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Morphological, pathological and molecular variability in *Botryodiplodia theobromae* (Botryosphaeriaceae) isolates associated with die-back and bark canker of pear trees in Punjab, India

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ABSTRACT. Thirteen isolates of *Botryodiplodia theobromae* collected from pear varieties grown in various regions of Punjab were studied for morphological, pathological and molecular characterization. The mycelial growth of *B. theobromae* isolates was classified as fluffy or depressed, uniform to irregular and cottony white turning to black. Colony growth rate varied from 19.1 to 24.9 mm per day. Pycnidia were produced either on the edge, centered or scattered on Petri dishes after 20 to 34 days of incubation. Pycnidia and pycnidiospores ranged in size from 118.0 to 240.0 µm and 14.5-35.5 x 6.5-14.5 µm, respectively. Lesion length produced by different isolates ranged from 1.9-7.2 x 0.8-

3.3 cm with 49.4-90.9% infection. Using nine SSR and seven RAPD markers, amplified DNA bands ranged from 0.2 to 1.5 and 0.18 to 2.0 kb, respectively. Polymorphism information content values ranged from 0.44 to 0.71 and 0.63 to 0.93 for SSR and RAPD markers, respectively. A dendrogram based on molecular data, grouped the isolates into three major clusters with 65 to 79.5% genetic similarity; most of the isolates showed variety-specific grouping. The isolates prevalent on pear cultivars 'Patharnakh' and 'Baggugosha' in Ludhiana, Amritsar and Hoshiarpur districts were found to have a high degree of similarity; these isolates were also considerably distant from mango isolates and from other isolates on other pear cultivars. The isolates from cultivars Punjab Beauty, LeConte and Kieffer also had a high degree of similarity. Isolates from cultivar Smith were different from other pear isolates but showed more similarity with mango isolates.

Key words: *Botryodiplodia theobromae*; Molecular markers; Pear; Punjab; Variability

INTRODUCTION

The fungal pathogen *Botryodiplodia theobromae* Patouillard [*Lasiodiplodia theobromae* (Patouillard) Griffon and Maublanc] represents the sexual state of *Botryosphaeria rhodina* (Berk. and M.A. Curtis) Arx. It is an important opportunistic pathogen with worldwide distribution in tropical and subtropical regions causing different types of diseases in many plant species. It is one of the economically important fungi that attack a wide range of hosts (Punithalingam, 1976). *B. theobromae* causes shoot blight, die-back, twig blight, cankers, etc., mainly in woody plants including fruit and tree crops such as pear, apple, *Albizia falcataria*, peach, mango, avocado, *Citrus* spp, *Eucalyptus* spp, *Azadirachta indica*, *Pinus* spp, etc. (Sharma et al., 1984; Verma and Cheema, 1984; Mattos and Ames, 1986; Sharma and Sankaran, 1987; Darvas and Kotze, 1987; Britton et al., 1990; Sangchote, 1991; Cedeno and Palacios-Pru, 1992; Cedeno et al., 1995; Mohali et al., 2005). Die-back and bark canker is extremely destructive in certain areas where climatic conditions are favorable for its development. The disease is confined to the current growth and 1- to 2-year-old twigs. The bark of infected parts shrinks considerably resulting in depressed lesions. Longitudinal and transverse cracks appear on the cankered bark of some older branches (Verma and Cheema, 1984). The pathogen causes distension, disrupts the cell walls and weakens the strength and toughness of wood (Shah, 2007).

DNA-based markers have been used to determine population genetic structures, gene flow and reproductive mode in many fungal pathogens such as *Botryosphaeria* spp and their anamorphs (Barnes et al., 2001; Zhou et al., 2002; Burgess et al., 2003; Slippers et al., 2004). Simple sequence repeat (SSR) markers represent a class of co-dominant molecular markers consisting of tandem repeat loci, rich in polymorphism with allele size determined by the addition or deletion of one or more repeats (Mohali et al., 2005). The molecular biology techniques are now used in conjunction with morphological and physiological markers for the analysis of populations. Few attempts have been made to organize *B. theobromae* isolates into groups on the basis of genetic characteristics. Mohali et al. (2005) studied genetic diversity and gene

flow between the populations of *L. theobromae* in Australia and observed that a small number of genotypes in Venezuela and South Africa populations showed low genotypic diversity. However, the genotypic diversity was very high due to very high gene flow between the populations from different hosts. Burgess et al. (2003) collected *B. rhodina* isolates from pines and *Eucalyptus* from South America, Mexico and Indonesia and found that 8 pairs were polymorphic among the isolates collected from *Pinus* spp. A further 5 pairs were polymorphic when isolates from *Pinus* spp were compared with those from *Eucalyptus* spp thereby revealing relationships among isolates to be more closely linked to the host than the geographical origin.

Botryodiplodia theobromae is one of the important pathogens in pear (*Prunus* spp)-growing areas of the Punjab State in India. Analysis of variability in this pathogen is of great significance to identify the race spectrum of the pathogen and no study has been carried out in India so far. Therefore, the aim of the present study was to evaluate the morphological and pathological variability in *B. theobromae* isolates collected from different pear-growing regions of the Punjab State, as well as their molecular characterization using SSR and random amplified polymorphic DNA (RAPD) markers.

MATERIAL AND METHODS

Source of *Botryodiplodia theobromae* cultures

Thirteen isolates of *B. theobromae* were collected from commercial pear-growing regions of the Punjab State. B-PL_1, B-PL_2, B-PL_3, B-PL_4, B-PL_5, B-PL_6, and B-ML were isolated from pear cultivars Patharnakh, Baggugosha, Punjab Beauty, Kiefer, Smith, LeConte, and mango cultivar Dashehri, respectively, in the district of Ludhiana. B-PA_1, B-PA_2 and B-PA_3 were isolated from pear cultivars Patharnakh, Baggugosha and Punjab Beauty, respectively, in the district of Amritsar, and B-PH_1, B-PH_2 and B-MH were isolated from pear cultivars Patharnakh, Baggugosha and mango cultivar Dashehri, respectively, in the district of Hoshiarpur (Table 1). Each isolate was maintained as a pure culture on potato dextrose agar (PDA) and stored at 4°C. The stock culture of each isolate was revived on PDA by placing pycnidia on PDA with the help of an inoculating loop. Petri dishes were incubated in a B.O.D. incubator at 30 ± 1°C.

Table 1. Source of *Botryodiplodia theobromae* isolates from pear growing regions of Punjab.

S. No.	Isolate number	Place of collection	Pear variety
Ludhiana District			
1	B-PL_1	New orchard, PAU	Patharnakh
2	B-PL_3		Punjab Beauty
3	B-PL_2	Old orchard, PAU	Baggugosha
4	B-PL_4		Kiefer
5	B-PL_5		Smith
6	B-PL_6		LeConte
7	B-ML		Mango cultivar Dashehri
Amritsar District			
8	B-PA_1	Manawala	Patharnakh
9	B-PA_2		Baggugosha
10	B-PA_3		Punjab Beauty
Hoshiarpur District			
11	B-PH_1	Hoshiarpur	Patharnakh
12	B-PH_2		Baggugosha
13	B-MH		Mango cultivar Dashehri

PAU = Punjab Agricultural University.

Morphological characterization of isolates

Isolates of *B. theobromae* were sub-cultured on PDA medium on Petri dishes (90 mm in diameter) for comparison of morphological and growth characters. Eight-millimeter discs from margins of actively growing 7-day-old fungal culture were inoculated on the center of Petri dishes containing PDA medium. Three plates were used for each isolate. Colony growth rate was recorded by measuring colony diameter after 24, 48, 72, 96, 120, and 144 h. Colony color and texture observations were recorded after 7 days of incubation.

Pycnidial characters of each isolate *viz*. pycnidial number, arrangement, size, and color were observed after 30 days of incubation. Pycnidiospore size, color and septation were also recorded using a compound microscope.

Pathogenic behavior of isolates

The detached twig inoculation technique was used for studying the pathogenic behavior of *B. theobromae* isolates on pear cultivar Baggugosha. A moist chamber was prepared by placing a blotter sheet on a plastic tray (45 x 35 x 7.5 cm). Pear twig pieces of 6-8 cm long and approximately 2 cm thick of similar age were taken from healthy branches, washed twice with distilled water, air dried on sterilized blotting paper, and again surface sterilized with absolute alcohol. An injury was inflicted on the surface of twigs with the help of a cork borer. Inoculation was done by placing 15-day-old 8-mm culture discs of each isolate in an inverted position both on injured and uninjured sites of inoculation. A check was maintained in a similar manner on twigs, which were not inoculated. Five twigs were used for each isolate. The trays with twigs were incubated in a growth chamber (at 30 ± 1°C, on a 16-h light cycle). The observations were recorded regularly for symptom development.

Molecular characterization of isolates

Isolation of genomic DNA

A mycelial culture of each isolate was grown on potato dextrose broth. The mycelium of 15-day-old culture was dried and ground to a fine powder in liquid nitrogen using pre-cooled pestle and mortar. Total genomic DNA of individual *B. theobromae* was extracted using the CTAB method (Murray and Thompson, 1980). Isolated DNA was purified by using 1 µL RNase A (50 µg/mL) and resolved on 0.8% agarose gel. A working DNA solution was made by diluting the DNA stock to 25 ng/µL with distilled water for polymerase chain reaction (PCR) amplification.

PCR amplification

SSR and RAPD primers (Table 5) were used for fingerprinting of *B. theobromae* isolates. In preliminary experiments, 9 SSR primers were screened with one isolate of *B. theobromae* and the primers depicting the best amplification were selected and used for SSR analysis of all the isolates of *B. theobromae*. PCR was performed in 0.2-mL PCR tubes with a 20-µL reaction volume containing 2.0 µL 10X buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl and 1.5 mM $MgCl_2$), 4.0 µL dNTP mix (10 mM; Bangalore Genei, India), 1.6 µL primers (0.2

µM: 0.8 µL of each forward and reverse), 0.3 µL Taq polymerase (Bangalore Genei; 5 U/µL), 2.5 µL DNA template (25 ng/µL) and 9.6 µL sterilized distilled water. The reaction mixture was vortexed and centrifuged briefly in a micro-centrifuge (Bangalore Genei). Amplifications were performed using a thermal cycler (MJ Research PTC200 or Eppendorf Master Cycler) programmed for initial denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 30 s, annealing of primers at 52-68°C (depending on the primer used) for 40 s, extension at 72°C for 45 s (10 cycles) and for 50 s (25 cycles) and a final extension at 72°C for 10 min using fastest ramp time between the temperature transitions. The amplified PCR products were resolved by electrophoresis using 1.5% agarose gel in 0.5X Tris borate EDTA buffer (0.5 M Tris, 0.05 M boric acid and 1 mM EDTA, pH 8.0). DNA ladders of 100 bp (Bangalore Genei) and *Lambda* DNA/*Eco*RI-*Hin*dIII-double digest (MBI Fermentas) were used as markers. The gels were run at 5 V/cm for 2-3 h using a Bangalore Genei power system, stained with ethidium bromide (0.5 µg/mL), and visualized by images captured using a UVP gel documentation system (FOTODYNE) with the Foto/Analyst® PC Image software.

After screening 18 RAPD primers, 7 were selected for further analysis of all isolates. For RAPD analysis, a 20-µL PCR volume containing 2.0 µL 10X buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl and 1.5 mM $MgCl_2$), 4.0 µL dNTP mix (10 mM; Bangalore Genei), 6 µL primers (50 ng/µL), 0.3 µL Taq polymerase (Bangalore Genei; 5 U/µL), 2 µL DNA template (25 ng/µL) and 5.7 µL sterilized distilled water was used. The thermal cycling profiles for RAPD were denaturation of genomic DNA at 94°C for 5 min, primer annealing at 37°C for 1 min and extension of primer at 72°C for 2 min. Forty additional PCR cycles were carried out at 94°C for 1 min, 37°C for 1 min and 72°C for 2 min. An additional cycle at 72°C for 5 min was run at the end of these cycles. The PCR products were resolved by electrophoresis on 1.5% agarose gel in 0.5X TBE buffer at 50 V for 3 h.

Data analysis

DNA fingerprint data generated by SSR and RAPD markers were converted into binary matrix data and analyzed by the DICE coefficient and the UPGMA method with the NTSYS 2.0 pc software (Rohlf, 1997) and a dendrogram was constructed.

RESULTS

Morphological variability among isolates

Of the 13 isolates studied for their morphological characters, 6 isolates, namely B-PA_2, B-PH_1, B-PA_1, B-PL_1, B-PL_2, and B-PH_2, showed a colony growth rate of 24.0 to 24.9 mm/day indicating their fast growing nature (Table 2). These isolates filled the entire 90-mm diameter Petri dish surface in 96 h. Six isolates, i.e., B-PL_3, B-PL_5, B-PA_3, B-PL_4, B-PL_6, and B-MH, exhibited a moderate growth rate that ranged between 21.6 and 23.5 mm. The lowest growth rate was observed by the isolate B-ML. The majority of the isolates (8) showed vegetative growth, 3 showed fluffy colony growth and 2 isolates were appressed to the culture medium. Almost all the isolates showed a uniform growth pattern, except B-PL_4 and B-PL_6, which exhibited an irregular growth pattern. The colony color of isolates was dull white (B-PA_1, B-PH_2, B-PL_3, and B-MH), cottony white (B-PL_5), cottony white changing to black (B-PL_4, B-PL_6 and B-PH_1),

Table 2. Colony characters of *Botryodiplodia theobromae* isolates collected from different pear growing regions of Punjab.

Isolates	Colony* growth rate (mm/day**)	Sporulation#	Colony texture and color
B-PL_1	24.3	+	Raised, uniform, cottony to greyish black
B-PL_2	24.0	+	Fluffy, uniform, cottony to greyish black
B-PL_3	23.5	+	Appressed, uniform, dull white
B-PL_4	22.1	+	Raised, irregular, cottony white changing to black
B-PL_5	23.2	-	Fluffy, uniform, cottony white
B-PL_6	20.8	-	Appressed, irregular, whitish in color
B-ML	19.1	+	Raised, uniform, cottony white changing to black
B-PA_1	24.7	+	Raised, uniform, dull white
B-PA_2	24.9	+	Raised, uniform, greyish white in color
B-PA_3	23.2	+	Fluffy, uniform, greyish white in color
B-PH_1	24.8	+	Raised, uniform, cottony white changing to black
B-PH_2	24.3	+	Raised, uniform, dull white in color
B-MH	21.6	+	Raised, uniform, dull white
CD (P = 0.05)	0.9		

*Mean of three replications; **24 h (based on growth at 96 h); #Sporulation after 35 days of incubation (+ = observed; - = not observed); B = *Botryodiplodia theobromae*; P = pear; M = mango; L = Ludhiana; A = Amritsar; H = Hoshiarpur; 1 = Patharnakh; 2 = Baggugosha; 3 = Punjab Beauty; 4 = Kieffer; 5 = Smith; 6 = LeConte. CD = critical difference.

cottony greyish black (B-PL_1 and B-PL_2), greyish white (B-PL_1 and B-PL_2) and whitish (B-PL_6).

The isolates varied considerably with respect to pycnidial and pycnidiospore characters and 2 isolates (B-PL_5 and B-PL_6) did not sporulate on culture medium (Table 3). Time taken for pycnidial production varied from 20 to 34 days under artificial conditions. Minimum time for pycnidial production was observed for the isolate B-PL_2 (20 days) and maximum time for the isolate B-ML (34 days). Pycnidial arrangement was on the periphery in 6 isolates, scattered in 3 and centered in 2. Pycnidial diameter in isolates varied from 118 to 240 µm. The pycnidiospore size varied from 14.5-35.5 x 6.5-14.5 µm and was maximum in the isolate B-PL_1 and minimum in B-PL_4 and B-PL_3 isolates.

Table 3. Pycnidial and pycnidiospore characters of *Botryodiplodia theobromae* isolates collected from different pear growing regions of Punjab.

Isolates	Pycnidial characters*			Pycnidiospore size (µm)
	Time taken (days)	Diameter (µm)	Arrangement	
B-PL_1	22	142-228	Periphery	27.0-35.5 x 10.0 -14.5
B-PL_2	20	122-239	Periphery	21.5-32.0 x 11.5-13.5
B-PL_3	29	126-230	Centered	8.0-23.0 x 6.5-9.0
B-PL_4	31	131-209	Centered	14.5-21.0 x 7.0-8.5
B-ML	34	124-179	Scattered	15.5-22.0 x 9.0-13.5
B-PA_1	24	118-240	Periphery	22.5-27.0 x 11.5-13.0
B-PA_2	23	122-238	Periphery	24.5-25.0 x 10.7-8.0
B-PA_3	28	127-218	Scattered	19.0-22.5 x 11.0-13.5
B-PH_1	22	131-235	Periphery	24.0-26.0 x 6.5-9.0
B-PH_2	21	126-237	Periphery	20.5-22.5 x 7.5-12.5
B-MH	33	125-189	Scattered	17.2-20.5 x 8.5-9.5
CD (P = 0.05)	4.3	6.8		

*Mean of three observations after 35 days of incubation at 30 ± 1°C; B = *Botryodiplodia theobromae*; P = pear; M = mango; L = Ludhiana; A = Amritsar, H = Hoshiarpur; 1 = Patharnakh; 2 = Baggugosha; 3 = Punjab Beauty; 4 = Kieffer; 5 = Smith; 6 = LeConte. CD = critical difference.

Pathological variability among isolates

Morphologically similar *B. theobromae* isolates responded variably with respect to the pathogenicity test on pear cultivar 'Baggugosha' under controlled conditions (Table 4). The least similar relationship was observed between morphological traits of the isolates and their pathogenic behavior. The isolates were able to infect the injured bark surfaces only, and no infection was observed in uninjured bark. Isolates B-ML and B-MH were able to infect mango cultivar Dashehri only. However, all the isolates from pear were able to infect both pear and mango hosts. The symptoms developed on healthy twigs after 3-7 days of inoculation. The percent infection in twigs by different isolates was in the range of 49.4-90.9% with a mean of 77.5%. It was the highest (90.9%) in isolates B-PL_1 and B-MH and the lowest (49.4%) in isolate B-PL_5. The average lesion size was in the range of 1.9-7.2 x 0.8-3.3 cm with an overall mean of 5.5 x 1.9 cm in different isolates. The largest lesion size (7.2 x 2.3 cm) was noticed in isolate B-PL_1 and the smallest (1.9 x 0.8 cm) in isolate B-PL_6.

Table 4. Pathogenic behavior of *Botryodiplodia theobromae* isolates collected from different pear growing areas of Punjab following detached twig method on pear cultivar Baggugosha.

Isolates	Incubation period (days)	Percent of infection	Average lesion size (cm)*
B-PL_1	3	90.9	7.2 x 2.3
B-PL_2	3	82.8	7.1 x 2.4
B-PL_3	4	65.6	4.2 x 1.3
B-PL_4	6	69.7	3.6 x 1.4
B-PL_5	7	49.4	2.9 x 1.0
B-PL_6	6	62.7	1.9 x 0.8
B-ML	7	82.5	6.3 x 1.9
B-PA_1	4	89.3	7.0 x 2.3
B-PA_2	4	81.8	6.8 x 2.5
B-PA_3	5	72.7	5.1 x 1.5
B-PH_1	3	87.5	6.5 x 2.8
B-PH_2	5	81.8	6.2 x 1.4
B-MH	4	90.9	6.2 x 3.3
Mean	4.7	77.5	5.5 x 1.9
CD (P = 0.05)	-	6.8	-

*After 15 days of incubation. Values are means of three replications. B = *Botryodiplodia theobromae*; P = pear; M = mango; L = Ludhiana; A = Amritsar; H = Hoshiarpur; 1 = Patharnakh; 2 = Baggugosha; 3= Punjab Beauty; 4 = Kieffer; 5 = Smith; 6 = LeConte. CD = critical difference.

DNA fingerprinting of *Botryodiplodia theobromae*

Genetic diversity was detected in 13 *B. theobromae* isolates by SSR and RAPD markers. On the basis of the amplification produced, 9 SSR and 7 (of 18) RAPD primers were screened to study the genetic variability. For genetic analysis of 13 *B. theobromae* isolates, SSR and RAPD primers produced multiple PCR products. A range of 2 to 4 bands with a total of 27 bands of 0.2 to 1.5 kb were obtained mainly with SSR primers. The banding pattern obtained by the primer *LAS15*and*16*, with all isolates, is depicted in Figure 1a. This primer produced 4 polymorphic bands in different isolates in the range of 0.2-0.6 kb. The primer *BOT17*and*18* produced 3 polymorphic bands in different isolates in the range of 0.3-0.9 kb, indicating diversity among all the isolates (Figure 1b). The PCR products of 0.2-0.5,

specifically present in isolates B-PL_1, B-PL_2, B-PL_8, B-PL_9, B-PL_{11}, and B-PL_{12}, were collected from different districts of the Punjab State. The PCR products of 0.6-1.5 were specifically present in isolates B-PL_3 and B-ML_{13}. However, isolates B-PL_4, B-PL_5, B-PL_6, B-PA_3, and B-ML_7 presented banding patterns of varying size with different primers used.

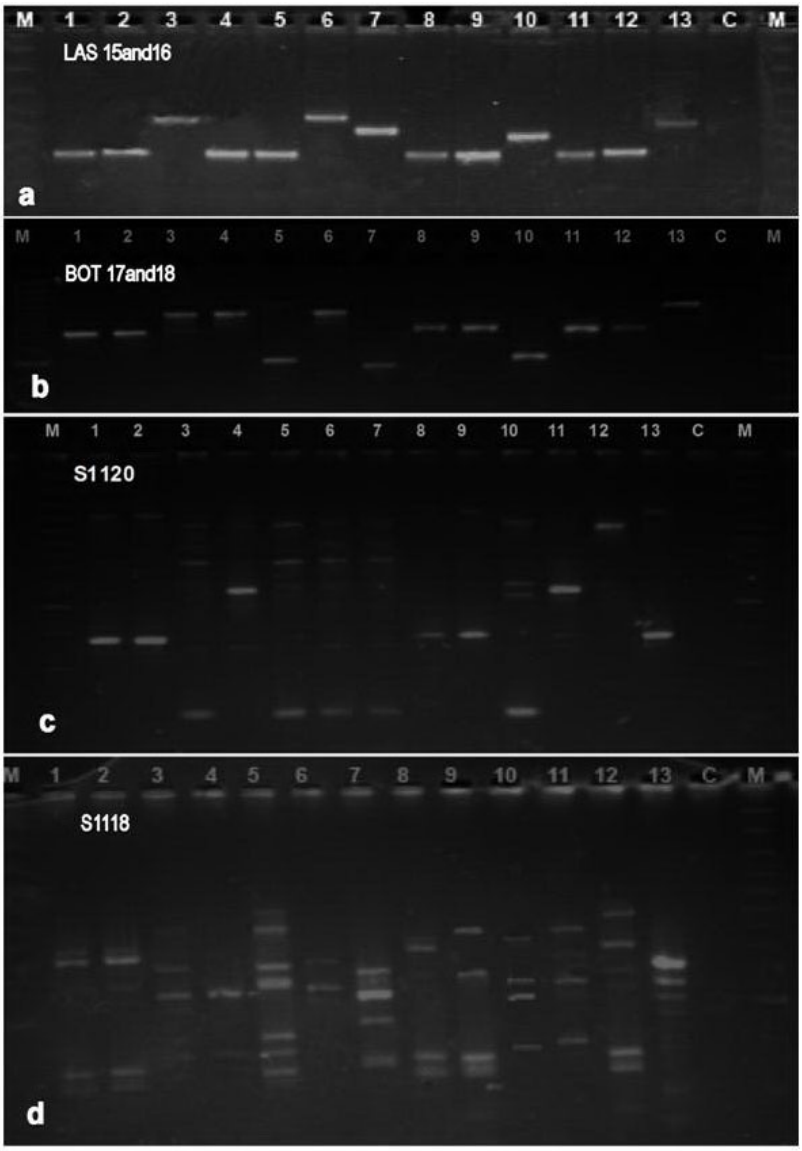

Figure 1. Ethidium bromide-stained DNA amplification profile of 13 isolates of *Botryodiplodia theobromae* using (a and b) microsatellite (SSR) and (c and d) randomly amplified polymorphic DNA (RAPD) markers. **a.** *LAS15*and*16*. **b.** *BOT17*and*18*. **c.** S1120. **d.** S1118. M = DNA marker; C = negative control; *lane 1* = B-PL_1; *lane 2* = B-PL_2; *lane 3* = B-PL_3; *lane 4* = B-PL_4; *lane 5* = B-PL_5; *lane 6* = B-PL_6; *lane 7* = B-ML; *lane 8* = B-PA_1; *lane 9* = B-PA_2; *lane 10* = B-PA_3; *lane 11* = B-PH_1; *lane 12* = B-PH_2; *lane 13* = B-MH.

Of the 18 RAPD primers used in the preliminary analysis, 7 were selected for genetic analysis of 13 *B. theobromae* isolates on the basis of good amplification. Seven RAPD primers produced 3-14 bands with a total of 51 bands. The primer S1120 yielded 7 bands in the range of 0.18-2.0 kb showing polymorphism among all the 13 isolates. The primer S1118 also produced 7 bands in the range of 0.5-2.4 kb, showing polymorphic DNA banding pattern. The primer S111 followed by S1110 produced the highest number of bands (14 and 10, respectively).

Polymorphic information content values

The polymorphic information content (PIC) values provide an estimate of the discriminatory power of a locus by taking into account not only the number of alleles that are detected but also the relative frequencies of these alleles (Table 5). The PIC values for SSR markers ranged from 0.44 for marker *LAS27*and*28* to 0.71 for marker *BOT19*and*20* with an average of 0.57 for all 9 markers. The PIC values of RAPD markers ranged from 0.63 in marker S1104 to 0.93 in marker S111 with an average of 0.81 for all 7 markers. Marker S111 amplified a total of 14 loci in 13 isolates of *B. theobromae* with a PIC value of 0.93, whereas marker S1110 amplified only 10 alleles with a PIC value of 0.88. Thus, in the present set of genotypes, SSR marker *BOT19*and*20* followed by marker *LAS15*and*16* and RAPD marker followed by marker S1110 were the most informative as compared to all other selected markers.

Table 5. Primer sequences, number of polymorphic bands, percent of polymorphism, and polymorphic information content (PIC) values obtained with SSR and RAPD markers.

Primer	Primer sequence (5' to 3')	Annealing temperature (°C)	Total No. of bands	Polymorphic bands (No.)	Percent of polymorphism	PIC values
SSR						
*LAS13*and*14*	F: 5'-GAGTTGTTAGTGCGGGCGCC-3' R: 5'-GCAGCCCCACAATTCACCAG-3'	58	3	2	67	0.59
*LAS15*and*16*	F: 5'-GCCAGATCCGTGCCCACTG-3' R: 5'-CATGCAGAGGTCGCAAAGTG-3'	58	4	4	100	0.65
*LAS21*and*22*	F: 5'-GGAAGATGATGGGATGGTTGC-3' R: 5'-GTACAAGAACGAACTCCGGGT-3'	55	2	1	50	0.50
*LAS27*and*28*	F: 5'-CGAACAGGGTTTCGTGACGT-3' R: 5'-CTCATATCTCGCCGGTTGCC-3'	52	2	1	50	0.44
*LAS35*and*36*	F: 5'-GGCATCACAACGACCAACCC-3' R: 5'-GCGAGAGTCGCAAGTACAGC-3'	58	2	1	50	0.48
*BOT11*and*12*	F: 5'-CGGCATGGTCTGCCGCTCC-3' R: 5'-GCATCTCCGGCTACCAACCG-3'	68	3	2	67	0.61
*BOT17*and*18*	F: 5'-GGCGCAATCTCGATTCGAGC-3' R: 5'-CCACGATGTCCGTTCATCG-3'	57	3	1	33	0.62
*BOT19*and*20*	F: 5'-GGCGGTCGCAGATGCGGTC-3' R: 5'-GCCCTATTCTGCGTGCCTCC-3'	62	4	4	100	0.71
*BOT35*and*36*	F: 5'-CTCCATCCTGATCCAGGGTCC-3' R: 5'-GACGAATCAAGCGGGCTGCCC-3'	65	4	3	75	0.50
Total			27			5.10 (0.57)*
RAPD						
S111	5'-CTTTCCGCAGT-3'	37	14	14	100	0.93
S116	5'-TCTCAGCTGG-3'	37	5	4	80	0.77
S1104	5'-GAGGGACCTC-3'	37	3	2	67	0.63
S1109	5'-ACCACGAGTG-3'	37	5	3	60	0.75
S1110	5'-CAGACCGACC-3'	37	10	10	100	0.88
S1118	5'-ACGGGACTCT-3'	37	7	6	86	0.85
S1120	5'-ACCAACCAGG-3'	37	7	7	100	0.85
Total			51			5.66 (8.10)*

*Average. SSR = simple sequence repeat; RAPD = random amplified polymorphic DNA.

Phylogenetic relationship among *Botryodiplodia theobromae* isolates

The combined data set of DNA markers obtained from all the isolates with SSR and RAPD markers were analyzed with UPGMA by the NTSYS 2.0 software and a dendrogram was constructed (Figure 2). All the isolates were divided into three major groups. Group I consisted of 6 isolates ($B\text{-}PL_1$, $B\text{-}PL_2$, $B\text{-}PA_1$, $B\text{-}PA_2$, $B\text{-}PH_1$, and $B\text{-}PH_2$) with 65% genetic similarity. Group II, comprised of 5 isolates ($B\text{-}PL_3$, $B\text{-}PL_6$, $B\text{-}PA_3$, $B\text{-}PL_4$, and $B\text{-}PL_5$), exhibited genetic similarity of 73-80%, whereas group III, comprised of 2 isolates (B-ML and B-MH), had genetic similarity of 79.5%. At 76% of genetic similarity coefficient, Group I was divided into 3 subgroups with 2 isolates in each subgroup, i.e., Ia ($B\text{-}PL_1$, $B\text{-}PL_2$), Ib ($B\text{-}PA_1$, $B\text{-}PA_2$) and Ic ($B\text{-}PH_1$, $B\text{-}PH_2$), while Group II was divided into subgroup IIa with 3 isolates ($B\text{-}PL_3$, $B\text{-}PL_6$, $B\text{-}PA_3$) and isolates $B\text{-}PL_4$ and $B\text{-}PL_5$ produced individual lineages at 76% similarity coefficient.

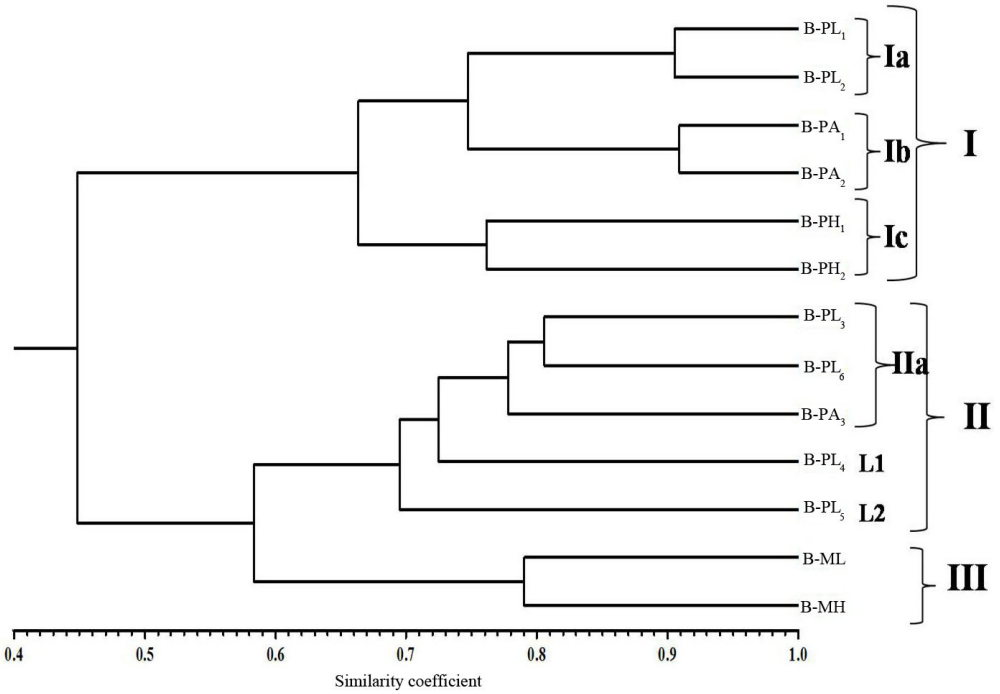

Figure 2. Dendrogram obtained from the combined data set of 13 *Botryodiplodia theobromae* isolates using SSR and RAPD markers.

DISCUSSION

Die-back and bark canker, caused by *B. theobromae*, is one of the important diseases of pear, which has become a major constraint to pear production during the last few decades (Verma and Cheema, 1984). The variability in disease symptoms, host range and geographical location of *B. theobromae* isolates suggests that there might be several strains of this species. The identification of *B. theobromae* isolates has proven to be tedious due to absence of stable morphological and physiological characteristics, which are influenced by the environment.

The differentiation of fungal isolates has been eased by the use of molecular markers as being stable and highly polymorphic (Barnes et al., 2001; Zhou et al., 2002; Burgess et al., 2003; Slippers et al., 2004).

The morphological and pathological characterizations of 13 isolates of *B. theobromae* collected from different pear growing regions of the Punjab State revealed that these isolates were fast to moderately fast growing, produced raised to fluffy colonies and dark black pycnidia arranged either on the periphery, centered or scattered in the colonies. These isolates showed varied pycnidia and pycnidiopore characters and demonstrated differences in their pathogenic potential. Some other authors have also reported morphological and pathological variability among isolates of *B. theobromae* from different hosts (Luke and Paul, 1982; Sabalpara et al., 1991; Cedeno et al., 1995; Ko et al., 2004; Mali et al., 2005). Although morphological and pathological variability existed among the isolates studied, the least similar relationship existed between morphologically similar isolates and their pathological behavior.

Based on molecular characterization of *B. theobromae* isolates, it is evident that the isolates prevalent on pear cultivars Patharnakh and Baggugosha in Ludhiana, Amritsar and Hoshiarpur districts have a high level of similarity and are grouped in one cluster. The isolates collected from cultivars Punjab Beauty, LeConte and Kieffer also showed a high level of similarity. Isolate from cultivar Smith was different from the isolates collected from other pear cultivars but showed more similarity with *B. theobromae* isolates, collected from mango cultivar Dashehri. The isolates collected from Patharnakh and Baggugosha exhibited a very high level of variation from the isolates collected either from mango cultivar Dashehri or from other pear cultivars. It was observed that different isolates of *B. theobromae* collected from pear and mango were not location specific, rather they were genotype specific. The results are more or less similar to those obtained from a morphological and cultural basis. Burgess et al. (2003) also found relationships among *Botryosphaeria rhodina* isolates, from pines and *Eucalyptus*, to be more closely linked to the host than to the geographical origin by analyzing the isolates from South America, Mexico and Indonesia. Mohali et al. (2005) observed low genotypic diversity in *Lasiodiplodia theobromae* populations from Venezuela and South Africa. However, the genotypic diversity was very high due to very high gene flow between the populations from different hosts.

RAPD markers were found to be more efficient in detecting polymorphism among the isolates than SSR. RAPD markers showed the best polymorphic DNA banding pattern. We observed that *B. theobromae* isolates collected from pear growing regions of the Punjab State are quite variable at the genetic level and variety specific grouping could be achieved to a greater extent.

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