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National Academy of Agricultural Science (NAAS) Rating : 3. 03

Genetic Variation Amongst the Isolates of *Fusarium Incarnatum* (Desm.) Sacc., Incitant of Wilt in Crossandra

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ABSTRACT: *Fusarium incarnatum is the important pathogen causing crossandra wilt. In the present investigation fifteen representative isolates of F.incarnatum, collected from different places of Tamil Nadu. Randam amplified polymorphic DNA (RAPD) marker used to estimate genetic variability among 15 isolates of pathogen. Based on the pathogenicity test, PDI on crossandra plants, isolate F-ISO-07 categoried as avirulent (less than 20 %), F-ISO-01, F-ISO-06, F-ISO-08, F-ISO-09, F-ISO-11, F-ISO-12, F-ISO-13 and F-ISO-14 as moderately virulent (20-40%), F-ISO-02, F-ISO-03, F-ISO-10 and F-ISO-15 as virulent (40-60%), F-ISO-04 and F-ISO-05 as highly virulent(60% and above) isolates. These isolates were characterized using 16 random primer, out of which 13 primers gave a total of 110 amplified products. Genetic distance between each isolate was calculated, and cluster analysis was used to generate a dendrogram showing relatedness among them. Isolates clustered in to two major groups, first Cluster has only one isolate F-ISO-12, which is a main branch of dendrogram and having its second arm with another cluster having two major sub-clads. First sub clad was divided into two clades having grouping of isolate number F-ISO-11, F-ISO13 and F-ISO-14. Second sub clad having 11 isolates of which, one is constituted of 4 isolates and another of 7 isolates. Similarity matrix thus produced indicated the maximum genetic similarity of 69.6 per cent between isolate F-ISO-13 and F-ISO-14, followed by 69.1% between F-ISO-01 and F-ISO-02, where as minimum similarity index was recorded at 42.1 per cent between F-ISO-04 and F-ISO-13.*

Key words: Crossandra, Fusarium incarnatum, Genetic diversity, RAPD, virulenceSecondary metabolites.

Crossandra (*Crossandra infundibuliformis*) is an important commercial flower, mainly grown in India, is affected by various fungal diseases. Among the various fungal diseases wilt disease caused by *Fusarium spp.*is one of the major problem in crossandra production and limits the crop cultivation. Crossandra wilt is a soil borne disease and causes yield losses up to 92 per cent(1,2,3). In nature, plant pathogens exists as different strains that exhibit variations in their morphology, culture, pathogenicity and virulence. To understand the present plant disease situation and to predict the possible future development, it is essential to know as much as possible more accurately about the variability in fungi that are pathogenic to plants and also the knowledge about pathogen population structure is pre-requisite for designing cost effective management strategies. Generally *Fusariam* identification is based on morphological criteria such as shape of micro and macroconidia, structure of conidiophores and the formation and disposition of chlamydospores but these are less accurate. Identification of pathogenic races is usually done by field experiments on selected host genotypes but it is of more time taking. So recent developments in biotechnology particularly genetic marker based methods such as RAPD, RFLP and SSR been used to determine the variability in pathogenic population of *Fusarium* spp such as *F.oxysporum* f.sp.*ciceri*.(4,5,6). RAPD has been also used to analyze genetic variation of phytopathogenic fungi like *F.avenaceum, F.chlamydosporum, F.graminearum, F.moniliformae* and *F.solani* (7). For plant pathogenic fungi,RAPD analysis can provide marker to differentiate isolates of *Fusarium solani* (8). RAPD

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analysis is very easy technique since previous knowledge of DNA sequences is not required, any random primer can be tested to amplify any fungal DNA. Hence keeping in view the importance of this crop and severity of the disease, present investigation was taken up at Department of Plant Pathology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai to study the genetic and pathogenic variability of isolates of *F.incarnatum* collected from different districts of Tamil Nadu state using the randomly amplified polymorphic DNA markers to assess the virulence pattern of *F.incarnatum* isolates*.incarnatum.*

MATERIALS AND METHODS

Isolation and Identification of Fungal isolates

An intensive systemic survey was conducted during the 2013 - 2014 to assess the wilt incidence in different crossandra growing areas of Tamil Nadu. Wilted crossandra plants manually sampled and infected plants were packed in paper bags and brought to laboratory. The pathogen was isolated from the diseased tissues of crossandra by tissue segment method (9). Colonies exhibiting the taxonomic features of *Fusarium spp.* were identified (10). Identification was based on characteristics of the macroconidia, phialids, microconidia, chlamydospores and colony growth traits. Further, the culture identity was confirmed by National Fungal Culture Collection of India- A National Facility (NFCCI & FIS), Mycology and Plant Pathology Group, Agarkar Research Institute, Pune. Pure cultures of all isolates were stored at 4ºC on PDA and used in further studies.

Virulence of different isolates of *F.incarnatum*

All the 15 isolates of *F.incarnatum* were examined for their virulence analysis and confirmed by soil inoculation method. The earthen pots with uniform size of 30 cm diameter were filled with five kg of garden land soil. The soil was sterilized in an autoclave at 1.4 kg cm^{-2} pressure for two h on two successive days and inoculated by mixing the inoculum of each isolates (multiplied on sand maize medium) of the pathogen. Two plant of 25-30 days old were sown in each pot and replicated 5 times and each replication maintained with three pots. The pots were maintained in green house by regular, uniform and judicious watering and then growth was constantly observed for development of the disease symptoms. The per cent disease incidence of each isolate was recorded.

Mycelium production and DNA extraction

The isolates of *F.incarnatum* were cultured in potato dextrose broth for eight days. One gram mycelium of each isolate was harvested by filtration and grinded to a fine powder using liquid nitrogen.100-200 mg of mycelial powder was taken and macerated with CTAB buffer. After maceration 700 µl of solution was transferred into 1.5 ml centrifuge tube and incubated at 65ºC for 10 minutes. After incubation equal volume of chloroform: isoamyl alcohol (24:1) was added. Then the mixture was kept in centrifuge at 10000 rpm for 10 minutes. The upper aqueous solution was taken and transferred into 1.5ml centrifuge, then equal volume of chloroform: isoamyl alcohol (24:1) was added. The mixture was again centrifuged at 10000 rpm for 10 minutes. After centrifugation 300 µl of aqueous was taken to which 5M sodium chloride: ice cold ethanol (5:2) was added. This mixture was kept at -27ºC for 1 hr or overnight. This was followed by centrifugation at 13000 rpm at 4ºC for 10 minutes. After centrifugation equal volume of ethanol was added and kept for pellet drying. Then 50 µl sterile water was added into the pellet than the genomic DNA was checked by agarose gel electrophoresis and stored at -20°C for further use.

PCR primers: Sixteen arbitrary RAPD PCR primers(10-mer) were used for amplification of DNA and all the RAPD primers were purchased from Sigma Aldrich, Bangalore, India.

RAPD-PCR analysis of *F.incarnatum*

Genotypic characterization of the *F.incarnatum* isolates was done by using a PCR-based fingerprinting method with randomly amplified polymorphic DNA (RAPD) markers (11). PCR amplification was performed using a Eppendorf nexus gradient master cycler and a 20 ìl total volume containing 2.0 units of Taq polymerase (Bangalore Genei Pvt Ltd, India), 2 ìl of 10X buffer, 1.5 ìl of 2.5 mM MgCl2, 1 ìl of 2.5 mM dNTP, 2 ìl of 10 ìM primer, 4 ìl of genomic DNA and sterile distilled water. The PCR was performed, using Eppendorf – Master Cycler nexus gradient S (Eppendorf, A G, Hamburg, Germany), with an initial denaturation step for 5 min at 94°C, followed by 40 cycles of 1 min for denaturation at 94°C, 1 min for annealing at 37°C and 2 min for extension at 72°C, with a final extension for 10 min at 72°C. Following amplification, 20ìl of each PCR product was separated by electrophoresis in 1.2% (w/v) agarose gel in Tris-acetate-EDTA (TAE) buffer. To visualize amplified DNA, fragments gels were stained with an ethidium bromide (0.1 mg

l^{-1}) and then photographed under transmission ultraviolet light, using an Alpha Imager 2000 (Alpha Innotech, San Leandro, CA, USA). The whole RAPD analyses experiment was repeated at least three times for all primers and isolates, only the RAPD bands which appeared consistently were evaluated for polymorphism.

Analysis of RAPD-PCR Results

Gel images were scored manually and recorded as presence and absence of bands in each isolate, coded as 1 and 0 respectively. The scores were used to create a data matrix to analyze genetic relationship using the NTSYS-pc program version 2.02 (Exeter Software, New York, USA) (12). A dendrogram was constructed based on Jaccard's similarity coefficient (13) using the marker data from *F.incarnatum* isolates with UPGMA.

RESULTS AND DISCUSSION

Virulence ability of *F.incarnatum* based on PDI. From the reaction of *F.incarnatum* isolates tested on crossandra it was clear that among the fifteen isolates screened F-ISO-04 collected from Thiminayakarbatti village of Dindugal district recorded maximum of 66.7 percent disease incidence where as infection in other isolates ranged from 13.3 to 60.0 per cent (Table 1). Based on the PDI, isolates are grouped in to four groups as avirulent, moderately virulent, virulent and highly virulent. The isolats F-ISO-04 and F-ISO-05 are found to be highly virulent(PDI e" 60%), the isolates,F-ISO-02, F-ISO-03, F-ISO-10 and F-ISO-15 as virulent (40-60%), the isolates,F-ISO-01, F-ISO-06, F-ISO-08, F-ISO-09, F-ISO-11, F-ISO-12, F-ISO-13 and F-ISO-14 as moderately virulent (20-40%), where as isolate F-ISO-07 as avirulant with PDI less than 20 per cent.

Table 1
Virulence of different isolates of *F. incarnatum* on crossandra plants in pot culture

S.No	*Place of collection*	*District*	*Isolate*	**Wilt incidence (%)*	*Category*
1	Ottupatti	Dindugal	F-ISO-1	33.3(18.36)**	Moderatly virulent
2	Puduchukkapuram		F-ISO-2	40.0(20.21)	Virulent
3	Metupatti		F-ISO-3	56.7(24.28)	Virulent
4	Thiminayakarbatti		F-ISO-4	66.7(26.51)	Highly virulent
5	Sempatti		F-ISO-5	60.0(25.07)	Highly virulent
6	Nelakottai		F-ISO-6	30.0(17.37)	Moderatly virulent
7	Pallipatti		F-ISO-7	13.3(11.46)	Avirulent
8	Madurai	Madurai	F-ISO-8	26.7(16.33)	Moderatly virulent
9	Melur		F-ISO-9	36.7(19.35)	Moderatly virulent
10	Saruvaraypatti		F-ISO-10	53.3(23.50)	Moderatly virulent
11	Kottampatti		F-ISO-11	23.3(15.31)	Moderatly virulent
12	Pallipatti	Karur	F-ISO-12	20.0(14.14)	Moderatly virulent
13	Trichy	Trichy	F-ISO-13	30.0(17.37)	Moderatly virulent
14	Navalurkottapatti		F-ISO-14	36.7(19.35)	Moderatly virulent
15	Thiruvallur	Thiruvallur	F-ISO-15	40.0(20.21)	Virulent
	CD (P=0.05)			2.54(2.24)	
	SE (m)			0.89(1.117)	

* Mean of five replications
** Figure in the parentheses are arc sine transformed values

MOLECULAR CHARACTERIZATION BY RAPD

A total of fifteen isolates of *F.incarnatum* were tested for their genetic variability by RAPD analysis, using 16 random primers. Among these, 13 random primers produced easily scorable and consistent banding patterns, which were used for RAPD analysis. The generated DNA finger prints were evaluated for overall clearness of the banding pattern. The number of scorable bands produced for primer ranged from 7 to 11 (Plate, 1a). The highest number of bands generated by primer OPA06 and minimum bands by OPB15. The primer OPA 10 generated highest polymorphic bands followed by OPA01, OPA7 and OPD05, where as minimum number of poly bands with OPA09 (Table. 2). Polymorphism with different primers ranged from 50 to 100 percent. Primer OPA10 produced the maximum of 100 per cent poly bands, where as primer OPA07 produced only 50 per cent of polymorphic bands. A total of 930 fragments were amplified with thirteen isolates.

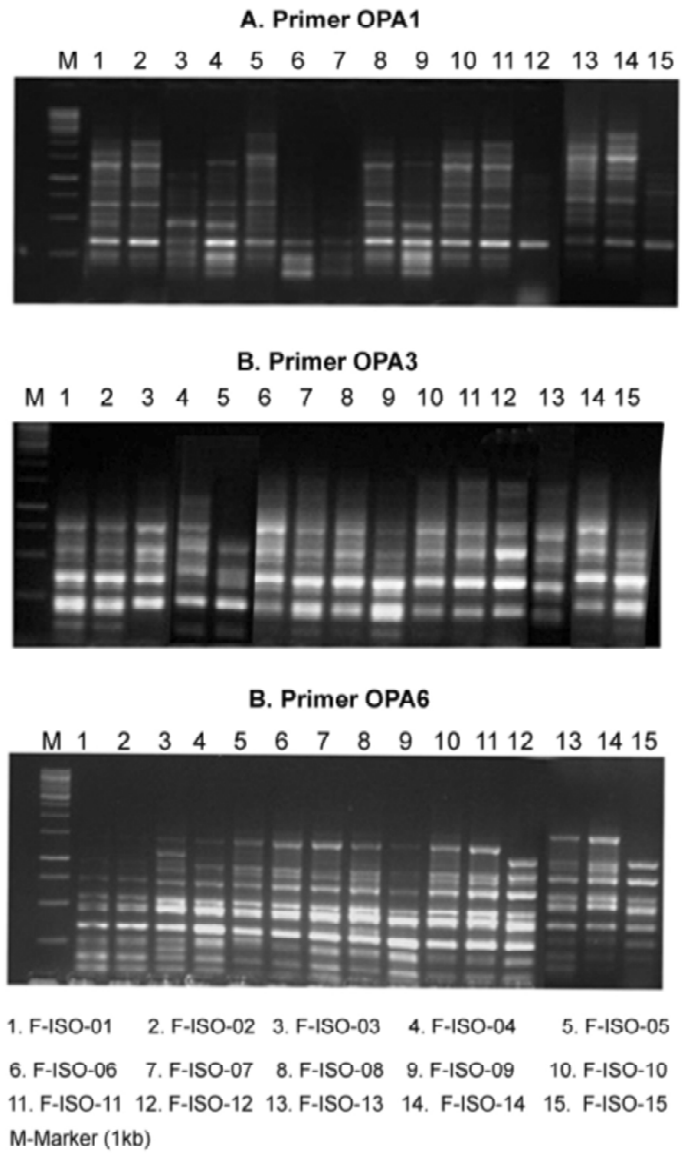

Plate 1a. RAPD analysis of *F. incamatum* isolates with random primerr

GENETIC SIMILARITY BASED ON RAPD PRIMERS

The maximum genetic similarity of 69.6 per cent was recorded between isolate F-ISO-13 collected from Trichy and F-ISO-14, collected from Navalurkottapatti, followed by 69.1% between F-ISO-01 collected from Ottupatti and F-ISO-02, collected from Puduchukkapuram (Table 3). Minimum similarity index was recorded at 42.1 per cent between F-ISO-04 collected from Thiminayakarbatti and F-ISO-13 collected from trichy confirming that the *F.incarnatum* isolates collected from entirely different geographical areas can also share some genetic relatedness.

PHYLOGENETIC TREE DENDROGRAM

The phylogenetic relationship among the isolates examined was represented by a dendrogram by using UPGMA was shown in Fig. 1. The results showed that two RAPD major clusters were detected among different isolates of *F.incarnatum*. First Cluster has only one isolate (F-ISO-12) from pallipatti village of Karur district, which is a main branch of dendrogram and having its second arm with another cluster having two major sub-clads. First sub clad was divided into two clades having grouping of isolate number F-ISO-11, F-ISO13 and F-ISO-14 from the adjoining areas. Second sub clad having 11 isolates of which, one is constituted of 4 isolates and another of 7 isolates.

Table 2
RAPD primers used to detect polymorphism, number of bands for polymorphism among *F.incarnatum* isolates per primer.

Name of the primer	*Sequence(5′-3′)*	*No. of amplicons*	*Monomor-phic bands*	*% Monomor-phism detec-ted by RAPD*	*Polymor-phic bands*	*% polymor-phismdetec-ted by RAPD*	*Total amplified fragments*
OPA1	5′- CAGGCCCTTC - 3′	*9*	*1*	11.1	*8*	88.9	*85*
OPA3	5′- AGTCAGCCAC - 3′	*8*	*3*	37.5	*5*	62.5	*59*
OPA4	5′- AATCGGGCTG - 3′	-	-	-	-	-	-
OPA5	5′- AGGGGTCTTG - 3′	*7*	*3*	42.9	*4*	57.1	*82*
OPA6	5′- GGTCCCTG AC - 3′	*11*	*5*	45.5	*6*	54.5	*120*
OPA7	5′- GAAACGGGTG - 3′	*9*	*1*	11.1	*8*	88.9	*62*
OPA9	5′- GGGTAACGCC - 3′	*8*	*4*	50.0	*4*	50.0	*82*
OPA10	5′- GTG ATCGCAG - 3′	*9*	*0*	0.0	*9*	100.0	*78*
OPB01	5′- GTTTCGCTCC - 3′	*8*	*2*	25.0	*6*	75.0	*72*
OPB03	5′- CATCCCCCTG - 3′	*8*	*1*	12.5	*7*	87.5	*61*
OPB11	5′- GTAGACCCGT - 3′	-	-	-	-	-	-
OPB15	5′- GGAGGGTGTT - 3′	*7*	*1*	14.3	*6*	85.7	*68*
OPC07	5′- 5TCCCGACGA3 - 3′	*8*	*1*	12.5	*7*	87.5	*58*
OPC08	5′- TGGACCGGTG- 3′	*9*	*2*	22.2	*7*	77.8	*49*
OPD05	5′- TGAGCGGACA - 3′	*9*	*1*	11.1	*8*	88.9	*54*
OPL8	5′- AGCAGGTGGA - 3′	-	-	-	-	-	-
	Total amplified fragments						*930*

Table 3
Similarity matrix among fifteen isolates of *F.incarnatum* using RAPD primers

Isolates	*F-ISO -01*	*F-ISO -02*	*F-ISO -03*	*F-ISO -04*	*F-ISO -05*	*F-ISO -06*	*F-ISO -07*	*F-ISO -08*	*F-ISO -09*	*F-ISO -10*	*F-ISO -11*	*F-ISO -12*	*F-ISO -13*	*F-ISO -14*	*F-ISO -15*
F-ISO-01	1.000														
F-ISO-02	*0.691*	*1.000*													
F-ISO-03	*0.471*	*0.458*	1.000												
F-ISO-04	*O.541*	*0.570*	0.713	1.000											
F-ISO-05	*0.570*	*0.506*	0.621	0.655	1.000										
F-ISO-06	*0.436*	*0.500*	0.439	0.476	0.404	1.000									
F-ISO-07	*0.506*	*0.514*	0.603	0.620	0.483	0.535	1.000								
F-ISO-08	0.564	0.597	0.506	0.617	0.551	0.555	0.608	1.000							
F-ISO-09	0.474	0.500	0.513	0.592	0.453	0.594	0.603	0.623	1.000						
F-ISO-10	0.573	0.505	0.602	0.655	0.591	0.506	0.534	0.588	0.471	1.000					
F-ISO-11	0.500	0.506	0.573	0.517	0.581	0.548	0.558	0.557	0.527	0.600	1.000				
F-ISO-12	0.427	0.447	0.500	0.500	0.444	0.507	0.540	0.481	0.507	0.444	0.475	1.000			
F-ISO-13	0.482	0.434	0.483	0.421	0.463	0.506	0.447	0.465	0.432	0.527	0.628	0.423	1.000		
F-ISO-14	0.477	0.519	0.478	0.495	0.430	0.500	0.494	0.477	0.446	0.571	0.617	0.563	0.696	1.000	
F-ISO-15	0.530	0.463	0.623	0.639	0.591	0.463	0.476	0.494	0.481	0.591	0.438	0.525	0.472	0.500	1.000

Values are calculated using software NTSYS PC

In present study, quantum of disease and RAPD analysis were correlated and it was observed that isolates from Dindugal districts ranged from avirulent to highly virulent (13.3 to 66.7%), indicating even though they are from same district forming into different virulent groups (F-ISO-01 and F-ISO-07) as well as clustered in to different clads, however some isolates like F-ISO-03, F-ISO-04 and F-ISO-05 from same geographical area exhibited similar virulence group and clustered together with highest genetic similarity when compared with other isolates. But the similar trend was not true for all isolates, indicating there is no proper correlation between virulence, molecular marker used and geographic origin of the isolate. This fact was also reported in Egyptian cotton (14,15). Ninety-nine isolates of *Fusarium oxysporum* f. sp. *ciceris* were characterized by RAPD marker and cluster analysis showed three groups of isolates (16). UPGMA cluster analysis divided *Fusarium oxysporum* f. sp. *ciceris* isolates in to seven distinct clusters by RAPD analysis with a set of 40 ten-mer primers (17). No apparent correlation found between geographical origin and virulence of isolates in *Fusarium oxysporum* f. sp. *ciceris* in India (18).

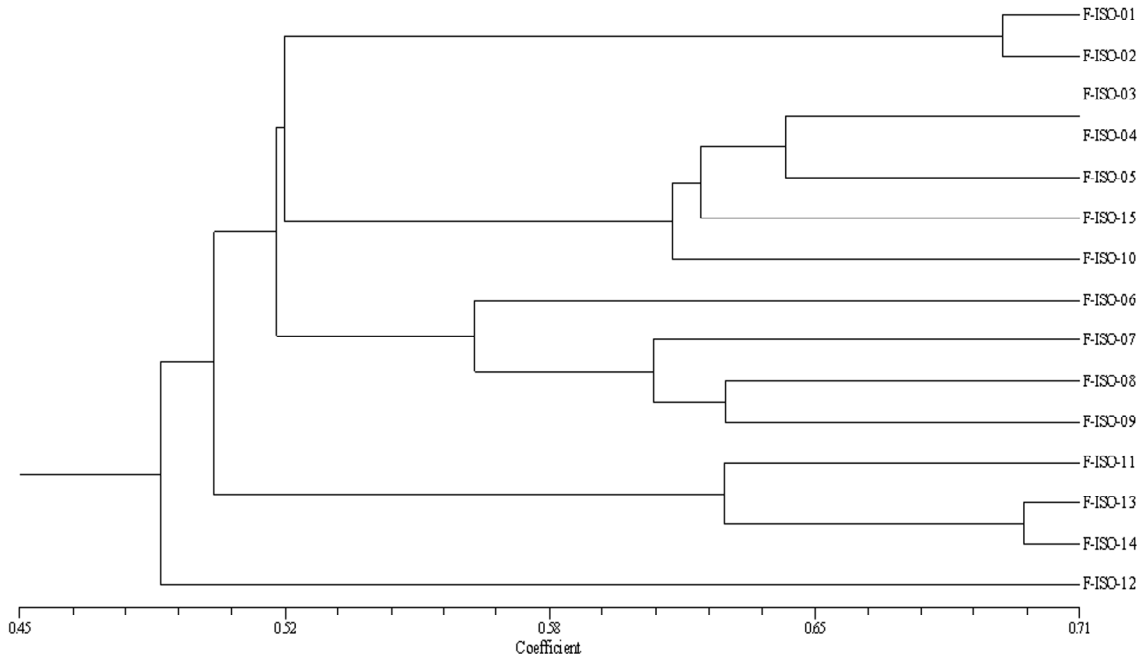

Figure 1. UPGMA dendrogram showing genetic relationship among *F. incarnatum* isolates based on Jaccard's similarity coefficient from RAPD data.

RAPD analysis offers the possibility of creating polymorphism 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 number of primers screened. In the present investigation it was found that out of 16 primers screened for amplification of DNA of *Fusarium* isolates, 13 were found to produce reproducible and scorable bands with high percentage of polymorphism. In a similar study, race characterization was established by DNA fingerprinting with RAPD marker for pathogenic and genetic diversity with in a collection of 46 isolates (19). Thirty six isolates of *Fusarium udum* collected from four pegionpea growing states in India, and analyzed the genetic variability by using RAPD and AFLP technique(4).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 a country. RAPD and oligonucleotide finger printing has been used to study the genetic and pathogenic diversity with in *Ascholyte rabei* of chickpea in Pakistan (20).

Conclusion

The present study generated significant information in terms of pathogenic and genetic variability of *F.incarnatum*, which could be used further for development of resistant varieties of crossandra. The study also highlights the facts that both pathogenic and virulence analysis and RAPD markers are useful tool for analyzing the structure of the pathogen population, but further studies are needed to make them complementary to each other.

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