INTERNATIONAL JOURNAL OF PLANT PROTECTION
VOLUME 9 | ISSUE 1 | APRIL, 2016 | 8-14

● e ISSN-0976-6855 | Visit us : *www.researchjournal.co.in*

IJPP

RESEARCH PAPER

DOI : 10.15740/HAS/IJPP/9.1/8-14

Efficacy of certain species of *Trichoderma* against blight of *Vigna radiata*

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ARITCLE INFO

Received : 13.05.2015
Revised : 01.02.2016
Accepted : 15.02.2016

KEY WORDS :
Alternaria, Chitinase, Laminarinase, Non-volatile, Volatile, *Trichoderma* spp. *Vigna*

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ABSTRACT

Alternaria sp attacks *Vigna radiata* causes many losses in yield. The antagonistic efficiency of *Trichoderma* spp evaluated in *in vitro* and in *in vivo* study against blight disease of *Vigna*. *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. Effect of non-volatile substances seems to be more effective on mycelial growth of the pathogen. *T. viride* was most promising to check the radial growth of the pathogen (100%) wherein *T. harzianum* exhibited 97.78 per cent inhibition. 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 54.11per cent.

How to view point the article : Bhattacharya, D., Chakraborty, M.R., Chatterjee, N.C. and Sengupta, C. (2016). Efficacy of certain species of *Trichoderma* against blight of *Vigna radiata*. *Internat. J. Plant Protec.*, **9**(1) : 8-14.

INTRODUCTION

Vigna radiata (L.) R. Wilczek a member of the family Fabaceae is an important pulse and has attained a prime position in the pulse economy of the country. 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 radiata* or mung bean 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 effective and widely recommended methods of 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 (Bhagat and Pan, 2012). 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.

MATERIAL AND METHODS

Alternaria sp was isolated from blighted leaves of *Vigna* plants and maintained in pure line on potato dextrose agar (PDA) slants at 4^0 C till used. 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 Indian Agricultural Research Institute (IARI), New Delhi, another two were isolated from rhizosphere of *Vigna* 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° ± 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:

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

where, I = per cent 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 and b) and Whipps (1987) with slight modification.

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 g) [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 β-1, 3-glucanase (laminarinase) and chitinase activity.

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

Activity of β-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 per cent 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.

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 2011 and 2012. Field trial was set up at Nabadwip area in Nadia district of West Bengal in farmers' field, where the incidence of wilt disease was very severe for the last few years.

For mass multiplication, the antagonists were grown in conical flasks containing 250 ml Czapek's synthetic medium at 26° ± 1°C for 18 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 x 26 cm) for 14 days at 28° ± 1°C (Biswas and Sen, 2000). Field application with this mass inoculum was done @ 300 g/ m^2 (4.5 x 10^7 propagules / g), 5 days before transplantation of tomato seedlings in the field. Light irrigation was given to maintain humidity.

RESULTS AND DISCUSSION

The result (Table 1) showed that out of the total 9 fungi isolated from rhizosphere of *Vigna* plants, seven were found to be antagonistic towards the growth of *Alternaria* sp. Among the isolates, *Aspergillus niger, Penicillium citrinum* showed 38.55 per cent and 19.34 per cent growth inhibition of the pathogen, respectively. Table reveals that five *Trichoderma* spp, (tested previously) were the most effective antagonists against the test pathogen. Highest response was performed by *T. viride* (87.33%) followed by *T. harzianum* (80.66%), *T. koningii* (79.56%) and *T. hamatum* (70.67%). The other isolates did not show any remarkable response. *Trichoderma* attached 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. The following consists of the production of various CWDEs and peptaibols (Howell, 2003).

The data presented in Table 2 indicates that all the four *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 (78.55%) wherein *T. harzianum* exhibited 76.29 per cent inhibition. This was followed by 74.11 per cent 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 97.78 per cent inhibition. Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that impede colonization by antagonizing micro-organisms; among these metabolites, the production of harzianic acid, tricholin, peptaibols, 6-pentyl-α-pyrone, massoilactone, viridian, glioviridin, glisopenins, heptilidie acid have been described (Vey *et al*., 2001). In addition to these suzukacillin, alamethicine, demadin, trichodermin (Chakraborty and Chatterjee, 2008) are some of the antibiotics extracted from culture filtrates of *Trichoderma* spp. A new antifungal compound *viz.* 6-substituted 2H-pyran-2-one named viridipyronone has been isolated from the culture filtrate of a strain of *T. viride* showing *in vitro* activity towards plant pathogenic fungi (Evidente *et al*., 2003).

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 (laminarinase) 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* sp, 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 (1014 units) than *T. harzianum* (876.33 units) and *T. koningii* (715.33 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

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

Fungi	Radial growth of the pathogen (cm)	Radial growth of the isolates (cm)	Growth inhibition of pathogen (%)*
Penicillium citrinum	7.26	1.74	19.34± 0.23
Aspergillus niger	5.53	3.47	38.55 ± 0.23
Trichoderma virens	2.0	7.0	77.78 ± 0.55
T. harzianum	1.74	7.26	80.66 ± 0.33
Trichoderma viride	1.14	7.86	87.33 ± 0.41
Trichoderma koningii	1.84	7.16	79.56 ± 0.39
T. hamatum	2.64	6.36	70.67 ± 1.22
Fusarium oxysporum	9.0	0.0	0
Helminthosporium sp.	9.0	0.0	0
Control	9.0	0	0
S.E.±	± 3.21		
C.D. (P=0.05)	6.8373		

*Data are the mean values of three replicates

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

Antagonists	Radial growth of the pathogen (cm)	Growth inhibition of the pathogen (cm)	Growth inhibition of the pathogen* (%)
T. viride	1.93	7.07	78.55 ± 0.43
T. harzianum	2.14	6.86	76.29 ± 0.32
T. koningii	2.93	6.07	67.44 ± 0.22
T. hamatum	2.33	6.67	74.11 ± 0.58
Control	9	0	0
S.E.±	± 5.36		
C.D. (P=0.05)	11.4168		

*Data are the mean values of three replicates

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

Antagonists	Radial growth of the pathogen (cm)	Growth inhibition of the pathogen (cm.)	Growth inhibition of the pathogen* (%)
T. viride	0	9	100 ± 0
T. harzianum	0.203	8.8	97.78 ± 0.35
T. koningii	2.9	6.1	67.78± 0.20
T. hamatum	2.5	6.5	72.22± 0.34
Control	9	0	0
S.E.±	± 0.47		
C.D. (P=0.05)	1.001		

*Data are the mean values of three replicates

Table 4 : Levels of production of laminarina (-1, 3 glucanase) enzymes by *Trichoderma* spp.-

Antagonists (*Trichoderma* spp.)	β-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	808 ± 1.03	734.66 ± 0.918	1014 ± 1.33
T. harzianum	552±1.5	302 ± 1.44	876.33±1.05
T. koningii	577 ± 0.77	387 ±1.04	715 ± 1.44
T. hamatum	431 ± 0.76	325±1.09	645± 1.22

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

	S.E.M	SEM	SEM	C.D. (P=0.05)	C.D. (P=0.05)	C.D. (P=0.05)
	CDB	CDB + ACW	CDB – S + ACW	CDB	CDB + ACW	CDB – S + ACW
β-1, 3glucanase	12.55	13.41	34.33	26.73	28.563	73.122

*Data are the mean values of five 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	126.40 ± 0.33		89.6 ±1.33		171.66 ± 0.92	
T. harzianum	91 ± 1.11		69.66 ± 1.29		121.33 ± 1.75	
T. koningii	73.33±0.58		42.00 ± 0.97		107.66 ± 0.73	
T. hamatum	83.66±0.23		52.33± 0.97		131.67± 1.63	
	SEM	SEM	SEM	C.D. (P=0.05)	C.D. (P=0.05)	C.D. (P=0.05)
	CDB	CDB + ACW	CDB – S + ACW	CDB	CDB + ACW	CDB – S + ACW
Chitinase	7.66	8.90	6.78	16.31	18.957	14.4414

* 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 infected plants	% of infection	Treatments	Total no. of plants#	No. of infected plants#	% of infection	Reduction of infection (%)*
2011	78	34	43.59 ± 0.87	*T. viride*	56	12	21.42	50.86 ± 1.28
	71	32	45.07± 0.37	*T. harzianum*	58	23	39.65	13.25 ± 4.22
	70	32	45.71± 0.72					
2012	70	30	42.85 ± 1.66	*T. viride*	60	14	23.33	46.49 ± 0.88
	68	30	44.11± 1.26	*T. harzianum*	58	18	31.03	26.85 ± 1.87
	70	34	48.57± 1.06					
						S.E.±		C.D. (P=0.05)
2011: Reduction of infection (%)						± 4.93		10.5009
2012: Reduction of infection (%)						± 5.20		11.076

Average data of three fields
*Data are the mean values of threes replicates.

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. It may be noted from the result that a large interpsecific differences in *Trichoderma* spp. existed in respect of the production of chitinase and β-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 the *Trichoderma* spp. indicating thereby their varying degree of biocontrol efficacy. Chitinase and β-1, 3-glucanase also known to release fungal cell wall fragments which elicit other defense responses. Production of lytic enzymes and the factors which influence their production are therefore, the aspects which will determine the potential 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 54.11 per cent and 46.49 per cent as against 13.25 per cent and 26.85 per cent reduction of infection with *T. harzianum*, respectively of the trial years (2011 and 2012).

Trichoderma strains are plant symbiont opportunistic aviruent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms (Harman *et al*., 2004 and Hussein *et al*., 2014). Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stresses and the uptake and use of nutrients. Together with the synthesis of stimulation of phytohormone production, most *Trichoderma* strains acidify their surrounding environment by secreting organic acids, such as gluconic, citric or fumaric acid (Gomez-Alarcon and de la Torre, 1994). These organic acids result from the metabolism of other carbon sources, mainly glucose, and, in turn, are able to solubilize phosphates, micronutrients and mineral cations including iron, manganease and magnesium (Harman *et al*., 2004). Biological control offers an environmentally friendly approach to the management of plant disease and can be incorporated into cultural and physical controls and limited chemical usage for an effective integrated disease management system.

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