J. Mycopathol, Res, 50(1) : 55-59 (2012)
© Indian Mycological Society, Department of Botany,
University of Calcutta, Kolkata 700 019, India

PCR-RFLP marker based DNA amplified fragments and diversity assessment of *Trichoderma* spp. from Andaman and Nicobar Islands

ISRAR AHMAD, SOMESHWAR BHAGAT, KRISHNA KUMAR, AJANTA BIRAH, A.K. TRIPATHI, K. MADHURI AND R.C. SRIVASTAVA

Division of Field Crops, Central Agricultural Research Institute, P.B.No.181, Port Blair 744101, Andaman and Nicobar Islands, India

Received : 10. 08 2011 Accepted : 21. 10. 2011 Published : 30.04.2012

The biocontrol fungi, *Trichoderma* spp. are the most important biocontrol agent and have been used extensively worldwide for the management of various soil plant pathogens. Eight isolates of *Trichoderma (T. harzianum, T. viride* and *T. hamatum)* were isolated from rhizosphere of different crops and lactation of South Andaman district, Andaman and Nicobar Island, and tested *in vitro* against *Sclerotium rolfsii* causing stem rot of brinjal. *In vitro* test of these antagonists against test pathogen revealed that all isolates significantly inhibited the *S. rolfsii in vitro* but the isolate Tv-2 was most effective in parasitizing the test pathogen with 69.1% inhibition followed by Tv-3 (68.5%) and Tv-5 (67.8%). PCR- RFLP amplified fragments with ITSI and ITS4 primer of these isolates showed clear difference among the *Trichoderma* isolates with overall 75% similarity except Thr-1. However, the *Trichoderma* isolates Tv-2, Tv-3, and Tv-5 of *T. viride* were found to have 90% similarity, whereas other isolates Thr-5B and Thr-5M of *T. harzianum* were very close in genetic similarity to these isolates.

Key words: Andaman and Nicobar Islands, *Trichoderma, Sclerotium rolfsii,* PCR-RFLP

INTRODUCTION

Biological control is a great renaissance of interest and research in microbiological balance in relation to soil borne disease,and in the development of more enduringly profitable and wiser farming system. The balancing dogma in nature remains unaffected as the principle of biological control involved every component from the nature, by the nature and within the nature through a little human interference as per requirement. The situation has become alarming and it has now been imperative to understand the role of soil microorganism in sustainable crop productivity without further deterioration of soil health, as biological control offers the chances of improving crop production with existing resources, avoiding the problems of development of pathogen resistance to chemical and offering a relatively pollution and risk free control.

Trichoderma (teleomorph *Hypocrea*) is a genus of asexual fungi found in soils of all climatic zones. *Trichoderma* is a secondary opportunistic invader, a fast growing fungus, a strong spore producer, a source of cell wall degrading enzymes, and an important antibiotic producer. The main biocontrol mechanisms that *Trichoderma* utilizes indirect confrontation with fungal pathogens are mycoparasitism (Papavizas,1985; Howell, 2003; Bhagat and Pan, 2008) and antibiosis (Howell, 1998; Reino *et al.*,2008). The abundance of *Trichoderma* in various soil, coupled with their ability to degrade various organic substrates in soil, their metabolic versatility, and their resistance to microbial inhibitors, suggests that they may possess the ability to survive in many ecological niches depending on prevailing conditions and the species or strains involved (Papavizas, 1985). *Trichoderma* spp. are among the most frequently isolated soil fungi and present in plant root systems. These fungi are opportunistic, avirulent plant symbionts (Harman *et al.*, 2004). and functions as parasites and antagonists of many phytopathogenic fungi, thus protecting plants from disease. So far, *Trichoderma* spp. are among the most studied fungal BCAs and commercially marketed as biopesticides, biofertilizers and soil amendments (Harman *et al.*, 2004; Lorito *et al.*, 2004). The studies on

Trichoderma in relation to its antagonistic potential and diversity have not been carried out in the Bay Islands (Bhagat and Pan, 2008). Therefore, present research aimes to isolate *Trichoderma* isolates from different crop rhizospheres and evaluate their antagonistic potential against *Sclerotium rolfsii* and study their diversity by PCR-RFLP.

MATERIALS AND METHODS

Antagonist and pathogen

Eight isolates of *Trichoderma* were isolated from different locations of Andaman and Nicobar Islands by following soil dilution (Dhingra and Sinclair, 1995) and plated on *Trichoderma* specific medium (TSM) (Elad and Chet, 1983). Pure culture of *Trichoderma* isolates were maintained in PDA slants and preserved at 4°C.

The pathogen, *Sclerotium rolfsii* Sacc. was isolated from infected part of brinjal(collar) following tissue segment method (Rangaswami, 1958). Pure culture of *S. rolfsii* was maintained in PDA slants and preserved at 4°C.

Dual culture test

Antagonistic activity of *Trichoderma* isolates were tested by dual culture test (Morton and Stoube, 1955) against *S. rolfsii.* Mycelial plug (6 mm) of *S. rolfsii* was inoculated in the periphery region of Petriplate previously poured with PDA and incubated for 3 days at 28±1°C.The mycelial plug of *Trichoderma* isolates were also inoculated in the same plate opposite to *S. rolfsii* and incubated at 28±1°C for 7 days. The Petriplates with only mycelial plug of pathogen served as control.

DNA extraction

Trichoderma isolates were maintained on Potato Dextrose Agar (PDA (Hi Media) at 28±1°C and these isolates were grown in Potato Dextrose Broth (PDB) for 5 days for DNA extraction. Fungal mat was collected on filter paper, washed with distilled water, air dried and grounded in Liq. N_2 Total genomic DNA was extracted by using the method of (Reader *et al.*, 1985). Pure DNA was dissolved in 100 µl 1xTAE buffer. Purity of DNA was quantified by UV spectrophotometry and ethidium bromide fluorescence.

PCR amplification

Primer ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC -3') were synthesized by Gene@ amplify a fragment of rDNA. PCR amplification were performed in a total volume of 20 µl by mixing 50 ng of genomic DNA, 0.2 µM conc. of each primer (Gene @), 2.5 mM dNTPs (Gene@), and 1 unit of *Taq* DNA polymerase (Gene@) in 1x PCR buffer. The reaction mixture was subjected to initial denaturation of 95°C for 5 min followed by 45 cycle of 95°C for 1 min, 55°C for 30 sec, 72°C for 1.20 min, with a final extension of 72°C for 10 min. in a thermocycler. PCR amplified products were analysed by running in 1% Agarose gel (Gene @), prepared in 1xTAE buffer containing 0.5 µg ethidium bromide and photographed over a transilluminator.

RFLF analysis

PCR amplified fragments by ITS1 and ITS4 primers from all the eight samples were subjected to restriction digestion by 3 different restriction endonuclease (RE) (*Eco* R1, *Hinf II,* and *Hae III,)* in a final reaction mixture containing PCR products 12 µl, RE 1.5 unit (Gene @),and 10x Buffer 1µl (Gene@). These digestion mixtures were incubated at 37°C for two hr and were mixed with 2 µl of bromophenol blue dye and run in 1% Agarose gel (Gene@), prepared in 1xTAE buffer containing 5 µl ethidium bromide.

Data analysis

All the genotypes were scored for presence and absence of the RFLP bands, and the data were entered into a binary matrix as discrete variables-1 for presence and 0 for absence of character and these data matrix was subjected to further analysis. The 0/1 matrix was used to calculate similarity as Jaccard coefficient using SIMQUL subrouline in similarity routine. The resultant similarity matrix was employed to construct dendrogram using SAHN based UPGMA to infer genetic relationship (Rohlf *et al.*, 1998).

RESULTS

Dual culture test

Eight isolates of *Trichoderma* were evaluated *in*

vitro against *Sclerotium rolfsii.* by dual culture test. All isolates significantly inhibited the test pathogen but the isolate TV2 was most effective in inhibition of mycelial growth of *S. rolfsii* followed by TV3 and TV5 which statistically did not differed. The antagonistic activity of other isolate was found to have intermediate effect and the isolated Thm- 1 recorded with least percentage inhibition of pathogen.So from this dual culture test we presumed that TV3, TV2 and TV5 can be used as a very good biocontrol agent against *S. rolfsii.*The growth of pathogen was completely stopped after 12 days of incubation in the isolates of *Trichoderma* except with Thr- 1.

PCR- RFLP analysis

The primer ITSI and ITS 4 amplified ITS regions in all the isolates. The sizes of the amplified fragements ranged from 590 to 600 bp (Fig. 1). Length of amplified fragement in all the eight isolates of *Trichoderma* were same. All eight isolates showed 2 and 3 RFLP patterns with enzyme *Hinf II, Hae III.* respectively (Fig. 2). There was no intraspecific differences among isolates on band patterns by *Eco* RI. A UPGMA dendrogram from combined restriction pattarn is presented in Fig. 3. Dendrogram clearly differentiated all isolates of *Trichoderma* on 50% similarity in four different clusters.Cluster one contained only simgle culture isolate of *Trichoderma harzianum* i.e. Thr5B. This isolate of *Trichoderma* shows 79 % similarity with other isolate of same species (Thr 5M). Cluster two contained all the isolate of *T. viride* i.e. TV2, TV3, TV5 and one isolate of Thr5M. TV3 and TV5 showed 100% similarity while both these isolates were 90% similar to TV2. All the isolates of *T.viride* showed 88% similarity with Thr5M that is different from *T. viride* species. Cluster three contained only two isolates but both from different species (*T. viride* and *T. hamantum*), showing 90% similarity. Cluster four contained only single isolate of *T. harzianum* i.e. Thr1 This isolate of *Trichoderma* showed entirely different antagonistic activity also.This isolate exhibited 50% similarity with other 7 isolate of *Trichoderma* i.e. Thr 5B and Thr 5M.

DISCUSSION

The use of fungal biocontrol agents is becoming an increasingly important alternative to chemicals in crop protection against insects and diseases in both agriculture and forestry. *Trichoderma* strains can act as a biocontrol agent (BCAs) through different synergic mechanism. However it is difficult to predict the degree of synergism and the behavior of a BCA in a natural pathosystem. Several researchers have reported that the *Trichoderma* can effectively suppress the growth and development of many soil borne plant pathogens *in vitro* and under field condition (Bell *et al.*,1982., 2007; Pan and Bhagat, 2008). Present investigation revealed that all the isolates did not inhibited the *S. rolfsii,* this findings are in accordance with Bell *et al.* (1982), Pan and Bhagat (2008)and Dubey *et al.*,(2007),where they comprehensively reported the variability in antagonistic potential among the different species of *Trichoderma* against plant pathogens. Bell *et al.* (1982) screened 77 isolates of *T. harzianum* against six fungal pathogens and recorded significant differences between pathogen -antagonist interactions. Mathew and Gupta (!998) showed that *T. harzianum* exhibited the maximum antagonistic activity causing 58.3% inhibition of pathogen followed by *T. hamatum, T. viride* and *T. virens* inhibiting 48.3, 46.1 and 44.9%, respectively. Mycoparasitism,the direct attack of one fungus on another, is a very complex process that involved sequential events, including recognition ,attack and subsequent penetration and killing of the host. *Trichoderma* spp. may exert direct biocontrol by parasitizing a range of fungi, detecting other fungi and growing towards them (Harman *et al.*,2004).

PCR-RFLP analysis can readily distinguish a given species or isolates from others. PCR amplification of ITS region of 5.8SrRNA gene of all the isolates yielded an ITS fragment of 600 bp length in all the isolates of *Trichoderma.* No intra-species ITS length diversity was detected. This is due to the fact that 5.8SrRNA gene is highly conserved at genus level and this confirmed that all the isolates belong to a single genus. Functionally and evolutionary conserved rRNA gene blocks contain both highly non conserved sequences which have been used in various studies to determine phylogenic relationships (Forster *et al.*, 1990, Gaudet *et al.*, 1989, Sreenivasaprasad *et al.*, 1994). In the present study all the isolates of *T. harzianum* were grouped together in one cluster and distributed randomely at 50% similarity. This could be due to intra-species variation amongs the isolates. Earlier report based on the RAPD profiles and ITS sequencing of the conserved gene was also suggested similar type of the observation amongst the isolates of *T.*

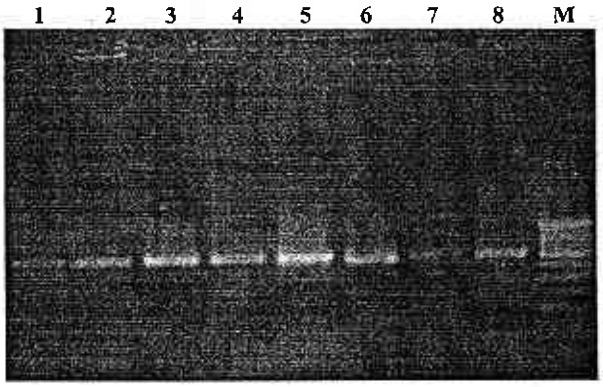

Fig. 1 : 600 bp PCR amplified fragments from primer ITS and ITS 4. M is marker

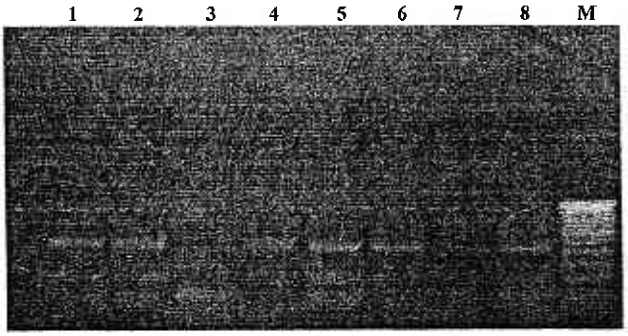

Fig. 2 : *Hinf* II digested DNA of all the isolates. M is marker DNA

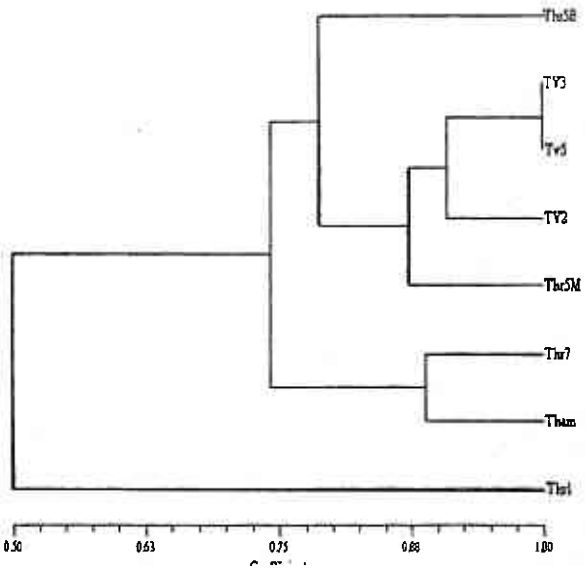

Fig. 3 : Antagonistic activity of *Trichoderma* isolates TV3 Thr7 & TV5.CDendogram showing the relationship among 8 *Trichoderma* spp. based on the PCR-RFLP profiles of 16S rDNA regions.

harzianum (Choi *et al*, 2003, Samuels *et al*, 2002). Genotyping of certain antifungal compounds producing *Trichoderma* spp.indicated that organisms having similar properties might share some genetic relationship. The high genetic similarity between TV2, TV3, and TV5 could be due to their similar type of habitat ecology and other edaphic factors. Very high similarity was supported by almost similar level of antagonistic activity. The variability of the antagonistic isolates also corroborated with different levels of antagonism exhibited by them.The dendrogram showed grouping of the isolates by the level of antagonism. However, relationships among the polymorphism existent and the source (host plant) of the isolates were not found. Furthermore, the grouping of isolates of different rhizospheres indicated the possibility of non-influence of host exudates.

ACKNOWLEDGEMENT

The authors are grateful to Indian Council of Agricultural Research for the financial support. Thanks are also due to P. Simachalam and Sheela Pal for their help rendered.

REFERENCES

Bell, D.K., Wells, H.D. and Markham, C.R. 1982. *In vitro* antagonism of *Trichoderma* spp. against six fungal plant pathogens. *Phytopathology* **72:** 379-382.

Choi, I..Y., Hong, S..B. and Yadav, M.C. 2003. Molecular and morphological characterization of gree mold, *Trichodrema* spp.isolated from oyster mushrooms. *Mycobiology,* **31:** 74-80.

Dhingra, O.P and Sinclair, J.B. 1995. *Basic Plant Pathology Methods,* 2nd edn. CRC press, Bocca Raton, America.

Dubey, S.C., Suresh, M. and Singh, B. 2007. Evaluation of *Trichoderma* spp against *Fusarium oxysporum* f sp *ciceris* for integrated management of chickpea wilt. *Bio. Cont* **40** : 118-127.

Elad, Y. and Chet, I. 1983. Improved selective media for isolation of *Trichoderma* spp. and *Fusarium* spp. *Phytoparasitica* **11** : 55-58.

Forster, H., Coffey, M.D., Ellwood, H. and Sogin, M. L. 1990. Sequence analysis of small subunit ribosomal RNAs of three zoosporic fungi and implications for fungal evolution .*Mycologia,* **82:** 306-312.

Gaudet, J., Julien, J., Lafay, J.F. and Brygoo, Y. 1989. Phylogeny of some *Fusarium* species, as determined by large subunit rRNA sequence comparison. *Mol. Biol. Evol.* **6:** 227-242.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., and Lorito, M. 2004. *Trichoderma* species-opportunistic, avirulent plant symbionts. *Nat. Rev.* **2:** 43-56.

Howell, C.R. 1998. The role of antibiosis in biocontrol. In: Harman GE, Kubicek CP (eds) *Trichoderma & Gliocladium,* vol.2. Taylor & Francis, Padstow, pp 173-184.

Howell, C.R. 2003. Mechanisms employed by *Trichderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Pl. Dis.* **87:** 4-10.

Lorito, M., Woo, S.L. and Scala , F. 2004. Le biotechnologie utili alla difesa sostenible delle piante: i funghi. *Agroindustria* 3: 181-195.

Mathew, K.A. and Gupta, S.K. 1998. Biological control of rot of French bean caused by *Rhizoctonia solani*. *J. of Myco. and*

Pl. Path. **28:** 202-205.

Morton, D.T. and Stroube, N.H. 1955. Aantagonistic and stimulatory effect of microorganism upon *Sclerotium rolfsii. Phytopat.* **45:** 419-420.

Pan, S. and Bhagat, S. 2008. Characterization of *Trichoderma* species from West Bengal. *J of Bio Cont,* **22:**43-49.

Papavizas, G .C.1985. Trichoderma and Gliocladium :biolgy and potential for biocontrol. *Annual Review of phytopathology* **23:** 23-54.

Rangaswami, G. 1958. An agar block techniques for isolating soil microorganism with special reference to Pythiacious fungi. *Sci. and Cul.* **24:** 85.

Reader, U., and Broda, P. 1985 Rapid prepration of DAN from filamentous funji. *Lett. Appl. Mic.***1:** 17-20.

Reino, J.L., Guerrero, R.F., Hernandez-Galan, R. And Collado, IG. 2008. Secondary metabolites from species of the biocontrol agent *Trichoderma. Phytochemisty Review* **7:** 89-123.

Rohlf, F.J. 1998. *NTSYS:numerical taxonomy and multivariate analysis system,* 2nd ed. Exeter Software, State University of New York, Stony Brook, N.Y.

Samuels, G. L., Dodd, S. L., Gams, W., Castlebury, L.A. and Petrini, O. 2002. *Trichoderma* species associated with the green mold epidemic of commercially grown *Agaricus bisporus. Mycologia,* **94:** 146-170.

Sreenivasrprasad, S., Mills, P.R. and Brown, A. E. 1994. Nucleotide sequence of the rDNA spacer 1 enables indentification of isolates of *Colletotrichum* as *C. aculatum. Mycol. Res.* **98:** 186-188.

White, T.J., Bruns, T., Lee, S. and Taylor, J.W. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics.In : *PCR Protocols: A guide to methods and application* (ed. M.A. Innis, D. H Gelfand, J.J. Sninsky & T.J. White), pp. 315-322. Academic Press: San Diego, U.S.A.