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Screening of *Trichoderma* Strains as a Biological Control Agent against *Fusarium solani* Causing Root Rot of Ashwagandha [*Withania somnifera* (L.) Dunal]

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Abstract

Five *Trichoderma* strains such as *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433, *T. harzianum* IMI-392434 were tested for efficacy to inhibit and overgrowth of mycelia of *Fusarium solani*, a causal agent of root rot of Ashwagandha on potato dextrose agar (PDA) medium. Duel culture technique showed that all *Trichoderma* strains effectively inhibited and overgrew mycelia of the pathogen, especially *T. harzianum* IMI-392432 providing the highest percent of inhibition of radial growth (PIRG) (69.32 %) and overgrowth mycelia (59.28 %). Liquid culture filtrates having 75% concentration extracted from 30-day-old *T. harzianum* IMI-392432 showed the highest percent inhibition of mycelia growth (PIMG) value of 76.23% by using normal poison agar technique. Further, highest percent inhibition of conidial germination (PICG) (82.86 %) of the pathogen was exhibited by *T. harzianum* IMI-392432 at same culture and concentrations. In this study it was observed that different *Trichoderma* strains have good antagonistic effect on Mycelial growth and conidial germination of *F. solani*. In each case, *T. harzianum* IMI-392432 performed the best bio-control agent against *F. solani* causing root rot disease of Ashwagandha.

সারসংক্ষেপ

অশ্বগন্ধার শিকড় পচনের জন্য দায়ী ছত্রাক *Fusarium solani* এর মাইসেলিয়ামের বৃদ্ধির প্রতিবন্ধকতা এবং অতিরিক্ত বৃদ্ধি জানার জন্য কক্ষ তাপমাত্রাতে পটেটো ডেকস্ট্রোজ এগার (পিডিএ) মিডিয়ামে *Trichoderma* এর পাঁচটি স্ট্রেইন যেমন *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433 এবং *T. harzianum* IMI-392434 এর কার্যকারিতা যাচাই করা হয়েছিল । দ্বৈত চাষ পদ্ধতিতে দেখা গিয়েছিল যে *Trichoderma* এর সমস্ত স্ট্রেইন জীবাণুটির মাইসেলিয়ামের বৃদ্ধি এবং অতিরিক্ত বৃদ্ধিতে বাধাদান করে, বিশেষ করে *T. harzianum* IMI-392432 সর্বোচ্চ মাত্রায় মাইসেলিয়ামের বৃদ্ধি (৬৯.৩২%) এবং অতিরিক্ত বৃদ্ধিতে (৫৯.২৮%) বাধাদান করে। নরমাল পয়জন এগার পদ্ধতিতেও ৩০ দিন বয়সী *T. harzianum* IMI-392432 এর কালচার ফিলট্রেট ৭৫% ঘনত্বে সর্বোচ্চ ৭৬.২৩% মাইসেলিয়ামের বৃদ্ধিতে বাধাদান করে। তাছাড়াও ৩০ দিন বয়সী কালচার ফিলট্রেট ৭৫% ঘনত্বে জীবাণুটির সর্বোচ্চ মাত্রায় কনিডিয়ামের জার্মিনেশনে (৮২.৮৬%) বাধা প্রদান করে। এই গবেষণায় দেখা গিয়েছিল যে, *F. solani* এর মাইসেলিয়ামের বৃদ্ধি এবং কনিডিয়ামের জার্মিনেশনের উপর বিভিন্ন *Trichoderma* স্ট্রেইন এর ভাল বিরোধী প্রভাব আছে। প্রত্যেক ক্ষেত্রে *T. harzianum* IMI-392432 অশ্বগন্ধার শিকড় পচন রোগের জীবাণু *F. solani* এর বিরুদ্ধে খুব ভাল জৈব নিয়ন্ত্রক এজেন্ট হিসেবে কাজ করেছিল।

Keywords: *Withania somnifera*; Antagonism; Biocontrol; *Fusarium solani;* Culture filtrate; Root rot; *Trichoderma*

Introduction

Ashwagandha [*Withania somnifera* (L.) Dunal] is an important medicinal plant native to the Indian-sub continent and it is considered as aphrodisiac and rejuvenating; and useful in the treatment of inflammatory and anti tumor agent

medicines for hiccups, several female disorders, bronchitis, rheumatism, dropsy, stomach and lung inflammation, skin diseases, curing disability and sexual weakness in males (Joshi *et al*. 2010). In ayurvedic system of medicine, there are several products where Ashwagandha used as single plant based formulation and therefore, there is huge demand for root raw material in industries necessitating its cultivation in large scale. Root rot of Ashwagandha caused by *Fusarium solani* is a major concern in many medicinal plant growing areas in Bangladesh leading to enormous plant losses. The pathogen is known to be very persistent in soil and capable of surviving in infested fields for very long period and is difficult to control. The root of infected plant showed pulpiness with brownish color. White cottony growth of the fungus was observed at the basal part of infected plants near ground level. Diseased areas of the plant enlarge with age and gradually turn brown. Longitudinal cracks may develop in older lesions and the cortical tissues are discolored and decayed. The plants in the nurseries also showed symptoms of yellowing, drooping and decay at seedling stage leading to 30-50% mortality (Gupta *et al*. 2004). Disease management options include crop rotation, improving soil fertility levels, use of resistant cultivars, use of fungicides and biological control. The impetus for developing biological control agents has been the public perception of pesticide toxicity in the environment. Residue-free produce has become a valuable commodity. Biological control is compatible with pesticide-free agriculture and the environment. Roots of plants support the growth of a complex of microorganisms that can have a profound effect on the growth and survival of the plant. *Trichoderma* spp. have been found as effective bio control agent of many soil borne plant pathogenic fungi such as *Fusarium*, *Sclerotium*, *Rhizoctonia* etc. (Chet and Inbar 1994). *Trichoderma* genus is known to improve the nutritional status of their host, provide alterations in the host's physiology and exudation from roots (Abeysinghe 2007). The genus *Trichoderma* is not only one of the most common, isolated from various habitats but also known to be secreting to the environment various secondary metabolites of a wide spectrum of effects on various fungal groups. Among the metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-á-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described (Vey *et al.* 2001), which provide to protect plant from disease (Chet *et al*. 1997). Therefore, the aims of this research work were to observed the effect of five *Trichoderma* strains on mycelial growth inhibition and overgrow mycelia of *F. solani* and to evaluate the efficacy of antifungal metabolites of *Trichoderma* strains on mecelial growth and spore germination of *F. solani*.

Materials and Methods

Isolation and identification of pathogen

Withania somnifera (L.) Dunal plant showing root rot was collected from Bangladesh Forest Research Institute (BFRI) nursery. For the isolation, infected roots of the plant were excised with a sterile scalpel and, surface sterilized with 0.1 % $HgCl_2$ for one minute. Sterilized pieces were washed twice with sterile water for one minute, dried in a laminar air flow cabinet, and cut into small pieces (1 cm length) and transferred on to antibiotic amended PDA plates. Plates were incubated at room temperature for 4 days, and white mycelium growth from the portion of infected roots was transferred to fresh PDA media. After incubation for 5 days, a single spore or mycelia was isolated and cultured on fresh PDA media. All the cultures were stored at 4°C for further study. The pathogen was identified based on the characteristics described by Booth (1971). The pathogenecity of *F. solani* was confirmed on local ashwagandha cultivar.

Sources of *Trichoderma* strains

Five *Trichoderma* strains namely; *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *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.

Mycelial growth inhibition and over-growth test

For mycelial growth inhibition and overgrowth test, dual culture test was performed. *Trichoderma* strains and *F. solani* was sub cultured onto PDA for 4 days. The margin of colony of *F. solani* was cut with sterile cork borer (0.6 cm diameter) and placed on agar surface at 1.5 cm from a margin of 9 cm diameter petri dish. Another agar disc of the same size of *F. solani* was also placed at the periphery but on the opposing end of the same petri dish. As a control, *F. solani* was placed in a similar manner on a fresh PDA media (Figure 1). All pairings were carried out in quadruplicate and incubated at 28±2°C. Antagonistic activity was tested 5 days after incubation by measuring the radius of the *F. solani* colony in the direction of the antagonist colony (R_2) and the radius of the *F. solani* colony in the control plate (R_1). The dishes were incubated for 10 days at room temperature, and then the ability of *Trichoderma* strains to overgrow the colony of *F. solani* were observed and compared with the control treatment. The inhibition percentage of radial growth (PIRG) were calculated by using the formula; $[(R_1-R_2)/R_1] \times 100$, when R_1 was the mean of colony radius of *F. solani* in the control dish and R_2 was the mean of colony radius of *F. solani* 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.

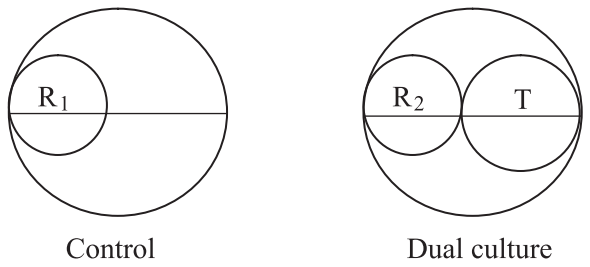

Figure 1. Method of dual culture. R_1/R_2: Mycelial growth of *F. solani* and T: *Trichoderma.*

Preparation of culture filtrates of *Trichoderma*

Two hundred milliliters of Potato Dextrose Broth (PDB) was prepared and poured into 500 ml conical flask and autoclaved for 15 minutes at 121°C/1.05kg/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 and the flasks were incubated at 28±2°C (Dennis and Webster 1971). The metabolites were collected after 30 days and filtered through 2 layers of Whatman No. 1 filter paper to remove hypal fragments and finally filtrated using a 0.22 ìm sized membrane filter. The filtrate was heated at 100°C for two minutes to inactivate the enzymes (Mustafa 2009). The inhibitory activity of the culture filtrate of *Trichoderma* strains was deter-mined for mycelial growth by poison agar technique and conidial germination of the test pathogen.

Determination of inhibitory activity of culture filtrate using normal poison agar technique

The sterilized culture filtrate of *Trichoderma* was mixed with PDA to make 25, 50 and 75 % concentrations in petri plates. The agar plates in

triplicates were inoculated at the centre with 6 mm diameter mycelial disc of pathogen and incubated at 28±2°C for 10 days. The plates without filtrate served as control. 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 10 days of inoculation. The readings were calculated for the percentage inhibition of mycelial growth (PIMG) based on the formula by Skidmore and Dickinson (1976). Where, PIMG = R_1-R_2/R_1´100; R_1= Radius of *F. solani* colony in control plate; R_2= Radius of *F. solani* colony in dual culture plate.

Effect of culture filtrates on conidial germination

Conidia obtained from 7-days-old PDA cultures of *F. solani* were suspended in a full strength (100%) (Undiluted) broth culture, and also in 25, 50 and 75 % dilution of the culture. The conidia concentration was readjusted with haemocytometer to 10^3/ml. Four replicate drops of the suspension were taken separate grove slide and kept at 25±2°C for 24 hours. Conidia suspension of the test fungi incubated in sterile PDB served as control. After incubation period, a drop of lactophenol cotton blue was placed over conidial suspension on the slide and examined under (×400) power microscope for recording the percentage of conidial germination. The two readings C_1 (Control) and C_2 (treated) of conidial germination were transformed into percent inhibition of conidial germination (PICG) using the formula by Skidmore and Dickinson (1976). Where, PICG = C_1-C_2/C_1´100.

Statistical analysis

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

Results and Discussion

Morphological and microscopic characteristics of *Fusarium solani*

Colonies grown on PDA became whitish to brown after 7 days of incubation, and produced macro and micro conidia (Figure 2). Macroconidia

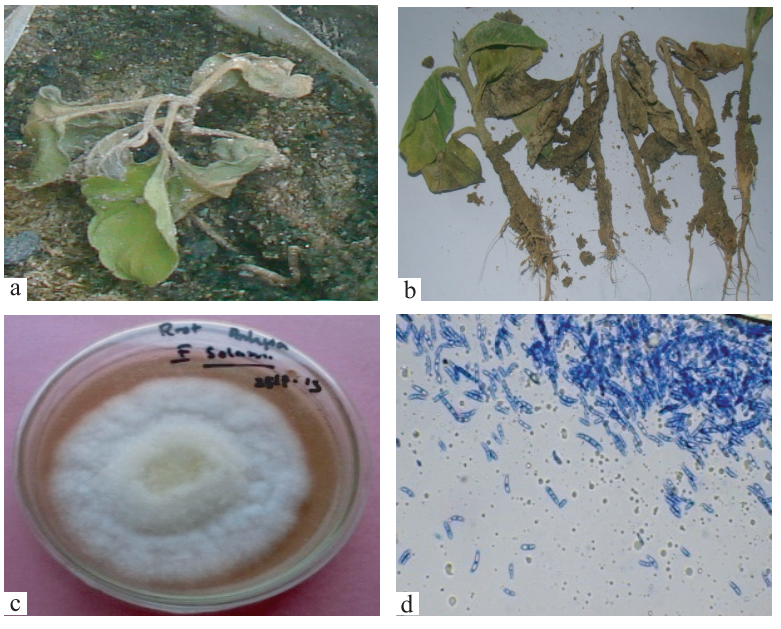

Figure 2. a & b: Root rot symptoms of ashwagandha in nursery. c: colonies of *F. solani* on PDA after 7 days. d: conidia of *F. solani*.

sickle-shaped, mostly with an elongated apical cell and pedicellate basal cell. Microconidia were 1- to 2-celled, hyaline, pyriform, fusiform to ovoid, straight or curved. The chlamydospores located in middle of hyphae (intercalary), on tip of the hyphae (terminal) and some chlamydospores were seen in middle of macro conidia. The chlamydospores were thick walled, rough, globose to oval shaped and measured 8.95-12.65 x 6.10-9.95 mm.

Pathogenecity test of *Fusarium solani*

Koch's postulates were demonstrated for the pathogen and confirmed as the causal agent of root rot of *Withania somnifera*. The pathogenicity tests for the *F. solani* isolate were examined with *Withania somnifera* local cultivar (data not shown).

Mycelial growth inhibition and overgrow test

All the five *Trichoderma* strains showed significantly antibiotic potential against *F. solani* by inhibiting its mycelial growth in dual culture technique (Figure 3). Five days after inoculation, growth of *F. solani* was found to be inhibited by *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433, *T. harzianum* IMI-392434 strains and attained a inhibition percentages of 59.36, 57.39, 69.32, 65.63, and 61.66, respectively. Strains *T. harzianum* IMI-392432 showed the highest PIRG value of 69.32. This indicates that *T. harzianum* IMI-392432 strain had maximum antifungal activity against *F. solani* compared to the other *Trichoderma* strains (Table 1). The results were fully agreement with Sharma and Trivedi (2010) who were evaluated eleven different fungal strains for their antagonistic activity against *F. oxysporum* under *in vitro* condition causing wilting of ashwagandha and *Trichoderma harzianum* showed the best antagonistic activity against this pathogen. In

Table 1. Efficacy of *Trichoderma* strains on mycelial growth inhibition and overgrowth of *Fusarium solani* in dual culture

Strains	Efficacy of *Trichoderma*[1]	
	Percentage of inhibition of radial growth (PIRG) [2]	Mycelial overgrowth (%) [3]
T. virens IMI -392430	59.36 c	52.54 c
T. pseudokoningii IMI -392431	57.39 d	45.5 d
T. harzianum IMI -392432	69.32 a	59.28 a
T. harzianum IMI -392433	65.63 b	54.23 c
T. harzianum IMI -392434	61.66 c	56.78 b

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

[2] PIRG value of *Fusarium solani* by *Trichoderma* strains on PDA in dual culture test calculated from four replications.

[3] Means of inhibition of mycelia over growth of *Fusarium solani* by *Trichoderma* strains on PDA in dual culture test calculated from four replications.

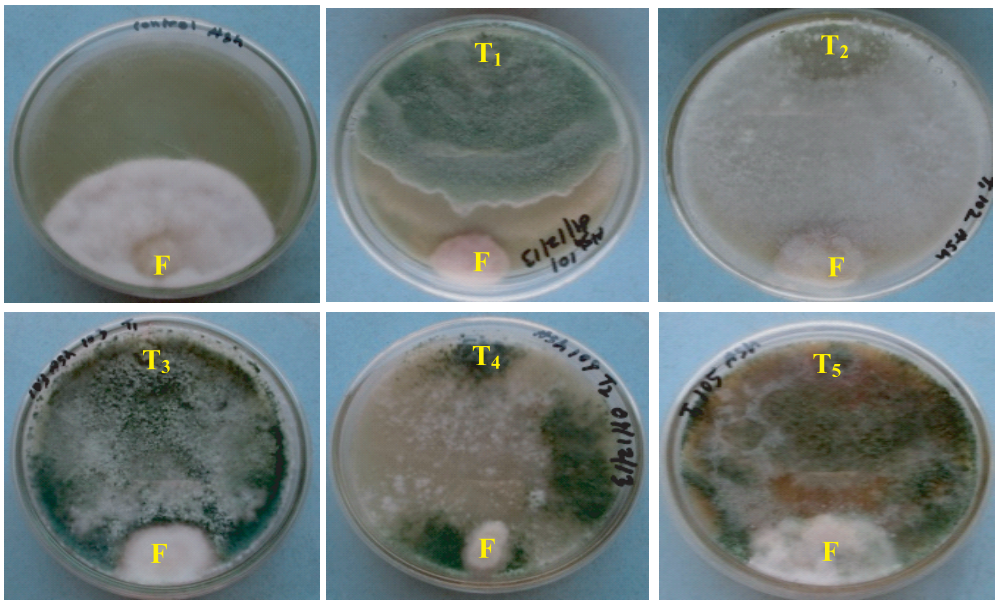

Figure 3. Effects of *Trichoderma* strains against *F. solani* in dual culture technique. F, T_1, T_2, T_3, T_4 and T_5 indicates *F. solani*, *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI- 392432, *T. harzianum* IMI-392433, and *T. harzianum* IMI-392434, respectively.

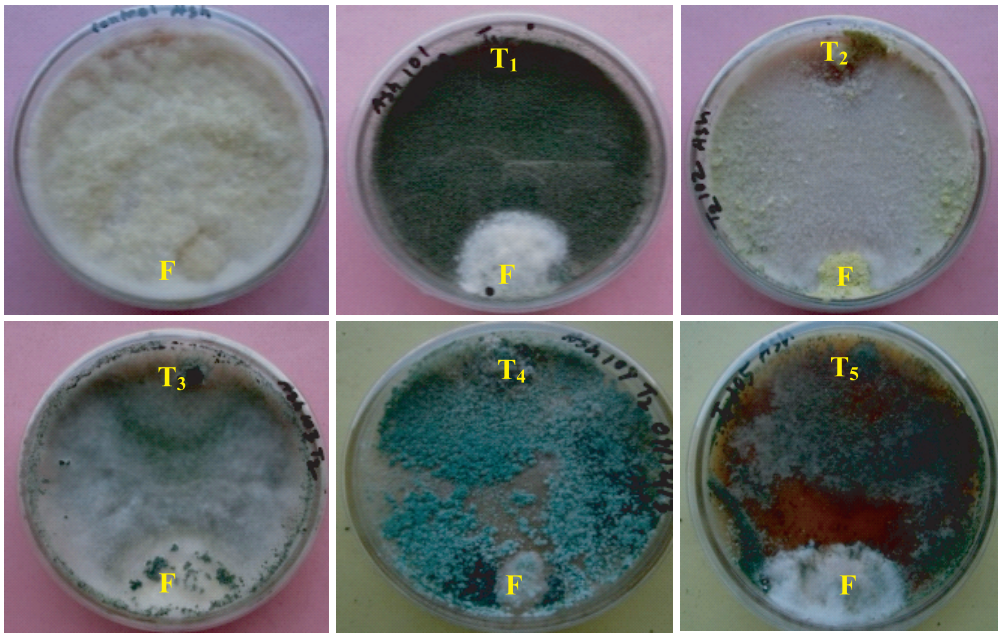

Figure 4. Overgrowth of *Trichoderma* covering the *F. solani* colony after 7 days of inoculation in dual culture. F, T_1, T_2, T_3, T_4 and T_5 indicates *F. solani*, *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI- 392432, *T. harzianum* IMI-392433, and *T. harzianum* IMI-392434, respectively.

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. In overgrowth test, all strains of *Trichoderma* of *F. solani* showed overgrowth percentages ranging from 45.5 to 59.28 (Figure 4 and Table 1). Strain *T. harzianum* IMI-392432 provided the highest percent overgrowth about 59.28% followed by *T. harzianum* IMI-392434 (56.78%), *T. harzianum* IMI-392433 (54.23%), *T. virens* IMI-392430 (52.54%) and *T. pseudokoningii* IMI-392431 (45.5%). This overgrowth may be due to its fast growing nature, rapid sporulation or secretion of cell wall lytic enzymes in dual culture (Sharma and Trivedi 2010). Elad (1996) stated that the mechanisms of the antagonism of *Trichoderma* spp. against different pathogens may be due to mycoparasitism, competition and antibiosis. In a previous study, 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 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.

Normal poison agar technique test

The three concentrations i.e. 25, 50 and 75% of culture filtrates of five *Trichoderma* strains were tested against mycelial growth of *F. solani* in normal poison agar technique. The PIMG values by culture filtrates of *Trichoderma* strains varied significantly (P=0.05) at different concentrations after 10 days of incubation (Table 2 and Fig 5). The highest PIMG value (76.23 %) was achieved at 75% concentration by culture filtrate of *T. harzianum* IMI-392432 followed by *T. harzianum* IMI-392433 (73.54%), *T. harzianum* IMI-392434 (71.36%), *T. virens* IMI-392430 (69.73%) and *T. pseudokoningii* IMI-392431 (64.65%). The lowest PIMG value 39.12% was recorded at 25% concentration of *T. pseudokoningii* IMI-392431. Among five *Trichoderma* strains, culture filtrate of *T. harzianum* IMI-392432 was the best for PIMG. In this case, the actual effect and mechanism involved was 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). Dennis and Webster (1971) and Jinantara (1995) showed that culture filtrate produced by *Trichoderma* contained inhibitory substances against microorganisms. Among the antibiotics produced by *T. harzianum* were 6-n-pentyl-2H-pyran-2-one, 6-n-pentyl-2H-pyran-2-one,pyridine, anthraquinones, butenolides, isonitrin D and F, trichorzianines and furanone (Claydon *et al*. 1987). Growth inhibition of the pathogens by the *Trichoderma* metabolites has been reported by several workers (Dennis and Webester 1971; Howell and Stipanovic 1983; Sivan *et al*. 1984; Claydon *et al*. 1987; Ghisalberti and Sivasithamparam 1991; Howell 1998). The metabolites released in the culture filtrates by *Trichoderma* strains in the present investigation may be toxic to *F. solani* that inhibited mycelia growth of the pathogen. This suggests that the antibiotics possibly play an important role in suppressing infection by the pathogen.

Efficacy to inhibit conidial germination

Percent inhibition of conidial germination (PICG) of *F. solani* varied significantly different (P = 0.05) by the application of different concentrations of culture filtrate of each *Trichoderma* strains at 12 hours of incubation (Table 3). The highest percent inhibition of conidial germination (82.86%) was achieved at 75% concentration of *T. harzianum* IMI-392432 followed by *T. harzianum* IMI-392433 (77.91%), *T. harzianum* IMI-392434 (73.85%), *T. virens* IMI-392430 (71.98%) and *T. pseudokoningii*

Table 2. Effect of culture filtrate of *Trichoderma* strains on the percentage inhibition of mycelia growth (PIMG) of *Fusarium solani* after 10 days of incubation

Strains	Percent inhibition of mycelia growth (PIMG)[1]		
	Culture filtrate of *Trichoderma* (%) [2]		
	25	50	75
T. virens IMI -392430	42.54 d	54.03 d	69.73 c
T. pseudokoningii IMI -392431	39.12 e	50.17 e	64.65 d
T. harzianum IMI -392432	53.69 a	62.88 a	76.23 a
T. harzianum IMI -392433	49.64 b	60.25 b	73.54 b
T. harzianum IMI -392434	47.37 c	58.37 c	71.36 c

[1]Means of inhibition of mycelia growth percentage calculated from three replications

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

Figure 5. Effects of culture filtrate of *Trichoderma* strains in PDA on mycelial growth of *F. solani* by normal poison agar technique at 25, 50 and 75% concentration after 10 days of incubation. CF= culture filtrate of *Trichoderma*, T_1= *T. virens* IMI-392430, T_2= *T. pseudokoningii* IMI-392431, T_3= *T. harzianum* IMI- 392432, T_4= *T. harzianum* IMI- 392433, T_5= *T. harzianum* IMI- 392434

Table 3. Efficacy of culture filtrates of *Trichoderma* on the percentage inhibition of conidial germination (PICG) of *Fusarium solani* within 12 h of incubation

Strains	Percent Inhibition of Conidial Germination[1]		
	Culture filtrate of *Trichoderma* (%)[2]		
	25	50	75
T. virens IMI-392430	43.84 c	63.47 d	71.98 d
T. pseudokoningii IMI-392431	39.68 d	57.53 e	66.26 e
T. harzianum IMI-392432	53.28 a	72.33 a	82.86 a
T. harzianum IMI-392433	49.26 b	68.94 b	77.91 b
T. harzianum IMI-392434	47.51 b	65.68 c	73.85 c

[1]Means of inhibition of spore germination calculated from four replications (100 spores per replication)

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

IMI-392431 (66.26%). Least percent inhibition (39.68%) of conidial germination of *F. solani* was recorded at 25% concentration of *T. pseudokoningii* IMI-392431 (39.68%). The strain *T. harzianum* IMI-392432 was found to be more potential, due to their highest conidial inhibition percentage. This result were in conformation with Intana *et al*. (2007) who tested two wild type strains of *T. harzianum* for efficacy test to inhibit conidial germination and germ tube formation of *Colletotrichum capsici* causing chili anthracnose and observed that antifungal metabolite extraction of these strains completely inhibited both the spore germination and germ tube formation of the pathogen.

Conclusion

In vitro results obtained in this study suggest that *T. harzianum* IMI 392432 is the best for inhibition of the mycelial growth and conidial germination of *F. solani*. This strain can be used as potential biological control agent to control of root rot disease of Ashwagandha. The bio-agents might be exploited for future plant disease management that enhances our economic loss through overcoming any environmental risk.

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