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Assessment of biocontrol potential of *Trichoderma* isolates against wilt in pulses

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ABSTRACT

Potentiality of *Trichoderma* isolates was assessed against wilt pathogen of pulse crops. *Trichoderma* spp. were identified by DNA bar-coding based on the sequences of internal transcribed spacers regions; and characterization of ech-42 and xyn-2 gene was also done for the *Trichoderma* spp. Among the 14 tested *Trichoderma* isolates, IIPRTh-33, IIPRRTas-8 and IIPRTas-13 showed the presence of xyn-2 genes. While the seven isolates (IIPRTh-33, IIPRTas-8, IIPRTas-13, IIPRTh-31, IIPRTg-3, IIPRTh-38, IIPRTh-20) showed the presence of ech-42 gene. The highest chitinase and xylanase activity was observed in IIPRTh-33 and IIPRTh-31. All the isolates were also screened for siderophore and IAA production. Ethyl acetate extract of two *Trichoderma* isolates (IIPRTh-31 and IIPR.Tg-3) yielded more than 43 metaboilites out of which 2H-Pyran-2- one and 1,2-benzenedioxylic acid esters, Benzaldehyde, 4-nitro- tetradecanoic acid3-methyl-heptadecanol, methyl cyclohexane, 6-nonylene alcohol, methyl-cyclopentane, 2-methyl heptadecanol, N-methyl pyrollidine, dermadin, ketotriol, koningin-A, palmitic acid, 3-(2'-hydroxypropyl)-4-(hexa-2'-4- dineyl)-2-(5H)-furanone, Phenylethyl Alcohol and 3-(propenone)-4-(hexa- 2'-4'-dineyl)-2-(5H)-furanone were reported to have antifungal activity of *Trichoderma* isolate. *Trichoderma* isolates IIPRTh-31 was tolerant to heat shock of 50°C and observed to be superior salt-tolerant among all the tested isolates.

Key words: Antibiosis, Biocontrol, Metabolites, Mycoparasitism, *Trichoderma*, Wilt disease

Trichoderma is known as well-established promising biological control agents worldwide and they also produced several secondary metabolites with potential applications as novel antibiotics. They are well known producers of chitinolytic enzymes and are used commercially as a source of these enzymes. Additional interest in these enzymes is stimulated by the fact that chitinolytic strains of *Trichoderma* are among the most effective biological control agents of many plant pathogens (Harman *et al*. 1993, Vinale *et al*. 2009, Agrawal & Kotasthane 2009, Karlsson *et al*. 2010, Singh *et al*. 2016). More than 100 species of *Trichoderma* have been phylogenetically characterized. There are different formulations which are available in market commercially for crop production worldwide (Harman 2000). *Trichoderma* have been widely used for the management of several phytopathogens. Wilt is the most difficult and challenging soilborne disease in terms of management. Chemicals are widely used for the control of this disease but they cause a great loss to the environment also. So, there is a need to explore *Trichoderma* species effective against Fusarium wilt. Among various biocontrol agents (BCAs) *Trichoderma* spp. has been widely exploited for their biocontrol ability in different crop to manage the different pathogens (Abd El-Khair *et al.* 2010, Mohiddin *et al.* 2010, Papavizas *et al.* 1984, Papavizas 1985, Harmon 2006, Harman *et al.* 2004, Verma 2007, Mishra and Gupta 2012, Mukherjee *et al*. 2013, Mishra *et al.* 2016, 2017, 2018a, 2018b). *Trichoderma* isolates trigger induce resistance in plants and protect the plant from pathogens. During the induced systemic resistance plants produce and accumulate metabolites and enzymes which play important role in defense mechanisms such as PAL, SOD, PR-Protein etc. The indigenous strains of *Trichoderma* spp. seems to function better as they are widely adapted to local environmental conditions. No significant studies pertaining to identification of indigenous potential *Trichoderma* spp from pulses rhizosphere and their exploitation. Therefore, the aim of the present manuscript was to identify and characterize the potential *Trichoderma* species and to check their efficacy against wilt pathogen of chickpea, pigeonpea and lentil.

MATERIALS AND METHODS

A total 13 isolates of *Trichoderma* (Table 1) collected from the pulse rhizosphere of different locations of Uttar Pradesh and stored in the Crop Protection Division of ICAR-IIPR Kanpur. *Fusarium oxysporum* f. sp. *ciceri, Fusarium udum* and *Fusarium oxysporum* f. sp. *lentis* were isolated from the research farm of ICAR-IIPR Kanpur. Molecular strain identification was done on the basis of ITS region of ribosomal RNA gene cluster amplification (Hassan et al., 2014). The ITS region was amplified using the following programme 3 min at 94 °C followed by 35 cycles each of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C and, finally extension of 10 min at 72°C. The PCR products were checked on 1% agarose gel, sequencing of the PCR products was done from Chromus Biotech Pvt. Ltd. Isolated *Trichoderma* species along with standard *Trichoderma* strains were checked for their antagonistic potential against the pathogen by binary culture test. For the test we take a 7mm disc of actively growing *Trichoderma* from the culture plate and inoculate it on a fresh and sterile PDA plate and on the opposite end we inoculate the test pathogen (Upadhyay and Rai, 1987). The main aim of this study was to check the

mycelial growth inhibition of Fusarium spp. by *Trichoderma* isolates. The experiment was replicated thrice and percent growth inhibition was calculated.

The endochitinase gene was amplified using the primer 5'- CTTGTAGTCCCAAATACCGTTCTCCCA -3' and R: 5'- GCAAACGCCGTCTACTT CACCAACTGG -3'. The PCR cycle was as follows: 5 min at 94°C,1 min 95 °C ,2 min at 50°C, and 2 min at 72°C for 30 rounds. The extension period was 7 min at 72°C (Carolina Carsolio et al 1994) and xyn-2 gene using the primer 5'-GTAGGTTACGTCTGACGG-3' and R: 5'-CCGTGAGGAAGCCCAGTC-3. The PCR conditions was as follows: 5 min at 94°C,1 min 94 °C,2 min at 51°C, and 1 min at 72°C and The extension period was 7 min at 72°C for 30 rounds. The raw sequence reads of ITS, ech42 and xyn-2 were checked for quality, trimmed, manually edited and assembled using CLC Genomics Workbench 7.5 (CLCBio, Aarhus, Denmark). To conduct taxonomic identification, publicly available sequences deposited at NCBI (www.ncbi.nlm.nih.gov) by using a basic local alignment search tool (BLAST) (Altschul et al. 1997).

Trichoderma sp isolated from the chickpea rhizosphere were maintained on agar. For enzyme production test the TLE medium containing 1% of either chitin, potato starch, cellulose, xylan (pH 5) was inoculated with spore conc. of 3 x 10^7 spores (in one mL of saline). Cultures were then incubated for 7 days at rotatory shaker (120rmp) at 28°C. After 7 days culture supernatant was filtered with what man filter paper and centrifuged to remove spores and stored at -21°C until the use. Chitinase activity was assayed at 37°C by monitoring the amount of reducing sugar N-acetyl glucosamine using p-nitro phenol. β-1,3 glucanase activity was determined based on the release of reducing sugar laminarin. One unit of enzyme was defined as the amount of enzyme necessary to produce 1 µmol of reducing sugar in one minute (chitinase and β-1,3-glucanase). Total cellulase activity was determined by measuring the amount of reducing sugar formed from filter paper (Ximenes *et al*. 1995). One unit of total cellulase activity correspond to the amount of reducing sugar produced in one minute. Endoglucanase activity was determined using the method of Janice *et al.* 2003. One unit of endoglucanase activity was defined as the amount of protein necessary to produce one mmol of reducing sugar in one minute. β-glucosidase activity (cellobiosidase) was assayed by measuring production of glucose from cellobiose (Ximenes *et al.* 1995). One unit of cellobiosidase or aryl- β-glucosidase activity was defined as the amount of protein necessary to produce 1 mmol of glucose or p-nitrophenol respectively, in one minute. Xylanase activities were assayed by measuring the amount of reducing sugars released from xylan, respectively, using dinitro salicylic acid (DNS) assay as described by Bailey et al. 1992. Briefly, 0.5 mL of culture supernatant was added to 1 mL of 0.05 M citrate buffer of *p*H 4.8. To this mixture, 0.5 mL of 1% w/v beech wood xylan was added as a substrate for xylanase assay. All the samples were incubated at 50°C for 30 min. To this, 2 mL of DNS reagent was added, heated in water bath at 90°C for 10 min and cooled immediately and the absorbance was measured in a spectrophotometer at 550 nm.

The IAA production ability of *Trichoderma* was assayed supplementing the basal media with 0.5 $mgmL^{-1}$of L–tryptophan and incubating at 28ºC for 3 days under shaking conditions (120 rpm). After 3 days culture broth was obtained and centrifuged for the removal of spores. Sopre free supernatant was used for the IAA test as described by Nathan Vinod Kumar *et al.* 2017. Quantification of siderophore enzyme was done by CAS shuttle assay (S. M. Pyne 1995). 0.5 mL of culture supernatant was mixed with 0.5 mL of CAS reagent and absorbance was measured at 630 nm against a reference consisting of 0.5 mL of uninoculated broth and 0.5 mL of CAS reagent. Siderophore content was calculated by using following formula:

$$\% \text{ Siderophore content} = AC–AT/AC \times 100$$

Where AC=Absorbance value of Control and AT= Absorbance value of treatment

For the extraction of volatile compounds two most effective *Trichoderma* species (IIPRTg-3 and IIPRTh-31) were inoculated into 500 ml of PDB medium and incubated 28°C for 25 days. After incubation period completed contents of the flasks were filter through muslin clothes. The liquid phase obtained after filtrations was used for extraction. Extraction was done with ethyl acetate (1:1). Upper phase of the solvent which contain volatile compounds was collected through the separating funnel. Solvent was removed from the collected phase and obtained residue is dissolved in acetone. This sample was then used for GC-MS analysis. To identify the thermo tolerant *Trichoderma* isolate conidial thermotolerance test was used. A conidial suspension containing 1×10^{10} conidia per ml was prepared and inoculated into the culture vials containing PDB. This inoculated PDB was exposed to heat shock for 1, 2 and 4 h at 48,50 and 52ºC (Sowmya Poosapati *et al.* 2014). Three replicates for each treatment were used. After heat shock treatment 1ml from each culture vial was serially diluted and plated on the PDA plates.

RESULTS AND DISCUSSION

Bio-control agent *Trichoderma* has gained importance as a substitute of chemical pesticides and hence an attempt was intended to corroborate the positive relatedness of molecular and morphological characters. Molecular identification of *Trichoderma* isolates was done using ITS primers (Anderson and Stasovski, 1992). Obtained Nucleotide sequences were used for the species level identification (Table 1). All 13 isolates belong to species *T. harzianum*, *T. longibrachiatum*, *T. asperellum* and *T. afroharzianum.*

Table 1. Origin of the *Trichoderma* strains used in this study and sequences from NCBI GenBank accession numbers

Isolates Name	Name of Species	NCBI GenBank accession number	
		ITS	Isolation sources
IIPRTh-33	*T. afroharzianum*	MN186847	Chickpea
IIPRTas-8	*T. asperellum*	KX681721	Pigeonpea
IIPRTas-13	*T. asperellum*	KX681731	Pigeonppea
IIPRTh-31	*T. asperellum*	MK968811	Chickpea
IIPRTg-3	*T.longibrachiatum*	MH511661	Pigeonpea
IIPRTh-38	*T. harzianum*	MK970735	Chickpea
IIPRTh-20	*T. harzianum*	MH511669	Pigeonpea
IIPRTh-3	*T. harzianum*	KX681710	Fieldpea
IIPRTh-23	*T. harzianum*	MH511673	Pigeonpea
IIPRTas-1	*T. asperellum*	KX681709	Lentil
IIPR-59	*T.longibrachiatum*	MK849898	Chickpea
IIPR-72	*T.longibrachiatum*	MK849904	Chickpea
IIPR-74	*T. asperellum*	MK849905	Chickpea
TH-10 NBAIR	-	-	-

Most of the *Trichoderma* isolates studied in this work were able to control the growth of tested pathogen. The mycelial inhibition percentage was found maximum for Th-31 (80%, 82.30 and 83.75), for remaining isolates it was found between 65 to 75% (Table 2). The differences observed in vitro assays might be due to the variability in genotypes. In vitro antagonistic activity of *Trichoderma* isolates indicates their potential to inhibit pathogen growth in field. However, the in vitro activity of *Trichoderma* isolates does not correlate directly with the in vivo ability to control pathogens since many other factors influence the activity of pathogens (Anees *et al.* 2010).

Trichoderma species are well known for their biocontrol potential. In the present study tested *Trichoderma* species shows the production of chitinase and xylanase enzyme. Chitinase and xylanase enzyme are thought to play an important role in mycoparasitism between phytopathogens and *Trichoderma*. In the present study isolated *Trichoderma* species found to produce the substantial amount of these enzymes (Table 3). Chitin and xylan are the main components of fungal cell walls and *Trichoderma* species are the potent producer of these enzymes.

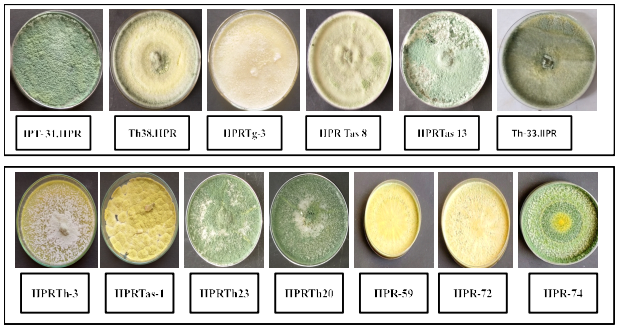

Fig. 1: Growth pattern of *Trichoderma* colonies in PDA medium

Table 2. In vitro antifungal activity of *Trichoderma* isolates against *Fusarium* spp.

Sl No	*Trichoderma* isolate	Inhibition in mycelial growth of *Fu* through binary culture assay	Inhibition in mycelial growth of *Foc* through binary culture assay	Inhibition in mycelial growth of *Fol* through binary culture assay
1	**IIPRTh-31**	80.6	82.3	83.75
2	**IIPRTh-33**	75.89	83.56	85.67
3	**IIPRTh-38**	75.90	74.90	72.15
4	**IIPRTg-3**	68.90	70.16	76.34
5	**IIPRTas-13**	69.00	65.89	73.45
6	**IIPRTas-8**	61.32	66.75	65.40
7	**IIPRTh-3**	68.79	68.90	73.67
8	**IIPRTh-23**	72.30	73.21	69.80
9	**IIPRTh-20**	71.45	74.40	70.78
10	**IIPRTas-1**	76.21	74.89	72.67
11	**IIPR-59**	64.44	62.44	71.11
12	**IIPR-72**	68.89	64.44	66.67
13	**IIPR-74**	73.32	61.63	62.79
14	**TH-10 NBAIR**	60.15	64.33	69.60

Trichoderma species have been reported for the biostimulation of plant growth and development of a wide variety of plants (Harman *et al.* 2004; Bhardwaj *et al.* 2014; Kumar *et al.* 2020). There are several mechanisms which are involved in the growth promotion like mineral solubilzation and uptake and increasing plant nutrient uptake (Altomare *et al.* 1999), secretion of phytohorrmones like IAA and enzymes leading to stronger root and shoot development. (Vinale *et al.* 2008a; 2012) in the present study isolated *Trichoderma* species shows the production of siderophore and IAA (Table 2).

Among the 13 tested *Trichoderma* isolates with one check, 7 isolates (IIPRTh-33, IIPR.Tas-8 and IIPR.Tas-13) showed the presence of ech-42 and xyn-2 genes. While the remaining three isolates (IIPRTh-33, IIPRTas-8 and IIPRTas-13) showed the presence of only xyn-2 gene. The *Trichoderma* chitinase and xylanase enzyme activity was monitored for all isolates under study. The highest chitinase and xylanase activity was observed in IIPRTh-33 and IIPRTh-31. All the isolates were also screened for plant growth promotion enzyme production (Siderophore and IAA) Table 3. In *Trichoderma* there are many volatile metabolites which have been reported to play an important role in the mycoparasitic action there are around more than 40 metabolites in *Trichoderma* which help in mycoparasitism (Sivasithamsaran and Ghiberti in 1998). The GC-MS analysis of partially purified crude extract of Th-.31.IIPR and IIPR.Tg3 yields around 43 compounds out of which 1, 2- benzenedicarboxylic acid reported in this study is well known for its antimicrobial activity and 2H-Pyran-3-ol, which act as plant growth regulator and helpful in mycotoxin detoxification also identified from culture filtrate of *Trichoderma* strain. Results of conidial thermo tolerant test clearly indicate that IIPRTh-31 and IIPRTas-1 are different from all the other tested isolates IIPRTh-31 was able to grow after the heat shock treatment at 50ºC for 4

Table 3. Plant growth promoting enzymes and Mycoparasitic enzyme quantification of *Trichoderma* isolates

Isolate Name	Growth promoting enzyme		Mycoparasitic enzyme					
	Sidrophore (%)	IAA (ug/ml)	Xylanase (IU/ml/min)	Chitinase (mg/ml)	Endoglucanase (IU/ml/min)	FPAase (IU/ml/min)	B-glucosidase (IU/ml/min)	B-1,3 glucanase activity (IU/ml/min)
IIPRTh-33	6.54	2.8	1.813	110	1.168	2.594	0.578	0.867
IIPRTas-8	22.55	2.8	1.013	50	0.827	2.057	0.620	0.743
IIPRTas-13	4.00	2.03	1.253	50	0.0651	2.16	0.651	2.52
IIPRTh-31	24.168	3.2	2.344	98	0.640	2.057	0.661	0.671
IIPRTg-3	14.55	2.6	1.529	45	0.671	2.170	0.609	0.836
IIPRTh-38	19.17	3.6	1.469	50	1.064	5.68	0.630	1.41
IIPRTh-3	28.84	4.0	0.999	50	0.827	1.943	0.630	0.960
IIPRTh-23	19.54	1.6	1.410	40	0.713	2.067	0.584	0.836
IIPRTh-20	27.82	2.8	1.43	45	0.651	2.046	0.568	1.260
IIPRTas-1	22.40	3.2	1.328	40	0.992	2.057	0.568	1.01
IIPR-59	14.96	4.6	0.121	38	0.568	2.098	0.532	1.177
IIPR-72	11.01	2.8	0.061	45	0.889	2.86	0.558	1.446
IIPR-74	13.04	3.2	0.087	60	0.723	1.974	0.568	0.775
TH-10 NBAIR	8.10	1.6	1.678	42	0.068	1.343	0.558	0.360

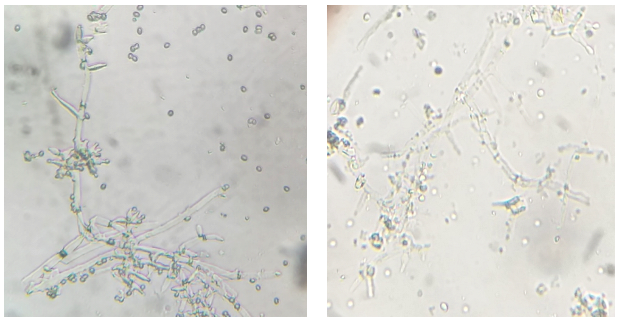

Fig. 2: Microscopic variability between control (A) and heat treated *Trichoderma* isolate (B)

hours while IIPRTas-1 was able to sustain heat shock at 50ºC for 2 hours. The heat tolerant colonies when culture on PDA plates it shows morphological features different from the wild type reduced sporulation, and decreased hyphal growth were also observed in the heat tolerant isolate after heat shock (Fig. 1).

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