.81±0.89 hi	53.78±1.57 e	63.70±0.52 g	68.09±1.07 gh	72.74±1.35 f
	7	19.99±0.45 efg	23.12±1.07 gh	27.88±0.41 ij	38.33±0.39 lm	27.92±0.77 gh	42.37±0.85 ij	58.56±0.43 efg	59.02±0.88 i	47.68±0.36 f	60.13±0.34 hi	65.57±1.38 ij	69.83±0.48 gh
	8	18.09±0.22 hij	20.52±0.99 j	27.23±0.45 j	37.18±0.54 mm	27.08±0.75 hij	38.81±0.92 mn	54.36±0.54 h	54.67±1.13 k	44.82±0.51 hi	57.35±0.59 j	62.64±0.93 k	67.69±0.39 ij
	9	14.23±0.14 lm	17.42±1.01 kl	23.21±0.45 l	36.05±0.79 no	23.18±0.85 mm	37.50±1.0 no	50.09±0.82 k	51.76±0.62 lm	43.76±0.62 hij	54.73±0.36 k	60.33±0.69 l	64.59±0.52 lm
	10	11.39±0.43 no	13.88±0.45 n	19.54±0.36 m	32.73±0.86 q	21.43±0.29 n	37.04±0.46 no	46.48±0.78 l	50.19±0.75 mm	41.65±0.52 kl	53.61±0.51 kl	59.64±0.45 l	62.91±0.62 m

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

**Table 3. Mean PIRG values of *A. tenuis* by modified bilayer poison agar using *Trichoderma* metabolites.**

No. of days	10-days-old metabolites				20-days- old metabolites				30-days-old metabolites					
	20%	40%	60%	80%	20%	40%	60%	80%	20%	40%	60%	80%		
<i>Trichoderma</i> isolates	4	20.67±0.96cde	24.54±0.83n	43.35±0.85fg	51.15±1.11ghi	37.33±1.03jkl	55.18±1.77jk	60.96±0.83k	65.38±1.29kl	57.42±1.73kl	61.69±0.79l	65.64±0.63o	67.46±0.44n	
	5	18.58±0.74efghi	23.40±0.85no	40.78±1.01ij	47.52±1.19lm	34.61±0.88l	51.96±1.44l	58.27±0.86k	62.25±1.03l	56.09±1.89l	58.01±0.62p	62.58±0.71p	65.49±0.35o	
	6	17.63±0.97defghij	21.91±0.98o	38.51±0.82k	45.27±1.33n	31.18±1.15m	49.03±1.19m	55.17±0.55m	59.99±0.8m	54.17±2.04m	54.69±0.69qr	59.23±0.84q	62.57±0.89p	
	7	14.45±1.02 ghijkl	19.90±1.06p	36.01±1.03l	42.01±1.71o	29.07±0.71o	46.19±1.14n	51.51±0.84n	56.85±1.31p	51.09±1.81n	53.48±0.58rs	55.06±0.93r	59.36±0.45q	
	8	12.79±0.89ijklm	17.88±1.02q	33.13±1.17n	38.63±1.39p	26.57±0.44o	43.16±1.53o	48.73±1.21o	54.01±1.59r	47.80±2.04o	51.96±0.66st	53.31±1.03s	56.06±0.76rs	
	9	11.86±1.03klm	15.87±0.84r	30.47±1.14m	35.35±1.29qr	23.76±0.64pq	40.19±1.75p	45.19±0.87p	51.51±1.16s	44.19±1.79p	49.42±0.45u	51.16±0.47u	53.61±0.58t	
	10	10.49±0.99klmn	14.03±0.87st	26.21±0.92p	30.63±1.08s	21.37±0.43r	36.69±2.13q	41.68±0.54q	49.34±1.29t	40.41±0.88q	46.21±0.83v	49.45±0.62u	51.58±0.52u	
	<i>T. vires</i> IMI-392430	4	18.34±0.96defghi	22.87±0.82no	35.80±1.56l	38.92±0.73p	30.23±0.93mnn	45.73±1.43n	52.03±0.81n	55.54±1.42pq	50.48±1.11n	56.29±0.91lq	59.79±0.39q	62.01±1.18p
		5	16.32±0.92fghijk	20.18±0.76p	33.74±1.51m	36.71±0.72q	25.98±0.92o	42.67±1.07o	48.93±0.69o	52.89±1.32rs	47.47±0.34o	51.56±0.77t	55.49±1.07t	59.55±1.29q
		6	14.01±0.49hijkl	17.94±0.73q	30.96±1.41m	34.12±1.76r	24.95±0.84op	38.81±0.88p	46.49±0.66p	49.70±1.36t	43.22±0.62p	49.31±0.43u	49.44±0.77u	54.69±1.91st
7		11.47±0.56klmn	15.52±0.57rs	28.44±1.86o	30.97±1.39s	22.25±0.89qr	34.54±0.71r	41.89±0.75q	46.52±1.22u	39.99±0.78qr	47.37±0.44v	49.44±0.77u	54.69±1.91st	
8		9.53±0.37mnn	12.63±0.75t	25.64±1.17p	29.75±0.53s	18.24±0.94s	30.97±0.96s	38.88±0.65r	43.53±1.81v	38.59±0.85r	44.26±0.44v	46.91±0.56v	50.90±1.42u	
9		7.51±0.61 mnn	10.69±0.97u	20.55±1.65q	22.81±0.92t	15.35±0.71t	27.47±0.38t	34.21±0.46s	41.05±1.41x	32.98±0.94s	42.47±0.89x	44.67±0.95w	48.51±2.07v	
10		5.35±0.47n	7.76±0.39v	14.35±0.93r	18.78±0.58u	10.21±0.47u	23.93±1.53u	29.43±0.23t	34.77±0.92x	24.67±1.01t	38.52±1.09y	40.97±1.31x	44.73±1.99w	
<i>T. pseudokoningii</i>		4	28.82±0.72a	40.31±0.62a	53.09±1.22a	59.76±0.59a	49.72±0.35a	67.03±0.84a	73.72±0.69a	77.04±0.84a	71.91±0.77a	82.39±0.88a	82.65±0.54a	85.94±0.44a
		5	27.06±0.59ab	38.98±0.64ab	50.78±1.03b	58.36±1.14ab	48.67±0.37ab	65.67±0.49ab	72.30±1.03ab	75.64±0.34b	71.36±0.85a	81.19±0.69ab	81.58±0.36ab	83.86±0.47bc
		6	25.15±0.51abc	37.47±0.59bc	48.45±0.99c	57.33±1.41bc	47.32±0.65bcd	64.71±0.59bc	71.03±1.13bc	74.82±0.55bc	69.57±0.54bc	79.79±0.52bcd	82.49±0.73cde	83.86±0.47bc
	7	22.69±0.36abcde	36.12±0.73cde	47.05±1.89cd	56.39±1.48cd	46.30±0.65cde	63.79±0.51cde	69.49±1.16cd	73.46±0.65cd	68.50±0.62cd	78.03±1.26de	77.64±1.03efg	80.84±0.89ef	
	8	21.56±0.37bcdef	35.17±0.52efg	46.02±0.78de	55.37±1.49de	45.36±0.64def	62.32±0.82ef	68.14±1.06de	72.36±0.43def	67.13±0.39def	76.36±1.09efg	76.24±0.77ghi	79.04±0.64gh	
	9	20.23±0.72cde fgh	34.07±0.48fgh	45.11±0.79ef	53.67±1.94ef	44.23±0.71f	60.81±0.88fg	66.58±0.69ef	70.69±0.56fgh	66.37±0.41ef	75.41±0.83gh	74.51±0.69ij	77.16±0.54jkl	
	10	18.56±1.01defghij	32.51±0.71hij	43.81±0.62fg	52.43±1.33fgh	41.69±0.65gh	59.69±0.73gh	65.69±0.35f	69.09±0.32hij	68.89±0.45cd	73.86±1.29hi	73.08±1.13jk	75.48±1.01k	
	<i>T. harzianum</i> IMI-392432	4	27.16±1.13ab	39.43±0.45a	52.67±0.89a	56.71±0.79bcd	48.41±0.25abc	64.09±0.74bcd	70.99±0.86bc	75.71±0.95ab	70.85±0.43ab	80.39±0.55bc	80.16±0.55bc	84.39±1.43ab
		5	25.12±0.56abc	35.57±0.6def	50.48±0.76b	55.32±1.08de	46.29±0.14de	62.12±0.79ef	69.51±0.41cd	72.74±0.94de	68.64±0.87cd	77.69±1.18def	78.22±1.21def	83.24±1.76bcd
		6	23.55±0.56abcd	33.59±0.28gh	48.36±1.05c	52.91±1.18fg	44.26±0.32 f	60.27±0.79g	68.08±0.32de	71.21±0.86efg	67.87±0.91cde	75.45±1.73gh	76.76±1.18fgh	82.79±1.22bcd
7		21.28±0.77bcdef	32.46±0.45hij	44.39±1.44ef	51.59±0.99gh	42.39±0.87gh	59.35±0.67gh	65.88±0.41f	70.52±1.03ghi	66.62±0.88ef	73.69±1.26hi	74.93±1.16hij	79.58±1.12fgh	
8		19.74±0.66cde fgh	31.69±0.51jkl	42.62±1.13gh	50.84±1.01hij	40.26±0.47hi	57.93±0.51hi	64.03±0.14g	69.07±0.72hij	65.39±1.11fg	72.68±1.31ij	73.26±1.32jk	77.69±1.18hij	
9		16.70±0.84efghijk	30.47±0.31k	41.07±1.39hij	49.15±0.83jkl	38.76±0.48ij	56.26±0.53ij	62.71±0.59gh	67.39±0.63j	64.45±1.06gh	71.55±1.49jk	71.85±1.21kl	76.35±1.25jk	
10		15.71±0.82fghijkl	28.61±0.49l	38.49±0.67k	48.07±0.72l	37.22±0.37kl	54.92±0.52kl	61.72±0.58hi	64.68±0.33k	63.11±1.25hi	69.78±1.69lm	70.42±1.51lm	73.70±0.13l	
<i>T. harzianum</i> IMI-392433		4	25.68±0.35abc	37.22±0.55cd	50.62±0.27b	52.90±1.05fg	46.82±0.49cde	62.87±0.95de	68.82±0.55d	71.39±1.05efg	65.32±0.94fg	76.19±0.96fg	79.44±0.39cde	81.57±0.58de
		5	24.91±0.46abc	35.20±0.81efg	48.32±0.56c	51.49±1.27ghi	44.45±0.62f	60.04±1.01g	66.41±0.38ef	68.82±1.21ij	62.83±1.29hi	74.99±0.94gh	77.71±0.81efg	78.78±0.71ghi
		6	23.82±0.51abcd	32.89±1.05hi	46.85±0.14cd	50.56±1.24ijk	42.53±0.32g	57.04±0.99j	63.38±0.52gh	65.63±1.22k	61.54±1.43j	72.33±1.35j	75.49±0.61hi	76.46±0.98ijk
	7	21.89±0.76bcdef	30.97±1.09jkl	44.54±0.51ef	48.91±1.16kl	40.62±0.56h	54.99±0.52kl	60.26±0.32j	64.56±1.14k	60.39±1.44j	70.45±1.19kl	73.62±0.65jk	72.36±1.22l	
	8	18.31±1.29defghi	28.73±0.94l	42.06±0.56gh	47.61±0.81lm	38.65±0.39k	54.16±0.76k	58.73±0.51jkl	62.20±0.67l	58.12±1.66k	68.23±1.35m	70.79±0.61l	69.85±0.63m	
	9	15.72±1.35fghijkl	27.78±0.79lm	40.18±0.67kl	46.00±1.01mn	36.56±0.64j	52.24±0.37l	57.09±0.49kl	59.55±0.51lm	56.74±1.51kl	66.03±1.57n	68.94±0.82mn	69.11±0.64mn	
	10	14.89±1.25fghijkl	26.26±0.78m	36.56±0.79l	45.48±1.05n	33.85±0.95l	50.71±0.52l	55.62±0.55lm	58.22±0.17no	56.36±1.52kl	63.23±2.03o	67.47±0.45n	67.40±0.45n	

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

**Table 4.** Mean PIGW values of *A. tenuis* using culture filtrates of *Trichoderma*.

<i>Trichoderma</i> isolates	10-day-old metabolites			20-day-old metabolites			30-day-old metabolites					
	20%	40%	60%	80%	20%	40%	60%	80%	20%	40%	60%	80%
<i>T. virens</i> , IMI-392430	51.21±0.42 c	53.63±0.21 c	55.99±0.33 d	61.12±0.76 d	54.87±0.56 c	57.93±0.17 d	60.70±0.23 c	66.37±0.76 d	57.07±0.58 d	61.03±0.17 c	64.29±0.31 d	69.83±0.41 d
<i>T. pseudokoningii</i> IMI-392431	44.58±1.27 d	47.12±2.06 d	51.57±0.54 e	56.08±1.29 e	48.13±1.43 d	51.76±0.41 e	56.53±0.34 d	59.57±0.34 e	51.41±1.17 e	56.07±0.51 d	59.58±0.25 e	63.51±0.25 e
<i>T. harzianum</i> IMI-392432	65.51±1.68 ab	71.22±0.46 a	75.15±0.55 a	78.22±0.36 a	71.06±0.97 a	75.06±0.74 a	77.88±0.34 a	82.78±0.43 a	74.34±0.73 a	77.50±0.85 a	80.14±0.31 a	84.63±0.65 a
<i>T. harzianum</i> IMI-392433	67.14±0.53 a	69.39±0.38 b	72.35±0.38 b	74.19±0.45 b	70.36±0.52 a	72.97±0.38 b	75.63±0.38 b	78.41±0.45 b	72.42±0.35 b	76.30±0.58 a	77.91±0.69 b	79.66±0.34 b
<i>T. harzianum</i> IMI-392434	64.29±1.04 b	68.01±0.64 b	69.31±0.49 c	71.09±0.55 c	67.55±1.01 b	70.02±0.64 c	73.89±0.28 b	76.56±0.34 c	69.21±0.75 c	73.19±1.16 b	74.80±1.01 c	77.48±0.81 c
<b>Second method</b>												
<i>T. virens</i> , IMI-392430	50.26±0.58 c	54.43±0.79 c	55.24±1.13 d	57.93±1.07 c	52.15±0.44 d	53.65±0.56 c	54.97±0.77 c	56.12±0.51 c	53.69±0.66 d	55.21±0.53 d	56.79±0.41 c	57.80±0.25 d
<i>T. pseudokoningii</i> IMI-392431	35.04±1.45 d	38.66±2.38 d	42.02±2.17 e	46.60±2.41 d	48.91±1.74 e	49.82±1.79 d	50.77±1.79 d	52.45±2.06 d	50.43±2.08 e	51.71±1.55 e	52.49±1.51 d	53.68±1.43 e
<i>T. harzianum</i> IMI-392432	56.98±1.29 a	59.82±2.15 a	63.35±1.93 a	66.22±1.22 a	67.87±1.68 a	69.32±1.46 a	70.91±1.29 a	72.29±1.44 a	70.69±1.72 a	71.22±1.27 a	73.41±1.42 a	75.78±1.18 a
<i>T. harzianum</i> IMI-392433	54.18±1.78 b	56.34±1.42 b	60.87±1.29 b	62.58±1.15 b	65.33±1.51 b	65.50±1.15 b	67.88±1.19 b	69.56±1.44 b	67.44±1.45 b	68.03±1.31 b	69.15±1.26 b	71.79±1.21 b
<i>T. harzianum</i> IMI-392434	53.47±0.29 b	54.37±1.01 c	58.84±0.75 c	61.11±0.82 b	63.46±0.38 c	64.84±0.72 b	66.19±0.81 b	67.95±0.61 b	65.26±0.43 c	65.95±0.76 c	67.45±0.99 b	69.61±1.02 c

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

## DISCUSSION

Mycelial interaction is one of the basic methods to assess antagonistic of microorganisms. Two criteria were considered in dual culture methods PIRG of *A. tenuis* and colony overgrowth time of *A. tenuis* by *Trichoderma* isolates. The results revealed that all *Trichoderma* isolates showed various degrees of antagonistic against *A. tenuis* and different isolates within the same species also showed different degrees of inhibition. Jinantara (1995) reported that nine isolates of *T. harzianum* possessed different abilities to attack *Sclerotium rofsii* and this was in agreement with Henis et al., (1983) who found that different isolates of *T. harzianum* parasitized sclerotia of *S. rofsii* at varying percent inhibitions. Two comparative methods were followed to test for variation in screening results in the placement of fungal mycelial plug. Results showed that although the percentage values varied, the ranking of species antagonistic remained in the same order. Thus, whatever procedures were applied, the qualitative results were similar. However, for accurate measurement of radius of the test fungi within the dual culture plate, the first method is recommended, because when test fungi are placed on the margin of the plate, it is easier to take measurements from the margin towards the centre. Based on two criteria, the highest PIRG values and minimum colony overgrowth times, *T. harzianum* IMI-392432 was the best antagonist. Dharmaputra et al., (1994) tested two isolates of *T. harzianum* and one isolate of *T. viride* against *Ganoderma* and reported that recorded all isolates inhibited the mycelial growth of the pathogen but *T. harzianum* (isolate B10-1) showed best performance. Etbarian (2006) reported that *T. viridie* (MO) reduced the colony area of *Macrophomina phaseoli* by 19.2 and 34.9% in the dual culture and cellophane methods, respectively.

Other than mycelial interaction and hyperparasitism by the *Trichoderma* species, scientists have also considered the action 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 an effective inhibitor of mycelial growth of *A. tenuis*. The ability of *Trichoderma* species to produce inhibitory substances against microorganisms has been described by Jinantara (1995), Sivasithamparam and Ghisalberti (1998) and Reino et al., (2008). In the present study, *T. harzianum* IMI-392432 showed better performance in the poison agar method using different concentrations of metabolites of different days. To know whether the antibiotic action of secondary metabolites of *Trichoderma* was diffusible as well as antifungal, the bilayer agar technique experiment was carried out. The inhibition of radial growth of *A. tenuis* 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 ( $P=0.05$ ) role on the outcome of *A. tenuis* mycelia. It is confirmed by this experiment that the metabolites produced by *T. harzianum* is diffusible and could prevent, inhibit or suppress the growth of *Alternaria* in culture. Therefore, *T. harzianum* IMI-392432 has a high potential as biocontrol agent against *A. tenuis*. In previous studies, Magnus 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 that *T. polysporum* significantly reduced the growth of *Ceratocystis paradoxa*, followed by *T. viridie*, *T. hamatum* and *T. aureoviride*. The actual effect and mechanism involved are 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 *A. tenuis* were confirmed where culture filtrates of *Trichoderma* prevented the growth of *A. tenuis* in the direct assay method. The highest PIGW value was recorded at 80% metabolite concentration which indicates that high percentage of culture filtrates 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*. Based on PIGW values, *T. harzianum* IMI-392432 showed a better inhibitory effect on growth of *A. tenuis*. Filtrates from *Trichoderma* species have been reported to exhibit antifungal activities (Claudia et al., 1997). Doi and Mori (1994) found successful 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 pathogens, *Sclerotium cepivorum*. The results of the inhibition of mycelial growth of *A. tenuis* by culture filtrates of *Trichoderma* were very similar to the above findings. This result also suggests that *T. harzianum* IMI-392432 produced antifungal compounds as secondary metabolites and such compounds may play an active role in the inhibitory effects on colony growth of *A. tenuis*.

## CONCLUSION

Due to the variable antagonistic potentials of individual isolates, it is very important that *Trichoderma* isolates are screened first to select the most active antagonist against a particular pathogen, in order to use a particular species of *Trichoderma* as a biocontrol agent. As a follow up to the results obtained from different *in vitro* studies *T. harzianum* IMI-392432 was the best for inhibiting the mycelial growth of *A. tenuis*. Hence, this strain may be referred to as a potential biocontrol agent and recommended for further study and commercialization in future.

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