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Evaluation of Bio-Control Efficacy of *Trichoderma* Strains against *Alternaria alternata* Causing Leaf Blight of Ashwagandha [*Withania somnifera* (L.) Dunal]

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Abstracts

Ashwagandha is an important ancient medicinal crops, being affected with many diseases, among which leaf blight caused by *Alternaria alternata* has become the constraint resulting in huge yield losses. Continuous usage of chemical methods leads to environment, soil and water pollution. Whereas biological control of diseases is long lasting, inexpensive, eco-friendly and harmless to target organisms. In this context, it is aimed to evaluate five *Trichoderma* strains viz. *Trichoderma virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433 and *T. harzianum* IMI-392434 as bio-control efficacy against *A. alternata* and growth promoting effect in Ashwagandha. All the *Trichoderma* strains had varied antagonistic effects against the pathogen. In dual culture technique, the strain *T. harzianum* IMI-392433 showed maximum percentage inhibition of mycelial growth (54.89%) followed by *T. harzianum* IMI-392432 (53.83%), *T. harzianum* IMI-392434 (48.94%) and *T. virens* IMI-392430, (43.62%) against the pathogen, while the least inhibition percentage was observed with the *T. pseudokoningii* IMI-392431 (36.60%). The culture filtrate of the *Trichoderma* strain, *T. harzianum* IMI-392433 recorded highest inhibition on the mycelial growth (39.05%) and spore germination (80.77%) of pathogen and the lowest was recorded in *T. pseudokoningii* IMI-392431 (20.45 and 50%). Moreover, seeds treated with spore suspension of the strain *T. harzianum* IMI-392433 reduced the percentages of disease severity index significantly. The strain *T. harzianum* IMI-392433 also significantly increased seed germination %, seedling vigor and growth of Ashwagandha. The correlation matrix showed that root yield per plant of Ashwagandha had significant and positive correlation with plant height (r=0.726**), number of leaf (r=0.514**), number of primary branch (r=0.820**), number of secondary branch (r=0.829**), fresh plant weight (r=0.887**), plant dry weight (r=0.613**), root length (r=0.824**), root diameter (r=0.786**), root dry weight (r=0.739**) and fresh root weight (r=0.731**). The significant and negative correlation (r=-0.336**) was observed with the root yield and percentages of disease severity index. The study recognized that the *T. harzianum* IMI-392433 strain performed well in inhibiting the mycelial growth and reduced the percentages of disease severity index of pathogen as well as increased the plant growth in Ashwagandha.

Key Words: *Withania somnifera*, leaf blight, *Alternaria alternata*, *Trichoderma*, biological control

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Introduction

Ashwagandha [*Withania somnifera* (L). Dunal.] is an important medicinal plant belonging to the family Solanaceae. It grows well in dry and sub-tropical regions of India, Sri Lanka and Bangladesh (Agarwal et al. 2004). The plant is most important due to presence of alkaloids, withanoloids, steroids, lactones which have antimicrobial properties. It has high reputation in traditional Indian medicine and is one of the most extensively used plants in Ayurveda and Unani medicines (Bhat et al. 2014). The roots of this medicinal plants are prescribtible for curing general sexual weakness in human. Nowadays Ashwagandha is cultivated for medicinal purposes in fields and open grounds throughout the Bangladesh. Due to high demand of raw materials of this medicinal plant and also market assurance of some reputed pharmacital companies, the farmers of the northern districts of Bangladesh especially the Natore districts of Bangladesh have come forward to cultivate this important medicinal plant commercially. At present, farmers are cultivating Ahwagandha in different districts of Northern part of Bangladesh such as Natore, Bogra, Gaibandha, Joypurhat and Naogaon. At this time, farmers are facing various diseases at the field level, resulting in reduced yields. Among the various diseases, leaf blight of Ashwangandha caused by *Alternaria alternata* is the most prevalent disease, which is most severe in the plains of Bangladesh. Blight first appear on older, lower leaves, progressing upward to the new growth. Blight have yellow halo and concentric ring pattern of lines in tan centers. Application of the fungicides is not economical in the long time because they pollute the environment, leave harmful residues and can lead to the development of resistant strains of the pathogen with repeated use (Vinale et al. 2008). Furthermore, Ashwagandha plant parts is used directly as a medicine. Uses of chemical fungicides are extremely harmful to the human body. Replacement of chemical fungicides with bio-control agents are an alternative mean to; manage the plant pathogens, produce safely food and reduce the environment pollution (Barakat and Al-Masri 2005).

Trichoderma is a filamentous soil fungus that functions as a bio-control agent for a wide range of economically important aerial and soil borne plant pathogens (Harman et al. 2004). *Trichoderma* spp. also are commercially marketed as biopesticides, bio-fertilizers and soil amendments. The use of *Trichoderma* fungi in agriculture can provide numerous advantages such as; colonization of the root and rhizosphere of plant, control of plant pathogens by different mechanisms, improvement of the plant health by promote plant growth and stimulation of root growth (Harman et al. 2004). The genus *Trichoderma* is not only one of the most common, isolated from various habitats soil fungi but also known to be secreting to the environment various secondary metabolites. 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 (Vey et al. 2001), which provide to protect plant from disease (Chet et al. 1997). Therefore, the objectives of this investigation were to assay the effect of five *Trichoderma* strains against leaf blight pathogen of *A. alternata* under *in vitro* condition. Plant growth and disease incidence percentage were also investigated to evaluate their performance under *in vivo* conditions.

Materials and Methods

Isolation and identification of pathogen

A. alternata was isolated from naturally infected *W. somnifera* plants in the field plantation. *W. somnifera* plants showing typical leaf blight symptoms were collected from Forest Protection Division (FPD) nursery at Bangladesh Forest Research Institute (BFRI), Chattogram, Bangladesh. The infected leaves were collected and put in poly bags and transferred to the laboratory. The infected leaves were cut into 5 mm pieces and surface sterilized with 0.5% sodium hypochloride solution for 5 minutes and rinsed trice with sterilized water. The pieces were dried with sterile filter paper and placed (3 pieces per plate) on fresh Potato Dextrose Agar (PDA) medium impregnated with streptomycin (0.5mg mL^{-1}) to prevent bacterial growth and incubated for 7-10 days at 28 to 30°C. The resulting *A. alternata* colonies were sub cultured by transferring small mycelia plugs from the colony margin. For storage, the fungal isolates were maintained on PDA slant. The fungal isolates were identified based on their colonial morphology. Microscopic examination of fungal isolates was carried at both vegetative

and sporulating stages.

Morphology of the fungal pathogen

Morphological characters of the fungal pathogen infecting *W. somnifera* were studied from the culture growth on PDA for 5 to 10 days at 27±1°C. As suggested by Chowdhry and Varshney (2000), observations regarding morphological characters of different structures viz., mycelium (young and matured), conidiophores, conidia, and chlamydospores were noted by adopting slide culture technique. The microscopic measurements were taken with the help of ocular micrometer. The measurements for young and old mycelium were recorded from five and ten day's old cultures, respectively.

Pathogenicity test

Pathogenicity of the fungus was confirmed by inoculating healthy leaves Ashwagandha plants grown in an experimental field. One month old healthy Ashwagandha saplings of local cultivar were selected for proving the pathogenicity of the fungal pathogen. Distinctly, ten leaves per experiment were sprayed with @ 5 mL of conidial suspension (1×10^5 conidia mL^{-1}) obtained from 7-day-old culture plates. Before inoculation, all plants were disinfected with 0.1% sodium hypochlorite solution for 1 min and rinsed out thoroughly with sterile distilled water. Only water was sprayed for control treatment. After inoculations, control and inoculated leaves were covered with polythene bags for 15 days.

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 and were verified by CABI Bioscience, Surrey, U.K.

Antagonism of Trichoderma in dual culture on A. alternate

The dual culture test was performed to test the ability of *Trichoderma* strains against *A. alternata* for mycelial growth inhibition and over growth. *Trichoderma* strains and *A. alternata* was sub cultured onto PDA for 7 days. The margin of colony of *A. alternata* 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 *A. alternata* was also placed at the periphery but on the opposing end of the same Petri dish. As a control, *A. alternata* was placed in a similar manner on a fresh PDA plate. All pairings were carried out in quadruplicate and incubated at 28±2°C. Antagonistic activity was tested 7 days after incubation by measuring the radius of the *A. alternata* colony in the direction of the antagonist colony (R_2) and the radius of the *A. alternata* 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 *A. alternata* were observed and compared with the control treatment. The inhibition levels were calculated by using the formula; $[(R_1-R_2)/R_1]\times100$, when R_1 was the mean of colony radius of *A. alternata* in the control dish and R_2 was the mean of colony radius of *A. alternata* 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; $[(D_1-D_2)/T_d]\times100$, 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.

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

The inhibition of the mycelial growth of *A. alternata* was tested by metabolites secreted by *Trichoderma* in liquid culture medium as per method given by Dennis and Webster (1971). Fifty mL of sterilized 1/5 strength potato dextrose broth (PDB) were dispensed into 250 mL Erlenmeyer flasks and inoculated with a 5 mm diameter disc from edge of four days old culture of the *Trichoderma* culture plates. Each flask was inoculated each in triplicate and set up was shaken at 100 rpm for 15 days at 28+2°C on Thermostat Culture Shaker. After 15 days, the culture filtrates were filtered through Whatman No.1 filter paper and sterilized by

millipore membrane filtration of 0.25 μm and stored at 4°C for further use. The sterilized culture filtrates of *Trichoderma* and molten double strength PDA were mixed together in equal proportion (1:1). The medium was then poured into the Petriplate @ 20 mL/plate. After solidification, the Petriplates were carefully inoculated with 5 mm discs of the test pathogen cut from the four day old culture. PDA plates inoculated with the test pathogen but not amended with culture filtrate were maintained as control. Plates were then incubated in an incubator at 28±2°C. Four replications were maintained for each treatment, one dish per a replicate. Periodic observations on radial growth of mycelium were recorded after 10 days of inoculation (Khan and Sinha 2007). Inhibition percentage of mycelial growth of test pathogen was calculated by the formula: I=(C−T)/C ×100 Where, I=Percent inhibition in growth of test pathogen, C=Radial growth of pathogen (mm) in control, T=Radial growth of pathogen (mm) in treatment.

Effect of culture filtrates on conidial germination of pathogen

Conidia obtained from 10-day-old PDA cultures of *A. alternata* were suspended in sterilized distilled water. The concentration of spore suspension was adjusted with a haemacytometer to 1×10^5 spores per mL. Two hundred μL of a mixture containing: 80 μL of conidial suspension, 100 μL of 1/5 strength Potato Dextrose Broth (PDB) and 20 μL of culture filtrate of *Trichoderma*, were pipette into each well of the microtitration plate and kept at 25±2°C for 24 hours. One plate row was filled with untreated spore suspension in 1/5 strength Potato Dextrose Broth (PDB) as negative control. Conidial germination 24 hours after inoculation was assessed by mounting 10 μL samples on a glass slide and counting the geminated spores at 100× magnifications. The percentage germination recorded for the four wells was averaged. 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 $PICG=C_1-C_2/C_1\times100$

Effect of Trichoderma strains on growth of Ashwagandha in vivo

To evaluate the efficacy of *Trichoderma* strains at controlling *Alternaria* leaf blight disease and growth promoting effect of Ashwagandha under *in vivo* condition, a pot trial experiment was conducted at the Forest Protection Division Laboratory, Bangladesh Forest Research Institute (BFRI), Chattogram, Bangladesh from January 2017 to June 2017.

Preparation of pot

Soil was collected from the research field of Bangladesh Forest Research Institute and mixed with sand at the portion of 3:1. Then autoclaved at 120°C for 15 minutes. The plastic pots of 10×30 cm diameter were filled with sandy loam soil (Ahmed and Meisner 1996).

Preparation of inoculum

Mycelial disc (5 mm diameter) of *Trichoderma* strains and *A. alternata* were obtained from 4-5 days old culture and separately transferred to 50 mL PDA in a 250 mL conical flask and incubated at 28°C. After incubation, 30 mL of sterile distilled water was added to each culture and the flask was shaken at 50 rpm for 30 min in an orbital shaker. Then the content of each conical flask was filtered through muslin cloth. The culture filtrate, containing the spores, was collected, and a concentration of 5×10^5 spores/mL was obtained by dilution with sterilized distilled water.

Seed treatment

Local variety of Ashwagandha seeds were collected from Forest Protection Division, Bangladesh Forest Research Institute (BFRI), Chattogram, Bangladesh. Pathogen-free healthy seeds were selected for use in this experiment.

For seed treatment, 10 to 15 seeds were dipped in the spore suspension (5×10^5 spores/mL) of 4-5 days old *Trichoderma* strains for about 20 min, and the treated seeds were dried by laminar air flow. After that, both *Trichoderma* treated and untreated seeds were again dipped in the spore suspension (3×10^5 spores/mL) of 7 days old culture of *A. alternata* for about 20 min and then dried by laminar air flow. After germination of treated seeds, the pot soil was treated with 30 mL of conidial suspension (combination of *Trichoderma* strains and *A. alternata*) according to respective treatment. The treatment was continued up to harvesting with seven days interval.

The experiments were design with the following combinations:

T_0=Control (untreated foliage and untreated seeds); T_1=*A. alternata* (3×10^5 spores/mL); T_2=*T. virens* IMI-392430 (5×10^5 spores/mL)+*A. alternata* (3×10^5 spores/mL); T_3=*T. pseudokoningii* IMI-392431 (5×10^5 spores/mL)+*A. alternata* (3×10^5 spores/mL); T_4=*T. harzianum* IMI-392432 (5×10^5 spores/mL)+*A. alternata* (3×10^5 spores/mL); T_5=*T. harzianum* IMI-392433 (5×10^5 spores/mL)+*A. alternata* (3×10^5 spores/mL); T_6=*T. harzianum* IMI-392434 (5×10^5 spores/mL)+*A. alternata* (3×10^5 spores/mL); T_7=*T. virens* IMI-392430 (5×10^5 spores/mL); T_8=*T. pseudokoningii* IMI-392431 (5×10^5 spores/mL); T_9=*T. harzianum* IMI-392432 (5×10^5 spores/mL); T_{10}=*T. harzianum* IMI-392433 (5×10^5 spores/mL); T_{11}=*T. harzianum* IMI-392434 (5×10^5 spores/mL).

Foliar spray

Thirty days old Ashwagandha seedlings were sprayed with spore suspension @ 5 mL of *Trichoderma* strains (5×10^5 spores/mL) with 0.05% Tween 20. After 24 h, the plants were sprayed with the spore suspension of *A. alternata* @ 5 mL of conidial suspension (3×10^5 spores/mL) obtained from 15-day-old culture plates. The seedlings which were sprayed with *A. alternata* alone (3×10^5 spores/mL), served as control. Observations on the leaf blight incidence were recorded seven days interval up to six month after inoculation of pathogen and the percentage of disease severity index was calculated for each individual treatment (Desai et al. 2004).

Data recording

All experiments were established as a completely randomized design with three replications and ten plants were used as each replication.

Seed germination percentages and vigor index was recorded after 3 to 10 days.

Vigour index for each treatment was determined using the following formula developed by Abdul-Baki and Anderson (1973).

Vigour index=[Mean of root length (cm)+Mean of shoot length (cm)]×percentages of seed germination.

Ashwagandha growth parameters namely; plant height, number of leaf, number of primary branch, number of secondary branch, fresh plant weight (gm), plant dry weight (gm), root length (cm), root diameter (cm), root dry weight (gm), fresh root weight (gm) and percentages of disease severity index (PDSI) were determined 180 days after sowing.

Assessment of the disease severity index of *Alternaria* leaf blight of Ashwagandha was made with the help of the descriptive scale developed by Sharma and Kolte (1994), using 0-5 scale rating. Where, 0=no symptoms of leafs; 1=1-10% leaf area infected; 2=11-25% leaf area infected; 3=26-50% leaf area infected; 4=51-75% leaf area infected; 5=>75% leaf area infected. For epidemiological studies, this scale was used throughout the investigation. For disease severity 10 plants (100 leaves) per replication

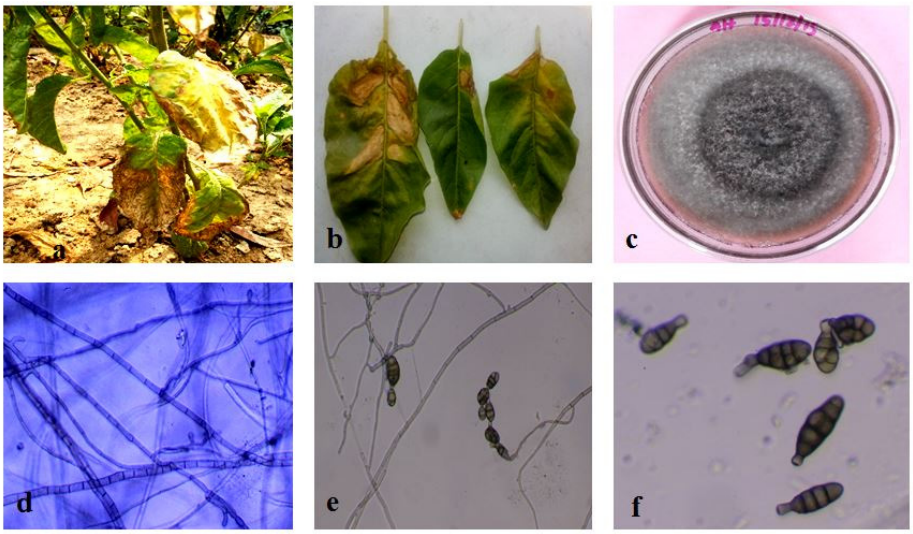

Fig. 1. Disease symptoms colony and conidia of *A. alternata*. (a, b) Leaf blight symptoms of Ashwagandha. (c) Colonies of *A. alternata* on PDA after 7 days. (d) Mycelium of *A. alternata*. (e, f) Conidia of *A. alternate*.

was randomly selected and percentages of disease severity index (PDSI) was calculated.

The percentages of disease severity index (PDSI) was calculated using the formulas of the Mckinney (1923).

Percentages of disease severity index (PDSI)=(sum of all disease rating/total no. of rating×maximum disease grade)×100.

Statistical analysis

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

Results and Discussion

Morphological and microscopic characteristics of A. alternata

Morphological observations of the fungus were recorded by adopting slide culture technique. The fungus produced profuse mycelial growth on PDA (Fig. 1c). Initially, the mycelium was hyaline that turned to grey-brownish, multicelled, septate and irregularly branched. Conidia were produced in long chains, pale to light brown, obpyriform, with a beak one to seven transverse and up to three longitudinal septa, and measured 10 to 45 μm long×7 to 22.5 μm wide. Conidiophores were straight, septate, light to olive golden brown with conidial scar, and measured 35 to 100 μm long×2 to 5 μm wide (Fig. 1d-f). The morphological descriptions and measurements of the fungus are similar to *A. alternata* (Simmons 2007).

Pathogenicity tests of A. alternata

Pathogenicity tests conducted on healthy leaves of *W. somnifera* resulted in the development of typical symptoms of the disease on leaves after 10-12 days, whereas no symptoms were observed on control plants. The same pathogen was consistently re-isolated and identified from all inoculated plants, confirming Koch's postulates (Data not shown).

Antagonism of Trichoderma in dual culture on A. alternate

All the five *Trichoderma* strains significantly exhibited antibiotic potential against *A. alternata* by inhibiting its mycelial growth in duel culture technique (Fig. 2a). Seven days after inoculation, growth of *A. alternata* was found to be inhibited by *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433, and *T. harzianum* IMI-392434 strains and attained a inhibition percentages of 43.62, 36.6, 53.83, 54.89 and 48.94 respectively. Strains *T. harzianum* IMI-392433 showed the highest PIRG value of 54.89%, while the least inhibition percentage was observed with the isolate *T. pseudokoningii* IMI-392431 (36.60%). This indicates that *T. harzianum* IMI-392433 strain had maximum antifungal activity against *A. alternata* compared to the other *Trichoderma* strains (Table 1). In a similar study, Akbari and Parakhi (2007) reported that *T. viride*-I and *T. hamatum*-IV&V isolates showed strong antagonism against *Alternaria alternata* causing blight of sesame. Ambuse et al. (2012) tested three *Trichoderma* species viz., *T. viride*, *T.*

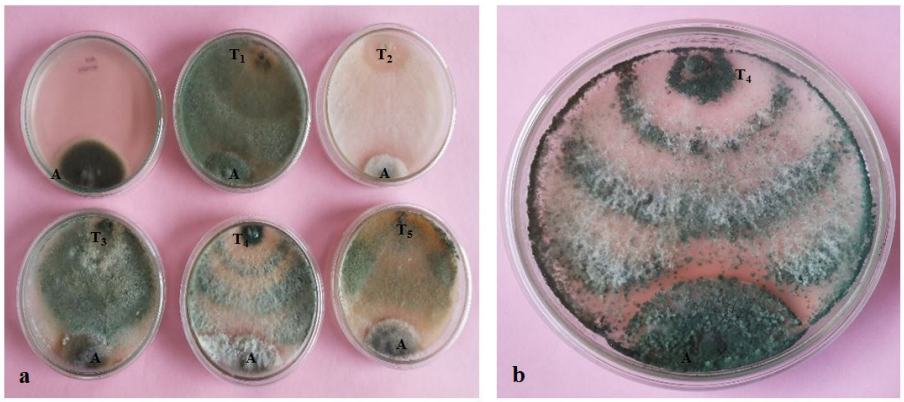

Fig. 2. (a) Antagonistic effects of *Trichoderma* strains against *A. alternata* in dual culture technique. F, T_1, T_2, T_3, T_4 and T_5 indicates *A. alternata*, *T. virens* IMI-392430, *T. pseudokoningii* IMI-392431, *T. harzianum* IMI-392432, *T. harzianum* IMI-392433, and *T. harzianum* IMI-392434, respectively. (b) Overgrowth of *Trichoderma* covering the *A. alternata* colony after 7 days of inoculation in dual culture.

Table 1. *In vitro* screening and comparative effect of culture filtrates of *Trichoderma* strains on growth and spore germination of *A. alternata*

Strains	Dual culture technique[1]			Culture filtrates of *Trichoderma*[1]		Conidial inhibition (%)[1]	
	Radial growth of *A. alternate* (mm)	Percent inhibition over control[2]	Mycelial overgrowth (%)[3]	Radial growth of *A. alternate* (mm)	Percent inhibition over control	Number of conidia germinated	% inhibition of conidia
T. virens IMI-392430	2.65b	43.62c	51.75d	3.65a	25.36d	18c	65.38c
T. pseudokoningii IMI-392431	2.98ab	36.60d	48.32e	3.89a	20.45e	26b	50d
T. harzianum IMI-392432	2.17b	53.83a	57.89ab	3.28a	32.93c	12d	75.92b
T. harzianum IMI-392433	2.12b	54.89a	59.31a	2.98a	39.05a	10e	80.77a
T. harzianum IMI-392434	2.4b	48.94b	56.29bc	3.17a	35.17b	12d	76.93b
Control	4.7a	00e	00f	4.89a	00f	52a	00e

[1]Means in a column followed by the same letter(s) are not significantly different according to DMRT ($p=0.05$); [2]means of inhibition of mycelia growth of *A. alternata* by *Trichoderma* strains on PDA in dual culture test calculated from four replications; [3]means of inhibition of mycelia over growth of *A. alternata* by *Trichoderma* strains on PDA in dual culture test calculated from four replications; [4]means of inhibition of mycelia growth of *A. alternata* by *Trichoderma* culture filtrates on PDA calculated from four replications.

koningii and *T. pseudokoningii* against *Alternaria tenuissima* and found 80% antagonistic activity of *Trichoderma* sp. against *Alternaria tenuissima*.

All strains of *Trichoderma* overgrew mycelia of *A. alternata* with overgrowth percentages ranging from 48.32 to 59.31 (Fig. 2b and Table 1). Strain *T. harzianum* IMI-392433 provided the highest percent overgrowth about 59.31% followed by *T. harzianum* IMI-392432 (57.89%), *T. harzianum* IMI-392434 (56.29%), *T. virens* IMI-392430 (51.75%) and *T. pseudokoningii* IMI-392431 (48.32%), respectively. 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).

Determination of inhibitory activity of culture filtrates of Trichoderma using normal poison agar technique

The culture filtrates of five *Trichoderma* strains were tested against mycelial growth of *A. alternata* in normal poison agar technique. The Percent Inhibition of Mycelial Growth (PIMG) values by culture filtrates of *Trichoderma* strains varied significantly ($p \leq 0.05$) after 10 days (Table 1 and Fig. 3b). The highest PIMG values (39.05%) were achieved by culture filtrate of *T. harzianum* IMI-392433 followed by *T. harzianum* IMI-392434 (35.17%), *T. harzianum* IMI-392432 (32.93%), *T. virens* IMI-392430 (25.36%) and *T. pseudokoningii* IMI-392431 (20.45%), respectively. The lowest PIMG values, 20.45%, were recorded at *T. pseudokoningii* IMI-392431. Among five *Trichoderma* strains, culture filtrate of *T. harzianum* IMI-392433 was the best for PIMG of *A. alternata*. It is clear from the present study that the antagonistic fungus inhibited the growth of test pathogens by the production of nonvolatile compounds indicating the main mechanism involved in biocontrol is antibiosis (Shanmugam and Varma 1999; Hazarika et al. 2000). In a similar study, Dennis and Webster (1971) and Jinantara (1995) reported 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, pyridine, anthraquinones, butenolides, isonitrin D and F, trichorzianines and furanone (Claydon et al. 1987). The metabolites released in the culture filtrates by *Trichoderma* strains in the present investigation may be toxic to *A. alternata* that inhibited mycelia growth of the pathogen. This suggests that the antibiotics possibly play an important role in suppressing infection by the pathogen.

Effect of culture filtrates on conidial germination

Percent inhibition of conidial germination (PICG) of *A. alternata* varied significantly different ($p \leq 0.05$) by the application of culture filtrate of each *Trichoderma* strains at 24 hours of incubation (Table 1). The highest percent in-

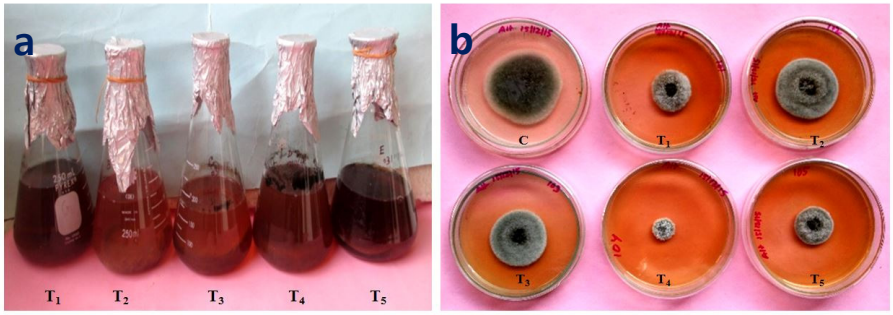

Fig. 3. (a) Culture filtrates of *Trichoderma* strains. (b) Effects of culture filtrates of *Trichoderma* strains in PDA on mycelial growth of *A. alternata* by normal poison agar technique at 80% concentration after 10 days of incubation. C=control; T_1= *T. virens* IMI-392430; T_2= *T. pseudokoningii* IMI-392431; T_3= *T. harzianum* IMI-392432; T_4= *T. harzianum* IMI-392433; T_5= *T. harzianum* IMI-392434.

Table 2. Effect of *Trichoderma* strains on seed germination % and vigour index of Ashwagandha under different treatments

Treatments	Seed germination (%)	Shoot length (cm)	Root length (cm)	Vogour index
T_0	68.58g	2.42ab	2.88abc	372.69j
T_1	45.28h	1.16b	1.86c	354.18k
T_2	70.32g	2.89ab	2.53bc	601g
T_3	69.61g	2.87ab	3.52abc	540i
T_4	73.41f	3.29a	3.12abc	681h
T_5	87.38b	3.64a	3.89ab	776e
T_6	78.46e	3.54a	3.87ab	775e
T_7	83.95c	3.86a	3.86ab	741f
T_8	81.58d	3.79a	3.97ab	785c
T_9	87.51b	3.88a	3.97ab	782d
T_{10}	90.43a	3.97a	4.76a	840a
T_{11}	88.98ab	3.96a	4.16ab	813b

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

T_0=Control (untreated foliage and untreated seeds); T_1=*A. alternate*; T_2= *T. virens* IMI-392430+*A. alternate*; T_3= *T. pseudokoningii* IMI-392431+*A. alternate*; T_4= *T. harzianum* IMI-392432+*A. alternate*; T_5= *T. harzianum* IMI-392433+*A. alternate*; T_6= *T. harzianum* IMI-392434+*A. alternate*; T_7= *T. virens* IMI-392430; T_8= *T. pseudokoningii* IMI-392431; T_9= *T. harzianum* IMI-392432; T_{10}= *T. harzianum* IMI-392433; T_{11}= *T. harzianum* IMI-392434.

hibition of conidial germination (80.77%) was achieved at *T. harzianum* IMI-392433 followed by *T. harzianum* IMI-392434 (76.93%), *T. harzianum* IMI-392432 (75.92%), *T. virens* IMI-392430 (65.38%) and *T. pseudokoningii* IMI-392431 (50%), respectively. Least percent inhibition of conidial germination of *A. alternata* was recorded of *T. pseudokoningii* IMI-392431 (50%). The strain *T. harzianum* IMI-392433 was found to be more effective, due to their highest conidial inhibition percentage. This result were in conformation with Rahman et al. (2014), who tested five *Trichoderma* strains for efficacy to inhibit conidial germination and germ tube formation of *Fusarium solani* causing root rot Ashwagandha and observed that antifungal metabolites extractions of these strains completely inhibited both the spore germination and germ tube formation of the pathogen.

Plant growth promotion

The effects of spore suspension of the *Trichoderma* strains on Ashwagandha seed germination and growth promotion are presented in Tables 2 and 3. The *Trichoderma* strains increased the germination percentages, besides increasing the shoot length, root length and vigor index of Ashwagandha seedlings after 10 days of sowing. Among the treatments, *T. harzianum* IMI-392433 (5×10^5 spores/mL) (T_{10}) recorded maximum germination percentage (90.43%), shoot (3.97 cm) length, root length (4.76 cm) and vigor index (840) compared to *A. alternate* (3×10^5 spores/mL) (T_1) treatment (45.28, 1.16, 1.86 and 354.18, respectively).

After 180 days, plant height, number of leaf, number of primary branch, number of secondary branch, fresh plant weight, plant dry weight, root length, root diameter, root dry weight, fresh root weight, and root yield were highest for T_{10} (*T. harzianum* IMI-392433 and lowest for *A. alternate* (T_1) treatment with a significant difference ($p<0.05$)

Table 3. Effect of seed treatment with *Trichoderma* strains on growth characteristics of Ashwagandha at 180 DAS

Treatments	Plant height (cm)	Number of leaf /plant	Number of primary branch	Number of secondary branch	Fresh plant weight (gm)	Plant dry weight (gm)	Root length (cm)	Root diameter (cm)	Root dry weight (gm)	Fresh root weight (gm)	Root yield/plant (gm)
T_0	15.28h	9.27ef	2.13 a	3.47 cd	4.89de	0.58de	7.39e	2.53d	1.32j	12.79f	1.28ab
T_1	13.78h	8.31f	2.12 a	2.73 d	4.42e	0.18e	7.34e	2.28e	0.89k	8.94g	0.89b
T_2	17.83f	11.26cd	2.16 a	4.86 abc	5.86cde	0.69cd	8.34cde	2.64d	1.43i	16.83e	1.68ab
T_3	15.95g	8.48f	2.14 a	4.42 bcd	5.23de	0.74cd	7.86de	2.53d	1.54h	14.28f	1.43ab
T_4	23.64d	11.73bcd	2.18 a	5.74 ab	6.52bcd	0.72cd	8.96bcde	2.74d	1.63g	20.64d	2.12ab
T_5	26.42c	12.48bc	2.28 a	5.98 ab	6.86abc	0.87bcd	9.34bcd	2.89d	1.75e	23.75c	2.38ab
T_6	20.74e	10.36de	2.12 a	5.14 abc	6.75abc	0.79cd	9.34bcd	2.75d	1.69f	19.63d	1.98ab
T_7	26.87c	11.16cd	2.53 a	6.14 ab	7.57abc	1.21bc	10.21abc	3.76cd	1.79d	26.46b	2.46ab
T_8	22.28de	11.29cd	2.27 a	5.97 ab	7.31abc	0.98bcd	9.37bcd	3.18cd	1.78d	24.62c	2.48ab
T_9	31.14ab	12.51bc	2.86 a	6.48 a	7.64abc	1.32bc	10.34 ab	4.92bc	1.86c	29.58a	2.96a
T_{10}	32.74a	15.49a	3.84 a	6.74 a	8.53 a	1.98a	11.27a	6.76a	2.65a	30.12a	3.04a
T_{11}	29.96b	13.37b	2.63 a	6.32 ab	7.98 ab	1.54b	10.47ab	5.84ab	1.97b	28.42a	2.86a

In a column same letters are not significantly different by DMRT at 5% level.
T_0=Control (untreated foliage and untreated seeds); T_1=*A. alternate*; T_2=*T. virens* IMI-392430+*A. alternate*; T_3=*T. pseudokoningii* IMI-392431+*A. alternate*; T_4=*T. harzianum* IMI-392432+*A. alternate*; T_5=*T. harzianum* IMI-392433+*A. alternate* ;T_6=*T. harzianum* IMI-392434+*A. alternate*; T_7=*T. virens* IMI-392430; T_8=*T. pseudokoningii* IMI-392431; T_9=*T. harzianum* IMI-392432; T_{10}=*T. harzianum* IMI-392433; T_{11}=*T. harzianum* IMI-392434.

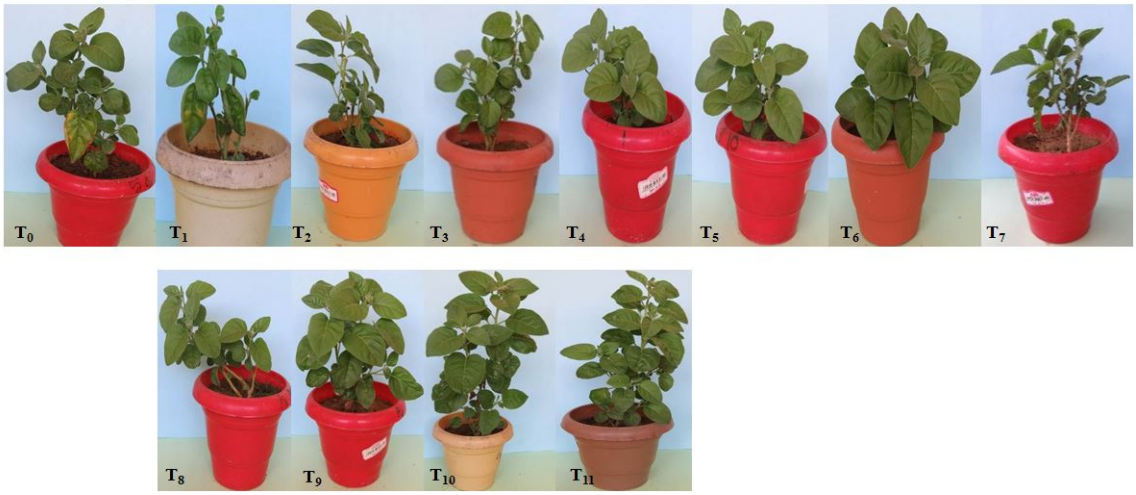

Fig. 4. Effect of *Trichoderma* strains on growth characteristics of Ashwagandha after 180 DAS.
T_0=Control (untreated foliage and untreated seeds); T_1=*A. alternate* (3×10^5 spores/mL); T_2=*T. virens* IMI-392430 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_3=*T. pseudokoningii* IMI-392431 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_4=*T. harzianum* IMI-392432 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_5=*T. harzianum* IMI-392433 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_6 =*T. harzianum* IMI-392434 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_7 = *T. virens* IMI-392430 (5×10^5 spores/mL); T_8=*T. pseudokoningii* IMI-392431 (5×10^5 spores/mL); T_9=*T. harzianum* IMI-392432 (5×10^5 spores/mL); T_{10}=*T. harzianum* IMI-392433 (5×10^5 spores/mL); T_{11}=*T. harzianum* IMI-392434 (5×10^5 spores/mL).

(Table 3 and Fig. 4). These results indicate that *T. harzianum* IMI-392433 has growth-promoting effect on Ashwagandha. The growth promotion exerted by *Trichoderma* might be due to the production of auxin-like compounds (Vinale et al. 2008). Similar beneficial effects on seed germination and seedling vigour was observed with inoculation

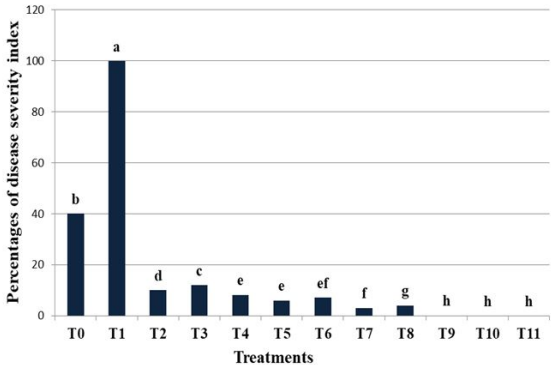

Fig. 5. Effect of *Trichoderma* strains on leaf blight disease severity index of Ashwagandha after six month of sowing. Bars marked by the same letters are not significantly different ($p<0.05$) by DMRT analysis.
T_0=Control (untreated foliage and untreated seeds); T_1=*A. alternate* (3×10^5 spores/mL); T_2=*T. virens* IMI-392430 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_3=*T. pseudokoningii* IMI-392431 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_4=*T. harzianum* IMI-392432 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_5=*T. harzianum* IMI-392433 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_6=*T. harzianum* IMI-392434 (5×10^5 spores/mL)+*A. alternate* (3×10^5 spores/mL); T_7=*T. virens* IMI-392430 (5×10^5 spores/mL); T_8=*T pseudokoningii* IMI-392431 (5×10^5 spores/mL); T_9 = *T. harzianum* IMI-392432 (5×10^5 spores/mL); T_{10}=*T. harzianum* IMI-392433 (5×10^5 spores/mL); T_{11}=*T. harzianum* IMI-392434 (5×10^5 spores/mL).

of *Trichoderma* spp. has been previously reported (Jadhav and Ambadkar 2007).

Percentages of disease severity index (PDSI) of Ashwagandha

Application of *Trichoderma* strains was significantly ($p\leq0.05$) intimidated the disease severity index (%) compared to *A. alternata* (T_1) treatment (Fig. 5). The highest percentages of disease severity index (%) was recorded in T_1 (*A. alternata*) treatment and the lowest was recorded at *T. harzianum* IMI 392432 (T_9), *T. harzianum* IMI 392433 (T_{10}) and *T. harzianum* IMI 392434 (T_{11}) treatments, respectively. In a similar study, Begum et al. (2010) were evaluated five *Trichoderma* strains to assay their efficacy in suppressing *Alternaria* fruit rot disease and promoting chili plant growth under field conditions. Their result demonstrated that application of *Trichoderma harzianum* IMI 392432 significantly ($p=0.05$) suppressed the disease compared to *Alternaria tenuis* treatment.

Correlation matrix

The correlation matrix among different parameters are presented in Table 4. The correlation matrix showed that root yield per plant of Ashwagandha had significant and

Table 4. Correlation matrix among different parameters of Ashwagandh as influenced by treatments

Parameters	Plant height	Number of leaf/ plant	Number of primary branch	Number of secondary branch	Fresh plant weight (gm)	Plant dry weight (gm)	Root length (cm)	Root diameter (cm)	Root dry weight (gm)	Fresh root weight (gm)	Percentages of disease severity index	Root yield/plant
Plant height	1.00											
Number of leaf	0.894**	1										
Number of primary branch	0.502**	0.708**	1									
Number of secondary branch	0.798**	0.821**	0.698**	1								
Fresh plant weight	0.856**	0.897**	0.780**	0.931**	1							
Plant dry weight	0.894**	0.817**	0.464**	0.723**	0.770**	1						
Root length	0.822**	0.810**	0.713**	0.872**	0.941**	0.698**	1					
Root diameter	0.782**	0.816**	0.773**	0.759**	0.841**	0.786**	0.875**	1				
Root dry weight	0.400**	0.626**	0.769**	0.671**	0.679**	0.323**	0.598**	0.538**	1			
Fresh root weight	0.970**	0.400**	0.461**	0.841**	0.881**	0.897**	0.838**	0.749**	0.395**	1		
Disease incidence %	-0.514**	-0.669**	-0.079**	-0.569**	-0.502**	-0.509**	-0.464**	-0.216**	-0.346**	-0.596**	1	
Root yield/plant	0.726**	0.514**	0.820**	0.829**	0.887**	0.613**	0.824**	0.786**	0.739**	0.731**	-0.336**	1

**Significant at 1% level.

positive correlation with plant height (r=0.726**), number of leaf (r=0.806**), number of primary branch (r=0.820**), number of secondary branch (r=0.829**), fresh plant weight (r=0.887**), plant dry weight (r=0.613**), root length (r=0.824**), root diameter (r=0.786**), root dry weight (r=0.739**) and fresh root weight (r=0.731**). The significant and negative correlation (r=-0.336**) was observed with the root yield and percentages of disease severity index. These results indicated that root yield per plant of Ashwagandha were dependence on growth characteristics of the plant. The higher root yield related to plant growth parameters were negatively influenced by the disease incidence percentages. The results are fully agreement with the findings of Rahman et al. (2012).

Conclusion

In the present study *T. harzianum*, IMI 392433 was found to be highly effective in inhibiting the mycelial growth and conidial germination of *A. alternata* and increased the germination percentages, seedling vigor, and plant growth. This strain can be used as potential biological control agent to control the leaf blight disease and increased plant growth of Ashwagandha.

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