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Isolation of *Trichoderma harzianum* and Evaluation of Antagonistic Potential against *Alternaria alternata*

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A B S T R A C T

Chilli is one of the important spices and cultivated around the world for its peculiar hot taste. Chilli leafs prone to affect by *Alternaria alternata* (leaf spot). In order to overcome this problem, efforts have been made to evaluate the efficiency of the bio-control agent *Trichoderma harzianum* for controlling the leaf spot disease in chilli. *Trichoderma harzianum* was isolated from the field soil and identified through microscopic and recommended standard methods. The antagonistic activity of the *Trichoderma harzianum* was evaluated against the pathogenic fungi responsible for leaf spot disease. *Alternaria alternata* belongs to the sub-division Deuteromycotina, class Dothideomycetes, family Pleosporaceae. The mycelium of *Alternari alternata* is septate, brown to brownish grey in colour. The conidiophores are dark, septate, arise in fascicles, measuring 14-74 × 4-8 µm. Conidia are brownish black, obclavate, borne singly or sparingly in chains of 2-4, muriform with long beak and the overall conidial size ranges between 148-184 × 17-24 µm with 10-11 transverse and *Alternaria alternata* proved the efficiency to inhibit. The antagonistic activity of *Trichoderma harzianum* was screened *in vitro* against *Alternaria alternata* by dual culture plate technique on PDA media for 7days. The results revealed *Trichoderma harzianum* was shown inhibition of 60.71% over *Alternaria alternata*.

Introduction

Chilli is one of the important spices and cultivated around the world for its peculiar hot taste. Chilli leafs prone to affect by *Alternaria alternata* (leaf spot). In order to overcome this problem, efforts have been made to evaluate the efficiency of the bio-control agent *Trichoderma harzianum* for controlling the leaf spot disease in chilli. *Trichoderma harzianum* was isolated from the field soil and identified through microscopic and recommended standard methods. The antagonistic activity of the *Trichoderm aharzianum* was evaluated against the pathogenic fungi responsible for leaf spot disease *i.e.*, *Alternaria alternata* belongs to the sub-division Deuteromycotina, class Dothideomycetes, family Pleosporaceae. Species of the genus are cosmopolitan, surviving both as saprophytes as well as weak parasites. The genus is characterized by the formation of polymorphous conidia either singly or in short or longer chains and provided with cross, longitudinal as well as oblique septa and having longer or short beaks

(Nayyar *et al.*, 2014). Yellow-brown spots with target-like concentric rings appear on leaves, as well as dark brown sunken spots on heads of Brussels sprouts, broccoli, and cauliflower, chilli. These spots contain many spores which are spread by wind, rain, or on equipment and people. Spores require at least 9 hrs. of moisture to germinate and infect the plant. Older, senescing plant parts are more susceptible to infection (Wukasch *et al.*, 1985).

The mycelium of *Alternari alternata* is septate, brown to brownish grey in colour. The conidiophores are dark, septate, arise in fascicles, measuring 14-74 × 4-8 µm. Conidia are brownish black, obclavate, borne singly or sparingly in chains of 2-4, muriform with long beak and the overall conidial size ranges between 148-184 × 17-24 µm with 10-11 transverse and *Alternaria alternata* proved the efficiency to inhibit (Subash *et al.*, 2014).

The use of various herbal extracts and natural products is being encouraged because these cause no health hazard or pollution. The extracts of *Canna indica*, *Convolvulus arvensis*, *Ipomoea palmata*, *Cenchrus catharticus*, *Mentha piperita*, *Prosopsis spicigera*, *Allium cepa*, *Allium sativum*, *Lawsonia inermis*, *Argemone mexicana*, *Datura stramonium* and *Cleroden droninerme* completely inhibited the spore germination of *Alternaria brassicae*, *Alternaria altenata* was isolated from leaves of cauliflower and chilli (Sheikh and Agnihotri, 1972).

The maximum residue limit (MRL) applied by legislation and buyers in the food industry is also increasingly strict, there is the risk of plant pathogens developing resistance to chemical control products. Biological control is therefore a useful and necessary part of growers' crop protection programmers. Biological control is based upon the natural enemies of harmful organisms, usually bacteria or fungi. These natural enemies are multiplied by manufacturers and sold as ready to- use control products. Growers can use biological control to replace (part of) their chemical control.

Several fungicides and botanicals belonging to various groups are recommended for the management of *Alternaria* leaf spot of chilli. Now-a-days farmers are using only the chemical fungicides for managing the disease, but it has the negative impact on the environment and develops resistant in pathogen. So due to these reasons use of specific chemical fungicides with recommended dosages bio-agent will not harm the environment and also will be effective in controlling the disease. So the studies are conducted to know the efficiency of fungicides, bio-agent in managing the *Alternaria* leaf spot of chilli caused by the *Alternaria alternata*.

Materials and Methods

Isolation of *Trichoderma harzianum* from Agriculture soil

The soil was collected from the Agriculture field and serially diluted till 10^{-5} dilutions separately and transfer 0.1ml of each dilution was placed on PDA media separately using spread plate technique. The plates were incubated at 25ºC for 4 to 5 days and then analysis of morphological study (Aneja *et al.*, 2003) was carried out.

Lacto Phenol Cotton Blue staining of *Trichoderma harzianum*

The lacto phenol cotton blue (LPCB) wet mount preparation is the most widely used method of staining and observing fungi. The preparation has three components, phenol, which was killed any live organisms, lactic acid which preserves fungal structures, and

cotton blue which stains the chitin in the fungal cell walls. Place a drop of 70% alcohol on a separate microscope slides and immerse the fungal specimens in the drop of alcohol and add one or two drops of the lacto phenol /cotton blue stain before the alcohol dries out. Holding the cover slip between forefinger and thumb, touch one edge of the drop of mountant with the cover slip edge, and lower gently, avoiding air bubbles and observe under microscope (Aneja *et al.,* 2003*).*

Characterization of *Trichoderma harzianum*

The thallus typically consists of a highly branched network of multi-nucleate. Many-branched conidiophores sprout on the mycelia. The conidiophores are the main dispersal route of the fungi, and often are green in color identified as *Trichoderma harzianum.*

Biochemical tests

Amylase production test

The isolate strains were inoculated into starch agar plates. All the inoculated as well as uninoculated (control) plates were incubated at 25ºC for 7 days. After incubation period, surface of the plates with iodine solution with a dropper for 30 seconds and pour off the excess iodine solution. A typical positive starch hydrolysis reaction (*i.e.,* clear zone surrounding the microbial colonies) shown by the *Trichoderma harzianum* (Azevedo *et al.,* 2000).

Cellulase production test

The isolate strains were inoculated into Czapek-mineral salt medium plates. All the inoculated as well as un-inoculate (control) plates were incubated at 25ºC for 7 days. After incubation period, surface of the plates with Hexadecyltrimethyl ammonium bromide (1% solution) with a dropper for 30 seconds and pour off the excess Hexadecyltrimethyl ammonium bromide solution. A typical positive cellulase production test (*i.e.,* clear zone surrounding the microbial colonies) shown by the *Trichoderma harzianum* (Gajera and Vakharia 2012).

Isolation and identification of *Alternaria alternata*

Sample collection

Soil samples from the chilli rhizosphere infested with *Alternaria alternata* was collected from 3 places of the field and mix well to make fine particles. Collection of soil samples were taken from the root zone at 5-15 cm depth.

Morphological characteristics of *Alternaria alternata*

The mycelium of *Alternaria alternata* is septate, brown to brownish grey in colour. The conidiophores are dark, septate, arise in fascicles. Conidia are brownish black, obclavate, borne singly or sparingly in chains with long beak.

This species represent slow and rudimentary growth in media and forms chlamydospores in less frequency (Kolte, 1985).

Antagonistic activity of *Trichoderma harzianum* against *Alternaria alternata* under *in-vitro*

Trichoderma harzianum was evaluated for its antagonistic activity against the *Alternaria alternata*. In Petri dishes containing PDA medium, inoculate *Trichoderma harzianum* and *Alternaria alternata* at 28^{o}c for 7 days. *Trichoderma harzianum* was inhibiting the growth of the pathogen. The bio-control agent, *Trichoderma harzianum* colonize over the pathogen (Kumar *et al.,* 2011).

Inhibition growth percentage (%) = $\frac{R1-R2}{R1}$ X100

Where,

R_1 = Growth of *Trichoderma harzianum* in petridish (mm).

R_2 = Growth of *Alternaria alternata* in petridish (mm).

Results and Discussion

Isolation and biochemical tests of *Trichoderma harzianum*

In the present study *Trichoderma harzianum* was isolated by serial dilution method from the soil sample collected from the Agricultural field of SHIATS, Allahabad. In PDA medium individual colonies of filamentous fungi were picked up and purified by streaking on Agar medium and incubate at 25ºC at 5-7 days. The fungal isolates were identified on the basis of macroscopic analysis in PDA medium. The fungal cultures were all incubated followed the recommendations of Pitt *et al.*, (1979). The fungal isolates have on PDA the conidiophores are the main dispersal route of the fungi, and often are green in colour identified. The biochemical tests (Amylase Production test, Cellulose Production test) used to identify the *Trichoderma harzianum.*

Biochemical tests

Isolation and morphological characteristics of *Alternaria alternata*

Soil samples from the chilli rhizosphere infested with *Alternaria alternata* was collected from 3 places of the field and mixed well to make fine particles. Collection of soil samples was taken from the root zone at 5-15 cm depth. The mycelium of *Alternaria alternata* is septate, brown to brownish grey in colour. The conidiophores are dark, septate, arise in fascicles, measuring 14-74 × 4-8 µm.

Conidia are brownish black, obclavate, borne singly or sparingly in chains of 2-4, muriform with long beak and the overall conidial size ranges between 148-184 × 17-24 µm with 10-11 transverse and 0-6 longitudinal septa. This species represent slow and rudimentary growth in media and forms chlamydospores in less frequency.

Table.1 Cultural characteristics of *Trichoderma harzianum*

Organism	Media	Cultural characteristics		
		Color	Texture	Hyphae
Trichoderma harzianum	PDA	Greenish white lower side cream	floccose	Vegetative aerial hyphehyline or trichotomously branched

Table.2 Morphological characteristics of *Trichoderma harzianum*

Organism	Morphological characteristics				
	Spore				
	Type	Size	Shape	Septation	Arrangement
Trichoderma harzianum	Conidia	Conidiophore52-120µ×7.-11.5µ,conidial head 26.5-28µ,spore 3-6µ	Spores are spherical or oval	Septate	Spores scattered or clusters

Table.3 Biochemical test of *Trichoderma harzianum*

Biochemical identification	Positive	Negative
Amylase production test	√	×
Cellulase production test	√	×

Table.4 Cultural characteristics of *Alternaria alternate*

Organisms	Media	Cultural characteristics		
		Color	Texture	Hyphae
Alternaia alternata	PDA	Light olive green to brown	Smooth or verrucose	Branched hyphae

Table.5 Morphological characteristics of *Alternaria alternate*

Organisms	Morphological characteristics				
	Spore				
	Type	Size	Shape	Septation	Arrangement
Alternaia alternata	Chlamydospores	Hyphae diameter: 5.25-8.6µ. Conidia about 25.5-46.75 µ×15-17.75µ	Club- shaped spores single or form chains	Alternate septate	Conidia borne in long chain

Table.6 Antagonistic activity of *Trichoderma harzianum* against *Alternaria alternata* under *in-vitro*

Media	Growth of *Trichoderma harzianum*	Growth of *Alternaria alternata*	Inhibition growth (%)
Potato Dextrose Aar	28 (mm)	11 (mm)	60.71 %

Plate.1 Cultural characteristics of *Trichoderma harzianum*

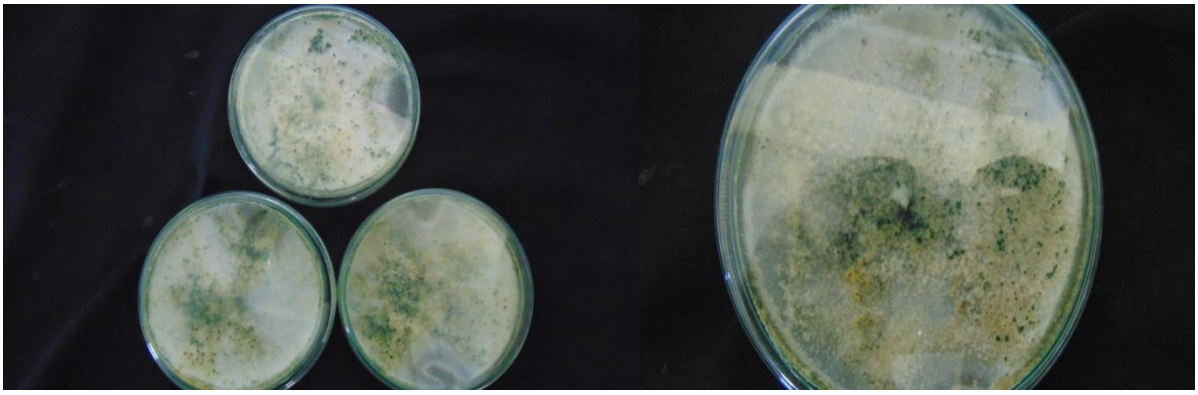


Plate.2 Morphological characteristics of *Trichoderma harzianum* (40X)

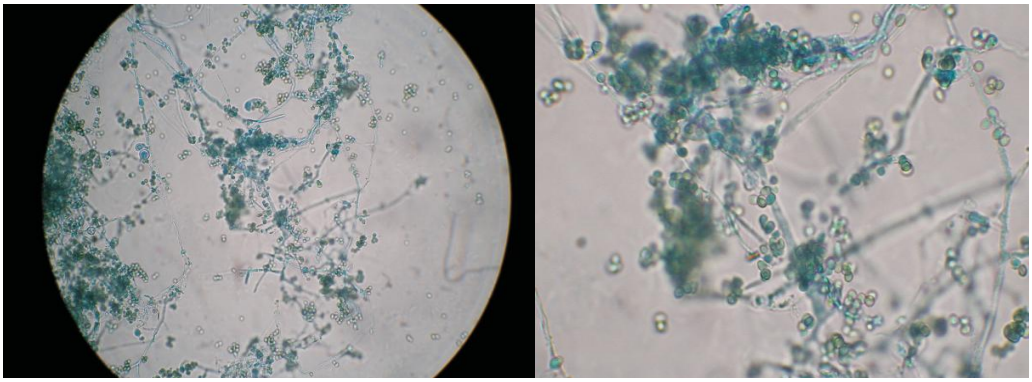


Plate.3 Amylase production test

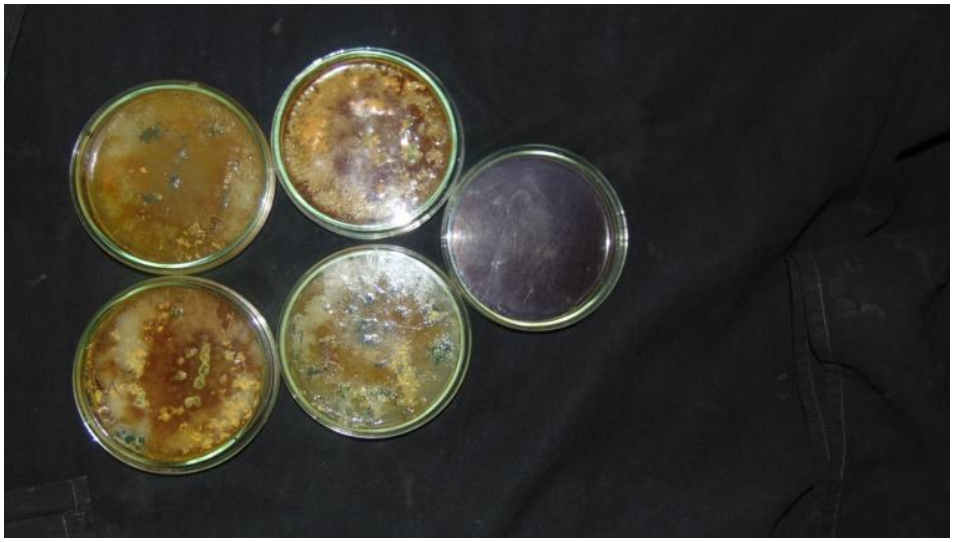


Plate.4 Cellulase production test

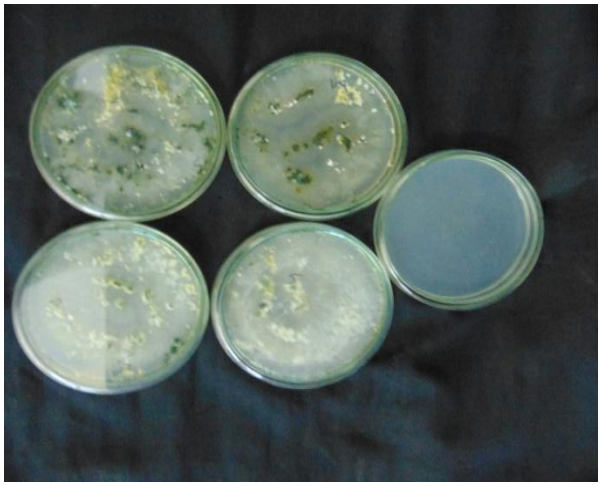


Plate.5 Cultural characteristics of *Alternaria alternata*

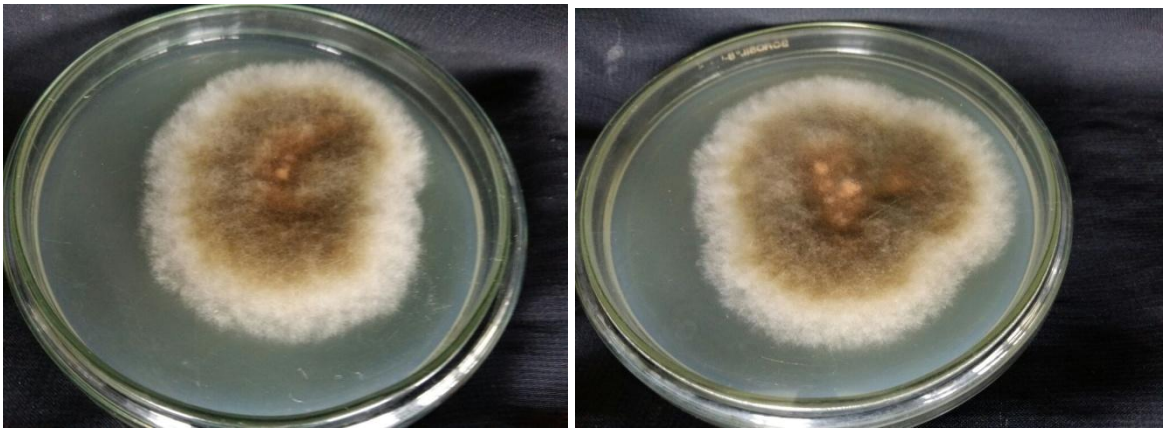


Plate.6 Morphological characteristics of *Alternaria alternata* (40X)

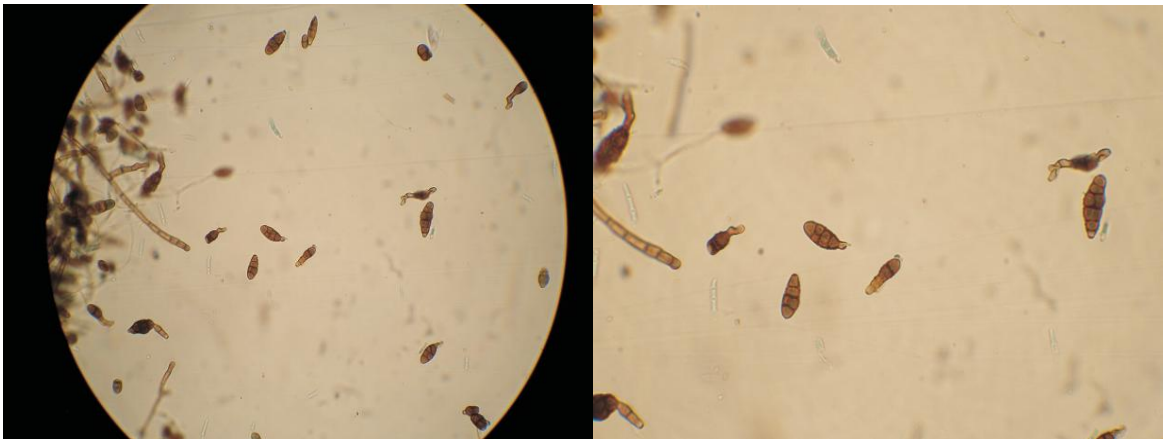
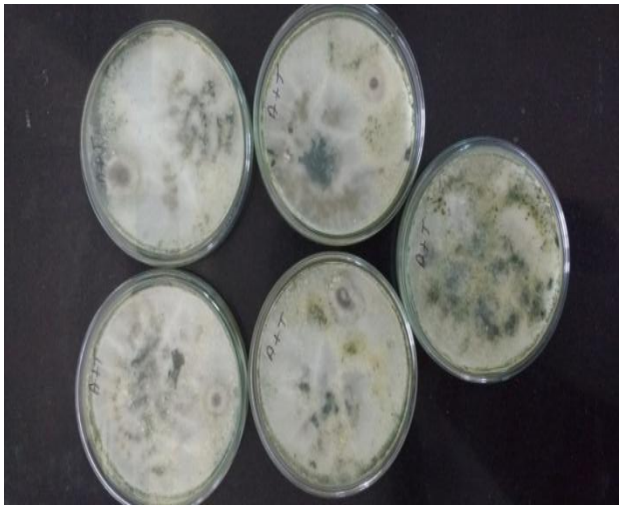


Plate.7 Antagonistic activity of *Trichoderma harzianum* against *Alternaria alternata* under *in-vitro*



Antagonistic activity of *Trichoderma harzianum* against *Alternaria alternata* under *in-vitro*

The antagonistic activity of *Trichoderma harzianum* was screened *in vitro* against *Alternaria alternata* by dual culture plate technique on PDA media for 7days. The results revealed *Trichoderma harzianum* was shown inhibition of 60.71% over *Alternaria alternata.* Where Growth of *Trichoderma harzianum* and *Alternaria alternata* 28 (mm), 11 (mm) respectively. Similarly, Bardia and Rai (2007) showed antagonistic effect of *Trichoderma viride* and *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *cuminis* by 51.15% and 58.41% inhibition of mycelial growth respectively. Rehman *et al.*, (2010) showed efficacy of *Trichoderma viride* and *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *Ciceris* by inhibition of mycelial growth 81% and 83.33% respectively. Cherkupally *et al.*, (2017) evaluated the efficacy of *Trichoderma viride* and *Trichoderma harzianum* against *Fusarium oxysporum* f. sp. *Melongenae* by inhibition of mycelial growth 78.88% and 81.11% respectively.

The use of chemical fungicides to control plant diseases caused by pathogenic fungi which constrain the yield. However, overuse of these synthetic chemicals causes hazardous to both environment and health the alternative method for replacement of chemical fungicides has led to the use of biological control agents. Microorganisms that grow in the rhizosphere are ideal for use as biocontrol agents. The studies proved that *Trichoderma harzianum* have the potential to control *Alternaria alternata* under *in vitro* to the extent of 60.71% by dual culture plate technique. It may be therefore a promising ecofriendly bio controlling sources and cost effective for the safe agricultural practices as well as to farmers.

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