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EVALUATION OF GENETIC DIVERSITY AMONG PHYTOPATHOGENIC ISOLATES OF *FUSARIUM SOLANI* COMPLEX CAUSING SHISHAM DIEBACK DISEASE IN PAKISTAN

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ABSTRACT

The aim of this study was to investigate the regional dependant genetic variation among different pathogenic *Fusarium solani* isolates and a link to persistence cause of shisham dieback in various agro ecological zones. A total of 23 pathogenic representative isolates of *Fusarium solani*, collected from various agro ecological zones were subjected to analysis of genetic variability in terms of DNA polymorphism using RAPD-PCR. Results were obtained with the help of 10 randomly amplified polymorphic DNA markers (OPA1-OPA10) to test genetic variability in *F. solani* isolates. A total of 238 amplified products generated with the primers and 23 isolates of *F. solani*. The pattern of genetic variability in the isolate was also supported by the analysis of the similarity indices and UPGMA dendrogram. Twenty three isolates of *F. solani* recovered from different areas showed a significant genetic variation durning RAPD analysis. It seems that the genetic variability among fungal isolates is regional dependent and reason of persistent cause of shisham dieback in Punjab, Pakistan. Current findings showed the variation among pathogenic isolates of *F. solani* and ravealed that persistent cause of shisham dieback is due to *F. solani* complex in different shisham growing regions in province Punjab, Pakistan.

Keywords: *Fusarium solani*, dieback, RAPD-PCR, shisham, agroecological zones.

INTRODUCTION

Shisham (*Dalbergia sissoo* Roxburgi) is an important timber tree found in India, Nepal, Bhutan, Bangladesh, Pakistan, and Afghanistan. It is also found under cultivation in tropical to subtropical regions of Africa and Asia viz. Java, Nigeria, Mauritius, Srilanka, Kenya, Northern Zimbabwe, Palestine and South Africa (Tewari, 1994). Due to its fast growth rate, high timber value, simple propagation and drought-resistant qualities, this species is considered as the suitable tree species for both private and government plantations. However, dieback is one of the important diseases of shisham with specialized symptoms like thinning of leaves in crown, drying up of ends of branches, stag headness in extreme cases (Khan and Khan, 2000) and it progresses from top to bottom (Bajwa *et al.*, 2003). After sings of crown thinning, death of the tree occurs in 3-5 years is evident. In recent past, phenotypically different varieties of shisham have also been grouped under resistance, susceptible and unsusceptible varieties on the basis of their relative resistance to dieback disease (Javaid *et al.*, 2003& 2004). However, during 1998 to 2009 the disease was noted as epidemic in various shisham growing area of Punjab province (Pakistan) especially on canal banks and forest sites followed by road sides and farmer's fields (Bajwa and Mukhtar, 2006; Mukhtar, 2012).

Fusarium solani causal pathogen of shisham dieback disease and has afflicted this tree species thought out Pakistan (Rajput *et al*., 2008); Mukhtar, 2012). However, a mix pattern of genetic diversity in shisham plantation (Ashraf *et al*., 2010), raised a question towards specificity of *F. solani* as causal pathogen of dieback disease of shisham. *Fusarium solani* is widely found in soil and constitutes one of the most important phytopathogens worldwide. In recent past, molecular phylogenetic analyses of DNA sequences, however, indicate the *F. solani* species complex comprises phylogenetically distinct species (O'Donnell, 2000). Therefore, it was assumed that the knowledge of the genetic diversity within this pathogen can be helpful to develop a deep understanding for dieback disease in various agroecological zones in Punjab. Thus, current study was designed on different pathogenic isolates associated with shisham dieback were collected from different agro ecological zones of Punjab, in order (i) to determine genetic diversity among pathogenic *F. solani* isolates and (ii) to find out cause of persistent reason of this disease in agroecological zones of Punjab.

MATERIALS AND METHODS

Collection of Pathogenic Fungal Isolates: Total of 23 confirmed pathologenic isolates of *F. solani* isolates (Mukhtar, 2012), collected from root and rhizospheric soil of infected dieback tree from various agro ecological zones of Punjab province (Pakistan) were used for DNA polymorphism (Table 1). These isolates were also deposited in First fungal Culture Bank of Pakistan (FCBP), institute of Agricultural Science, University of the Punjab, Lahore, Pakistan. All cultures in this study were derived from single spore culture and maintained on 2% Malt Extract Agar (MEA) medium for further study.

DNA Extraction: Pure cultures of the isolates were maintained on 2% MEA plates and incubated at $25^{o}\pm 2$ ^{o}C for 10 days. The modified CTAB (Cetyltrimethylammonium bromide) method of Lee *et al.* (1988) and Wu *et al.* (2001) was used for DNA isolation from fungal mass. RNase treatment was performed by adding 2 µl of RNase to each tube containing DNA and incubated for 30 minutes at 37°C in thermomixer. The concentration of DNA was determined using a UV–visible spectrophotometer (Thermo Spectronic, Madison, WI). DNA quantity was calculated with the following formula:

$$\text{DNA Conc. } (\mu g\ ml^{-1}) = \text{OD at 260 nm} \times \text{Dilution factor} \times 50$$

DNA concentration of 25-50 ng μl^{-1} was used for PCR reactions.

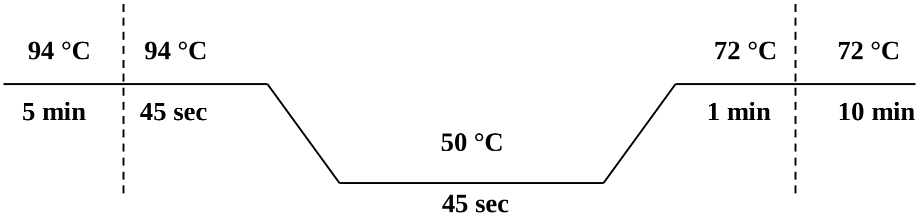
Selection of RAPD- Primers: Genus *Fusarium* specific 10 base oligonucleotide primers of series OPA-1 to OPA-10 (Table. 2) were employed for the genomic DNA amplification **(**Crowthurst *et al.* 1997; Zakaria *et al.*, 2009; Gupta *et al*., 2010**).**

Table 2. List of OPA decamer DNA primers

Primers	Sequence (5′ to 3′)	Primers	Sequence (5′ to 3′)
OPA-1	CAGGCCCTTC	OPA-6	GCTCCCTGAC
OPA-2	TGCCGAGCTG	OPA-7	GAAACGGGTG
OPA-3	AGTCAGCCAC	OPA-8	GTGACGTAGG
OPA-4	AATCGGGCTG	OPA-9	GGGTAACGCC
OPA-5	AGGGGTCTTG	OPA-10	GTGATCGCAG

Optimization and Amplification of RAPD-PCR: The RAPD-PCR amplification conditions were optimized in XP thermal cycler TC-XP-G (Bioer, Germany). RAPD-PCR was amplified in 20 µl reaction volume containing; 2.0 µl genomic DNA, 1.5 µl (10mM) primer, 0.2 µl dNTPs (25mM each), 3µl of 10X buffer (15mM), 2.5 µl $MgCl_2$ (1.5mM), 0.2µl (3U/µl) of Taq DNA polymerase (Fermentas.USA) and DDH_2O (10.6 µl).

The optimum RAPD- PCR was amplified by Thermo PXE 0.5 (Thermo Electron Corporation, USA) programmed at initial DNA denaturation at 94°C for 5 minutes. Afterward DNA denaturation followed by 35 cycles of amplification each consisting of 3 steps; DNA denaturation into single strands at 94°C for 45seconds; primer annealing at 50°C for 45 seconds and extension of complementary DNA strands at 72°C for 1 minute from each primer. The final extension period was 10 minutes at 72°C for *Taq* DNA polymerase to synthesize any un-extended strands left.



After the completion of the PCR, the products were stored at −20°C until the gel electrophoresis was done.

RAPD- PCR Analysis: The amplified products (5µl) from each sample along with 2µl of loading dye (bromophenol blue) were loaded in well on 2% agarose gel [containium bromide (0.5 µg ml^{-1})] dipped in 1X TBE buffer. A 100 bp DNA ladder (Fermentas.USA) was used as DNA marker. Gel electrophoresis (Electrophoratic gel System, EC 330, Thermo electron Corporation, USA) was conducted for 40 minutes at 120 V. The PCR products were visualized by placing the gel in UV documentation system (Biometra, Germany) and photographed.

Data analysis: The analysis of RAPD was done by comparison of molecular weights of bands with standard bands of DNA Ladder Marker. Presence of bands was scored from the gel photograph. Only clear and reproducible bands were given consideration. These bands were considered as polymorphic when they were absent in some sample in frequency greater than 1% (Jorde, 1995). Clear bands were scored as present (1) or absent (0) at particular position or distance migrated on the gel. Genetic similarity matrix was constructed by using Jaccard's similarity coefficient (Jaccard, 1908). Genetic similarities between these twenty three *F. solani* were determined using the Un- Weighted pair group method of arithmetic averages (UPGMA). These data patterns were analyzed through Minitab 14.1 (Minitab, 2004**)**. The results were presented graphically in the form of dendrogram.

RESULTS AND DISCUSSION

DNA-based techniques are important tools of choice for understanding the genetic diversity and phylogeny of *Fusarium* species (Arif *et al*. 2008). In present study, RAPD–PCR was carried out to determine the degree of genetic variability within pathogenic isolates of *F. solani*, obtained from dieback infected shisham trees. Among ten selected primers, six primers namely OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, and OPA-06 provided good amplification and revealed high polymorphic pattern among isolates (Fig. 1). Genetic similarity matrix was used for cluster analysis and to construct a dendrogram with the Unweighted Pair Group Method of Arithmetical Averages (UPGMA) algorithm (Sneath and Sokal, 1973) by using the NTSYS-software (Rohlf, 1998). The 23 pathogenic *F. solani* isolates were found to be highly heterogeneous due to presence of polymorphism product patterns with respect to RAPD analysis. The dendrogram depicted clustering from the pair wise matrix of genetic similarity between all pathogenic genotypes. In case of 23 isolates of *F. solani*, seven main clusters were identified in the homology tree (Fig 2). The genetic similarity for tested *F. solani* isolates ranged from 0-77%, indicating level of diversity. *Fusarium solani* isolate form Muzaffargharh (Sh-99) showed the maximum diversity with isolates from Sailkot (Sh-84 & Sh-82), Sheikhupur (Sh-72), Shahiwal (Sh-66), Faisalabad (Sh-59), Jehlem (Sh-36), Rawalpindi (Sh-32) and Bahawalpur (Sh-17) with 0.00% similarity value, followed by isolates from Sh-73 [0 % similarity with isolate from Multan (Sh-42) and Faisalabad (Sh-59)] and Sh-82 [0% similarity with R.Y. Khan (Sh-10) and Multan (Sh-42)] with maximum diversity. Although the pathogenicity test was able to clarify both similarities and differences to certain extent (Mukhtar, 2012), the cluster analysis of RAPD-PCR revealed clearer discrimination between these isolates. The variations of the banding patterns of *F. solani* isolates are sufficient to differentiate between all isolates tested. In recent past, Gupta *et al*. (2009) also reported the genetic polymorphism and diversity in isolates of *F. solani* isolated from wilt disease of Guava in India. Similarly, Hawa *et al*. (2010) also used RAPD analysis to study genetic variation among different isolates of *F. semitectum.* RAPD analysis also showed that *F. solani* isolates from dieback infected shisham trees were variable and the regional dependant genetic variation among these isolates is prominent with relation to shisham die back. However this finding is contradiction to Bereneuza *et al*. (2004), who emphasized that the geographical distribution has no effect on the genetic variation of *F. solani* isolates, however, this diversity may correlates with the host plant from which the isolates were isolated.

Several studies have indicated that *F. solani* is a species complex comprising several species and varieties (Booth 1971; Gerlach 1981; Nirenberg 1995). Sequence analysis of 28S rDNA indicated that *F. solani* isolates might comprised a group of species (Guadet *et al*., 1989). RAPD analysis suggests that RAPD markers can be a quick and reliable alternative for differentiating isolates of *Fusarium* spp., into their respective pathogenicity group which supports the results of our study. Genome analysis using RAPD-PCR indicated a promising way to assess the genetic variation and identification of *F. solani* isolates. Usefulness of such technique in differentiating between closely related strains is supported by many authors in the field of molecular biology (Nagarajan *et al*., 2004; Aiat, 2006; Gupta *et al*., 2009: Younes *et al*., 2013). Therefore, present work here strongly supports the conclusion that genetic variability among pathogenic isolates of *F. solani* (causal agent of shisham dieback) is a cause of persistence reason of shisham dieback in various agro ecological zones of Punjab.

Table 1. List of *Fusarium* species employed in RAPD analysis

Lane in Gel	Isolate Number	Location	Source
1	Sh-10	R.Y. Khan	Root
2	Sh-110	D.G. Khan	Root
3	Sh-16	Bahawalpur	Root
4	Sh-17	Bahawalpur	Root
5	Sh-19	Bahawalpur	Soil
6	Sh-22	Attock	Root
8	Sh-32	Rawalpindi	Root
9	Sh-36	Jehlem	Root
10	Sh-39	Jehlem	Soil
11	Sh-42	Multan	Root
13	Sh-47	Surgohda	Root
14	Sh-53	Shahiwal	Root
16	Sh-59	Faisalabad	Soil
17	Sh-61	Lahore	Root
18	Sh-66	Shahiwal	Root
20	Sh-72	Sheikhupur	Root
22	Sh-73	Sheikhupur	Root
23	Sh-81	Sailkot	Root
24	Sh-82	Sailkot	Root
25	Sh-84	Sailkot	Root
27	Sh-88	Gujaranwala	Soil
28	Sh-95	Mianwali	Root
29	Sh-99	Muzaffargharh	Soil

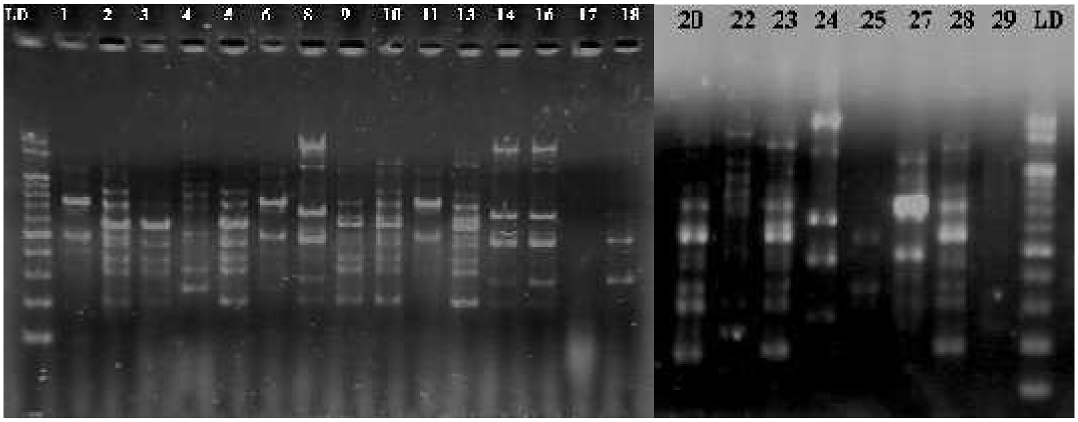


Figure. 1. RAPD DNA fragments amplified with decamer primer OPA1 showing monomorphism and polymorphism among *Fusarium* species isolates. Lane LD indicates DNA ladder

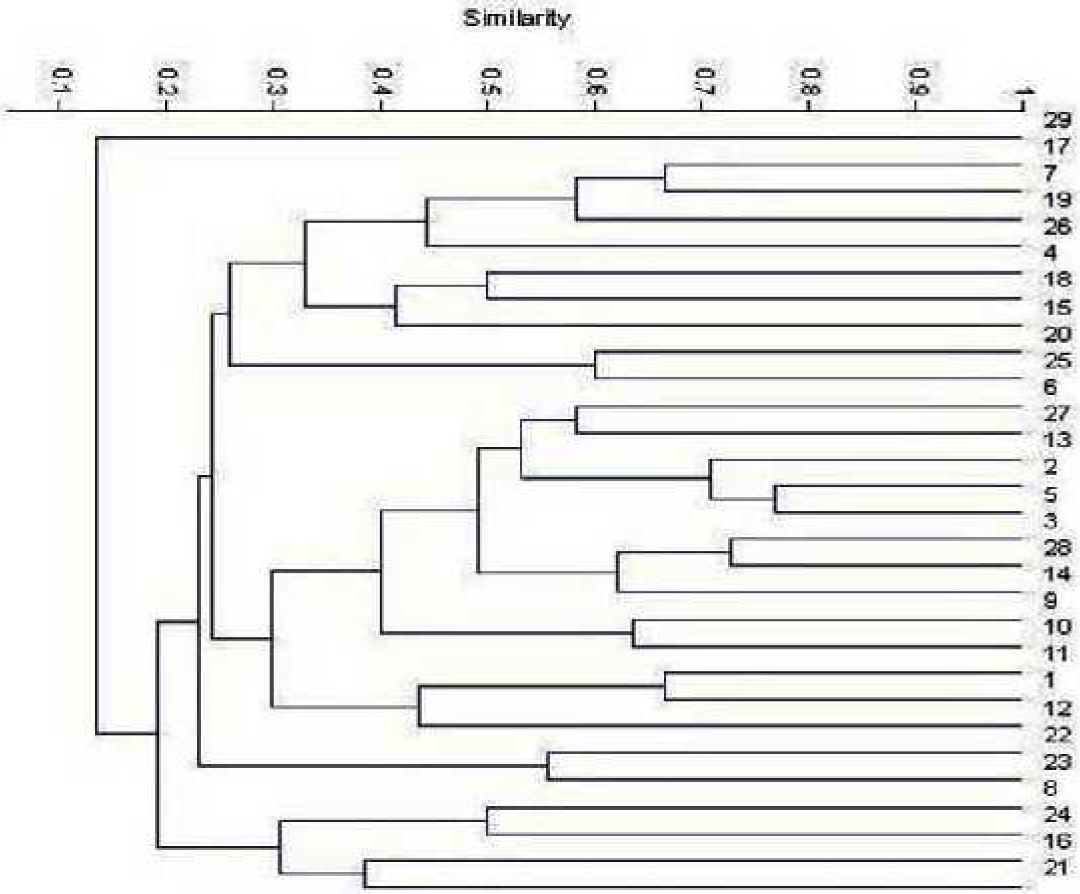


Figure. 2. Dendrogram of 23 aggressive isolates of *Fusarium solani* OPA-01, OPA-02, OPA-03, OPA-04, OPA-05, and OPA-06 primers.

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