Int.J.Curr.Microbiol.App.Sci (2018) *7*(6): 490-497

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 06 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.706.054

Molecular Characterization of *Trichoderma harzianum* and *Trichoderma viride* Isolated from Rhizosphere Soils by RAPD Markers

S.E. Navyashree[1*], Sumangala Koulagi[1], Sandhyarani Nishani[2], V. Kantharaju[3] and Chaya P. Patil[4]

[1]Department of Plant Pathology, [2]Department of Biotechnology and Crop Improvement, K. R. C. College of Horticulture, Arabhavi, Karnataka, India
[3]Department of Plant Pathology and Head AICRP on Fruits, KRCCH, Arabhavi, Karnataka, India
[4]KRCCH, Arabhavi, University of Horticultural Sciences, Bagalkot-587104, India

**Corresponding author*

Keywords

Trichoderma harzianum, *Trichoderma viride*, RAPD, Genetic diversity, PCR, Dendrogram

Article Info

Accepted:
04 May 2018
Available Online:
10 June 2018

A B S T R A C T

Twenty five isolates of *Trichoderma harzianum* and *Trichoderma viride* were obtained from rhizosphere soil of turmeric and ginger collected from Belagavi and Bagalkot districts of Karnataka and eight potential isolates based on antagonistic activity were studied using RAPD PCR. The genetic relatedness among two isolates of *Trichoderma harzianum* and six isolates of *Trichoderma viride* were analysed with five random primers. RAPD profiles showed genetic diversity among the isolates with the formation of two major clusters. Analysis of dendrogram revealed that similarity coefficient ranged from 0.15 to 0.95. Molecular variability among the potential isolates showed 81 amplified bands out of which 77 were polymorphic and 4 were monomorphic.

Introduction

The intensified use of fungicides in disease management has resulted in accumulation of toxic compounds potentially hazardous to environment and humans (Cook and Baker, 1983) and also in the build-up of resistance to the pathogens (Dekker and Georgopolous, 1982).

In view of this, effective alternatives to chemical control are being investigated and the use of biological control agents (BCAs) seems to be one of the promising approaches (Cook, 1985).

Among the BCAs, *Trichoderma* dominates the literature as successful antagonists and have gained wide acceptance as effective biocontrol agent against several soil borne plant

pathogens due to their ability to successfully antagonize other fungi (Joshi *et al.,* 2010; Hermosa *et al.,* 2012). *Trichoderma* commonly available in soil and root ecosystems has gained immense importance since last few decades due to its biological control ability against several soil borne plant pathogens (Kubicek and Harman, 1998).

These antagonistic microorganisms reduce growth, survival or infections caused by pathogens by different mechanisms like competition, antibiosis, mycoparasitism, hyphal interactions and enzyme secretion. In order to utilize the full potential of *Trichoderma* species in specific applications, precise identification and characterization of these fungi is vital (Druzhinina and Kubicek, 2005).

Several potential isolates for biological control are difficult to be distinguished from one other. Therefore in the present investigation, randomly amplified polymorphic DNA (RAPD) markers were used to estimate the genetic variations between the eight potential isolates of *Trichoderma* sp. which were previously isolated from the rhizosphere soil of turmeric and ginger.

Materials and Methods

Isolation and identification of *Trichoderma*

Rhizospheric soil samples were collected from turmeric and ginger growing areas of Belagavi (Gokak and Raybag Taluks) and Bagalkot (Mudhol and Jamakhandi Taluks) districts of Karnataka and collected soil samples were air dried for 4 h and used for the isolation of *Trichoderma* spp. by dilution plate technique using *Trichoderma* Selective Medium (TSM) which contained $MgSO_4.7H_2O$-0.2 g; K_2HPO_4-0.9 g; KCl-0.15 g; NH_4NO_3-1.0 g; Glucose-3.0 g; Chloramphenicol-0.2 g; Captan-0.2 g; Rose Bengal-0.15 g; Agar-agar-20 g; Distilled water-1000 ml (Elad and Chet, 1983).

Genomic DNA extraction from *Trichoderma* isolates

Each potential isolate was grown in 100 ml of potato dextrose broth (PDB) in 250 ml Erlenmeyer flasks. Cultures were maintained at 25±1°C with shaking (120 rpm) for three days. Mycelial mats were harvested by filtration, washed three times with sterile distilled water and powdered with liquid nitrogen using a pestle and mortar. Genomic DNA was extracted from the pulverized mycelium, using a modification of the Cetyl Trimethyl Ammonium Bromide (CTAB) extraction procedure, described by Alaei *et al.*, (2009).

A volume of 750 µl of extraction buffer (1.4 M NaCl, 50mM Tris-HCl pH 8, 0.01 M Na-EDTA, 1% β-mercapitoethanol and 2% CTAB) was added to 200 mg of each sample. The reaction mixture was briefly vortexed and incubated at 65°C in a water bath for 60 min. 100 µg proteinase K was added and samples were mixed by vortexing and incubated at 37ºC for 45 min. Then, 750 µl of Chloroform: Isoamyl alcohol [24:1 (vol:vol)] was added to the sample. The mixture was emulsified using a vortex and subsequently centrifuged at 13,000 rpm for 10 min. The clear supernatant was transferred to a new tube and the nucleic acids were precipitated with 600 µl isopropanol and centrifuged at 12,000 for 10 min. The pellet was washed in 70% ethanol and re-centrifuged. Finally, the pellets were dried at room temperature, suspended in 80 µl of Tris-EDTA buffer (10mM Tris-HCl, 1mM EDTA) and stored at -20°C.

RNAse treatment

The nucleic acid dissolved in TE buffer were treated with 3 µl of RNase A (20 mg/ ml) to remove any RNA contamination and incubated at 37ºC in a water bath and stored at - 20ºC until use.

Qualitative and quantitative estimation of DNA

The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The quantification of DNA was done by direct method by taking absorbance at 260 nm (OD_{260}). Absorbance of DNA samples dissolved in TE buffer was recorded against TE buffer as a blank using spectrophotometer. Concentration of DNA was calculated from optical density using following formula:

Concentration in µg/µl =

$$\frac{OD260 \times 50 \times \text{Dilution factor}}{1000}$$

DNA was also quantified by means of 0.8% agarose gel electrophoresis followed by ethidium bromide visualization using a 100 bp DNA ladder as DNA size markers. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

RAPD of *Trichoderma* isolates

For RAPD, five primers i.e., AA-04; OPA-4; AA-11; OPA-16 and OPC-05 were selected and their nucleotide sequences were given in the table 2.

PCR was programmed with an initial denaturing at 94°C for 4 minutes followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Thermocycler.

The reaction was carried out as follows

Twenty microliters of RAPD-PCR products were separated in a 2% agarose gel for electrophoresis with 1X TAE buffer, followed by staining with ethidium bromide and the bands were visualized under UV light. The image of the gel electrophoresis was documented through gel documentation system. All reproducible polymorphic bands were scored and RAPD profile patterns of eight potential isolates of *Trichoderma* were obtained by five RAPD primers.

Scoring and data analysis

The image of the gel electrophoresis was documented through gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed. The RAPD patterns of each potential isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendrograms using NTSYSpc. The analysis work was based on Jacquard's similarity coefficient given as:

Similarity coefficient =

$$\frac{\text{No.of polymorphic bands}}{\text{Total no.of bands}}$$

Results and Discussion

Twenty five isolates were obtained using the *Trichoderma* selective medium from the rhizosphere soil of turmeric and ginger (Table 1).

Among them twelve isolates were identified as *Trichoderma harzianum* and thirteen isolates as *Trichoderma viride*

Based on the antagonistic activity of all *Trichoderma* isolates tested against soil borne pathogens, eight isolates were found to show highest per cent inhibition and they were further characterised by using RAPD primers and were listed below

Fig.1 RAPD banding pattern in potential isolates of *Trichoderma* sp.

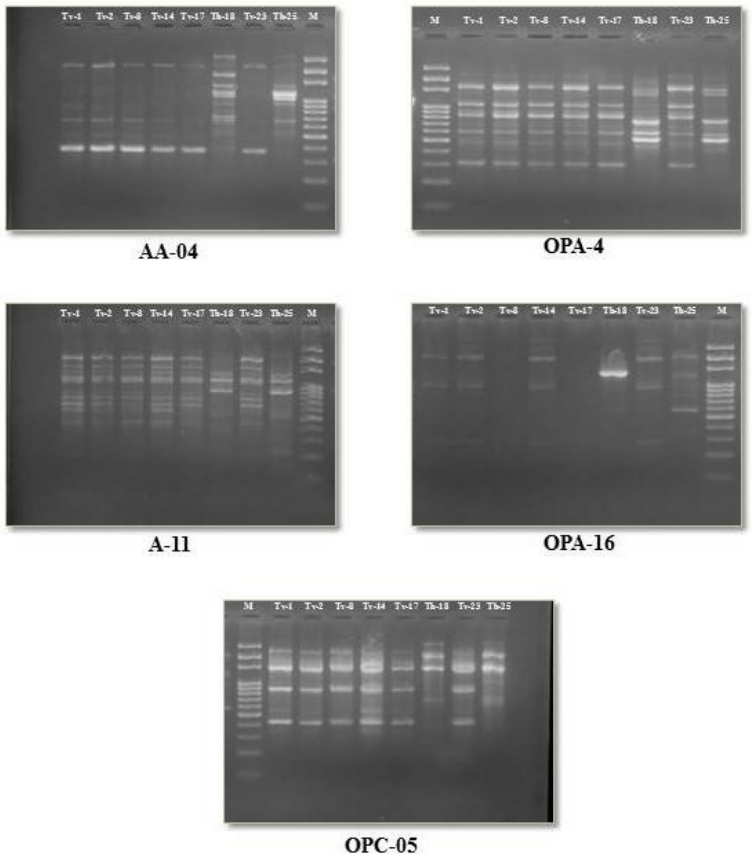

Fig.2 Dendrogram for eight potential isolates of *Trichoderma* sp. based on RAPD analysis

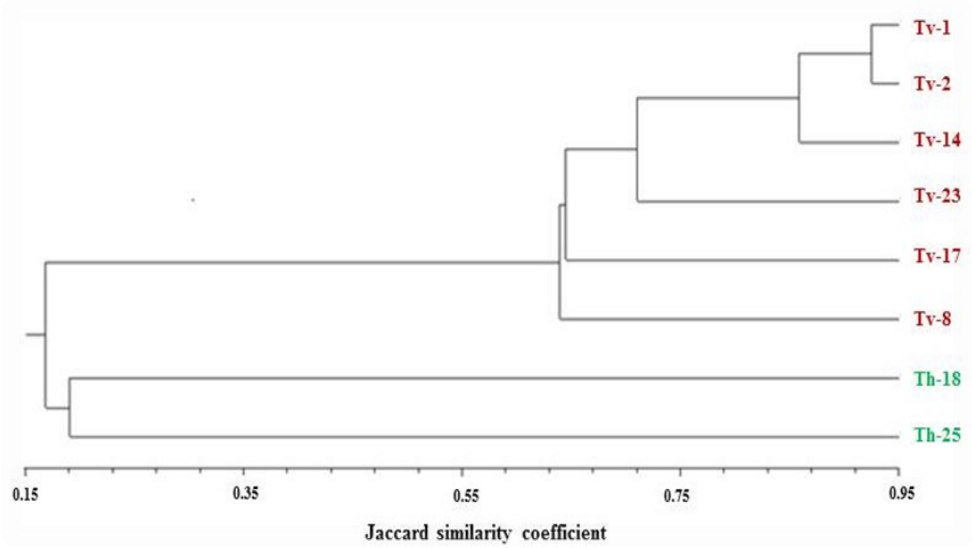

Table.1 Details of *Trichoderma* isolates collected from different locations

Sl. No.	District	Taluk	Location	Crop	Isolate No.
1	Belagavi	Gokak	Sangankere	Turmeric	Tv-1
2			KRCCH, Arabhavi	Turmeric	Tv-2
3			Kalloli	Ginger	Th-7
4			Sangankere	Ginger	Tv-8
5			Hallur	Turmeric	Th-12
6			Gurlapur	Ginger	Th-13
7			Tukanatti	Turmeric	Tv-22
8			Naganur	Turmeric	Tv-23
9		Raybag	Kaladal	Turmeric	Th-10
10			Itnal	Ginger	Tv-11
11			Kankanwadi	Turmeric	Tv-14
12			Kaladal	Ginger	Th-16
13			Devapur	Ginger	Tv-17
14			Halagawadi	Ginger	Tv-20
15			Hastuwada	Turmeric	Th-21
16	Bagalkot	Mudhol	Shirol	Turmeric	Tv-5
17			Mareguddhi	Turmeric	Tv-6
18			Siddapur	Turmeric	Tv-15
19			Mallapur	Turmeric	Th-24
20			Mudhol	Turmeric	Th-25
21		Jamakhandi	Rabakavi	Ginger	Th-3
22			Sasalatti	Turmeric	Tv-4
23			Bandigani	Ginger	Th-9
24			Madarakandi	Turmeric	Th-18
25			Aasangi	Turmeric	Th-19

Table.2 Amplification profile of RAPD primers for eight potential *Trichoderma* isolates

Sl. No.	Primer code	Primer sequence	Total Bands	Amplification product range (bp)	Polymorphic bands	Polymorphism (%)
1	AA-04	CAGGCCCTTC	21	430-3100	21	100
2	OPA-4	AATCGGGCTG	12	341-1500	11	91
3	A-11	AGGGGTCTTG	21	416-2300	21	100
4	OPA16	AGCCAGCGAA	13	296-3330	12	92
5	OPC-05	GATGACCGCC	14	336-3000	12	85

Table.3 Similarity coefficient of potential isolates of *Trichoderma* sp.

	Tv-1	Tv-2	Tv-8	Tv-14	Tv-17	Th-18	Tv-23	Th-25
Tv-1	1.00							
Tv-2	0.93	1.00						
Tv-8	0.68	0.70	1.00					
Tv-14	0.87	0.85	0.64	1.00				
Tv-17	0.70	0.71	0.64	0.66	1.00			
Th-18	0.18	0.19	0.15	0.15	0.18	1.00		
Tv-23	0.69	0.67	0.53	0.78	0.51	0.14	1.00	
Th-25	0.19	0.19	0.18	0.16	0.16	0.19	0.15	1.00

RAPD of *Trichoderma* isolates

Primer name	Sequence(5'-3')
AA-04	CAGGCCCTTC
OPA-4	AATCGGGCTG
A-11	AGGGGTCTTG
OPA-16	AGCCAGCGAA
OPC-05	GATGACCGCC

The reaction was carried out as follows

Cycles	Denaturation		Annealing		Extension	
First cycle	94^0C	4 min	-	-	-	-
35 Cycles	94^0C	1 min	36^0C	1 min	70^0C	90 s
Last cycle	-	-	-	-	72^0C	7 min

The antagonistic activity of all *Trichoderma* isolates

Sl. No.	*Trichoderma* species	*Trichoderma* isolates
1	*Trichoderma harzianum*	Th-18 and Th-25
2	*Trichoderma viride*	Tv-1, Tv-2, Tv-8, Tv-14, Tv-17 and Tv-23

RAPD-PCR analysis of potential *Trichoderma* isolates

The genetic relatedness among two isolates of *T. harzianum* and six isolates of *T. viride* were analysed by five random primers AA-04, OPA-4, A-11, OPA-16 and OPC-05 to generate reproducible polymorphism. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicated the genetic diversity of *Trichoderma* isolates (Figure 1). A total of 77 reproducible and scorable polymorphic bands ranging from approximately 296 bp to 3300 bp were generated with five primers among the eight potential isolates (Table 2). Jaccard's similarity coefficients were estimated for all eight potential isolates of *Trichoderma* sp. Relationships among the isolates were evaluated by cluster analysis of the data based on the similarity matrix (Table 3).

The Dendrogram was generated by NTSYSpc software (Figure 2) and similarity coefficient was ranged from 0.15 to 0.95 among the isolates. The similarity matrix indicated that isolates Th-18 and Tv-23 were genetically distinct as they showed only 14% similarity while the isolates Tv-1 and Tv-2 were found to be genetically similar, as 93% similarity was observed between them followed by 87% similarity between the isolates Tv-1 and Tv-14.

Based on the results obtained all the eight isolates were grouped into two major clusters. One cluster represents *T. viride* isolates *viz*., Tv-1, Tv-2, Tv-14, Tv-23, Tv-17 and Tv-8 and other major cluster represents *T. harzianum* isolates *viz*., Th-18 and Th-25. Again the *T. viride* cluster was sub grouped into two. The first subgroup included the isolates Tv-1, Tv-2, Tv-14, Tv-23 and Tv-17 while second subgroup comprised the single isolate Tv-8.

The first subgroup was again divided into two sub clusters. The first sub cluster comprised of isolates Tv-1, Tv-2, Tv-14 and Tv-23 while second sub cluster was included single isolate Tv-17. The first sub cluster was again sub divided into two which included the isolates Tv-1, Tv-2 and Tv-14 while second sub cluster included single isolate Tv-23. Again the first cluster was divided into two sub clusters *viz.*, Tv-1 and Tv-2 while second cluster included the single isolate Tv-23.

Lastly, the cluster was again divided into two sub clusters which include Tv-1 and Tv-2. The second major cluster of *T. harzianum* was divided into two different clusters contain isolates Th-18 and Th-25 respectively. The results of our studies validate the existence of significant intraspecific diversity in isolates of both *T. harzianum* and *T. viride*. The results were in accordance with the findings of Singh *et al.,* (2006), Shalini, *et al.,* (2006).

RAPD marker used to estimate the genetic variation among 8 potential isolates of *Trichoderma* sp. revealed 85-100 per cent polymorphism. The similarity matrix indicated that isolates Th-18 and Tv-23 were genetically distinct as they showed only 14 per cent similarity while the isolates Tv-1 and Tv-2 were genetically similar as 93 per cent similarity was observed between them followed by 87 per cent similarity between the isolates Tv-1 and Tv-14. Cluster analysis was used to generate a dendrogram, grouped isolates into two major clusters, the first major cluster consisted of six *T. viride* isolates (*viz.,* Tv-1, Tv-2, Tv-14, Tv-23, Tv-17 and Tv-8) and second major cluster included two *T. harzianum* isolates (*viz*., Th-18 and Th-25). In conclusion, the RAPD-PCR analysis used in the present study could successfully characterize the eight potential *Trichoderma* isolates.

References

Alaei, H., Baeyen, S., Maes, M., Hofte, M. and Heungens, K., 2009, Molecular detection of *Puccinia horiana* in Chrysanthemum × Morifolium through conventional and real-time PCR. *J. Microbiol. Methods*, 76: 136-145.

Cook, R. J. and Baker, K. F., 1983, The nature and practice of biological control of plant pathogens. St Paul, M. N., USA, APS Press.

Cook, R. J., 1985, Biological control of plant pathogens: theory to application. *Phytopathol*. 75: 25-29.

Dekker, J., Georgopolous, S. G., 1982, Fungicide resistance in crop protection. Wageningen, Netherlands, Pudoc.

Druzhinina, J. and Kubicek, C. P., 2005, Species concepts and biodiversity in *Trichoderma* and *Hypocrea*: from aggregate species to species clusters? J. Zhejiang Univ. Sci., 68 (2): 100-112.

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

Hermosa, M. R., Viterbo, A., Chet, I. and Monte, E., 2012, Plant beneficial effects of *Trichoderma* and of its genes. *Microbiol*., 58: 17-25.

Joshi, B. B., Bhatt, R. P. and Bahukhandi, D., 2010, Antagonistic and plant growth activity of *Trichoderma* isolates of Western Himalayas. *Environ. Biol*., 31: 921-928.

Kubicek, C. P. and Harman, G. E., 1998, *Trichoderma* and *Gliocladium*, Vol. I and II. Taylor and Francies Ltd. London, UK.

Shalini, Narayan, K. P., Lata and Kotasthane, A. S., 2006, Genetic relatedness among *Trichoderma* isolates inhibiting a pathogenic fungi *Rhizoctonia solani*. *African J. Biotechnol.*, 5(8): 580-584.

Singh, S. K., Sharma, V. P., Sharma, S. R., Satish Kumar and Tiwari, M., 2006, Molecular characterization of *Trichoderma* taxa causing green mould disease in edible mushrooms. *Curr. Sci.*, 90(3): 427-431.

How to cite this article:

Navyashree, S.E., Sumangala Koulagi, Sandhyarani Nishani, V. Kantharaju and Chaya P. Patil. 2018. Molecular Characterization of *Trichoderma harzianum* and *Trichoderma viride* Isolated from Rhizosphere Soils by RAPD Markers. *Int.J.Curr.Microbiol.App.Sci.* 7(06): 490-497.
doi: https://doi.org/10.20546/ijcmas.2018.706.054