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EVALUATION OF *TRICHODERMA* SPECIES AGAINST *ALTERNARIA* FOR MANAGEMENT OF BLIGHT DISEASE OF *VIGNA UNGUICULATA*

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Abstract

Alternaria sp attack *Vigna unguiculata* causes many losses in pulse production. The antagonistic efficiency of *Trichoderma* spp evaluated in *in vitro* and in *in vivo* study against blight disease of *Vigna unguiculata*. *Trichoderma* had shown significant antagonistic activity against *Alternaria*. *Trichoderma* species were capable of producing some volatile and non-volatiles substances which inhibit the growth of the pathogen. Studies on the production of hydrolytic enzymes *viz.* β-1, 3 glucanase and chitinase by four *Trichoderma* spp were also studied. Field trials with the application of two *Trichoderma* species were made where T. *viride* caused reduction of infection of plants to 43.62%.

Key words: *Alternaria*, chitinase, mycoparasitism, *Trichoderma*, *Vigna*.

Introduction

Vigna unguiculata, a member of the family Fabaceae is an important pulse and has attained a prime position in the pulse economy of the country. This species provides enormous benefits to human health as medicine. It prevents cancer, diabetes, supports immune system and health metabolism; controls blood cholesterol, removes free radicals, treats cardiovascular ailments and stomach, pancreatic and urination problems. Blight disease of *Vigna* caused by *Alternaria* sp is a serious problem posing a serious threat to its production and has been reported from almost all *Vigna* growing countries throughout the world. The disease causes blackening of the leaves with ring like spots resulting in enormous loss to the total photosynthates of the plant followed by reduced yield of the plant. Biological control is one of the most promising and widely recommended methods of plant disease control. *Trichoderma* spp are among the most frequently isolated soil fungi and present in plant root system, these fungi are opportunistic avirulent symbionts and function as antagonists of many phytopathogenic fungi (Chakraborty *et al*., 2008; Sharma *et al*., 2010). Therefore, present investigation has been carried out to evaluate the biocontrol agents particularly the *Trichoderma* spp both *in vitro* and field condition against blight disease.

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Materials and Methods

Alternaria sp was isolated from blighted leaves of *Vigna* plants and maintained in pure line on potato dextrose agar (PDA) slants at 4^0C till used. The identification of the pathogen has been confirmed by Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi (ITCC No 54424.03). Among the four species of *Trichoderma, T. viride, T. harzianum, T. koningii* and *T. hamatum*, two antagonists *viz*. *T. harzianum* and *T. hamatum* were procured from IARI, New Delhi, another two were isolated from rhizosphere of *Vigna unguiculata* plants.

Testing of mycoflora isolated from the rhizosphere *Vigna* plants against the test pathogen

Dual culture plate technique (Royse and Ries, 1978) was adopted for initial screening of the fungi isolated from the rhizosphere of *Vigna* plants against *Alternaria* sp. Inoculum disc (5 mm) was taken from 7 days old culture of *Alternaria* sp and transferred on one side of the PDA plate. Another mycelial disc (5mm) of each of the isolated fungi was placed just opposite to the first inoculum on the same plate. The plates were incubated at 26° $\pm$ 1°C for 7 days and the inhibition of mycelial growth of the test pathogen was measured. Plates without antagonist served as control. Growth inhibition of the pathogen by each antagonist was measured on the basis of radial growth of the pathogen in dual culture plate and

in control plate with the help of the following formula:

$I = (C – T/C) \times 100$

Where, I = percent inhibition, C = radial growth of pathogen in control, T = radial growth of pathogen in the treatment.

The percentage inhibition of the pathogen was presented in the Table 1. In the present study all the efficient antagonists i.e. four species of *Trichoderma* were again subjected to give trial for verification of their efficacy to check the growth of the test-pathogen.

Studies on the production of volatile compounds by *Trichoderma* spp.

This experiment was designed to identify the antagonists producing volatile and non-volatile antibiotics based on the tests performed by Dennis and Webster (1971a, b).

In order to study volatile antibiotics, 15 ml of PDA medium was poured both in the base and the lid of the petriplate. The medium was allowed to solidify. Then a 5 mm inoculum disc of the test-pathogen was placed at the centre of the lid of the petriplate and the bottom of the petriplate was inoculated with a 5 mm inoculum disc of the respective antagonist. The petriplate was sealed to one another and incubated at 26° ± 1°C for 7 days. The percentage inhibition of growth of the pathogen was recorded as the difference in radial growth of the pathogen in the presence or absence of *Trichoderma*.

Studies on the production of non-volatile compounds by *Trichoderma* spp.

To estimate the non-volatile antibiotic production, a 5 mm inoculum disc of each of the respective antagonists was placed centrally on dialyser bag (Sigma) covered PDA plate. After 2 days of incubation at 26° ± 1°C, the respective antagonist and the dialyser bag were removed. After that, a 5 mm inoculum disc of the test pathogen was placed centrally on the same PDA plate and incubated at 26° ± 1°C for 7 days. The results of this investigation were presented in Table 4.

Lytic enzyme production by the antagonists

Preparation of fungal cell wall material

The pathogen was grown in potato dextrose medium at 26° ± 1°C. After 20 days, the mycelia were removed by filtration and dried at 60°C. Cell wall material was then prepared following the method of Bruce *et al*., 1995. Mycelia were ground in a mortar and pestle before being exposed to ultrasonic disintegration (Braun-sonic 1510) for 3 minutes at 150 W. Then the resulting mycelial pastes were washed thrice (with repeated centrifugation at 5000 rpm) with 0.1 M NaCl in 0.5 M acetate buffer at pH 5.5 (0.5 M anhydrous sodium acetate and 0.5 M acetic acid mixed at 6.2 : 1 respectively) followed by three washes with deionized distilled water. The samples were then lyophilized overnight before being ground to a fine powder in a mortar and pestle.

Preparation of enzyme source

Enzyme source was prepared following the methods of Bruce *et al*., 1995 and Dutta and Chatterjee, 2004. The *Trichoderma* isolates *viz*. *T. harzianum, T. viride* and *T. hamatum* and *T. koningii* were cultured separately in 50 ml amounts of each of the three different media like Czapek's Dox Broth (CDB) as basal medium, CDB + *Alternaria* sp cell wall material (0.1 gm) [CDB + AOC] and CDB without sucrose + *Alternaria* sp cell wall material (0.1 gm) [CDB – S + AOC]. The flasks were incubated at 26°C for 10 days. After incubation, mycelial mat was removed by filtration and culture filtrates were sterilized by passing them through 0.45 µm membrane filters. The filtrates were then dialysed overnight (to remove residual sugars) in a continuous flow of cold water at 10-12°C using 2.4 nm pore size dialysis bag prior to assay for b-1, 3-glucanase (laminarinase) and chitinase activity.

Assay of b-1, 3-glucanase (laminarinase)

Activity of b-1, 3-glucanase or laminarinase i.e. enzymatic hydrolysis of laminarin (Sigma) was measured according to dinitrosalicylic acid method of Miller (1959). The reaction mixture contains 0.5 ml of enzyme source, 0.2 ml of citrate buffer (pH 4.8) and 1.6 mg of soluble laminarin. The reaction mixture was incubated at 40°C for 60 minutes. The reaction was stopped by boiling and the amount of reducing sugar was determined. The enzyme activity was expressed as release of µmol glucose/ml of filtrate/hour.

Assay of chitinase

The assay of chitinase is based on the estimation of reducing sugars released during the hydrolysis of swollen chitin. The reaction mixture, containing 1ml of 0.5% swollen chitin (suspended in 50 mM sodium acetate buffer at pH 5.21 containing 0.02% sodium azide) and 1 ml of enzyme source, was incubated at 40°C for 60 minutes with shaking, and then centrifuged at 4000 rpm for 5 minutes. The amount of reducing sugar released in the supernatant was determined by the method described by Miller (1959), using N-acetyl glucosamine as standard. One unit (u) of activity was defined as the amount of enzyme which catalyses the release of 1 µmol reducing sugar in 60 min at 40°C. The results are presented in Table 5.

Table 1: Trial with antagonistic fungi to select potent antagonist and their effect on growth of *Alternaria* sp following 'dual culture plating method'.

Antagonits	Radial growth of the pathogen (cm)	Radial growth of the isolates (cm)	Growth inhibition of pathogen *(%)
Trichoderma hamatum	2.0	7.0	77.72 ± 0.53
T. harzianum	1.68	7.32	82.58 ± 0.43
Trichoderma viride	1.18	7.92	88.32 ± 0.231
Trichoderma koningii	1.84	7.16	79.52 ± 0.09
Control	9.0	0	0
SEM	±0.0009		
CD at 5%	0.001917		

Data are the mean values of three replicates

Efficacy of potent antagonist to reduce the infection under field condition

Bioefficacy of potentially effective *Trichoderma* isolates namely, *T. viride, T. harzianum, T. hamatum* and *T. koningii* was evaluated in terms of control of the infection of *Vigna* plants caused by *Alternaria* sp under field condition for the year 2014 and 2015. Field trial was set up at Nabadwip area in Nadia district of West Bengal in farmers' field, where the incidence of disease was very severe.

For mass multiplication, the antagonists were grown in conical flasks containing 250 ml Czapek's synthetic medium at 26° ± 1°C for 14 days to obtain mycelial mats. After that, the mycelial mats were harvested and the culture filtrates were collected. The culture filtrates thus obtained were stored at 4°C and directly applied to the soil, 14 days before transplantation of seedlings. The mycelial mats were multiplied by growing on substrate consisting of 1:1 wood saw dust and wheat bran with small quantity of malt extract in sterilized plastic bags (30 × 26 cm) for 14 days at 28° ± 1°C (Howell, 2003). Field application with this mass inoculum was done @ 300 gm/m^2, 5 days before transplantation of *Vigna* seedlings in the field.

Table 2: Effect of volatile antibiotics produced by *Trichoderma* spp on growth of *Alternaria* sp.

Antagonits	Radial growth of the pathogen (cm)	Growth inhibition of the pathogen(cm)	Growth inhibition of pathogen *(%)
T. viride	1.96	7.12	79.14 ± 0.07
T. harzianum	2.16	6.9	77.29 ± 0.43
T. koningii	2.94	6.12	68.20 ± 0.09
T. hamatum	2.41	6.6	75.01 ± 0.35
Control	9	0	0
SEM	± 0.0003		
CD at 5%	0.000639		

Data are the mean values of three replicates.

Table 3: Effect of non-volatile antibiotics produced by *Trichoderma* spp. on growth of *Alternaria* sp.

Antagonits	Radial growth of the pathogen (cm)	Growth inhibition of the pathogen(cm)	Growth inhibition of pathogen *(%)
T. viride	0	9	100 ± 0
T. harzianum	0.103	8.9	97.88 ± 0.3
T. koningii	2.8	6.2	68.00± 0.04
T. hamatum	2.2	6.8	72.12± 0.09
Control	9	0	0
SEM	± 0.0008		
CD at 5%	0.001704		

Data are the mean values of three replicates

Results and Discussion

The result Table 1 showed that out of the total five species of *Trichoderma* was tested, and all five species were found to be antagonistic towards the growth of *Alternaria* sp. Highest response was performed by *T. viride* (88.32%) followed by *T. harzianum* (82.58%), *T. koningii* (79.52%) and *T. hamatum* (77.72%).

The data presented in table 2 and 3 indicates that all the four selected *Trichoderma* species were capable of producing some volatile and non-volatiles substances which inhibit the growth of the pathogen. The *Trichoderma* spp again showed interspecific variability in growth inhibition of the pathogen by volatile and non-volatiles antibiotics. The result demonstrated that the volatiles produced by *T. viride* was most promising to check the radial growth of the pathogen (79.14%) wherein *T. harzianum* exhibited 77.29% inhibition. This was followed by 75.01% growth inhibition with *T. hamatum*. *Trichoderma* sp was almost equally effective to check the growth the pathogen by non-volatiles. *T. viride* was most promising to check the radial growth of the pathogen (100%) wherein *T. harzianum* exhibited 97.88% inhibition. Probable mechanism of *Trichoderma* action is attachment to the pathogen with cell wall carbohydrates that bind the pathogen lectin. Once *Trichoderma* attaches itself, it coils around the pathogens hyphae and forms appressoria, which facilitates the entry of *Trichoderma* hyphae into lumen of the parasitized fungus and form appressoria (Biswas and Sen, 2000; Howell, 2003). Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that impede colonization by antagonizing microorganisms; among these metabolites, the production

Table 4: Levels of production of b-1, 3 glucanase by *Trichoderma* spp.

Antagonists (*Trichoderma* spp.)	**b-1, 3 glucanase (1 unit = 1µ mole glucose released / ml. of iltrate / min / mg protein)***		
	Carbon Source		
	CDB	**CDB + ACW**	**CDB – S + ACW**
T. viride	810 ±0.032	738 ± 0.39	1022 ±0.443
T. harzianum	532 ±0.07	312± 0.07	895.3± 0.3
T. koningii	577 ± 0.07	378 ±0.087	720 ± 0.09
T. hamatum	428 ± 0.09	322±0.093	615± 0.34

(CDB = Czapek's Dox Broth, ACW= Cell wall material of *Alternaria* sp, S = Sucrose)

	SEM CDB	**SEM CDB + ACW**	**SEM CDB – S + ACW**	**CD at 5% CDB**	**CD at 5% CDB + ACW**	**CD at 5% CDB – S + ACW**
b-1, 3gucanase	13.20	14.08	35.22	28.11	29.990	75.0186

Data are the mean values of three replicates

Table 5: Levels of production of chitinase by *Trichoderma* spp on CDB, CDB + ACW, CDB – S + ACW.

Antagonists (*Trichoderma* spp.)	Chitinase (1 unit = 1µ mole glucose released / ml. of iltrate / min / mg protein)*		
	Carbon Source		
	CDB	**CDB + ACW**	**CDB – S + ACW**
T. viride	125.30 ± 0.078	90.2 ±0.33	170.56 ± 0.23
T. harzianum	92 ± 0.088	69.82 ± 0.07	122.32 ± 0.09
T. koningii	74.10±0.12	43.00 ± 0.76	106.65 ± 0.35
T. hamatum	83.62±0.07	52.28± 0.076	131.62± 0.099

	SEM CDB	**SEM CDB + ACW**	**SEM CDB – S + ACW**	**CD at 5% CDB**	**CD at 5% CDB + ACW**	**CD at 5% CDB – S + ACW**
Chitinase	7.05	9.28	5.68	15.02	19.766	12.098

Data are the mean values of five replicates

Table 6: Field trials with potential *Trichoderma* spp on reduction of blight of *Vigna* caused by *Alternaria* sp.

Trial year	**Total no. of plants**	**No. of infe-cted plants**	**% of infection**	**Treatment**	**Total no. of plants#**	**No. of infec -ted plants#**	**% of infection**	**Reduction of infection (%)***
2014	75	32	42.80 ± 0.04	*T. viride*	58	14	24.13	43.62 ± 0..075
	72	34	46.94± 0.09	*T. harzianum*	56	14	25.00	26.82 ± 0.07
	70	32	45.71± 0.07					
2015	68	30	44.11 ± 0.43	*T. viride*	62	16	25.80	25.80 ± 0.80
	72	36	50.00± 0.23	*T. harzianum*	60	15	25.00	25.005 ± 0.09
	70	32	44.44± 0.07					
				SEM	CD at 5%			
2014: Reduction of infection (%)				± 0.0983	0.2093			
2015: Reduction of infection (%)				± 0.4172	0.8886			

*Average data of three fields

of harzianic acid, tricholin, peptaibols, 6-pentyl-a-pyrone, massoilactone, viridian, glioviridin, glisopenins, heptilidie acid have been described (Vey *et al*., 2001). In addition to these, suzukacillin, alamethicine, demadin, trichodermin are some of the antibiotics extracted from culture filtrates of *Trichoderma* spp (Chakraborty and Chatterjee, 2008).

Studies on the production of hydrolytic enzymes *viz.* β-1, 3 glucanase and chitinase by four *Trichoderma* spp table 4 and 5 have reflected their inducible nature. Wide variations existed in respect of the production of β-1, 3-glucanase among the different species but similar pattern of enzyme production was recorded in the media types used. In Czapek's Dox Broth (basal medium) supplemented with cell wall material of *Alternaria*, lower quantity of enzyme was excreted by all *Trichoderma* spp but when cell wall material of the pathogen alone was considered as the sole carbon source (i.e. in CDB – S + ACW), higher β-1, 3-glucanase activities of the antagonists were recorded where *T. viride* comparatively

produced better β-1, 3-glucanase (1022 units) than *T. harzianum* (895.3 units) and *T. koningii* (720 units).

On the other hand, various carbon sources influenced differently the production of chitinase enzyme by the antagonists, as there exist a parallel relationship between carbon source in the medium and *Trichoderma* species. All the *Trichoderma* spp. showed highest enzyme activity in medium containing Czapek's Dox Broth (CDB) omitting sucrose but substituted with cell wall material of the pathogen (CDB-S+ACW) and lowest in CDB with cell wall material of the pathogen (CDB+ACW). Different species of *Trichoderma* also showed variable degrees of chitinase production and *T. viride* was recorded to be the most efficient producer (170.56 units) followed by *T. hamatum* (131.62) and *T. harzianum* (122.32). It may be noted from the result that a large interpsecific differences in *Trichoderma* spp existed in respect of the production of chitinase and b-1, 3-glucanase in all the media tested and *T. viride* produced maximum units of both the enzymes. The difference in enzyme activity may be due to varying degree of substrate utilization by different *Trichoderma* species indicating thereby their varying degree of biological control efficiency. Chitinase and b-1, 3-glucanase is also known to release fungal cell wall fragments which elicit other defence responses by antagonists. Degree of production of lytic enzymes will determine the potentiality of any *Trichoderma* isolate selected for the biological control of plant pathogenic fungi (Karasuda *et al*., 2003; Chakraborty and Chatterjee, 2008).

Field trials (Table 6) with the application of two *Trichoderma* species were made where the rate of the intensity of infection by the pathogen varies. *T. viride* caused reduction of infection of plants to 43.62% as against 26.82% reduction of infection with *T. harzianum* of the trial year (2014). The competence shown by *Trichoderma* strains to inhibit the growth of the tested pathogen *in vitro* and field condition suggests that *Trichoderma* are implicated in the biocontrol mechanism and plant defense mechanisms (Harman *et al*., 2004; Hussein *et al*., 2014). Biological control offers an environmentally friendly approach to the management of plant disease and can be incorporated into cultural and physical controls for an effective integrated disease management strategy.

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