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GENETIC DIVERSITY AMONG *Botryodiplodia theobromae* ISOLATES CAUSING COLLAR/STEM ROT OF MANGO IN PAKISTAN

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ABSTRACT

The wide spread fungus *Botryodiplodia theobromae* (synonym: *Lasiodiplodia theobromae)* is a significant cause for low production in mango producing countries of the world. A total of 11 isolates collected from ten mango growing districts of two provinces of Pakistan showed typical pigmentation and cultural features on Potato dextrose agar (PDA) medium. The colonies of most of the isolates were grayish initially, which later turned blackish with prominent fruiting bodies on obverse of petridishes. Total genomic DNA of each isolate was used in the random amplified polymorphic DNA (RAPD) technique. The amplification with 20 primers detected scoreable polymorphism among the isolates. A genetic similarity matrix based on Nei and Li's index determined coefficients ranging from 9.76-65.05%. The isolates grouped into three clusters, A, B and C comprising 7, 2 and 2 isolates, respectively, at a genetic relatedness of 29%. RAPD proved successful in differentiating isolates of *B. theobromae* at molecular level. The present studies reveal extensive association of *B. theobromae* with mango in the orchards of the Punjab and Sindh provinces of Pakistan. In addition to aerial phase of the fungus devastation of collar region, eventually destined to decline, indicates probable spread through canal water.

Keywords: *Mangifera indica*, quick decline, *Lasiodiplodia theobromae*, RAPD

INTRODUCTION

Mango (*Mangifera indica* L.), an important fruit crop, is grown in tropical and sub-tropical regions of the world. It is a part and parcel of the cultural heritage of Indo-Pakistan and has been under cultivation in sub continent for 4000 years or more. There are more than 250 cultivars of mango grown in Pakistan cultivated on an area of 0.099 million ha with annual production of 1.0371million tonnes (Anonymous, 2004).

Its production remains hampered due to a number of diseases at all stages of its development and it is estimated that production could be increased by 28% if the crop is protected against various diseases (Rawal, 1998). Amongst these diseases, collar/stem rot commonly known as quick decline is becoming very serious. Apparently healthy looking plants die with in days resulting in complete destruction of the precious wealth of the country. Most of the workers

have reported *Botryodiplodia theobromae* (synonym: *Lasiodiplodia theobromae*) as cause of this decline disorder (Das Gupta and Zachariah, 1945; Mahmood and Gill, 2002; Al Adawi et al., 2003; Khanzada et al., 2004).

B. theobromae is an aggressive and wide host range fungus of Deuteromycetes class and genetic variability amongst the isolates of this fungus affecting mango is also remarkable (Al Adawi et al., 2003). The main objective of the present study was to employ random amplified polymorphic DNA (RAPD) (William et al., 1990) technique to investigate the genetic diversity in isolates of *B. theobromae* originating from collar/stem rot affected plants in Pakistan. This information will be helpful to understand the population structure, monitor the spread of the pathogen and devise future strategies for the control of this malady.

MATERIALS AND METHODS

Collection of Isolates

Diseased samples from collar/stem rot affected mango plants of different cultivars during a survey of 10 districts of Punjab and Sindh provinces of Pakistan were collected during the year 2002 and 2003. The origins and disease phenotypes associated with the isolates are indicated in Table 1. Samples were maintained on ice from collection to analysis (Iqbal et al., 2003). After initial culturing, duplicate samples were lyopholised and maintained at 8 ºC.

Morphological and Cultural Features of the Isolates

Five tissue pieces, 5 mm in length, were excised from each sample collected from collar/stem portion of the plant. These pieces were surface disinfested in 1% sodium hypochloride solution for two minutes and placed in Petri-dishes containing Potato dextrose agar (PDA) medium. All the plates were incubated at 25 ºC with 12 hrs alternate periods of light and darkness. The identified colonies of recovered isolates were purified on PDA and Basal Agar Medium (BAM) to note the cultural features of the isolates. The colony diameter of isolates was recorded after 2, 4 and 6 days of incubation. The following characters were recorded for each isolate to evaluate their colony appearance.

Sr. No	Characters	Categories
1	Color of mycelium on obverse side	0 = white 1 = grayish 2 = mouse gray 3 = black
2	Color of mycelium on reverse side	0 = white 1 = grayish 2 = mouse gray 3 = black
3	Color of pycnidia	0 = light grey 1 = medium gray 2 = dark gray 3 = black
4	Amount of pycnidia	0 = absent 1 = few 2 = moderate 3 = abundant
5	Status of pycnidia	0 = not grouped 1 = grouped 2 = globosed irregular 3 = irregular
6	Shape of pycnidia	0 = sub globose 1 = globose

		2 = globosed & irregular 3 = irregular
7	Conidial color	0 = hyaline 1 = cinnamon 2 = fawn
8	Conidial shape	0 = sub ovoid 1 = ellipsoid 2 = oblong

RAPD Analysis

Mycelial threads were multiplied in microfuge tubes containing 500µl of liquid potato dextrose medium and allowed to grow for 72 hours at 25 ºC. The mycelial mat was pelleted by centrifuging for 5 min at 10000 rpm, washed with 500µl of TE buffer and again pelleted. The TE buffer was decanted and 300µl of extraction buffer (200 mM Tris Hcl, pH 8.5, 250 mM Nacl, 25 mM EDTA, 0.5% SDS) was added. The mycelia of each isolate were crushed with a conical pestle following the addition of 150µl of 3M sodium acetate (pH 5.2). The tubes were placed at -20 ºC for 10 min and centrifuged (10,000 rpm for 5 min). The supernatant was transferred to a clean tube and an equal volume of isopropanol was added. After 5 min, the precipitated DNA was pelleted by centrifugation. The pellet was washed with 70% ethanol, vacuum dried and dissolved in 20µl TE buffer (Cenis, 1992).

DNA was quantified spectrophotometrically and by electrophoresis on 0.8% agarose gel. Total genomic DNA of each isolate was diluted in sterile distilled water to a concentration of 5.0ng/µl for RAPD analysis. PCR reactions were performed in a 25µl reaction volume containing 10 mM Tris-HCl, 50 mM KCl, 3 mM $MgCl_2$, 100 µM each of dATP, dCTP, dGTP, dTTP,, 30 ng of primer, 0.001% gelatin, 30 ng of genomic DNA and 1 unit of *Taq* polymerase. A total of 20 random decamer primers obtained from Operon Technologies Inc. USA were surveyed. Amplifications were performed in a thermal cycler (Master cycler gradient, Eppendorf, Germany) programmed for one cycle at 94 ºC for 5 min followed by 40 cycles at 94 ºC for 1 min, 36 ºC for 1min, 72 ºC for 2 min and one cycle at 72 ºC for 10 min. PCR products were separated on 1.2% agarose gel stained with ethidium bromide and photographed on a UV transilluminator.

Only reproducible fragments were scored and all polymorphic loci were marked as present/absent. Bands of the same length were scored as identical (Assigbets et al., 1994). A binary matrix combined the data records for all the 20 isolates. Nei and Lei's (1979) coefficients of similarity were used to construct a dendrogram using the unweighted pair group of arithmetic means (UPGMA, Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

All the fungal isolates originating from different cultivars of mango affected with collar/stem rot, exhibited morphological features typical of the species *B. theobromae* (Ellis, 1971). Colonies initially originating showed grayish coloration, which subsequently turned black on PDA as well as BAM, spreading across the whole under surface. The upper surface gradually developed prominent fruiting bodies. These distinctive cultural characteristics were also retained when single spores were sub-cultured. Black color of mycelium on obverse and reverse sides of Petri plates was observed in the colonies of maximum isolates (63.63%). As for as amount and color of pycnidia are concerned, these two features were noted in category of 2 i.e. few and medium grey respectively. Not grouped but sub globose pycnidia were observed in maximum colonies of the isolates with the percentages of 36.36 and 54.54 respectively. Similarly, conidia in fawn color and ellipsoid in shape were examined in abundance by 54.54 and 63.63 percent, respectively (Table 2). Concisely, this study presented black colored mycelia with few; medium grey, not grouped, subglobose, fawn colored and ellipsoid conidia as main morphological and cultural features of most of *B. theobromae* isolates (Mirzaee et al., 2002). Minor differences in

morphological characteristics and physical features among isolates were detected. Considerable differences between isolates were encountered in culture. Similar results consisting of limited morphological differences in isolates of *B. theobromae* have already been reported (Al Adawi et al., 2002). The growth characteristics of the isolates on PDA and BAM are summarized in Table 3. Maximum mean colony diameters i.e. 38.08 and 37.05 mm were exhibited after two days by BD-8 on PDA and BAM, respectively. Similarly, maximum colony growth of the same isolate was observed after four and six days of incubation on the same media. Least growth of 21.55 and 20.51 after two days and 49.46 and 48.42 after four days was shown by BD-5 on PDA and BAM, respectively. After six days, least growth of 77.33 and 77.29 was reflected by BD-6 on PDA and BAM, respectively. The differences in growth characteristics of all the isolates in three different periods of incubation and two media were found significant. The correlation between the isolates and days was also remarkable but for the interaction between isolates and medium, days and medium, isolates, days and medium were found as insignificant. This clearly indicated that both the media proved to be best for the growth of all the isolates of *B. theobromae*. Further, all the isolates promoted their growth as the time of incubation increased.

Classical techniques used to define *Botryodiplodia* strains are technically demanded and time consuming. RAPD provided a relatively quick and easy alternative for the characterization of isolates. Total genomic DNA of 11 isolates was analyzed using 20 ten mer random primers. The bands showed variation in the profiles depending on the primer and type of isolate tested reflecting scoreable polymorphism. The size of the amplified products was in the range of 250 bp to 3 kbp. A similarity matrix, generated from RAPD data, was used in unweighted pair group method of arithmetic means UPGMA cluster analysis (Table 4). The resulting dendrogram groups isolates into three distinct clusters designated as A (containing 7 isolates) B (2 isolates) and C (2 isolates), respectively finally related at a genetic relatedness of 29% (Fig. 1). The genetic similarities between isolates ranged from 9.76 to 65.05%. Isolates BD-2 and BD-3 were the most similar (65.05% genetic similarity) where as isolates BD-6 and BD-8 were least similar (9.76%) and grouped in separate clusters.

Isolates BD-1 and BD-2 obtained from two closely situated districts Multan and Khanewal although fell in the same cluster A but shared different subgroups having 57% similarity between themselves. BD-1 and BD-6 identified from two other quite close districts Multan and Muzaffargarh joined two different clusters A and C, respectively showing similarity of only 15%. Two isolates BD-4 and BD-11 were collected from Bahawalpur and Rahim Yar Khan Districts of the Punjab province which are not much distant and located on the same track. Both the isolates were collected from the collar rot affected parts of the cultivars S-S-I and S-S-II which developed from the same ancestry but the isolates joined different subgroups in the same cluster indicating 39% similarity. Isolates BD-5 and BD-11 originated from Rahim Yar Khan District but were placed in different clusters B and A, respectively.

Two isolates of Sindh province viz. BD-9 and BD-10 joined cluster A and led to a joint subgroup with 64% similarity. But BD-7 and BD-8 belonging to the same province diverted to different clusters i.e. C and B, respectively. It means no site specific correlation could be found.

It is evident that some isolates from nearest areas were almost closely related. Still other from the same places differed significantly. Data revealed that the isolates might have distributed across the country through canal water or air born conidia from the place of origin because short distance dispersal was possible through water. Moreover, isolates appeared to have broad cultivar spectrum and not specialized on a single cultivar. Genetic variability amongst the isolates of fungus *B. theobromae* affecting mango has already reported to be remarkable (Al Adawi et al., 2003).

The isolates of ecological proximity or different geographical origins may be differentiated through DNA finger printing technique. Composite RAPD data supports the view that isolates from different cultivars and origin do not form a homogenous group. Detection of significant differences at molecular level and realization of unique population structures would

provide a base to devise target specific disease management strategies to minimize the spread of pathogenic forms to new areas.

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TABLES

Table 1: Origin of *Botryodiplodia theobromae* isolates

Sr. No	Isolates	Districts	Variety	Status
1	BD-1	Multan	Malda	Local
2	BD-2	Khanewal	Sanglakhi	Local
3	BD-3	Lodhran	Sensation	Local
4	BD-4	Bahawalpure	SS-I	Local
5	BD-5	Rahim Yar Khan	Fajri	Local
6	BD-6	Muzaffargarh	Langra	Local
7	BD-7	Hyderabad	Dusehri	Local
8	BD-8	Mir Pur Khas	Chaunsa	Local
9	BD-9	Nawab Shah	Saroli	Local
10	BD-10	Sangar	Sindhri	Local
11	BD-11	R.Y.Khan	SS-II	Local

Table 2: Morphological and cultural features of *Botryodiplodia theobromae* isolates

Category	Color of mycelium (%)		Pycnidia (%)				Conidia (%)	
	Obverse	Reverse	Amount	Color	Status	Shape	Color	Shape
0	(9.09)	9.09	27.27	0.0	36.36	54.54	9.09	18.18
1	0.0	0.0	45.45	45.45	27.27	18.18	36.36	63.63
2	63.63	63.63	27.27	36.36	9.09	18.18	54.54	18.18
3	27.27	27.27	0.0	18.18	27.27	9.09	0.0	0.0

Table 4: Similarity matrix of eleven *Botryodiplodia theobromae* isolates collected from different locations of Pakistan

	BD-1	BD-2	BD-3	BD-4	BD-5	BD-6	BD-7	BD-8	BD-9	BD-10	BD-11
BD-1	1.0000										
BD-2	0.5712	1.0000									
BD-3	0.4654	0.6505	1.0000								
BD-4	0.3688	0.5459	0.5926	1.0000							
BD-5	0.2891	0.3592	0.4224	0.4010	1.0000						
BD-6	0.1510	0.2805	0.3283	0.3068	0.3166	1.0000					
BD-7	0.3440	0.4420	0.5094	0.3801	0.4048	0.3255	1.0000				
BD-8	0.3314	0.3901	0.4094	0.4603	0.3710	0.0976	0.3099	1.0000			
BD-9	0.4112	0.5261	0.5152	0.4032	0.3658	0.2660	0.4272	0.4667	1.0000		
BD-10	0.4874	0.5814	0.5262	0.3829	0.3757	0.1693	0.3684	0.4139	0.6492	1.0000	
BD-11	0.4639	0.4632	0.5273	0.3962	0.3533	0.1917	0.3042	0.4435	0.5010	0.5395	1.0000

FIGURES

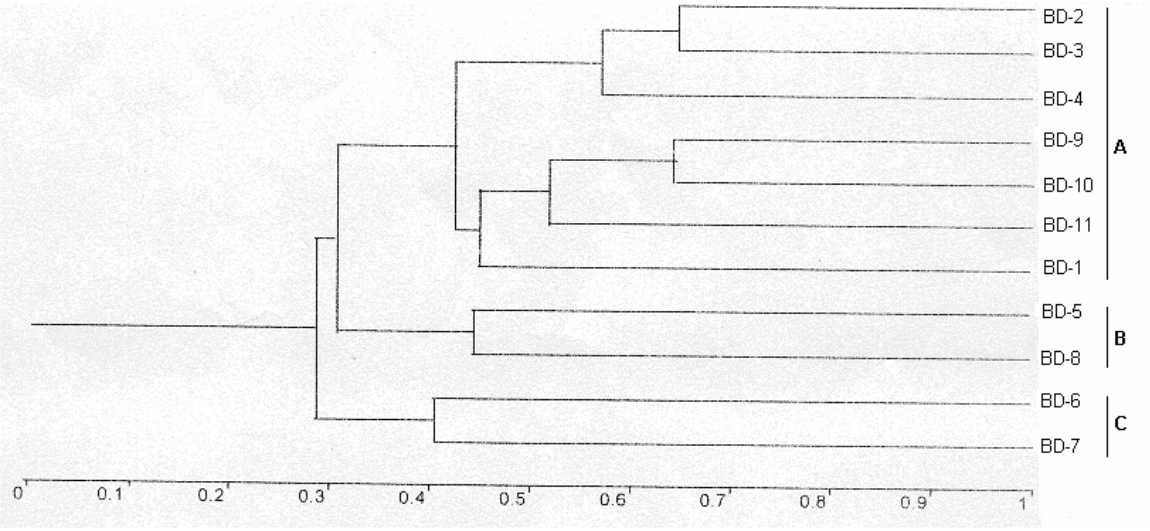

Figure 1: Dendrogram of 11 fungal isolates developed from RAPD data using the unweighted pair group method of arithmetic means (UPGMA). The scale is based on Nei and Li's (1979) coefficients of similarity