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RESEARCH ARTICLE

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RAPD-PCR technique for molecular detection and diversity of *Fusarium oxysporum* f.sp. *dianthi* causing vascular wilt of carnation

PONNUSAMY RAJESHKUMAR, PASUVARAJI ADHIPATHI AND SEVUGAPERUMAL NAKKEERAN

ABSTRACT : *Fusarium* wilt disease is one of the major economic constraint which hampers carnation cut flower production. Thirteen isolates of *Fusarium oxysporum* f.sp. *dianthi* inciting vascular wilt of carnation were evaluated for their molecular characterization using random amplified polymorphic DNA (RAPD- PCR). The isolates were categorized based on the morphological characteristics, produced colonies with circular pattern growth. However, differences were obtained in colony colour, shape and size of conidia. The 5.8S-rDNA of ITS region was amplified the specific amplicon size of 398 bp which confirmed the pathogen as *Fusarium oxysporum*. The molecular polymorphism among isolates were analysed by means of RAPD-PCR and the genetic co-efficient matrix derived from the scores of RAPD profile showed that minimum and maximum per cent similarities and diversity among isolates were in the range of 70 to 96 per cent, respectively. The cluster analysis by un-weighted pair-group method with arithmetic average (UPGMA), separated the isolates into four clusters which confirming the genetic diversity among isolates.

KEY WORDS : Carnation, *Fusarium oxysporum* f.sp.*dianthi*, Molecular diversity, RAPD, Vascular wilt

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INTRODUCTION

The major impediment in the cultivation of carnation is realized due to the infection of soil borne pathogen namely *Fusarium oxysporum* Schlechtend: Fr. f.sp. *dianthi* (Prill and Delacr.) W.C. Snyd. and H.N.Hans. *Fusarium oxysporum* f.sp. *dianthi* is an important soil borne pathogen causing substantial yield loss to carnation, leading to deterioration in quality and quantity of the marketable blooms and which hampers carnation cut flower production in major cut flower growing regions of Nilgiris, Kodaikanal in Tamil Nadu. Eight biological races of FOD has been reported. Among which race2 is most prevalent in carnation growing areas in the world (Denmik *et al*., 1989). Manulis *et al.*(1993) has suggested the use of RAPD technique for identification and diversity of

MEMBERS OF RESEARCH FORUM

Address of the Correspondence : **PASUVARAJI ADHIPATHI,** Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, COIMBATORE (T. N.) INDIA Email: adhiagri@gmail.com

Address of the Coopted Authors : PONNUSAMY RAJESHKUMAR AND SEVUGAPERUMAL NAKKEERAN, Department of Plant Pathology, Centre for Plant Protection Studies, Tamil Nadu Agricultural University, COIMBATORE (T. N.) INDIA

Fusarium oxysporum f.sp.*dianthi*. The use of molecular marker techniques were diversity among *Fusarium oxysporum* f.sp. *lentis* incited by lentils (Datta *et al.*, 2011). Migheli *et al*. (1998) has reported by RAPD fingerprint the genetic diversity among *Fusarium oxysporum* f.sp.*dianthi* races and development of forma specialis. Like in other pathogens systems, molecular techniques have become reliable and are highly suitable tools for identifying *Fusarium* species and for assessing genetic variation within collections and populations (Burgess *et al.*, 1989). RAPD (Randomly Amplified Polymorphic DNA) offers a promising, versatile and informative molecular tool to detect genetic variation within the populations of plant pathogens (Williams *et al.*, 1990). The study of *Fusarium* sp. has been greatly advanced by the adoption of molecular techniques like RAPD analysis (Voigt *et al.*, 2005).

EXPERIMENTAL METHODS

Collection and isolation of pathogen :

A total of 13 isolates of *Fusarium* wilt pathogen was recovered from 10 commercial cultivars grown in different horticultural farms in the major carnation-producing areas at Nilgiris, Coonoor and Kothagiri of Tamil Nadu, India. Root portion was excised from the infected plant. The infected roots were cut into 1cm bits and surface sterilized with 0.1 per cent mercuric chloride ($HgCl_2$) solution for 30 seconds and washed thrice in the series of sterile distilled water to remove the traces of mercuric chloride, and transferred to sterilized Petri plates containing potato dextrose agar (PDA) medium amended with 1000 ppm of streptomycin sulphate to avoid bacterial contamination. The Petri plates were incubated at room temperature (27±10°C) for 5 days and observed periodically for the growth of pure colonies. Isolates were purified by growing on plain agar medium. Individual conidia were observed under microscope and mono-conidial cultures were developed for all the isolates. Isolates were stored as conidial suspensions in glycerol at -80°C.

Morphological characterization of *Fusarium* wilt of carnation :

All the isolates were confirmed at the species level based on the morphological characters of as described by Burgess *et al.* (1994) and Koch's postulates were proved.

Molecular characterization of *Fusarium* isolates :

Isolation of genomic DNA of Fusarium :

Genomic DNA was extracted from the suspension culture of *Fusarium* by the Cetyl Trimethyl Ammonium Bromide (CTAB) method as described by Knapp and Chandlee (1996) and purified by RNAse treatment and quantified by UV spectrophotometer.

Detection of *Fusarium oxysporum* :

To confirm the 13 isolates as *Fusarium oxysporum* species, 5.8S-rDNA intervening sequence specific ITS FU F (5'CAACTCCCAAACCCCTGTGA3'); ITS FU R (5' GCGACGAT TACCAGTAACGA3') primers were used to get an amplicon of 389 bp size (Singh and Kumar, 2001). Amplification was conducted with a total reaction volume of 25µl in Eppendorf Master cycler, German. The PCR settings used were as follows: a hold of 2min at 95°C, 30 cycles of 1min at 94°C, 30 sec at 54°C and 1min at 72°C and a final extension of 10min at 72°C. The PCR products were resolved on two per cent agarose at 50 V, stained with ethidium bromide (0.5µg/ml) and photographed and analyzed using gel documentation system.

Molecular diversity analysis of *F. oxysporum* f.sp. *dianthi* using RAPD :

PCR amplification with 7 arbitrary primers for RAPD was carried out in 25 µl reaction containing 2 µl dNTP (250 µM each dNTP), 1µl primer (20ng/µl), 1µl template DNA (30 ng/µl), 2.5 µl reaction buffer (10X), 0.5 µl Taq DNA polymerase (3 U/µl), and de-ionized water 18.0 µl. PCR reactions were performed in a thermal cycler (Eppendorf, Nexus gradient) programmed for one cycle of denaturation at 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1 min, 72°C for 2 min and with final extension cycle at 72°C for 10 min. After completion of amplifications, 3 µl of gel loading dye was added to each sample and 25 µl total volumes were resolved on 1.5% agarose gel in 0.5X TBE buffer. The size of amplified DNA fragments was estimated with 100 bp ladders (Bangalore Genei Pvt. Ltd., India). The amplification products were visualized with a UV transilluminator and photographed in the gel documentation system (Alpha Innotech Corporation, San Leandro, California). All RAPD-PCR reactions were repeated atleast three times

and only the RAPD bands which appeared consistently were evaluated for polymorphism.

Data analysis :

DNA polymorphisms were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. Data were analysed to obtain Jaccard's co-efficients among the isolates by using NTSYS-pc (version 2.11V; Exeter Biological Software, Setauket, NY). Jaccard's co-efficients were clustered to genetrate dendrograms using the SHAN clustering programme, selecting the unweighted pair-group method with arithmetic average (UPGMA) algorithm version 2.11v in NTSYS-PC computer package (Exter software, NY) (Rohlf, 1993).

EXPERIMENTAL RESULTS AND ANALYSIS

The results obtained from the present investigation as well as relevant discussion have been summarized under the following heads :

Morphological characterization of *Fusarium oxysporum* f. sp. *dianthi* :

Thirteen different isolates of *Fusarium* spp., was isolated. The mycelium of the fungal culture on PDA medium was initially white and later turned; light pink to orange in different isolates. Macroconidia was sparse, and fusoid, 2-3 septate and measured 17.0-24.0 x 3.5-4.0 µm. Microconidia were abundant, hyaline, continuous, ovoid and measured 4.5-8.0 x 2.0-3.5 µm. Chlamydospores were hyaline, and spherical, measured 4.5 – 7.5 µm in diameter. Based on these phenotypic characters, the pathogen was confirmed as *Fusarium oxysporum* f.sp. *dianthi* (Table 1).

Pathogenecity :

Potting mixture comprising of laterite soil, sand and compost were mixed in the ratio of 3:1:1 and steam sterilized at 120lb pressure for 1hr on alternate days. The sterilized potting mixture was filled in to the plastic pots @ 5kg/pot. The vascular wilt pathogen *F. oxysporum* multiplied in potato dextrose broth, consisting of 10^6 conidia/ml was inoculated @ 1 per cent to the soil weight. Later the rooted cuttings of carnation were planted. Similarly, uninoculated control was also maintained. The pots were watered regularly upto saturation on alternate days.Observations were made regularly for the appearance and symptom development. Different isolates of CFODTNAU1-13 was inoculated to confirm the ability to cause the wilt symptom in carnation. Similarly, non-pathogenic isolate of *F. oxysporum* f. sp. *lycopersici* was also inoculated as a non pathogenic check. After symptom development, re-isolation was done and compared with the original culture for confirmation of the pathogen identity.

Molecular detection and molecular diversity analysis of *F. oxysporum* f.sp.*dianthi* using RAPD :

The present work was carried out to explore the possible utilization of random amplified polymorphic DNA (RAPD) technique for identifying *Fusarium* spp., either alternatively or complementary to those methods based upon morphological and pathological characteristics. The PCR amplification of Internal Transcribed Spacer (ITS) region of thirteen isolates of *F. oxysporum* using genus specific primers amplified at DNA fragment size of approximately 389 bp corresponding to the region of the 18S-23S rDNA. The DNA sequenceing results confirmed that all the isolates were 98-100 per cent sequence homology with the *F. oxysporum* f.sp. *dianthi* in the NCBI data base. Hence, the pathogen associated with Fusarium wilt of carnation is *F. oxysporum* f.sp. *dianthi*.

The thirteen isolates of *F. oxysporum* f.sp. *dianthi* from carnation were identified, purified and preserved in the PDA medium and their confirmed by ITS and RAPD. The 13 isolates of *F. oxysporum* f.sp. *dianthi* obtained from fusarium wilt of carnation samples were compared and categorized at molecular level using a simple technique RAPD-PCR. RAPD analysis was done with seven different 10-mer primers such as OPA-9, OPA-10, OPA-11, OPA-12, OPA-13, OPA-14 and OPA-15. RAPD analysis with these primers, enabled to identify the genetic variability among the 13 isolates tested in the present study (Fig. 1). Each of the primers varied greatly in their ability to resolve variability among the genotypes. All the primers were able to give high polymorphism among the isolates (Table 2).

Results of the cluster analysis using the RAPD polymorphic DNA products revealed high level of DNA polymorphism, which resulted in the possible occurrence of several distinct groups in relation to *F. oxysporum* f.sp. *dianthi* isolates. Four main groups of isolates were

Table 1 : Phenotypic characterization of the isolates of *Fusarium oxysporum* infecting carnation

Sr. No	Name of the isolates	Isolates submitted in NCBI genbank with accession number	Phenotypic characters of the isolates
1.	CFODTANU1	KC565709	The mycelium of the fungal culture on PDA medium was initially white and later turned, light pink. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.5-8.0 x 2.0-3.5µm. Macroconidia was sparse, and fusoid,2-3 septate and measured 17.0-24.0 x 3.5-4.0µm. Chlamydospores were hyaline, and spherical, measured 4.5 – 7.5 µm in diameter.
2.	CFODTNAU2	KC565710	The mycelium of the fungal culture on PDA medium was initially white and later turned, light pink. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.0-8.0 x 2.0-3.0µm. Macroconidia was sparse, and fusoid, and measured 16.0-25.0 x 3.8-4.0 µm. Chlamydospores were hyaline, and spherical, measured 4.4 – 7.2 µm in diameter
3.	CFODTNAU3	KC565711	The mycelium of the fungal culture on PDA medium was initially white and later turned, to orange. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.7-8.5 x 2.0-3.5µm. Macroconidia was sparse, and fusoid, 2-3 septate and measured 18.0-27.0 x 3.8-4.2 µm. Chlamydospores were hyaline, and spherical, measured 4.2 – 7.5 µm in diameter
4.	CFODTNAU4	KC565712	The mycelium of the fungal culture on PDA medium was white. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.2-8.5 x 2.0-3.3µm. Macroconidia was sparse, and fusoid, 2-3 septate and measured 19.0-25.0 x 3.8-4.2 µm. Chlamydospores were hyaline, and spherical, measured 4.3 – 7.4 µm in diameter
5.	CFODTNAU5	KC565713	The mycelium of the fungal culture on PDA medium white. Microconidia was sparse ,hyaline, continuous, ovoid and measured 4.5-8.0 x 2.0-3.5µm. Macroconidia was abundant, and fusoid, 2-3 septate and measured 17.0-24.0 x 3.5-4.0 µm. Chlamydospores were hyaline, and spherical, measured 4.5 – 7.5 µm in diameter
6.	CFODTNAU6	KC565715	The mycelium of the fungal culture on PDA medium was initially white and later turned, voilet. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.5-8.0 x 2.0-3.5µm. Macroconidia was sparse, and fusoid, 2-3 septate and measured 17.0-24.0 x 3.5-4.0 µm. Chlamydospores were hyaline, and spherical, measured 4.5 – 7.5 µm in diameter
7.	CFODTNAU7	KC565716	The mycelium of the fungal culture on PDA medium was white and later turned to violet. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.5-8.0 x 2.0-3.5µm. Macroconidia was sparse, and fusoid, 2-3 septate and measured 19.0-29.0 x 3.8-4.0 µm. Chlamydospores were hyaline, and spherical, measured 4.3 – 7.0µm in diameter
8.	CFODTNAU8	KC565718	The mycelium of the fungal culture on PDA medium was initially white and later turned, to violet. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.6-8.5x 2.2-3.4µm. Macroconidia was sparse, and fusoid, 2-3 septate and measured 21.0-27.0 x 3.0-4.0 µm. Chlamydospores were hyaline, and spherical, measured 4.8 – 7.3 µm in diameter
9.	CFODTNAU9	KC565719	The mycelium of the fungal culture on PDA medium was white. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.0-8.5 x 2.0-3.1µm. Macroconidia was sparse, and fusoid, 2-3 septate and measured 18.0-25.0 x 3.8-4.2 µm. Chlamydospores were hyaline, and spherical, measured 4.4 – 7.4 µm in diameter
10.	CFODTNAU10	KC565720	The mycelium of the fungal culture on PDA medium was initially white and later turned, to violet. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.6-8.5x 2.2-3.4µm. Macroconidia was sparse, and fusoid, 2-3 septate and measured 21.0-27.0 x 3.0-4.0 µm. Chlamydospores were hyaline, and spherical, measured 4.2 – 7.7 µm in diameter
11.	CFODTNAU11	KC565721	The mycelium of the fungal culture on PDA medium was white. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.2-8.5 x 2.0-3.3µm. Macroconidia was sparse, and fusoid, 2-3 septate and measured 18.0-25.0 x 3.8-4.2 µm. Chlamydospores were hyaline, and spherical, measured 4.1 – 7.8 µm in diameter
12.	CFODTNAU12	KC565722	The mycelium of the fungal culture on PDA medium was initially white and later turned, light pink. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.5-8.0 x 2.0-3.5µm. Macroconidia was sparse, and fusoid,2-3 septate and measured 17.0-24.0 x 3.5-4.0µm. Chlamydospores were hyaline, and spherical, measured 4.5 – 7.5 µm in diameter.
13.	CFODTNAU13	JX036529	The mycelium of the fungal culture on PDA medium was initially white and later turned, light pink. Microconidia was abundant,hyaline, continuous, ovoid and measured 4.0-8.0 x 2.0-3.0µm. Macroconidia was sparse, and fusoid, and measured 16.0-25.0 x 3.8-4.0 µm. Chlamydospores were hyaline, and spherical, measured 4.4 – 7.2 µm in diameter

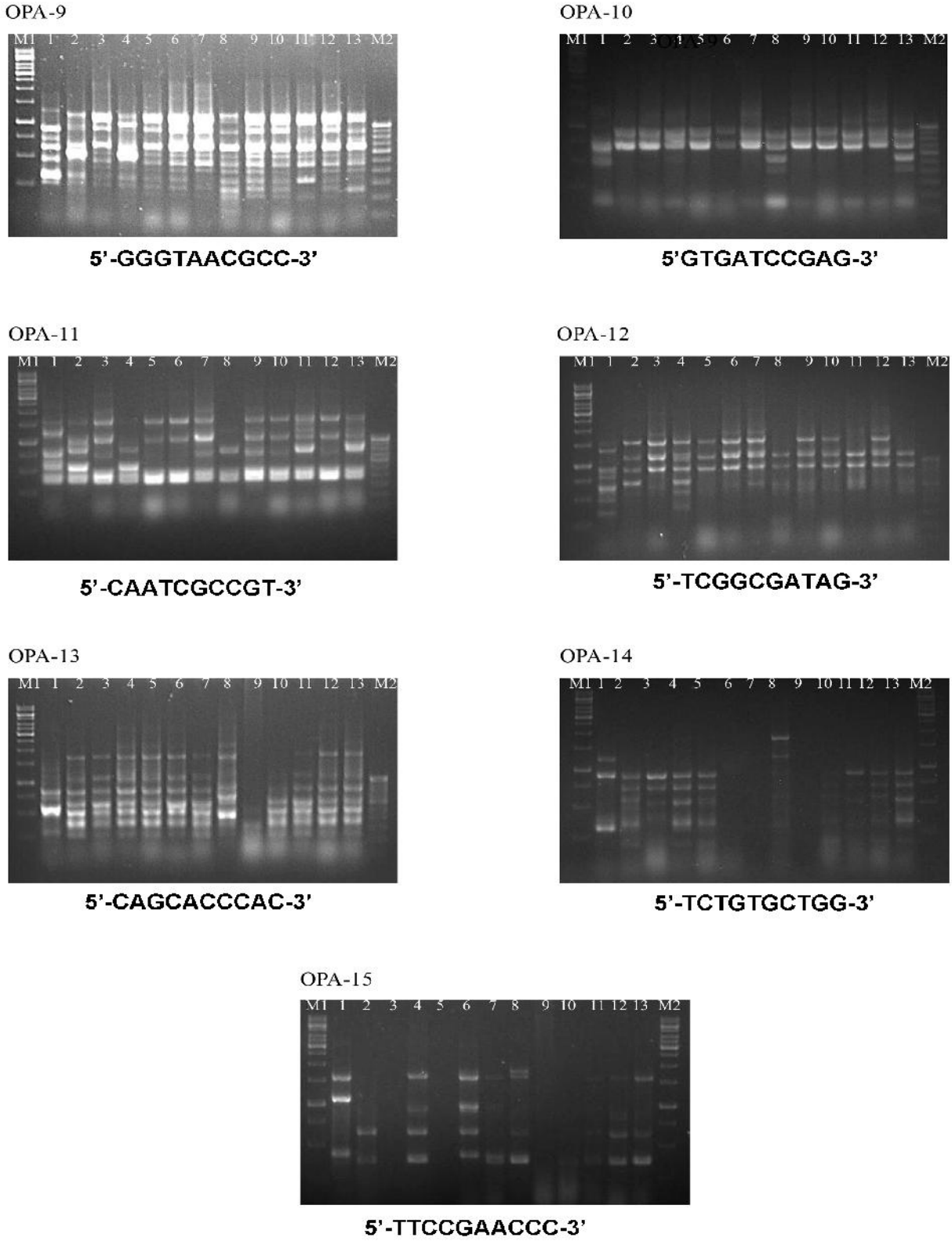

Fig. 1 : Random amplified polymorphic DNA of *Fusarium oxysporum* f.sp. *dianthi* isolates with random primers

resolved at 3.2 per cent similarity level. Isolates in group I include CFODTNAU1 and CFODTNAU8. Isolates in group 2 include CFODTNAU2 and CFODTNAU4. Isolates in group 3 include CFODTNAU3, CFODTNAU5, CFODTNAU6, CFODTNAU10, CFODTNAU12 and CFODTNAU13. Isolates in group 4 include CFODTNAU7, CFODTNAU11 and CFODTNAU9 (Fig. 2). Hence, all the analyzed isolates exhibit very high level of DNA polymorphism making a subdivision of main groups possible. Similarly, Zheng and Ploetz (2002) recorded wide variation in RAPD profiles of 74 isolates of *Fusarium* mango using 10-mer primers. Different workers have grouped between species of *Fusarium* from the different regions by using RAPD analysis (Voigt *et al.,* 1995; Yli-Mattila *et al.,* 1996 and Jana *et al.,* 2003). Molecular techniques based on DNA analysis help to determine a genetic variability of fungi (Martin *et al.,* 2000 and Taylor *et al.,* 2001).

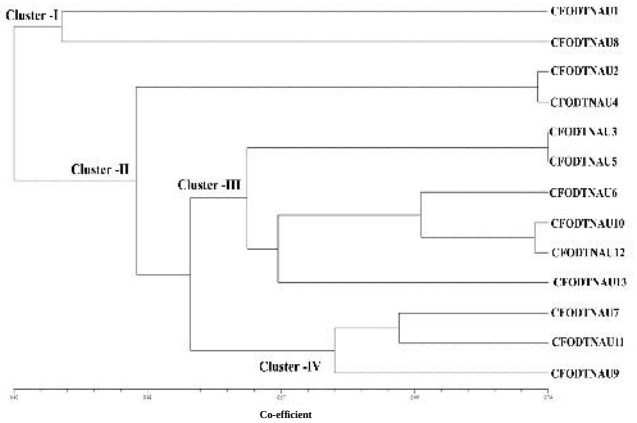

Fig. 2 : Genetic analysis *Fusarium oxysporum* f.sp. *dianthi* isolates and genetic distances were obtained by RAPD analysis

Molecular studies conducted with different techniques show that there are interspecific variation within *Fusarium* genus and also intraspecific differences within *F. oxysporum* (Kistler, 1997). Knowledge of genetic mechanisms underlying the variability in pathogen (*Fusarium* spp.) is almost invariably achieved through the use of molecular markers, that is, molecules which serve to distinguish one species or isolate it from another. There is considerable genotypic variability among *F. oxysporum* f.sp. *dianthi* isolates obtained from the infected wilt of carnation. Genetic characterization of European isolates of *Fusarium* species associated with FHB revealed large variation among the isolates of the *Fusarium* species. By using seven primers, all the isolates could be distinguished from each other. RAPD-PCR analysis gives comprehensive information regarding the genetic variability among the *Fusarium* community and can be used effectively as a DNA fingerprinting technique for *Fusarium* spp. Khalil *et al.* (2003) used RAPD in combination with pathogenicity assays to study the taxonomic kinships among five *Fusarium* species. Unfortunately, there was no clear-cut relationship between clustering in the RAPD dendrogram, pathogenicity test and geographic origin of tested isolates. This will be a valuable tool for epidemiological studies of these fungi and may be important for the development of

Table 2 : Genetic similarity co-efficient matrix of *F. oxysporum* f. sp. *dianthi* derived from RAPD analysis

ISOLATES	CFOD TNAU1	CFOD TNAU2	CFOD TNAU3	CFOD TNAU4	CFOD TNAU5	CFOD TNAU6	CFOD TNAU7	CFOD TNAU8	CFOD TNAU9	CFOD TNAU10	CFOD TNAU11	CFOD TNAU12	CFOD TNAU13
CFODTNAU1	1												
CFODTNAU2	0.261	1											
CFODTNAU3	0.307	0.405	1										
CFODTNAU4	0.390	0.705	0.365	1									
CFODTNAU5	0.325	0.500	0.793	0.450	1								
CFODTNAU6	0.292	0.500	0.529	0.567	0.500	1							
CFODTNAU7	0.350	0.527	0.558	0.512	0.617	0.571	1						
CFODTNAU8	0.351	0.378	0.361	0.486	0.342	0.378	0.368	1					
CFODTNAU9	0.416	0.333	0.562	0.400	0.529	0.444	0.656	0.324	1				
CFODTNAU10	0.230	0.484	0.516	0.472	0.531	0.580	0.562	0.437	0.342	1			
CFODTNAU11	0.432	0.459	0.575	0.414	0.542	0.542	0.617	0.307	0.575	0.400	1		
CFODTNAU12	0.268	0.514	0.593	0.540	0.606	0.656	0.588	0.428	0.500	0.714	0.472	1	
CFODTNAU13	0.325	0.567	0.555	0.550	0.567	0.567	0.552	0.410	0.513	0.472	0.611	0.583	1

selective pathogen management strategies. Similar work to related to the present investigation was also done by Patil *et al.* (2014); Chandel (2015); Madhuri *et al.* (2014) and Mahalakshmi and Yesuraja (2013).

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