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EXPLOITING THE BIOCONTROL ACTIVITY OF *Tricoderma* SPP AGAINST ROOT ROT CAUSING PHYTOPATHOGENS

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ABSTRACT

The main aim of this investigation was to determine the antagonistic activity of *Tricoderma* spp isolated from rhizosphere soil of sunflower. Two species namely *Trichoderma viride* and *Trichoderma koningii* were isolated. In dual culture method *Trichoderma viride* showed maximum growth inhibition of 72.20% against *Fusarium oxysporum* whereas, *Trichoderma koningii* effectively inhibited *Pythium debarianum* (57.42%). The result of volatile assay revealed *Pythium debarianum* was effectively inhibited by both antagonists and in non volatile assay *Pythium debarianum* was greatly inhibited followed by *Fusarium oxysporum*, *Fusarium javanicum*, *Macrophomina phaseolina*. The *Trichoderma viride* and *Trichoderma koningii* were recorded for their maximum cellulolytic and chitinolytic activity (3.6U/ml and 0.37IU/ml, 2.75 IU/ml and0.29IU/ml), respectively.

Keywords: *Tricoderma* spp, phytopathogens, biocontrol, chitinase, cellulase.

INTRODUCTION

Fungal Phytopathogens are the cause of many plant diseases and much loss of crop yields, especially in subtropical and tropical regions [1]. *Macrophomina phaseolina* (Tassi) Goid, a soil borne fungus causes charcoal rot over 500 plant species and has a wide geographic distribution [2]. Chemical fungicides are extensively used in contemporary agriculture. However these products may cause problems such as environmental pollution and have adverse effects on human health. Microorganisms as bio control agents have high potential to control plant pathogens and have no negative effect on the environment (or) other non target organisms. *Tricoderma* spp are used as effective biocontrol agents against several soil borne fungal plant pathogens including *Macrophomina phaseolina, Fusarium* spp, *pythium* spp [3]. The bio control exercised by *Tricoderma* can occur by several antagonistic mechanisms such as nutrient competition, antibiotic production and mycoparasitism. Mycoparasitism has been reported as the major antagonistic mechanism displayed by *Tricoderma* spp. After host recognition, *Tricoderma* spp attaches to the host hyphae via coiling and penetrate the cell wall by secreting cell wall degrading enzymes which allow them to bore holes into the fungal host and extracts nutrients for their own growth. Most phytopathogenic fungi have cell wall that contain chitin as a structural backbone arranged in an regularly ordered layers and β, 1-3 glucan as a filling material arranged in an amorphic manner. Chitinases and β, 1-3 glucanases have been directly involved the mycoparasitism interaction between *Tricoderma* spp and its host [4].

MATERIALS AND METHODS

Isolation of antagonist

The fungal antagonists were isolated from the rhizosphere soil of sunflower; using serial dilution and pour plate technique on potato dextrose agar medium (PDA). The antagonist were purified and identified based on morphological and miroscopical characters. The isolates were maintained on PDA slants at 4^0C through out the study.

Isolation of phytopathogens

Diseased plant tissues were washed under running tap water to remove surface soil and other contaminant. Infected tissues were cut into small pieces and placed in 1% sodium hypochloride for five minutes, placed on PDA plates and incubated at 28^0C. The cultures were purified by hyphal tip method [5] and maintained on PDA slants by storing it under refrigeration (4^0C). Phytopathogens isolated from infected tissues were identified based on morphological and microscopical charters.

Growth inhibition assay by dual culture method

Interaction between antagonistic fungi and pathogenic fungi were determined by the method of Dennis and Webster [6]. A 5 mm diameter mycelial disc from the margin of the *Trichoderma* one week-old culture and the pathogens *M. phaseolina*, *Fusarium javanicum*, *F. oxysporum*, *Pythium debarianum* were placed on the opposite side of the plate at equal distance from the periphery. In control plates (without *Trichoderma*), a sterile agar disc was placed at opposite side of the pathogen *M. phaseolina* inoculated disc. The plates were incubated at 28 ± 2°C and observed after 7 days.

Assay for volatile metabolites of *Trichoderma* spp

Productions of volatile metabolites by Trichoderma spp were assayed as described by Dennis and Webster [6] and Goyal *et al*., [7] with slight modifications. The *Trichoderma* isolates were centrally inoculated by placing 3mm disc taken from three days old cultures on the PDA plates and incubated at 28 ± 2° C for three days.

The top of each petridish was replaced with bottom of PDA plate inoculated centrally with the pathogen. Petridish with PDA medium without *Trichoderma* spp at the lower lid and the upper lid with pathogens was maintained as control. The pair of each petridishes were sealed together with paraffin tape and incubated for 4-6 days. After incubation the inhibition of mycilial growth was calculated.

Assay for non volatile metabolites of *Trichoderma* spp

The effects of non volatile metabolites produced by the *Trichoderma* spp were determined by following the methods of Dennis and Webster [6]. The isolates of *Trichoderma* spp were inoculated in 100ml sterile potato dextrose broth in 250ml conical flasks and incubated at 28 ± 2°C for 15 days. After incubation the cultures were filtered through Millipore filter and culture filtrates were added to molten PDA medium (40^0C) to obtain a final concentration of 10% (v/v). The medium was poured into petriplates and after solidification 3mm disc of the pathogens were placed centrally and incubated at 28 ± 2° C. Control plates were maintained without amending the culture filtrate. The percent of growth inhibition in all the above experiments were calculated by the formula

$$I = \frac{C - T}{C} \times 100$$

Where

I = Percentage of inhibition
C = Growth of mycelium in control
T = Growth of mycelium in treatment

Assay for enzyme activity

For assay of enzyme activity, *Trichoderma* species were grown on minimal synthetic medium (MSM) containing the following components (in grams per liter): $MgSO_4.7H_2O$, 0.2; $K2HPO_4$, 0.9; KCl, 0.2; NH_4NO_3, 1.0; FeSO4.7H2O, 0.002; $MnSO_4$, 0.002 and $ZnSO_4$, 0.002. The medium was supplemented with the appropriate carbon source for cellulose and chinase assay (commercially available Carboxy methyl cellulose, Chitin). The pH was set to 6.3 with 50mM phosphate buffer and autoclaved at 15 lps for 20 min. The medium was inoculated with a spore suspension to give a final concentration of ~5 $\times 10^6$ conidia per milliliter and placed on a rotary shaker at 150 rpm at 25^0C for different time intervals. The cultures were harvested at 24, 48, 72 hour of incubation and were filtered through Whatman No. 44 filter paper and finally centrifuged at 12000 rpm for 10 min at 4^0C to get cell-free culture filtrate which were then used as enzyme source.

Assay for cellulase activity

Cellulase activity was assayed following the method of Miller [8]. The assay mixture contained 1 ml of 0.5% pure cellulose (Sigma Co.) suspended in 50 mM phosphate buffer (pH 5.0) and 1 ml of culture filtrates of different *Trichoderma* strains. The reaction mixture was incubated for 30 min at 50°C. The blanks were made in the same way using distilled water in place of culture filtrate. The absorbance was measured at 540nm and the amount of reducing sugar released was calculated from the standard curve of glucose. One unit of cellulose activity is defined as the amount of enzyme that catalyzed 1.0 µ mol of glucose per minute during the hydrolysis reaction.

Assay for chitinase activity

It was measured using colloidal chitin as substrate [9]. Enzyme solution (0.5 ml) was added to 0.5 ml of substrate solution, which contained 1% colloidal chitin in phosphate buffer (0.05 M, pH 5.2) and 1 ml distilled water. The mixture was then incubated in shaking water bath at 50°C for 10 min, thereafter 3 ml of 3; 5-dinitrosalicyclic acid reagent was added. The mixture was then placed in a boiling water bath for 5 min, after cooling, the developed color, as indication to the quantity of released N -acetylglucosamine (NAGA), was measured spectrophotometrically at 575 nm. The amount of NAGA was calculated from standard curve of NAGA.

RESULTS AND DISCUSSIONS

Table-1. Percent of inhibition by *Trichoderma* isolates after 7 days of inoculation in dual culture.

Pathogens	Antagonists	
	T. viride	***T. koningi***
Pythium debarianum	66.24	57.42
Fusarium oxysporum	72.20	52.75
Fusarium javanicum	64.43	44.54
Macrophomina phaseolina	42.36	47.24

Table-2. Percent of inhibition by *Trichoderma* isolates in volatile assay method.

Pathogens	Antagonists	
	T. viride	***T. koningi***
Pythium debarianum	73.33	60.17
Fusarium oxysporum	60.28	54.20
Fusarium javanicum	68.2	59.4
Macrophomina phaseolina	49.10	40.9

Table-3. Percent of inhibition by *Trichoderma* isolates in non volatile (culture filtrates) assay method.

Pathogens	Antagonists	
	T. viride	***T. koningi***
Pythium debarianum	76.66	60.21
Fusarium oxysporum	62.10	57.21
Fusarium javanicum	66.76	54.43
Macrophomina phaseolina	39.67	37.67

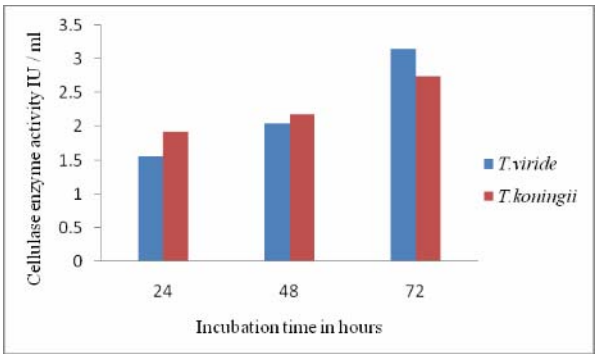

Figure-1. Cellulase enzyme activity of *T. viride* and *T. koningii.*

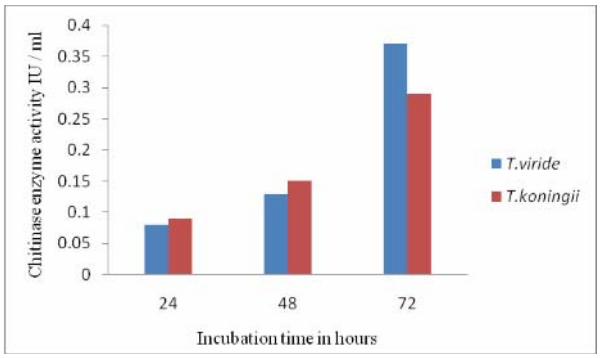

Figure-2. Chitinase enzyme activity of *T. virid* and *T. koningii.*

Growth inhibition assay by dual culture method

The antimicrobial metabolites produced by *Trichoderma* spp are effective against wide range of phytopathogens eg. *Fusarium moniliform*, *F. oxysporum*, *Rhizoctonia solani*, *and Colletotrichum capsici*, *Alternaria alternate*, *F. solani*, *Macrophomina phaseolina*, *Sclerotium rolfsii*, *Pythium* specieses [10-13]. In the present investigation two species of *Trichodema* were isolated and tested for their antagonistic activity against four different plant pathogens. The results of dual culture assay revealed that the maximum inhibitory activity of *Trichodema viride* was 72.20% against *Fusarium oxysporum* and minimum inhibition was 42.36% against *M. phaseolina*. *T. koningi* showed maximum inhibition against *Pythium debarianum* (57.42%) and minimum inhibition against *Fusarium javanicum* (44.54%) (Table-1). Similar result has been observed [14]. In their studies *T. viride* and *T. koningii* showed highest inhibition of 74.3%, 55.5% against *M. phaseolina* respectively (Figure-1).

Assay for volatile and non volatile metabolites of *Trichoderma* spp

In volatile assay it was observed that the isolates of *T. viride* and *T. koningi* effectively inhibited the Growth of *Pythium debarianum* (73.33%, 60.17%, respectively). *Trichoderma* produces several volatile compound such as Ethylene, Hydrogen cyanine, Aldehydes and Ketones which play an important role in controlling the plant pathogens [15]. The study non volatile assay revealed that both the antagonist effectively inhibited the mycelial growth of phytopathogen in the following order *Pythium debarianum* > *Fusarium javanicum* >, *Fusarium oxysporum* >, *Macrophomina phaseolina*. The volatile and non volatile copound from *Trichoderma* effectively inhibited the growth of *Colletotrichum capsici* [16]. Muthukumar *et al*., [17] and Christy Jeyaseelan *et al*., [18] recorded maximum growth inhibition of *T .viride* against *Pythium aphanidermatum* through more production of volatile and non volatile compounds.

Assay for cell wall degrading enzyme activity

Chitinolytic and glucanolytic (fungal cell wall-degrading enzymes) have been suggested to be the primary determinants of biocontrol by *Trichoderma* spp [19]. The highest cellulase activity of the isolate of *T. viride* and *T. koningii* observed were 3.15IU/ml and 2.75 IU/ml, respectively after 72 hours incubation. Vinit Kumar Mishra, [20] observed that the *T. viride* exhibited highest cellulase activity of 3.6U/ml and it was found to be suppress the mycelial growth of *Pythium aphanidermatum*. In this investigation the maximum chitinase enyme was 0.37IU/ml in the case of *T. viride,* whereas *T. koningii* exhibited 0.29IU/ml activity. Eman Faith Sharaf *et al*., [21] reported the maximum production of chitinase enzyme (23.8U/ml) by a most potent *T. viride* isolate using colloidal chitin prepared from Shrimp shell waste as sole carbone source. Sevugaperumal Ganesan and Rajagobal Sekar [22] explored the antagonistic activity of nine species of *Trichoderma* against *Rhizactonia solani*, and reported that the chitinolytic activity of the antagonists ranged from 5.51IU/ml to 0.12 IU/ml. *T. harizianum* culture filtrates, possessing chitinase and glucanase activities, were capable of hydrolyzing dried or fresh mycelium of the phytopathogenic fungus *Sclerotium rolfsii*. Growth of *Sclerotium rolfsii* was significantly inhibited by the enzyme preparations from *T. harizianum* [23]. Agrawal and Kotasthane [24] evaluated the production of chitinase enzyme by *Trichoderma* spp using colloidal chitin which was derived from the cell wall of plant pathogen *Rhizactonia* and commercial chitin.

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