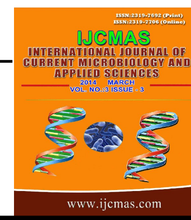

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Original Research Article

Molecular characterization of *Fusarium solani isolates* causing dry root rot of sweet orange (*Citrus sinensis* osbeck)

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Keywords

Polymerase chain reaction; *Fusarium* isolates; Random primers; Molecular characterization; RAPD.

A B S T R A C T

Fusarium solani is the important pathogen causing dry root rot disease of citrus in India. In the present investigation twelve representative isolate of *Fusarium solani,* collected from different places of Andhra Pradesh. Random amplified polymorphic DNA (RAPD) marker used to estimate genetic variation/variability among 12 isolates of the *Fusarium.* Based on pathogenicity test, per cent seedling mortality on Jambhiri species the isolates TCF_2 and TCF_{12} were categorized as slightly aggressive (1-25% seedling mortality), TCF_1, TCF_3, TCF_4, TCF_6, TCF_8 TCF_9 and TCF_{11} as moderately aggressive (26-50%). TCF_7 and TCF_{10}, as highly aggressive (51-75%), while TCF_5 as extremely aggressive (76-100%). These isolates were characterized using 20 random primers of OPM series, out of which 17 primers gave a total of 174 amplified products, showing 90.22% polymorphism. Genetic distance between each isolates was calculated, and cluster analysis was used to generate a dendrogram showing relationship among them. Isolates clustered into two major groups, first group having TCF_2 and TCF_3 in one cluster and remaining isolates in other cluster which in turn grouped into two sub-clusters seperating isolates TCF_6, TCF_7, TCF_8 formed one group and TCF_1, TCF_9, TCF_5 formed another group. However, TCF_4, $TCF_{10,}$ TCF_{11} and TCF_{12} did not resemble any isolate. Similarity matrix thus produced indicated TCF_6 and TCF_{12} were genetically distinct with only 28.6% similarity followed by TCF_2 and TCF_1 (30.2%). While the isolate TCF_7 and TCF_6 were found to be genetically similar as 60.4% similarity was observed between the isolates followed by 58.0% similarity between TCF_8 and TCF_7 isolates

Introduction

Citrus is considered as one of the most important tropical fruit crop in India. There are several species of this fruit crop grown in more than 50 countries of the world and it is one of the choicest fruits having highest consumer's preference both as fresh fruit as well as for its refreshing processed juice. It plays a vital role in the fruit economy of the country next to mango. Citrus cultivation in India is facing

plethora of production constraints viz., non-availability of disease free planting material, bud wood transmissible diseases, general neglect, irregular bearing, scarcity of water and poor management practices, cultivation in unsuitable soils. Beside this, *Citrus* spp. are prone to attack by more than 150 diseases and disorders caused by fungal, viral and few bacterial pathogens right from nursery level to bearing stage resulting in considerable losses. Among the diseases responsible for citrus decline, the dry root-rot is the major disease effecting both life and production.

Dry root rot of sweet orange is caused by the soil borne pathogen *Fusarium solani*. The disease incidence of dry root rot is reported to be 5 to 50 percent and about 10 to 15 per cent of the infected trees are being killed every year (Gopal *et* al., 2000). In India, this disease was reported mainly from Andhra Pradesh and Tamil Nadu on sweet orange (Ramakrishanan, 1954). Bender *et al.,* (1982) reported dry root rot of citrus seedling due to *Fusarium solani* (Mart) Sacc. The disease occurs both in nursery and main field (Gopal *et al.,* 2001). *Fusarium solani* is an important soil borne fungus attacking a wide range of host plants and has a worldwide distribution. Determination of genetic diversity in *Fusarium solani* is of great importance in plant breeding for resistance. Assessment of genetic diversity in *Fusarium solani* is needed to determine whether isolates constitute genetically distinct groups and to obtain molecular markers for differentiating them and also correlate their relationship with geographic distribution and/or pathogenic ability to cause quantum of disease.

The modified polymerase chain reaction (PCR) with single primers of arbitrary nucleotide sequence and requiring no prior sequence information have proved useful in detecting intraspecific polymorphism among organisms (Welsh and Mc Clelland, 1990 and Williams *et al.,* 1990). This amplification technique (arbitrarily primed PCR on RAPD) can generate specific DNA fragments useful for genome mapping, identification of isolates and application in molecular biology (Hadrys *et. al.,* 1992). For plant pathogenic fungi, RAPD analysis can provide marker to differentiate isolates of *Fusarium solani* f.sp. *cucurbitae* (Crowhurst *et al.,* 1991), *Fusarium oxysporum* f.sp. *pisi* (Grajal-Martin *et al.,* 1993) and isolates from different geographic origins of *Colletotrichum graminicola*(Guthrie *et al.,* 1992). The potential of this technique for identifying DNA marker related to the intraspecific diversification of the pathogen led us to investigate the genetic diversity within *F. solani*. The aim of the study was to examine the genetic variability and relationship between 12 isolates from Andhra Pradesh, India.

Materials and Methods

Twelve isolates of *Fusarium* spp. isolated from bits of infected sweet orange tree roots were selected for the present study. The details of the isolates are enlisted in Table 1.

Pathogenicity assay

Fusarium solani isolates were multiplied on Sorghum sand medium and added to soil in pots at 7.5 percent (W/W) by soil infestation following the procedure given by Haware (1980) and Gopal *et al.,* 2001). Soil mixture without inoculum served as control. Each pot was sown with 30 seeds of Jambhiri and germination percent was recorded 21 days after sowing (DAS).

Seedling mortality percent was recorded at 45 DAS. Based on percent mortality of seedlings the isolates were categorized into 4 groups as slightly aggressive (1-25% seedling mortality), moderately aggressive (26-50%), as highly aggressive (51-75%) and as extremely aggressive (76-100%) based on classification given by Chohan and Kaur (1976).

Nucleic acid extraction and Amplification using RAPD markers

Pure cultures of the isolates were maintained on PDA slants and incubated at 280±20C for 6 days under controlled temperature and mycelia were aseptically transferred to flasks of potato-dextrose broth (PDB, HiMedia) and incubated for 5 days at 280±20C without shaking. The mycelia were filtered from liquid medium through four cheesecloth layers. Total DNA was isolated according to the CTAB protocol of Murray and Thompson (1980) and Gopal *et al.,* 2007 with slight modifications. DNA concentration was determined spectrophotometrically at 260 nm and rechecked by running samples on 1% agarose gel along with 1kb molecular weight marker (Fermentas). The samples were diluted in ultrapure water and the concentration adjusted to 25-50 ng/µl.

Twenty random primers were purchased from Operon Technologies, USA. The PCR was performed in a total volume of 25µl, containing 50 ng of template DNA, 10 picomole of random primer, 0.2mM of dNTPs, 2mM of $MgCl_2$, 1.5U of Hot Start *Taq* DNA polymerase (Fermentas). Amplification was performed in Thermal Cycler (Corbett Research, Australia). Initial denaturation was for 2 min at 94ºC, followed by 45 cycles of 1min at 92 ºC, 1min at 37^0C and 2 min at 72ºC and 5 min at 72ºC for final extension step. The amplified products were separated on 1.0% agarose gels and stained with ethidium bromide. Images were captured using Vilber Lourmat, France gel documentation system.

Data Analysis

The amplified DNA fragments for each accession were scored as present (1) or absent (0). Data generated by 20 RAPD primers were used to compile a binary matrix for cluster analysis. Genetics similarity among accessions was calculated according to Jaccard coefficient (Jaccard, 1908). The similarity coefficients were then used to construct a dendrogram using the Unweighted Pair Group Method of Arithmetical averages (UPGMA) algorithm, as described by Sneath and Sokal (1973).

Results and Discussion

Categorization of different *Fusarium solani* isolates based on quantum of disease

The results revealed that TCF_5 recorded the maximum per cent seedling mortality of 81.66 where as TCF_{12} recorded the least per cent seedling mortality (18.33) (Table 1). The isolates were categorized into 4 groups based on per cent seedling mortality. TCF_2 and TCF_{12} were categorized as slightly aggressive (1-25% seedling mortality), TCF_1, TCF_3, TCF_4, TCF_6, TCF_8 TCF_9 and TCF_{11} as moderately aggressive (26-50%). TCF_7 and TCF_{10}, as highly aggressive (51-75% seedling mortality), and TCF_5 as extremely aggressive (76-100%). It was observed that isolates from Nellore and Anantapur district ranged from slightly to moderately aggressive except TCF_{10} collected from Peruvali, Anantapur district. All isolates collected from Prakasam district ranged from moderately

to extremely aggressive. Further, the isolate TCF_5 which showed maximum quantum of disease and categorized as extremely aggressive collected from Prakasam district. These finding are in agreement with Chandra *et al.* (1983) who studied three isolates of *Fusarium oxysporum* and 7 isolates of *F. solani* of tomato and reported that the isolates with in a species differed in their potential to cause pre or post emergence killing of seedlings and induce wilt in mature plants. Reddy and Raju (1991) found that Gwaliar isolate was most aggressive among 13 isolates of *Fusarium udum.* Among 9 isolates of *Fusarium oxysporum f.sp. cumine* causes wilt of cumin, T_4 (49.30%) was most virulent (Champawat and Pathak 1989). An isolate of Kyang withya thika caused highest (38.3%) wilt incidence in pigeon pea out of 12 isolates of *Fusarium udum* tried (Okiror and Kimani 1997).

Polymorphism detected by Random amplification of polymorphic DNA

Identification of genetic relationships in *Fusarium solani* isolates is very difficult because of the lack of morphological difference. The traditional methods for identifying the DNA profiling are largely used for identification because of several limitations of morphological data. RAPD is a rapid and sensitive technique which can be used to estimate relationship between closely and more distantly isolates. 20 arbitrary primers were used for RAPD analysis detected a total of 174 fragments with an average of 10.24 fragments per primers and 89.12% fragments were polymorphic. The number of scorable bands produced per primer ranged from 8 to 18 and size of the products ranged from 400bp to 1.8kb (Fig. 1).

The maximum number of polymorphic bands (18) obtained with OPM-04 and minimum number (6) was obtained with primer OPM-13 (Table 2). The polymorphism ranged from 75.00% (primer OPA-13) to as high as 100% for 4 primers (OPM-04, OPM-05, OPM-06 and OPM-12).

Genetic similarities base on RAPD markers

The maximum genetic similarity value was 0.604 between isolates TCF_7 and TCT_6 followed by 0.580 similarity between TCT_8 and TCT_7 isolates, while the lowest genetic similarity value of 0.286 was between isolates of TCF_6 and TCF_{12} (Table 3).

Phylogenetic analysis based on RAPD

The phylogenetic relationship among 12 isolates of Fusarium solani analyzed by an UPGMA method was shown in Fig.2. The cluster results showed that two major clusters having TCF_2 and TCF_3 in one cluster and remaining isolates in other cluster which in turn were grouped separately in to two sub-clusters separating isolates form one group and TCF_1, TCF_9, TCF_5 form another group. However, TCF_4, $TCF_{10,}$ TCF_{11} and TCT_{12} did not resemble any isolate. In present study, quantum of disease and RAPD analysis were correlated and it was observed that isolates from Nellore and Anantapur district ranged from slightly to moderately aggressive forming different groups. Whereas, isolates TCF_6, TCF_7 and TCF_8 collected from Prakasam district ranged from moderately to highly aggressive. Further, the isolate TCF_5 also collected from Prakasam district which showed

Table.1 *Fusarium* isolates obtained from rhizoplane of sweet orange from different locations in Andhra Pradesh and their pathogenicity test on Jambhiri seedlings.

Name of Location	Isolate	Seedling mortality (%) at 35 DAS	Class of aggressiveness
Chillapuram, Nellore	TCF_1	38.33(38.23)	Moderately aggressive
Malakapatnam, Nellore	TCF_2	21.66(27.76)	Slightly aggressive
Gudur, Nellore	TCF_3	32.33(34.63)	Moderately aggressive
Nandipadu, Nellore	TCF_4	41.66(40.22)	Moderately aggressive
Bestavaripeta, Prakasam	TCF_5	81.66(64.67)	Extremely aggressive
Srinivasanagar, Prakasam	TCF_6	43.33(41.15)	Moderately aggressive
Pusalapadu, Prakasam	TCF_7	59.33(50.33)	Highly aggressive
Pandillapalli, Prakasam	TCF_8	29.99(33.21)	Moderately aggressive
Hampapuram, Ananthapur	TCF_9	33.33(35.24)	Moderately aggressive
Peruvali, Ananthapur	TCF_{10}	63.33(52.71)	Highly aggressive
A.K. Agraharam, Ananthapur	TCF_{11}	31.66(34.27))	Moderately aggressive
Nagasamudram, Ananthapur	TCF_{12}	18.33(25.33)	Slightly aggressive
	Control	0.00 (0.00)	
SEm (±) CD at5%		2.720 7.960	

* Mean of three replications; TCF: Tirupati Citrus *Fusarium*

maximum quantum of disease and categorized as extremely aggressive did not resemble any other isolate.

Accurate, reliable characterization of variation is not always possible using morphological characters, even when it is, identification of variations at the intraspecific level can be difficult. In recent years, a number of techniques have been developed based on comparisons of nucleic acids, which have been successfully applied to distinguish strains of a particular microorganism. These techniques include, Restriction Fragment Length Polymorphism (RFLP) analysis, Amplified Fragment Length Polymorphism (AFLP), Rrandom Amplification of Polymorphic DNA (RAPD) etc. In the present investigations, RAPD, a PCR based technique has been deployed to assess variability at the intraspecific level. In this technique, arbitrary short oligonucleotide primers targeting unknown sequences are used to generate amplified products that often show polymorphisms within species (Welsh and Mc Clelland, 1990, Williams *et al.,* 1990). Random PCR approaches are being increasingly used to generate molecular markers, which are useful for taxonomy and for characterizing fungal populations. Since previous knowledge of DNA sequences is not required, any random primer can be tested to amplify any fungal DNA. The RAPD method has been successfully used to differentiate and to identify fungi at the intraspecific level (Gutheric *et al.,* 1992; Assigbetse *et al.,* 1994 and Nicholson and Rezanoor, 1994) and at the interspecific level (Lehman *et al.,* 1992). Differences between isolates from different areas are therefore more easily detected. The RAPD technique has been used to detect genetic variation among strains/isolates within a species (Cook *et al.,* 1996 and Boyd and Carris, 1997).

Table.2 RAPD Primer used to detect polymorphism, number of band for polymorphism between *Fusarium solani* isolates per primer

S.No	RAPD Primer	Sequence (5'-3')	Total No. of bands	Polymorphic bands	% Polymorphism
1	OPM-01	GTTGGTGGCT	10	9	90.00
2	OPM-02	ACAACGCCTC	12	11	91.67
3	OPM-03	GGGGGATGAG	8	7	87.50
4	OPM-04	GGCGGTTGTC	18	18	100.00
5	OPM-05	GGGAACGTGT	12	12	100.00
6	OPM-06	CTGGGCAACT	12	12	100.00
7	OPM-07	CCGTGACTCA	--	--	--
8	OPM-08	TCTGTTCCCC	9	8	88.89
9	OPM-09	GTCTTGCGGA	10	8	80.00
10	OPM-10	TCTGGCGCAC	10	8	80.00
11	OPM-11	GTCCACTGTG	9	8	88.89
12	OPM-12	GGGACGTTGG	11	11	100.00
13	OPM-13	GGTGGTCAAG	8	6	75.00
14	OPM-14	AGGGTCGTTC	--	--	--
15	OPM-15	GACCTACCAC	9	7	77.78
16	OPM-16	GTAACCAGCC	10	9	90.00
17	OPM-17	TCAGTCCGGG	9	8	88.89
18	OPM-18	CACCATCCGT	--	--	--
19	OPM-19	CCTTCAGGCA	9	8	88.89
20	OPM-20	AGGTCTTGGG	8	7	87.50
		Total	174	157	
		Mean	10.24	9.24	89.12

Table.3 Jaccard's similarity coefficient of 12 isolates of *Fusarium solani* based on polymorphism obtained with 20 random primers

Isolates	TCF_1	TCF_2	TCF_3	TCF_4	TCF_5	TCF_6	TCF_7	TCF_8	TCF_9	TCF_{10}	TCF_{11}	TCF_{12}
TCF_1	1.000											
TCF_2	0.302	1.000										
TCF_3	0.434	0.433	1.000									
TCF_4	0.422	0.448	0.430	1.000								
TCF_5	0.496	0.306	0.325	0.431	1.000							
TCF_6	0.439	0.348	0.369	0.460	0.378	1.000						
TCF_7	0.483	0.435	0.361	0.504	0.443	0.604	1.000					
TCF_8	0.405	0.389	0.336	0.505	0.359	0.509	0.580	1.000				
TCF_9	0.522	0.383	0.429	0.479	0.464	0.470	0.576	0.413	1.000			
TCF_{10}	0.360	0.327	0.324	0.381	0.372	0.396	0.408	0.339	0.455	1.000		
TCF_{11}	0.441	0.308	0.362	0.379	0.437	0.429	0.472	0.432	0.471	0.412	1.000	
TCF_{12}	0.397	0.330	0.339	0.347	0.430	0.286	0.397	0.331	0.441	0.417	0.474	1.000

Fig. 1 RAPD marker profies of 12 varieties of *Fusarium solani* generated by primers OPM-01, OPM-04 and OPM-05 in 1% agarose gel

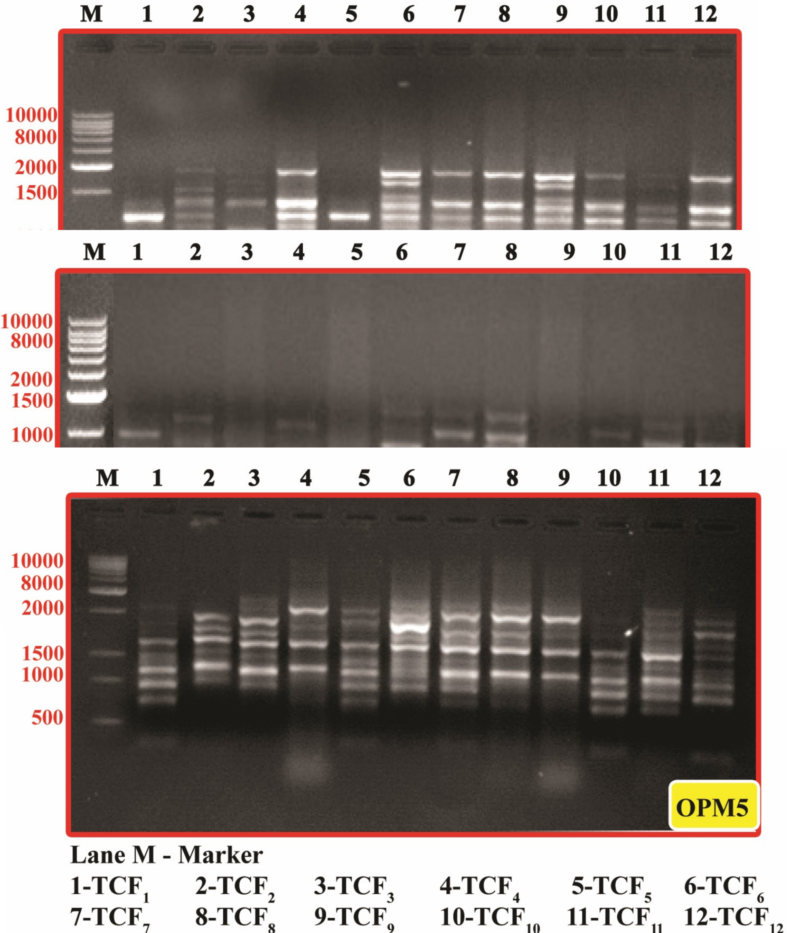
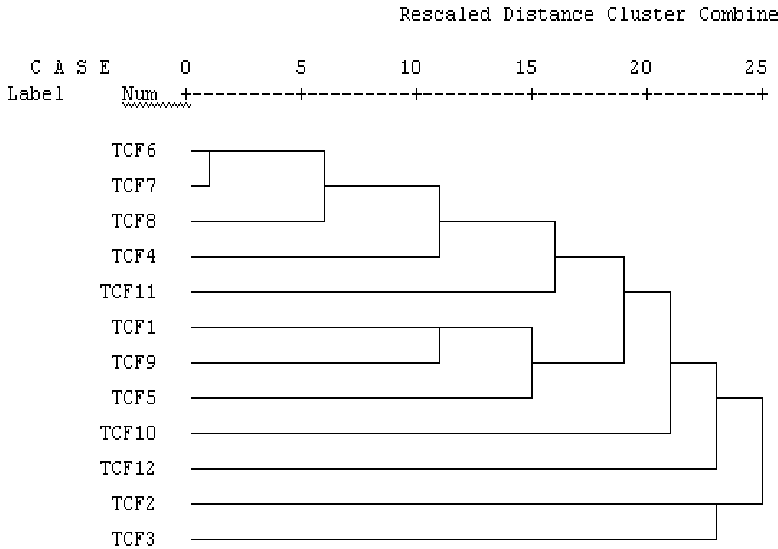


Fig.2 Dendrogram generated using UPGMA method illustrating the generic diversity relationships among 12 isolates of *Fusarium solani* by RAPD markers



RAPD analysis offers the possibility of creating polymorphisms without any prior knowledge of the DNA sequences of the organism investigated. The patterns produced are highly polymorphic, allowing discrimination between isolates of a species, if sufficient numbers of primers are screened. In the present investigations, it was found that once the optimal RAPD conditions for a given species are established, the method works-well for fungal samples, even on crude DNA extracts. Out of 20 primers screened for amplification of DNA of isolates of *Fusarium,* 17 were found to give reproducible and scorable bands with high percentage of polymorphism. Maximum polymorphism was showed in PCR reaction with OPM4, OPM5, OPM6 and OPM12. These primers showed 100 percent polymorphism as all these bands obtained were polymorphic with size ranging from 400 bp to 1.8 kb.

In a similarly study, Assigbetse *et al.,* (1994) observed genetic diversity with in a collection of 46 isolates of world wide origin, based on pathogenic and RAPD markers and established DNA fingerprinting for race characterization. Sivaramakrishnan *et al.,* (2002) studied genetic variability in 36 isolates of *Fusarium udum* collected from 4 pegionpea growing states in India and analyzed by using RAPD and AFLP technique. They suggested that PCR based method to identify the different races of *Fusarium* wilt pathogen will serve the purpose of routine analysis of field population and drawing a pathogen map of the country. Similarly opinion expressed by Jamali *et al.,* (2000) that RAPD and oligonucleotide finger printing has been used to study the genetic and pathogenic diversity with in *Ascholyte rabei* of chickpea in Pakistan. Harries (1999) suggested that though RAPD could be used for a quick screening of *Fusarium udum* isolates in the laboratory. However, one has to recognize the technology while making comparisons of results from different labs. Wechter *et al.,* (1995) employed RAPD analysis to identify genetic markers linked to race 1 *Fusarium* wilt resistance. Nelson *et al.* (1997) revealed that RAPD analysis could easily distinguished the isolates of *Fusarium oxyspoum* from *Erythoxylum coca var.* indicating its utility in DNA finger printing. Bohm *et al.* (1982) suggested the complete homogeneity in RAPD pattern with in the subpopulation contrasts with what has been observed for most other formae specialies of *Fusarium oxysporium.* Tantaoui *et al.* (1996) reported that no polymorphism was observed in RAPD analysis of 42 isolates of *Fusarium oxysporum* f.sp. *albenidis* from date palm and 7 got near identical RAPD patterns among 39 isolates of *Fusarium oxysprum* from angsana. This could be due to the pathogen that becomes wide spread relatively quicker as a result of an increase in product of host plant, which showed limited genetic variability. The present study can be concluded that the purification, cloning and sequencing of selected amplificans which are being performed will allow the development of *Fusarium solani* specific primers to be used in a fast and reliable screening of pathogenic isolates by PCR.

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