Molecular and Antagonistic Variability of *Trichoderma atroviride* against Legume Crop Pathogens in Uttar Pradesh, India

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Abstract

Antagonistic and molecular variability are reported to exist among eight isolates of *Trichoderma atroviride,* collected from rhizosphere soil of legumes from different places of Uttar Pradesh, India. Antagonistic variability of isolates of *T. atroviride* revealed significant suppression in the radial growth of *Fusarium oxysporum* f.sp. *ciceri*, *F. oxysporum* f.sp. *lentis* and *Fusarium udum*. The maximum inhibition of 47.91% of mycelial growth of *F. udum* was recorded in case of TAU_8 isolate, which is isolated from soil sample of Azeetmal. TSI_3 and TKD_3 isolates of Misihinrick and Maitha block of Sitapur and Kanpur dehat, respectively was found (41.66%) least effective against *F. udum*. Similarly, maximum inhibition of mycelial growth of *F. oxysporum* f.sp. *ciceri* was recorded as 55.08% by the TH_3 isolate, which was isolated from soil sample of Bilgram block of Hardoi district. The TS_5 isolate of Lainbua block of Sitapur was found 39.76% least effective against *Fusarium lentis* and TH_1 showing maximum inhibition of 60.26% myccelial growth against *Fusarium lentis*. Molecular variability among the isolates showed that total number of amplified bands was found 74 out of which 65 were showing polymorphic and 19 were monomorphic and the size of amplified product varied from 0.1 kb to 0.75 kb.

1. Introduction

Trichoderma species are free living rapid growing fungi that are common in soil and root eco-system. The teleomorph of this genus is *Hypocrea*. The fungi are exceptionally good model for biocontrol and more importantly as bioagent by means of mycoparasitism, antibiosis and competition (Mandal, 1995; Girdhari, 2006). *Trichoderma* spp. parasitize a wide range of fungi. Several species of *Trichoderma* have been isolated from various substrates and location (Bilgrami et al., 1971; Nagamani et al., 2002). Several articles have also been published to identify the *Trichoderma* spp., based on the molecular or physiological bases (Samuels, 1996; Hermosa et al., 2000). Kiffer and Morelet (2000) have recognized several species of *Trichoderma* based on molecular characters. Most of the species of *Trichoderma* are effective against soil borne pathogens that cause diseases in leguminous crop. Among them Fusarium wilt is most important one. There have been found a high level of genetic diversity in *Trichoderma* spp. (Chakraborty et al., 2010) and can be used to produce wide range of products of commercial and ecological interest.

2. Materials and Methods

2.1. Isolation of Trichoderma spp.

Soil samples were collected from rhizospheres of chickpea, pigeonpea and lentil from different places of Uttar Pradesh (Table 1) and *Trichoderma* spp. were isolated on PDA medium by following serial dilution plate technique as described by Johnson and Curl (1972). 10 g soil sample from well pulverized, air dried soil was added into 90 ml sterile water in a flask to make 1:10 dilution (10^{-1}).The mixture was vigorously shaken on a magnetic shaker for 20-30 minutes to obtain uniform suspension. One ml of suspension from flask was transferred into a test tube containing 9 ml sterile water under aseptic conditions to make 1:100 (10^{-2}) dilution. Further dilution 10^{-3} was made by pipetting 1 ml suspension into additional water as prepared The Petri plates were incubated at 25±2^{0}C for 7 days in an incubator. As soon as the mycelial growth were visible in the PDA culture medium, the hyphal tips from the advancing mycelium were cut and transferred into the culture slants containing PDA medium for further purification and identification of pathogen. The pure culture was obtained by

adopting single spore technique. The identity of the purified bioagents was then confirmed by ITCC, Division of Plant Pathology IARI, New Delhi-12.

2.2. Antagonistic variability of different isolates of T. atroviride against Fusarium spp.

The antagonistic potentiality of the isolates of *T. atroviride* was determined by dual culture technique (Morton and Stroube, 1955). A disc of 5 mm diameter was made from 7 days old culture of different isolates of *T. atroviride* and placed at one point leaving 1 cm distance from the periphery of one side of petri plate and on the opposite site, disc (5 mm diameter) of *Fusarium udum, F. oxysporum* f.sp. *ciceri* and *F. oxysporum* f.sp. *lentis* were placed separately. Plate was kept without antagonist to serve as control. The Petri plates were incubated at 25±2^{0}C for 7 days. Three replications were kept for each treatment. Observations on colony growth were recorded and % inhibition was measured by using the following formula:

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

Where, I: % inhibition in mycelia growth; C: Growth of pathogen in control plates; T: Growth of pathogen in dual culture plates.

2.3. Variability of T. atroviride based on RAPD

2.3.1. Production of mycelial mat

T. atroviride was grown in 1000 ml conical flask containing 400 ml of PDB medium. Two agar plugs from actively growing colony of *T. atroviride* were transferred to each flask aseptically in a laminar flow. The flask was incubated at 25^{0}C for 21 days. The mycelial mat was collected by passing the fluid through three layers cheese cloth.

2.3.2. Extraction of genomic DNA

The fungal cell wall was disrupted by grinding with pestle and mortar in liquid nitrogen. The DNA was extracted by CTAB method of fungal DNA extraction as used by Kumar et al. (2011). Quantification of DNA was done with 0.8% agarose gel electrophoresis. Working concentration of DNA was adjusted to 20 ng μl^{-1} and stored at 4^{o}C. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

2.3.3. Random amplified polymorphic DNA (RAPD)

The procedure described by Williams et al. (1990) with minor modification was done for carrying out PCR reaction to produce RAPD profiles. Amplification of DNA fragments was carried out by the PCR using 10-mer arbitrary primers. The reaction mixture consisted of 300 ng of 200 µmM of dNTP mix (Fermentas company), 15 pmol of primer (Operon), 5U μl^{-1} of Taq polymerase (Fermentas) and 25 mM $MgCl_2$. DNA amplifications were performed in thermocycler with one cycle of initial denaturation at 94^{o}C for 15 min, followed by 40 cycles of denaturation at 94^{o}C for 1 min, annealing at 35°C for 2 min, extension at 72°C for 1 min and final extension at 72°C for 10 min.

Amplified products together with uncut lambda marker double digest; Bangalore, Genei) were resolved by 1.5% agarose gel electrophoresis (60 V for 1 hr). Gels were photographed by Gel documentation system (Uvitec).

2.4. Data analysis

Comparison of each profile for each primer was done on the basis of the presence or absence (1/0) of amplified bands. Bands of the same size (in bp) were scored as identical. Analysis was based on the cluster analysis using UPGMA to identify the variability.

3. Results and Discussion

Soil samples were collected from different places of Uttar Pradesh and bio-agents were isolated by serial dilution method. The isolates were preserved in refrigerator on PDA and confirmation of the isolates was done by ITCC, Division of Plant Pathology, IARI, New Delhi as *T. atroviride* (Table 1).

3.1. Antagonistic evaluation of T. atroviride against Fusarium ssp.

In order to evaluate the antagonistic potential of *T. atroviride* dual culture technique was carried out with three legume pathogens viz., *F. oxysporum* f.sp. *ciceri*, *F. oxysporum* f.sp.

Table 1: Isolation of *T. atroviride* from different places of Uttar Pradesh

ID Number	Culture Number	Reference Number	Source	Fungus identified
ITCC-7442/09	6 CP	06	Sultanpur	*Trichoderma atroviride*
ITCC-7443/09	24 CP	07	Sitapur	*Trichoderma atroviride*
ITCC-7445/09	71 L	09	Hardoi	*Trichoderma atroviride*
ITCC-7446/09	115 L	10	Bahraich	*Trichoderma atroviride*
ITCC-7447/09	52 L	11	Unnao	*Trichoderma atroviride*
ITCC-7448/09	75 PP	12	Auriya	*Trichoderma atroviride*
ITCC-7449/09	126 PP	13	Kanpur Dehat	*Trichoderma atroviride*
ITCC-7451/09	105 CP	15	Etawah	*Trichoderma atroviride*

lentis and *F. udum* species. The effect of different isolates of *T. atroviride* with respect to suppression of mycelial growth of three test pathogens was recorded.

It is evident from the data that the *T. atroviride* suppressed the radial growth of *F. oxysporum ciceri* and *F. oxysporum lentil* and *F. udum* significantly (Table 2). The maximum inhibition of 47.9% of mycelial growth of *F. udum* was recorded in case of TAU_8 isolates, which is isolated from soil sample of Azeetmal block of Auraiya. TSI_3 and TKD_3 isolate of Mishriack and Maitha were found least effective against *F. udum* among all the isolates of *T. atroviride.*

Most of the isolates of *T. atroviride* were able to suppress myeclial growth of *F. oxysporum* f.sp. *ciceri* significantly as evident from the Table 3. The maximum inhibition of mycelial growth of *F. oxysporum* f.sp. *ciceri* was recorded as 55.8% by the TH_3 isolate, which was isolated from soil sample of Bilgram block of Hardoi district. TSI_4 isolate of Misirithrik was found least effective against *F. oxysporum* f.sp. *ciceri* among all the isolates. The findings clearly shows the wide range (22-53% inhibition over control) of antagonistic effect of different isolates of *T. atroviride.*

The antagonistic effect of different isolates of *T. atroviride* against mycelial growth of *F. oxysporum* f.sp. *lentis* shows that the maximum 60.26% inhibition of mycelial growth was recorded by the *T. atroviride* TH_1, isolated from soil sample of Bilgram block of Hardoi district. The TS_5 isolate of Lainbua block of Sultanpur was found least effective against the pathogen, showing 39.76% inhibition over control. Girdhari (2006) also found the antagonistic variability in different isolates of *Trichoderma* spp. collected from different places of India. The present finding was also supported by several workers (Fernandez, 1992; Santosh, 2004). Singh et al., 2013 also revealed that 30 isolates of *Trichoderma viride* collected from various district of U.P. were found highly antagonist against three test pathogens.

3.2. Variability of T. atroviride based on RAPD

The result presented on the Figure 1 showed that the total number of reproducible band amplified were 94 out of which 75 were found to be polymorphic and 19 were monomorphic, hence the % of polymorphism is 79.78. The number of bands primer^{-1} ranged from maximum of 12 (given by OPC-13) to a minimum of 3 (OPC-8) with an average of 7 bands primer^{-1}. The experimental findings also revealed that the 7 RAPD primers (OPC 10,11,13,14,15, 19 and 20) produced average or above average amplified products (Table 2). Moreover OPC 11,19 and 20 i.e 3 RAPD primers showed unique polymorphic bands and none of the OPC primers showed unique monomorphic bands. Among the tested RAPD marker

Table 2: OPC primers used for RAPD amplification and their corresponding PCR products for bio-agent *T. atroviride*

Name of Primer OPC	Sequence of Primer 5'-3'	Amplified product	Total number of bands	Number of poly-morphic band	Number of mono-morphic bands
OPC 1.	TTCGAGCCAG	Yes	6	2	4
OPC 2.	GTGAGGCGTC	No	0	0	0
OPC 3.	GGGGGTCTTT	Yes	4	3	1
OPC 4.	CCGCATCTAC	Yes	4	3	1
OPC 5.	GATGACCGCC	No	0	0	0
OPC 6.	GAACCGACTC	Yes	6	3	3
OPC 7.	GTCCCGACGA	No	0	0	0
OPC 8.	TGGACCGCTG	Yes	3	1	2
OPC 9.	CTCACCGTCC	No	0	0	0
OPC 10.	TGTCTGGGTG	Yes	7	2	5
OPC 11.	AAAGCTGCGG	Yes	0	10	0
OPC 12.	TGTCATCCCC	No	0	0	0
OPC 13.	AAGCCTCGTC	Yes	12	11	1
OPC 14.	TGCGTGCTTG	Yes	11	11	0
OPC 15.	GACGGATCAG	Yes	7	7	0
OPC 16.	CACACTCCAG	No	0	0	0
OPC 17.	TTCCCCCCAG	No	0	0	0
OPC 18.	TGAGTGGGTG	Yes	5	3	2
OPC 19.	GTTGCCAGCC	Yes	0	0	0
OPC 20.	ACTTCGCCAC	Yes	9	9	0
Grand Total			94	75	19

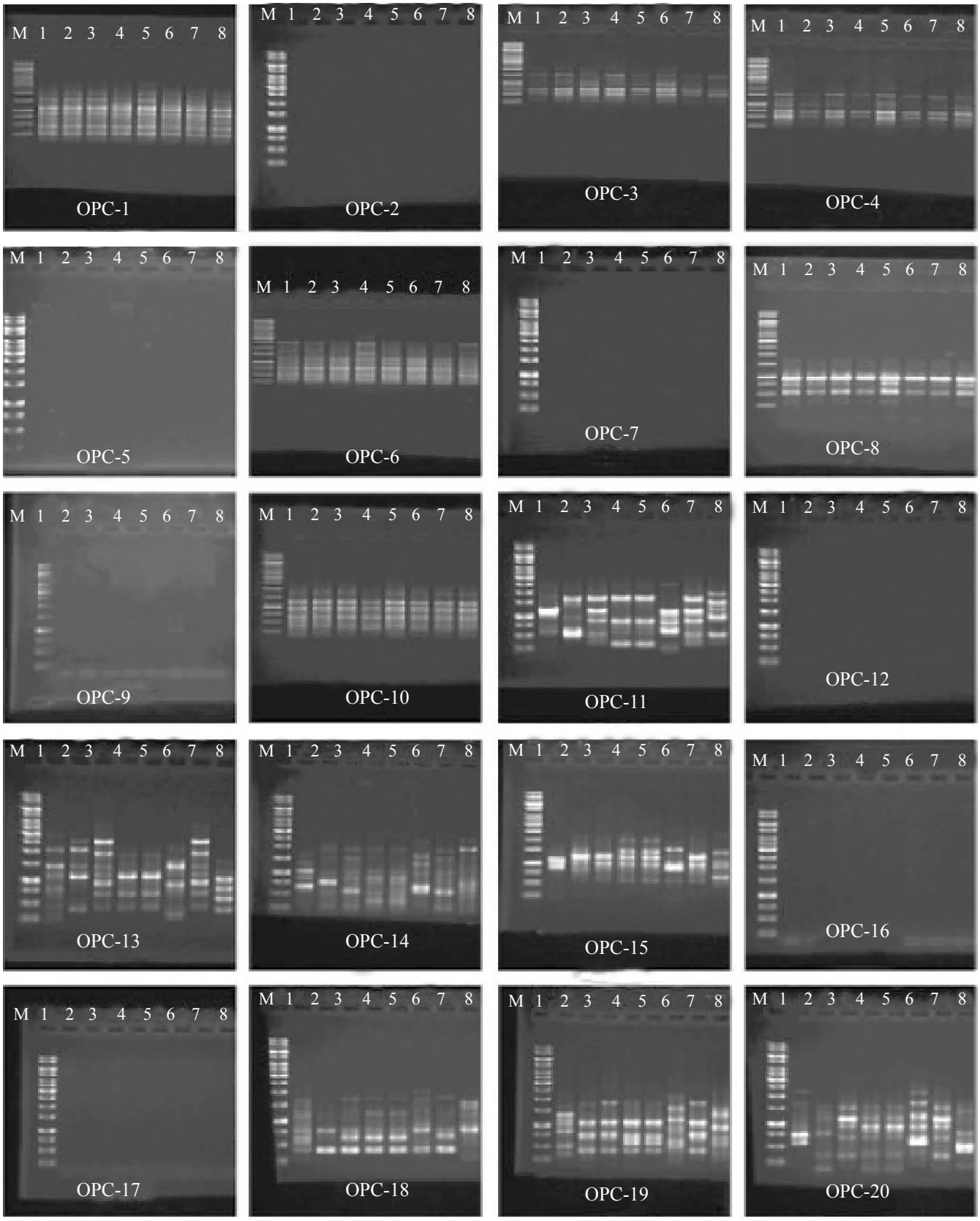

Figure 1: RAPD analysis of *Trichodrma atroviride* isolates (8) with 20 primers (OPC-01-OPC20).

Table 3: Antagonistic evaluation of different isolates of *T. atroviride* against *F. oxysporum* f.sp. *ciceri*, *F. oxysporum* f.sp. *lentis* and *F. udum*

I.D. Number		*In vitro* antagonistic effect of *Trichoderma* spp. against *Fusarium udum*				*In vitro* antagonistic effect of *Trichoderma* spp. against *Fusarium oxysporum* f.sp. *ciceri*				*In vitro* antagonistic effect of *Trichoderma* spp. against *Fusarium oxysporum* f.sp. *lentis*			
	Culture Number	Block	Name of Pathogen	Average growth (24 mm)	% inhibition	Block	Name of Pathogen	Average growth (23 mm)	% inhibition	Block	Name of Pathogen	Average growth (26 mm)	% inhibition
ITCC-7442/09	06 CP	Kurebhar	TS_7	13.0	45.83	Kadipur	TS_6	11.6	53.65	Lainbua	TS_5	15.6	39.76
ITCC-7443/09	24CP	Mishinrick	TSI_3	14.0	41.66	Misirithrik	TSi_4	14.3	37.69	Gondlamau	TSi_3	14.3	44.88
ITCC-7445/09	71L	Bilgram	TH_5	13.0	45.83	Bilgram	TH_3	10.3	55.08	Bilgram	TH_1	10.3	60.26
ITCC-7446/09	115L	Ballaha	TBa_1	12.6	47.25	Gosainganj	TB_1	11.0	52.17	Ballaha	TBa_4	15.0	42.30
ITCC-7447/09	52L	Bangarmau	TU_3	13.5	43.75	Fatehpur...	TU_2	13.8	40.00	Bangermau	TU_5	14.80	43.07
ITCC-7448/09	75PP	Azeetmal	TAU_8	12.5	47.91	Azeetmal	TAU_8	13.0	43.47	Auraiya	TAa_4	13.5	48.07
ITCC-7449/09	126PP	Maitha	TKD_3	14.0	41.66	Maitha	TKD_6	13.0	43.43	Maitha	TKD_4	13.8	46.92
ITCC-7451/09	105PP	Bharthna	TE_{10}	13.0	45.83	Bharthna	TE_4	12.9	43.91	Bharthna	TE_8	13.0	50.00
SEm±				0.23	1.28			0.25	1.19			0.38	1.38
CD (p=0.05)				0.508	2.71			0.55	2.52			0.81	2.93

7 OPC primers (OPC-2, 5, 7, 9, 12, 16 and 17) primers does not give any amplification. The size of the amplified product varied from minimum of 0.1 kb to maximum of 750 bp i.e 0.75 kb. Thus presence or absence of the bands mentioned in the Table 2 indicated that the variability existed among the isolates. Chakraborty et al. (2010) found the variability based on RAPD analysis among nineteen isolates of *T. viride* and *T. harzianum* obtained from rhizosphere soil of plantation crops, forest soil, and agricultural fields of North Bengal. Pervaiz et al. (1999) also found that in Precise Detection and Tracing of *Trichoderma hamatum* 382 in compost amended mixes by using molecular markers.

4. Conclusion

It may be concluded from the present findings that antagonistic and molecular variability exist among eight isolates of *T. atroviride,* collected from rhizosphere soil of different places of Uttar Pradesh. It is also concluded that there was good genetic diversity and these are strong possibility to get the isolates specific primers that will utilized for particular *Trichoderma* isolates with good biological potential form the field isolates without going the cumbersome bioassay.

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6. References

Bilgrami, K.S., Jamaluddin, Rizvi, M.A., 1971. Fungi of India. Today and Tomorrow Publication, New Delhi.

Chakraborty, B.N., Chakraborty, U., Saha, A., Dey, P.L., Sunar, K., 2010. Molecular characterization of *Trichoderma viride* and *T. harzianum* isolated from soil from North Bengal based on r DNA markers and analysis of their PCR-RAPD profiles. Global Journal of Biotechnology and Biochemistry 5(1), 55-61.

Fernandez, M.R., 1992. The effectiveness of *Trichoderma harzianum* on fungal pathogen infecting wheat and black oat straw. Soil Biology and Biochemistry 24, 1031-1034.

Girdhari, L.K., 2006. Effect of *Trichoderma* on *Drechslera oryzae* and its role in defense against brown leaf spot of paddy. M.Sc. Thesis, CSAUAT, Kanpur, 78.

Hermosa, M.R., Gronoda, I., Iturriaga, A., Diaz-Minguez, J.M., Castro, C., Monte, E., Garcia-Acha, I., 2000. Molecular Characterization and Identification of Biocontrol Isolates of *Trichoderma* spp. Applied and Environmental Microbiology 66(5), 1890-1898.

Johnson, L.F., Curl, E.A., 1972. Methods for Research on

the Ecology of Soil Borne Plant Pathogens. Burgess Publishing Company, Minneapolis, 247.

Kiffer, E., Morelet, E., 2000. The Deuteromycetes Mitosporic Fungi Classification and Generic Keys. Science Publications Inc., USA, 1152.

Kumar, Vipul., Shahid, M., Singh, A., Srivastava, M., Biswas, S.K., 2011. RAPD analysis of *Trichoderma longibrachiatum* isolated from pigeonpea fields of Uttar Pradesh. Indian Journal of Agricultural Biochemistry 24(1), 80-82.

Mandal, S., 1995. Effect of some antagonists on *Drechslera sorokiniana*, causal agent of spot blotch of wheat. M.Sc. Thesis, IARI, New Delhi, 74.

Morton, D.J., Stroube, W.H., 1955. Antagonistic and stimulating effects of soil micro-organism of Sclerotium. Phytopathology 45, 417-420.

Nagamani, A., Kunwar, I.K., Manoharachary, C., Reddy, P., 2002. *Trichoderma fllavofuscum*-a new record of India. Indian Phytopathology 55, 247-248.

Pervaiz, A., Abbasi, S.A., Miller, T.M., Harry, A.J., Hoitink., Jin Man Kim., 1999. Precise Detection and Tracing of *Trichoderma hamatum* 382 in compost amended mixes by using molecular markers, National University San 96-1, Doonduck-dong,Yosu, Cheon Nam 550-250.

Samuels, G.J., 1996. *Trichoderma*: a review of biology and systematic of the genus. Mycological Research 100, 923-935.

Santosh, B.P., 2004. Effect of some antagonists on *Drechslera oryzae*, causal agent of brown leaf spot of paddy. M.Sc. Thesis, CSAUAT, Kanpur, 49.

Singh, A., Shahid, M., Srivastava, M., Kumar, V., Bansal, A., 2013. Antagonistic activity of *Trichoderma viride* isolates against different pathogens of *Fusarium oxysporum* isolated from legume crop of U.P. Progressive Research 8(1), 47-50.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Research 18, 6531-6535.