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Antagonistic activities of *Trichoderma* strains against chili anthracnose pathogen

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Abstract

Antagonist activities of different *Trichoderma* strains were evaluated *in vitro* against *Colletotrichum capsici*, a causal agent of anthracnose fruit rot of chili. Dual culture test showed that *Trichoderma* strains effectively inhibited mycelia growth of the pathogen. *T. harzianum* IMI-392433 showed the highest inhibition (81.96 %) and mycelial overgrowth (78.98%). Also, metabolites having 80% concentration extracted from 30-day-old *T. harzianum* IMI-392433 revealed the highest PIRG (percentage inhibition of radial growth) value of 85.16 and 87.18% by using normal poison and modified bilayer poison agar technique, respectively. Further, metabolites extracted from 30-day-old *T. harzianum* IMI-392433 at a concentration of 2000 mgL^{-1} completely inhibited spore germination and germ tube elongation of the pathogen. Also, severity of anthracnose was significantly decreased as compared with the control (2% methanol) when chili fruits were soaked in 2000 mgL^{-1} of 30-day-old metabolites from *T. harzianum* strains. Importantly, metabolites extracted from *T. virens* IMI-392430 (71.09) and *T. pseudokoningii* IMI-392431 (69.52) were found to be most efficient in the inhibition of disease severity. Taken together, our data suggest that *Trichoderma* strains especially *T. harzianum* IMI-392433 is a potential antagonistic organism and can be used to control the anthracnose disease caused by *C. capsici*.

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Introduction

Chili (*Capsicum annuum* L.) is one of the most important spice crops in the world and grown in all seasons and areas of Bangladesh. The average yield of chili is 509 kg/acre, which is very low as compared to the yield of other chili growing countries of the world (B.B.S 2010). There are many factors responsible for the low yield of the chili. Among the various factors diseases are predominant. Fungal diseases play a vital role in reducing the yield of the chili. Out of the fungal diseases, anthracnose incited by *Colletotrichum capsici* is very important. Because it inflicts considerable quantitative and qualitative losses of the crop in the field as well as in pre and post harvest stages and these infections together account for more 50% of the crop losses (Rahman et al., 2004; Pakdeevaraporn *et al*., 2005). To date, no information is available on the biological control of this pathogen.

The fungus is both internally and externally seed-borne (Ramachandran et al., 2007). The black wound found on infected fruits will expand very quickly under high moisture condition, especially in tropical countries (Ramachandran *et al*., 2007). Fruiting bodies and spores of *C. capsici* will be abundantly produced on those black lesions. Sowing such contaminated seeds results in pre emergent and post emergent damping-off of seedlings in nursery and field (Zimand et al., 1996). The fungus survives in an active form on the stems and branches causing dieback symptoms. Management strategies reflect use of presumed disease free seeds and seedlings, resistant cultivars and fungicidal sprays. However, all approaches are not fully successful but have their disadvantages, such as the brief commercial life of resistant cultivars or occurrence of fungicide resistance (Basak *et al*., 1996). Therefore, alternate means and ways need to be finding out to control of the disease. Bio control is thus a promising and safe way for management of anthracnose disease.

Trichoderma and its products have been studied and used for bio control in many countries. The fungi *Trichoderma* has been an exceptionally good model to study bio control because it is ubiquitous, easy to isolate and culture, grows rapidly on many substrates, affects a wide range of plant pathogens, acts as a mycoparasite, competes well for food and growth sites, produces antibiotics and has an enzyme system capable of attacking a wide range of plant pathogens (Islam *et al*., 2008). Furthermore, *Trichoderma* inhibit or degrade pectinases and other enzymes that are essential for plant-pathogenic fungi, such as *Botrytis cinerea*, to penetrate leaf surfaces (Zimand *et al*., 1996). Most of effective *Trichoderma* strains produced cell wall lytic enzymes and volatile and non-volatile toxic metabolites that impede colonization by antagonized microorganisms. Among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described to protect plant from disease (Chet *et al*., 1997; Vey *et al*., 2001). Therefore, the purposes of this research work were (i) to observe the effect of five *Trichoderma* strains on mycelial growth inhibition and overgrow mycelia of *Colletotrichum capsici*. (ii) to evaluate the efficacy of antifungal metabolites extracted from selected five *Trichoderma* strains on mecelial, spore germination and germ-tube growth inhibition of *Colletotrichum capsici*.

Materials and methods

Sources of Trichoderma strains

Five *Trichoderma* strains namely; *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431 and *T. harzianum* IMI-392432, *T. harzianum* IMI-392433 and *T. harzianum* IMI-392434 were used in this study which was collected from Biotechnology and Microbiology Laboratory, Department of Botany, Rajshahi University, Bangladesh.

These strains were isolated from decomposed garbage and soil by Rahman (2009) and were verified by CABI Bioscience, Surrey, U.K.

Isolation and pathogenicity test of the pathogen

C. capsici, a causal agent of anthracnose was isolated from nine anthracnose infected chili fruits obtained, from the different chili planting areas in Pabna and Rajshahi districts, Bangladesh. Anthracnose fungus was isolated by tissue transplanting method described by Agrios (2005). For pathogenicity test, each of nine isolates of *C. capsici* was cultured on PDA for 3 days. Then 0.7 cm agar plug contained with mycelia of *C. capsici* was placed on pierced area on chili fruit (*Capsicum annum* L. var. *annum*) obtained from chili plantations area at Agriculture field, Khorkhori, Rajshahi district. All inoculated fruits were incubated in moist plastic chamber, kept at room temperature (27±°C). Disease severity of anthracnose infection was recorded at 5 days after incubation by measuring size of diseased lesion on chili fruit. The percentage of disease severity was calculated by using the formula; $((R_T-R_C)/R_T) \times 100$, when R_T was the mean of diseased lesion radius on chili in the tested treatment and R_C was the mean of diseased lesion radius on chili in the control (placed with an agar plug without C*. capsici*). For each treatment, there were four replicates and 5 chili fruits were used in each replicate. Then a most pathogenic isolate was used for further.

Mycelial growth inhibition and over-growth test

For mycelial growth inhibition and overgrowth test, dual culture test was performed. *Trichoderma* strains and *C. capsici* was sub cultured onto PDA for 4 days. The margin of colony of *C. capsici* was cut with sterile cork borer (0.7 cm diameter) and placed on agar surface at 1.5 cm from a margin of 9 cm diameter Petri dish. At 4 days after placing the plug of *C. capsici*, a plug of the *Trichoderma* strains was inoculated at the opposite direction, 6 cm apart from the *C. capsici* plug. The dishes were incubated for 5 days at room temperature, and then mycelial growth inhibition and the ability of *Trichoderma* strains to overgrow the colony of *C. capsici* were observed and compared with the control treatment (*C. capsici* grown on PDA without a *Trichoderma*). The inhibition levels were calculated by using the formula; $((G_C-G_T)/G_C) \times 100$, when G_C was the mean of colony radius of *C. capsici* in the control dish and G_T was the mean of colony radius of *C. capsici* in Petri-dish of dual culture test. Each treatment was performed with four replicates, one dish per a replicate. The overgrowth rates of *Trichoderma* strains were calculated by using the formula; $((D_1-D_2)/T_d) \times 100$, when D_1 was the mean of colony radius of the *Trichoderma* strains on the day of recording, D_2 was the mean of colony radius of the *Trichoderma* strains on the day before recording and T_d was the time (d) between before and after recording. Each treatment was performed with four replicates.

Preparation of culture filtrates of Trichoderma

Two hundred milliliters of Richard's solution (KNO_3: 1.0g, KH_2PO_4: 0.5g, $MgSO_4 \cdot 7H_2O$: 0.25g, glucose: 34g, and trace amounts of $FeCl_3$ in 1 liter distilled water, pH 6.5) was prepared and poured into 500 ml conical flasks and autoclaved for 15 minute at 121°C/1.05kg/cm^2 pressure. Six pieces of agar discs (6mm) 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 100rpm 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 normal 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 15minutes.

The sterilized different day's old and different concentrations *Trichoderma* 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 technique, seven-day-old culture discs (6mm) of *C. capsici* were inoculated at the centre of previously prepared normal poison agar plates and incubated at room temperature (28±2°C) for 10 days. For the modified bilayer poison agar technique, an agar disc (6mm) of *C. capsici* 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 *Trichoderma*, was poured over the *C. capsici* 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. capsici* 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).

Where, PIRG = R_1-R_2/R_1×100

R_1= Radius of *C. capsici* colony in control plate; R_2= Radius of *C. capsici* colony in dual culture plate.

Extraction of antifungal metabolites from Trichoderma strains for inhibition of spore germination of C. capsici

Five *Trichoderma* strains were separately cultured on PDA for 2 days, before 0.7 cm cork borer cut the margin of colony of each strain. Twenty-five plugs of each *Trichoderma* strains were put into 3 liter flask containing 1 liter of potato dextrose broth (PDB) and incubated at room temperature for 10, 20 and 30 days. After incubation of 10, 20 and 30 days, conidia and mycelia of *Trichoderma* strains were removed from broth culture by filtration. The culture filtrate was used for extraction of antifungal metabolites. The extraction of antifungal metabolites was repeated for three times in sequences with 350 ml of ethyl acetate (EtoAc). Separation of the two phases was facilitated by the addition of 5 g of sodium chloride to the first extraction. Then EtoAc was evaporated at 40°C in a rotary evaporator. Dry weights of antifungal metabolites were recorded (Intana *et al*. 2003).

Inhibition of spore germination of C. capsici by metabolites of Trichoderma strains

Spore suspension of *C. capsici* was prepared from 7-day-old culture growing on PDA, using PDB as diluents. Concentration of spores in a suspension was determined and adjusted with haemacytometer to10x10^5 spores/ml. Then, 5.0 ml of spore suspension was mixed with 5.0 ml of 1000, 1500 and 2000 mg/l of 10, 20 and 30 days old each antifungal metabolite in a test tube, respectively and kept for 24 h at room temperature before the sample was stained with lacto phenol cotton blue. Germinating spores and the length of germ-tube were observed and recorded under compound light microscope compared with a control treatment. The percentage inhibition of conidial germination of *C. capsici* was calculated by using the formula; ((R_C-R_T)/R_C) ×100, when R_T was the mean of spore germination on the tested treatment and R_C was the mean of spore germination on the control treatment (Yenjit et al., 2004).

Control of anthracnose on chili fruit

The chili fruits (*Capsicum anuum* L. var. *annuum*) collected from chili field at Agriculture field, Khorkhori, Rajshahi district were disinfested with 0.5 % sodium hypochlorite before making a tiny wound on each chili fruit with a sterilized needle.

Then, the wounded chili fruit was immersed in 2000 mg/l, 30 days old of each antifungal metabolite for an hour at room temperature. Then, 0.5 ml of spore suspension of *C. capsici* ($10x10^5$spores/ml) was dropped on the wounded area on chili fruit. The inoculated chili fruits were kept in plastic box and incubated for 4 days in plant growth chamber (25°C, 12 h of light and 80 % of water capacity content). The percentage of disease inhibition was then observed, recorded and compared with the control treatment (wounded chili fruits soaking in 2% methanol). Each treatment comprised of four replicates with ten fruits in each replicate. The percentage of disease inhibition was calculated by using the formula; $((R_C-R_T)/R_C) \times 100$, when R_C was the mean of diseased lesion radius on chili in the control and R_T was the mean of diseased lesion radius on chili in the treatment with antifungal metabolite.

Statistical analysis

All data were analyzed by DMRT using the help of computer package program SPSS (SPSS Inc., Chicago, IL, USA).

Results and discussion

Isolation and pathogenicity test

The results showed that all strains of *C. capsici* could cause disease on chili fruits at various levels of percent infection. Isolates Cca-AKR-01 and Cca-AKR-02 collected from Agriculture field, Khorkhori, Rajshahi provided 82.69 and 81.78 % of infection. While isolate Cca-ARU-01 provided only 48.12 % of infection (Table 1). Therefore, isolate Cca-AKR-01 was revealed to me most pathogenic.

Growth inhibition and overgrow test

All strains of *Trichoderma* significantly could inhibit mycelial growth of *C. capsici*. The inhibition percentages were ranging from 59.67-81.96. Strain of *T. harzianum* IMI-392433 showed the highest percentage inhibition (81.96 %) of mycelial growth and the lowest (59.67 %) was recorded in *T. pseudokoningii* IMI-392431 (Table 2). All *Trichoderma* strains provided significant higher percentages mycelial overgrowth of *C. capsici*. The mycelial overgrowth percentages were ranged from 55.69 to 78.98. Strain of *T. harzianum* IMI-392433 provided the highest percent (78.98 %) mycelial overgrowth and the lowest was recorded (55.69 %) in *T. pseudokoningii* IMI-392431 (Table 2). In a similar study, Rahman et al., (2009) were screened five *Trichoderma* strains against *Ceratocystis paradoxa* causing pineapple disease of sugarcane and reported that *T. harzianum* IMI 392432 reduced 63.80 % radial growth of the pathogen in dual culture technique.

Dharmaputra *et al.* (1994) tested two isolates of *T. harzianum* and one isolates of *T. viride* against *Ganoderma* and found that all isolates inhibited the mycelial growth of the pathogen, but *T. harzianum* (isolates B 10-1) 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. Intana *et al.* (2007) were tested three mutant and two wild type strains of *T. harzianum* for efficacy to inhibit and overgrow mycelia of *Colletotrichum capsici*, a causal agent of anthracnose of chili on potato dextrose agar (PDA) at room temperature. All strains effectively inhibited and overgrew mycelia of the pathogen, especially two mutant strains (T-35-co4 and T-35-co5) providing the high percent of inhibition of 83.00 and 75.50%, respectively. In addition, another mutant strain, T-50-co4, overgrew mycelia of the pathogen with the highest percentage of 69.50.

Table 1. Percent infection on chili fruits by *Colletotrichum capsici* isolated from anthracnose-infected chili fruits in various locations.

Strains	*Location of isolates*	*Infection (%)*
Cca-AIP-01	Agriculture field, Ishurdi, Pabna	61.47 e
Cca-AIP-01	Agriculture field, Ishurdi, Pabna	57.49 f
Cca-AKR-01	Agriculture field, Khorkhori, Rajshahi	82.69 a
Cca-AKR-02	Agriculture field, Khorkhori, Rajshahi	81.78 a
Cca-AKR-03	Agriculture field, Khorkhori, Rajshahi	75.24 b
Cca-ABR-01	Agriculture field, Budpara, Rajshahi	72.18 c
Cca-ABR-02	Agriculture field, Budpara, Rajshahi	70.48 cd
Cca-AMR-01	Agriculture field, Meahearchondi, Rajshahi	68.79 d
Cca-ARU-01	Agriculture research field, Rajshahi University Campus	48.12 g

Means in a column followed by the same letter(s) are not significantly
different according to DMRT (P=0.05).

Table 2. Mean PIRG values and colony overgrowth time of *Trichoderma* isolates against *C. capsici* by dual culture technique.

Strains of Trichoderma	*Mean % mycelial growth inhibition (PIRG)**	*Percent of mycelial overgrowth of Trichoderma strains**
T. virens IMI-392430	69.59 d	64.89 d
T. pseudokoningii IMI-392431	59.67 e	55.69 e
T. harzianum IMI-392432	79.38 b	72.89 b
T. harzianum IMI-392433	81.96 a	78.98 a
***T. harzianum* IMI-392434**	76.38 c	69.87 c

* mean of four replications
Means in a column followed by the same letter(s) are not significantly
different according to DMRT (P=0.05).

Screening by poison and modified bilayer poison agar technique

The PIRG (Percentages inhibition of radial growth) values by metabolites of *Trichoderma* strains varied significantly ($p \leq 0.05$) at different concentrations and different days. With the normal poison agar technique, the highest PIRG values (85.16%) were achieved at 80% concentration on the 4th day by 30-day-old metabolites of *T. harzianum* IMI 392433 (Table 3c). However, with the modified bilayer poison agar technique, the highest PIRG values (87.18%) were achieved at 80% concentration of 30-day-old metabolites of *T. harzianum* IMI 392433 (Table 4c). The lowest PIRG values, 8.36 and 12.88%, were recorded 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 techniques, respectively. The PIRG values of each strain were significantly different ($p \leq 0.05$) among different concentrations and different days of metabolites in both techniques. 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. capsici* was very pronounced compared to the growth of the un inoculated control bilayers.

It is clear that the presence or absence of *Trichoderma* metabolites can have a significant role on the outcome of *C. capsici* mycelia. This experiment confirmed that the metabolites produced by *T. harzianum* are diffusible and can prevent, inhibit, or suppress the growth of *C. capsici* in culture. Therefore, *Trichoderma* has a large potential as a biocontrol agent against *C. capsici*. 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 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 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).

Inhibition of spore germination and germ-tube elongation

Percent inhibition of conidial germination of *C. capsici* varied significantly different (p≤0.05) by the application of different day's old and different concentrations metabolites of each *Trichoderma* strains at different hours. The highest percent inhibition of conidial germination was achieved at 2000mgL^{-1} concentration after 2 to 24 hr by 30-day-old metabolites of *T. harzianum* IMI-392433 followed by *T. harzianum* IMI-392432, *T. harzianum* IMI-392434, *T. virens* IMI-392430 and *T. pseudokoningii* IMI-392431, respectively. The lowest percent inhibition of conidial germination of *C. capsici* was recorded at 1000 mgL^{-1} concentration after 2 to 24 hours of 10-day-old metabolites of *T. pseudokoningii* IMI-392431 (Table 5).

Table 3a. Mean PIRG values of *C. capsici* by normal poison agar technique using 10- day- old *Trichoderma* metabolites.

Trichoderma strains	***No of days***	**10- day- old metabolites***			
		20%	**40%**	**60%**	**80%**
T. virens IMI-392430	4	21.32 ef	28.16 e	40.74 d	43.18 e
	6	15.12 i	20.26 ij	34.69 fg	40.97 fg
	8	12.34 j	20.67 hi	30.79 h	31.11 i
	10	8.36 k	15.86 k	22.84 k	24.11 m
T. pseudokoningii IMI-392431	4	15.31 i	20.39 ij	31.16 h	39.56 gh
	6	13.14 j	16.38 k	28.21 i	37.41 i
	8	8.32 k	15.18 k	22.94 k	25.68 m
	10	5.34 l	12.26 l	19.22 l	20.39 n
T. harzianum IMI-392432	4	27.21 b	34.21 b	46.14 b	48.21bc
	6	24.31 d	27.18 ef	40.29 d	46.18 d
	8	18.12 g	25.94 f	35.46 f	39.16 h
	10	15.34 i	20.86 hi	28.94 i	35.14 j
T. harzianum IMI-392433	4	29.86 a	36.94 a	48.21 a	53.24 a
	6	25.34 cd	30.14 d	42.78 c	49.92 b
	8	20.36 f	27.18 ef	38.14 e	42.17 ef
	10	17.19 gh	22.29 h	31.28 h	38.76 hi
T. harzianum IMI-392434	4	26.91 bc	32.12 c	43.86 c	46.91cd
	6	22.14 e	24.17 g	37.29 e	43.82 e
	8	16.12 hi	23.98 g	33.16 g	37.14 i
	10	12.14 j	18.74 j	26.47 j	33.12 k

* mean of three replications
Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05

Table 3b. Mean PIRG values of *C. capsici* by normal poison agar technique using 20- day- old *Trichoderma* metabolites.

Trichoderma strains	No of days	20 –day- old metabolites*			
		20%	**40%**	**60%**	**80%**
T. virens	4	31.12 cd	40.19ef	55.94 d	62.14 ef
IMI-392430	6	26.84 f	35.14 g	49.38 f	58.97 g
	8	24.12 g	31.17 h	42.18 h	52.48 j
	10	22.84 g	24.22 k	34.34 k	49.16 k
T. pseudokoningii	4	29.14 e	35.54 g	41.16 h	57.44 gh
IMI-392431	6	24.34 g	31.17 h	36.28 j	50.11 k
	8	21.17 h	27.68 ij	36.13 j	41.26 l
	10	15.12 i	20.97 l	28.17 l	35.31 m
T. harzianum	4	37.14 b	46.62 b	60.92 b	69.84 b
IMI-392432	6	32.21 c	41.98 d	55.82 d	64.44 d
	8	29.64 de	38.64 f	48.38 f	60.94 f
	10	26.91 f	29.17 i	41.14 h	56.45 hi
T. harzianum	4	39.92 a	49.18 a	63.17 a	72.12 a
IMI-392433	6	36.68 b	45.14 bc	58.21 c	68.86 b
	8	31.19 cd	41.76 de	51.22 e	63.37 de
	10	28.14 ef	32.12 h	45.54 g	58.99 g
T. harzianum	4	35.52 b	44.28 c	58.81 c	66.64 c
IMI-392434	6	29.19 e	38.84 f	51.97 e	61.27 f
	8	26.99 f	35.18 g	46.65 g	57.74 gh
	10	24.46 g	26.14 j	38.77 i	54.96 i

* mean of three replications
Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).

Antifungal metabolites extracted from all *Trichoderma* strains at 2000 mgL^{-1} concentration provided markedly high inhibitive activities on germ-tube growth of *C. capsici*. The strongest inhibition of germ tube growth of *C. capsici* was recorded in 30-day-old metabolites of *T. harzianum* IMI-392433 at 2000 mgL^{-1} concentration and the lowest inhibition of germ tube growth was recorded at 1000 mgL^{-1} concentration in 10-day-old metabolites of *T. pseudokoningii* IMI-392431 after 2 to 24 hours of incubation (Table 5). In the present study, the antifungal metabolites of *Trichoderma* strains had the greatest effect on conidial germination and germ tube growth of *C. capsici*. Antifungal metabolites extracted from *T. harzianum* IMI 392433, 2000 mgL^{-1} provided markedly high inhibitive activities on spore germination and germ-tube growth of *C. capsici*. These inhibition efficacies were significantly different from those of a control. In a similar study, Intana et al. (2007) were tested two wild type strains of *T. harzianum* for efficacy to inhibit conidial germination and germ tube formation of *C. capsici* and observed that antifungal metabolites extractions of these strains completely inhibited both the spore germination and germ tube formation of the pathogen and 1000 mgL^{-1} of antifungal metabolites gave the promising result. Similar observations were also reported by Imtiaj and Lee (2008).

Govindasamy and Balasubramanian (1989) reported the reduction in germination rate and germtube growth from *Puccinia arachidis* urediospores when mixed with *T. harzianum* conidial suspensions.

Table 3c. Mean PIRG values of *C. capsici* by normal poison agar technique using 30- day- old *Trichoderma* metabolites.

Trichoderma strains	No of days	30- day- old metabolites*			
		20%	**40%**	**60%**	**80%**
T. virens	4	48.37 f	57.48 d	64.46 d	68.93 g
IMI-392430	6	45.46 g	54.42 f	57.87 f	65.48 h
	8	40.18 hi	45.76 i	50.86 i	60.18 j
	10	32.14 jk	39.83 k	46.74 j	59.94 j
T. pseudokoningii	4	41.14 h	48.22 h	52.99 h	62.14 i
IMI-392431	6	38.64 i	49.27 h	53.36 h	58.63 j
	8	33.24 j	39.84 k	43.64 k	55.49 k
	10	30.92 k	35.27 l	40.14 l	49.98 i
T. harzianum	4	56.34 b	64.28 b	71.29 b	78.19 c
IMI-392432	6	51.14 d	59.74 c	63.34 d	71.14 f
	8	48.86 ef	51.39 g	57.74 f	69.18 g
	10	40.14 hi	45.48 i	52.84 h	66.19 h
T. harzianum	4	59.91 a	67.82 a	76.74 bc	85.16 a
IMI-392433	6	54.12 c	61.18 c	70.21 bc	79.94 b
	8	50.34 de	55.28 ef	61.19 e	73.31 e
	10	48.31 f	48.47 h	53.77 h	69.37 g
T. harzianum	4	53.38 c	61.14 c	68.73 c	75.36 d
IMI-392434	6	48.92 ef	56.38 de	60.13 e	69.93 fg
	8	45.61 g	49.93 gh	55.86 g	66.91 h
	10	40.14 hi	43.17 j	49.82 i	62.21 i

Table 4a. Mean PIRG values of *C. capsici* by modified bilayer poison agar technique using 10- day- old *Trichoderma* metabolites.

Trichoderma strains	No of days	10- day- old metabolites*			
		20%	**40%**	**60%**	**80%**
T. virens	4	23.29 e	31.86 cd	42.38 ef	45.48 d
IMI-392430	6	19.98 f	23.29 g	38.94 h	42.24 e
	8	16.73 h	21.14 hi	35.34 i	35.45 g
	10	12.88 j	17.91 k	28.16 k	30.78 h
T. pseudokoningii	4	19.16 fg	26.54 e	38.84 h	42.14 e
IMI-392431	6	17.93 gh	19.26 jk	31.38 j	40.96 ef
	8	14.38 ij	18.82 jk	34.68 i	30.81 h
	10	9.53l k	14.74 l	25.28 l	24.68 i
T. harzianum	4	31.14 bc	36.54 b	47.91 b	51.12 b
IMI-392432	6	29.86 c	30.21 d	43.38 de	48.46 c
	8	22.16 e	20.46 ij	41.86 efg	42.68 e
	10	17.98 gh	22.71 gh	38.94 h	39.57 f
T. harzianum	4	33.14 a	39.98 a	50.51 a	56.91 a
IMI-392433	6	31.68 ab	32.93 c	48.97 ab	51.24 b
	8	26.54 d	25.28 ef	44.26 cd	48.85 c
	10	19.54 fg	24.16 fg	40.18 gh	42.79 e
T. harzianum	4	28.18 d	34.94 b	45.46 c	49.93 bc
IMI-392434	6	26.98 d	26.16 e	41.12 fg	45.74 d
	8	18.89 fg	22.63 gh	38.84 h	40.16 f
	10	14.74 i	20.54 ij	36.28 i	36.38 g

* mean of three replications

Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).

It also decreased the number of rust pustules/cm^2 leaf area. The inhibitory effect of the secondary metabolites of *Trichoderma* may be due to the production of metabolites, which has been reported by Horvath *et al.* (1998), Ghisalberti and Rowland (1993) and Iqbal *et al*. (1994). During plant pathogens attack, Trichoderma secreted many cell wall-degrading enzymes such as *endochitinase, chitobiosidase, N-acetyl-β-glucosaminidase and glucan 1, 3-β-glucosidase*. These enzymes strongly inhibit spore germination (or cell replication) and germ tube elongation (Tronsmo and Hjeljord, 1997). *Trichoderma* spp. can also produce different antibiotics against fungal phytopathogens. Among these antibiotics, the production of *gliovirin, gliotoxin, viridin, pyrones, peptaibols* and others have been described (Vey *et al., 2001).* Gliovirin from *T. virens* was active against *Pythium ultimum, gliotoxin from T. virens was very active against Rhizoctonia solani* (Howell *et al.,* 1993*).* This research indicated an additional successful use of antifungal metabolites from Trichoderma strains in controlling anthracnose on chili fruits caused by *C. capsici.* Higher efficacy of the metabolites of *Trichoderma* strains to inhibit spore germination and germ-tube growth of C. capsici usually resulting in better control of chili's anthracnose.

Control of anthracnose on chili fruit

Antifungal metabolites extracted from all *Trichoderma* strains significantly reduced percentages of inhibition of anthracnose disease severity on chili fruit by 69.52-81.39% at 30 days old metabolites, where as the control (2% methanol) was at 10.41% (Table 6).

The highest percent inhibition of disease severity was achieved at 2000 mg/l concentration by 30-day-old metabolites of *T. harzianum* IMI-392433 followed by *T. harzianum* IMI-392432, *T. harzianum* IMI-392434, *T. virens* IMI-392430 and *T. pseudokoningii* IMI-392431, respectively. The lowest percent inhibition of disease severity of *C. capsici* was recorded in *T. pseudokoningii* IMI-392431 (Table 6). In a similar study, Intana *et al*. (2007) were soaked chili fruits in 1000 mg/l of antifungal metabolites from three mutant strains of *T. harzianum* to control anthracnose disease and observed that the severity of anthracnose was significantly decreased as compared with the control (2% methanol).

Deore *et al. (2004)* reported that application of culture filtrates of *Trichoderma* spp*.* reduced the number of powdery patches and spots on the leaves and fruits There are many reports of successful use of antifungal metabolite extracted from *Trichoderma* spp*.* to control disease causing fungi such as *Sclerotium rolfsii* causing disease on vegetables (Maiti *et al.,* 1991), *Ceratocystis paradoxa* causing pine apple disease of sugarcane (Rahman *et al.*, 2009), *Pythium aphanidermatum* causing wilt of cotton and watermelon (Ordentlich *et al.*, 1992) and damping-off of cucumber (Intana, 2003) and *Phytophthora* sp. causing various plant diseases (Wilcox *et al.*, 1992).

Table 4b. Mean PIRG values of *C. capsici* by modified bilayer poison agar technique using 20- day- old *Trichoderma* metabolites.

Trichoderma strains	*No of days*	20 –day- old metabolites*			
		20%	**40%**	**60%**	**80%**
T. virens	4	35.42 de	44.91 c	58.45 e	65.57 c
IMI-392430	6	31.19 f	38.35 e	52.94 g	61.16 de
	8	26.94 ijk	36.95 ef	47.18 j	58.76 efg
	10	24.94 i	30.18 h	40.22 k	50.22 h
T. pseudokoningii	4	28.18 hij	35.16 g	50.24 hi	59.21 ef
IMI-392431	6	26.58 jkl	36.29 fg	48.98 i	59.14
	8	23.26 m	30.28 h	40.24 k	50.47 h
	10	18.64 n	28.98 h	38.78 k	40.61 i
T. harzianum	4	39.48 b	50.78 a	62.24 b	72.29 b
IMI-392432	6	36.91 cd	45.27 c	58.86 de	69.38 b
	8	31.48 f	44.21 c	51.22 gh	63.46 cd
	10	28.84 gh	40.28 d	52.37 g	58.89 efg
T. harzianum	4	42.28 a	52.31 a	65.83 a	75.81 a
IMI-392433	6	38.94 b	50.94 a	61.14 bc	71.49 b
	8	34.38 e	48.74 b	58.48 e	65.51 c
	10	30.26 fg	45.31 c	55.45 f	60.28 de
T. harzianum	4	37.18 c	48.61 b	60.48 cd	69.58 b
IMI-392434	6	34.48 e	41.14 d	55.11 f	65.54 c
	8	28.48 hi	40.58 d	49.94 hi	61.26 de
	10	26.18 ki	36.98 ef	50.18 hi	56.27 fg

* mean of three replications

Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).

Table 6. Inhibition of disease severity on chili fruits after applied with antifungal metabolites (2000 mg/l) from *Trichoderma* isolates in growth chamber at 25°C for 5 days.

Treatments	***Percentages of disease severity***	**Percentages of inhibition of disease severity**
T. virens IMI-392430	28.91 c	71.09 d
T. pseudokoningii IMI-392431	30.48 c	69.52 d
T. harzianum IMI-392432	14.89 f	85.11 a
T. harzianum IMI-392433	18.61 e	81.39 b
T. harzianum IMI-392434	22.38 d	77.62 c
2 % methanol	89.59 b	10.41 e
Control 1 (with pathogen)	100.0 a	0.0 f
Control 2 (without pathogen)	0.0	-

Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).

Table 5. Efficacy of different days and different concentrations of antifungal metabolites (1000, 1500 and 2000 mg/l) from five *Trichoderma* strains on the inhibition of spore germination and germ-tube elongation of *C. capsici* within 24 hr of incubation.

Concen. (mg/l)	*Strains of Trichoderma*	*Inhibition of conidial germination (%) after various incubation period **						Length of germ tube (μm) *
		2 h	**4 h**	**6 h**	**8 h**	**12 h**	**24 h**	
	10 days old metabolites							
1000	*T. virens* IMI-392430	21.97z	42.18yz	45.12 z	47.82 z	52.82 y	55.15 v	71.18 b - 96.68 b
	*T. pseudokoningii*IMI-392431	22.92z	41.18z	44.29 z	46.18 z	51.17 z	53.23 w	73.38 a - 98.44 a
	T. harzianum IMI-392432	26.62x	43.12y	48.81 y	50.24 y	55.92 x	58.86 tu	68.12 cd - 91.14 d
	T. harzianum IMI-392433	27.75wx	44.98x	49.92 x	52.26 y	56.68 x	59.92 t	67.18 de - 90.12 d
	T. harzianum IMI-392434	25.55y	42.48yz	47.74 y	49.48 y	55.84 x	57.77 u	69.46 c - 93.32 c
1500	*T. virens* IMI-392430	29.16w	47.97w	53.12 wx	56.68 w	58.86 yz	61.98 s	65.58 e - 87.33 e
	*T. pseudokoningii*IMI-392431	28.82w	46.32x	50.21 x	54.46 x	57.96 yz	60.14 t	66.15 e - 89.92 e
	T. harzianum IMI-392432	31.21v	51.17v	54.48 wx	59.98 tu	61.14 uv	63.38 rs	62.28 f - 84.47 f
	T. harzianum IMI-392433	32.17v	52.28v	55.48 w	60.84 tu	62.26 tu	64.64 qr	60.16 g - 82.28 g
	T. harzianum IMI-392434	30.92v	49.94w	53.22 xy	58.88 v	60.84 vw	63.12 rs	63.37 f - 86.64 e
2000	*T. virens* IMI-392430	36.48 t	54.22 u	59.82 u	62.28 t	64.45 tu	66.12 q	58.86 g - 79.98 h
	*T. pseudokoningii*IMI-392431	34.28 u	54.12 u	57.74 v	61.94 t	63.36 tu	65.58 q	59.98 g - 81.12 gh
	T. harzianum IMI-392432	41.17 r	59.94 s	62.19 st	65.54 s	67.84 rs	70.89 op	55.54 h - 76.68 i
	T. harzianum IMI-392433	42.28 qr	60.14 s	63.27 rs	67.74 r	68.44 rs	71.12 op	53.35 i - 74.48 j
	T. harzianum IMI-392434	39.98 s	57.92 t	61.12 tu	64.45 s	66.99 s	69.72 p	56.64 h - 77.38 i
	20 days old metabolites							
1000	*T. virens* IMI-392430	45.59 p	64.48 r	65.48 p	69.92 q	71.14 q	73.34 n	49.54 jk - 72.28 kl
	*T. pseudokoningii*IMI-392431	43.32 q	61.34 s	64.47 qr	68.24 r	69.33 r	72.18 no	51.16 j - 73.39 jk
	T. harzianum IMI-392432	49.89 n	66.18 qr	67.12 p	71.18 pq	72.12 q	76.68 r	47.22 lm - 69.78mn
	T. harzianum IMI-392433	50.42 n	67.75 pq	69.86 o	72.25 q	74.48 p	77.17 lm	46.29 m - 68.24 n
	T. harzianum IMI-392434	47.24 o	65.29 r	66.48 p	70.94 pq	71.98 q	75.58 m	48.18 kl - 71.12 lm
1500	*T. virens* IMI-392430	53.38 m	69.98 no	71.18 o	75.54 n	76.12 o	80.18 k	43.12 no - 65.54 op
	*T. pseudokoningii*IMI-392431	52.12 m	68.48 op	70.44 o	73.34 o	75.54 op	78.44 l	44.46 n - 66.48 o
	T. harzianum IMI-392432	56.92 kl	72.12 lm	74.26 mn	77.12 n	80.12 mn	82.18 j	41.19 pq - 62.28 q
	T. harzianum IMI-392433	57.12 kl	73.34 kl	75.54 m	79.94 m	81.21 lm	83.38 ij	40.12 qr - 61.12 qr
	T. harzianum IMI-392434	55.78 l	71.18 mn	73.33 n	76.58 n	79.29 n	81.94 j	42.28 op - 64.64 p
2000	*T. virens* IMI-392430	60.47 j	75.54 j	79.98 k	81.17 lm	83.38 k	85.54 gh	37.82 s - 57.72 s
	*T. pseudokoningii*IMI-392431	58.38 k	74.92 jk	77.74 l	80.26 m	82.21 kl	84.46 hi	39.48 r - 59.94 r
	T. harzianum IMI-392432	62.18 i	78.44 i	82.22 ij	83.44 jk	85.54 j	87.12 fg	34.48 t - 54.48 tu
	T. harzianum IMI-392433	63.38 hi	79.58 hi	83.48 hi	84.18 ij	86.64 ij	88.27 ef	33.18 tu - 53.37 uv
	T. harzianum IMI-392434	61.94 ij	76.39 j	81.12 jk	82.22 kl	85.14 j	86.98 fg	36.69 s - 55.44 t
	30 days old metabolites							
1000	*T. virens* IMI-392430	67.12 g	81.18 gh	85.48 fg	86.11 h	88.84 h	90.12 d	29.18 v - 48.86 w
	*T. pseudokoningii*IMI-392431	64.26 h	80.33 gh	84.28 gh	85.32 hi	87.72 hi	89.24 de	32.27 u - 52.44 v
	T. harzianum IMI-392432	70.89 f	84.12 f	86.23 f	88.68 fg	91.27 fg	92.12 c	24.16 w – 46.18 w
	T. harzianum IMI-392433	71.29 f	85.58 ef	86.47 f	89.73 fg	92.18 f	93.28 c	22.18 x - 39.14 y
	T. harzianum IMI-392434	68.37 g	81.39 g	86.18 f	88.47 g	90.47 g	91.89 c	26.26 w – 47.58 w
1500	*T. virens* IMI-392430	74.24 e	87.34 cd	88.47 e	92.24 e	95.52 de	96.46 b	17.78 x - 32.34 x
	*T. pseudokoningii*IMI-392431	72.14 f	86.32 de	86.61 f	90.18 f	94.29 e	95.44 b	19.18 x - 36.65 x
	T. harzianum IMI-392432	78.92 cd	88.76 bc	91.18 d	95.58 cd	97.32 c	99.12 a	12.29 y - 28.88 y
	T. harzianum IMI-392433	80.14 c	89.86 b	93.13 c	96.14 c	98.17 bc	99.86 a	10.19 y - 26.69 yz
	T. harzianum IMI-392434	77.38 d	87.79 cd	90.48 d	94.42 d	96.64 cd	98.47 a	14.97 x - 29.22 y
2000	*T. virens* IMI-392430	84.14 b	90.39 b	96.94 b	99.94 a	100 a	100 a	7.87 y - 22.72 yz
	*T. pseudokoningii*IMI-392431	83.28 b	90.18 b	94.16 c	97.84 b	99.38 a	100 a	9.89 xy - 24.78 yz
	T. harzianum IMI-392432	80.14 a	96.28 a	100 a	100 a	100 a	100 a	4.65 z - 17.48 z
	T. harzianum IMI-392433	89.23 a	98.78 a	100 a	100 a	100 a	100 a	3.18 z - 16.78 z
	T. harzianum IMI-392434	89.14 a	95.76 a	99.24 a	100 a	100 a	100 a	5.18 z - 19.44 z

* mean of three replications

Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).

Table 4c. Mean PIRG values of *C. capsici* by modified bilayer poison agar technique using 30- day- old *Trichoderma* metabolites.

Trichoderma strains	No of days	30- day- old metabolites*			
		20%	**40%**	**60%**	**80%**
T. virens	4	51.13 de	59.84 ef	68.29 e	72.48 f
IMI-392430	6	48.67 fg	57.22 g	61.18 gh	68.47 hi
	8	45.42 i	51.24 i	55.97 k	63.53 j
	10	38.27 k	41.16 l	49.47 m	61.19 k
T. pseudokoningii	4	48.98 fg	53.27 h	58.14 j	68.98 hi
IMI-392431	6	42.19 j	51.18 i	55.45 k	59.87 kl
	8	36.86 kl	48.37 j	50.28 m	58.23 lm
	10	35.58 l	38.28 m	45.78 n	56.67 m
T. harzianum	4	58.42 b	67.27 b	76.65 b	84.46 b
IMI-392432	6	55.28 c	62.85 d	72.94 c	76.89 d
	8	50.17 ef	60.96 e	62.48 g	71.45 fg
	10	48.79 fg	50.94 i	58.27 ij	67.57 i
T. harzianum	4	62.47 a	69.94 a	78.81 a	87.18 a
IMI-392433	6	58.73 b	65.54 c	75.12 b	81.24 c
	8	52.84 d	62.98 d	71.17 d	75.14 e
	10	50.39 ef	58.22 fg	65.48 f	71.38 fg
T. harzianum	4	56.69 c	65.41 c	73.33 c	80.27 c
IMI-392434	6	51.18 de	59.76 ef	69.28 e	72.46 f
	8	47.29 gh	58.28fg	59.88 hi	69.84 gh
	10	46.68 hi	45.53 k	52.68 l	64.42 j

* mean of three replications

Means in a column followed by the same letter(s) are not significantly different according to DMRT (P=0.05).

Conclusions

In vitro results obtained using different techniques suggest that *T. harzianum* IMI 392433 was the best for inhibition of the mycelial growth, conidial germination, germ tube elongation and disease severity of *C. capsicci*. This strain can be used as potential biological control agent to control the anthracnose disease of chili.

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