Applied Biological Research 14 (1): 39-46, 2012

MOLECULAR DIVERSITY AND GENE FLOW AMONG *Botryodiplodia theobromae* POPULATIONS IN PEAR USING RAPD MARKERS

M.D. Shah*[#], K.S. Verma, Kuldeep Singh**, M.A Khan** and A.A. Wani*

*Department of Plant Pathology, **School of Biotechnology, Punjab Agricultural University, Ludhiana - 141 004, Punjab (India)*
**Division of Plant Pathology, S.K. University of Agricultural Sciences and Technology of Kashmir, Shalimar, Srinagar - 191 121, J&K (India)*
[#]e-mail: *mehraj547@rediffmail.com*

(Received 13 August, 2011; accepted 22 January, 2012)

ABSTRACT

Different populations of *Botryodiplodia theobromae* causing pear die-back and bark canker in Punjab, India were analyzed for their diversity at molecular level, gene flow and heterogeneity. Molecular diversity was assessed on the basis of allele frequencies of randomly amplified polymorphic DNA (RAPD) primers using *POPGENE* software. Allele frequency ranged from 0.0 to 1.0 in 3 populations of *B. theobromae*. Mean genetic diversities within population (H_S) varied from 0.25 to 0.80 indicating a high diversity. Molecular diversity across the populations ranged from 0.09 to 0.50 with a mean of 0.39; and differed significantly from mean H_S (0.27). Diversity indices of RAPD loci varied from very low (< 0.09) to very high H_S values (>0.50) across the populations (G_{ST}) with a mean G_{ST} of 0.30. Lower G_{ST} values of 0.019, 0.036, 0.052, 0.074 and 0.097 were observed at S116, S1109, S1120, S1118 and S1110 loci, respectively, depicting high genetic diversity among all the isolates. However, at S111 locus the G_{ST} value was 0.80 indicating low diversity in population at this locus. Out of 51 loci 33 showed gene flow (*Nm*) < 1 indicating high genetic differentiation within the population, while rest with *Nm* >1 had frequent gene flow across the populations of *B. theobromae*. Pair-wise comparison (G_{ST}) values among all the loci ranged from 0.22 to 0.35 revealing low genetic differentiation among the populations. The observed high molecular diversity (H_S) within the population suggested that *B. theobromae* variability was high in Punjab. The RAPD-based dendrorgam divided isolates into 3 major clusters at 65% similarity coefficient. In general, 20-25% similarity was observed among all the isolates of *B. theobromae* in Punjab.

Keywords: Bark canker, *Botryodiplodia theobromae*, die-back, gene flow, molecular diversity, pear, RAPD markers

INTRODUCTION

Pear is one of the most important temperate fruit of the world and occupies position next to apple in terms of importance, area, production and varietal diversity (Rathore, 1991). The major producers of pear are China, Italy, USA, Spain, Argentina, Republic of Korea, Turkey, Germany, Japan, India and South Africa. In India, pear is cultivated on an area of 23000 ha with annual production of 2.0 lakh tonnes (Anonymous, 2004). In Indian state of Punjab pear cultivation has assumed great significance in its sub-mountainous and central humid districts of Amritsar, Gurdaspur, Jalandhar, Ropar and Hoshiarpur. However, the crop is vulnerable to attack by many fungal diseases like scab (*Venturia pirina*), leaf spots (*Alternaria alternata, Mycosphaerella chaubattiensis* and *M. pyrina*), shoot or fruit

blight (*Phoma glomerata*), root rot (*Polyporus palustris* and *Ganoderma lucidum*), die-back and bark canker (*Botryodiplodia theobromae*) (Anderson 1956; Verma and Cheema, 1984). *Botryodiplodia theobromae* Patouillard [*Lasiodiplodia theobromae* (Patouillard) Griffon & Maublanc] is an important opportunistic pathogen distributed worldwide in tropical and subtropical regions with a wide host range (Punithalingam, 1976). Its hosts are mainly woody plants including fruit and tree crops such as pear, apple, *Albizia falcataria*, peach, mango, *Citrus* spp. (Shah *et al*., 2007) causing shoot blight, die-back or bark canker in these trees. The pathogen causes distension and disruption of the cell wall which leads to weakening of the wood. The pathogen colonizes the healthy plant tissue without exhibiting symptoms and thus can be considered as a latent pathogen capable of endophytic survival (Shah *et al*., 2007). *Botryodiplodia theobromae* is one of the important pathogens in pear (*Prunus* spp.) growing areas of Punjab state of India, analysis of genetic diversity and gene flow in this pathogen is of great significance.

DNA-based markers have been used to determine population genetic structures, gene flow, genetic isolation and reproductive mode in many fungal pathogens such as *Botryosphaeria* spp. and their anamorphs (Burgess *et al.*, 1984; Zhou *et al.*, 2000; Barnes *et al*., 2001; Slippers *et al.*, 2004; Shah *et al.*, 2010). Therefore, the present study was planned to study the genetic diversity and gene flow within and amongst three populations of *B. theobromae* collected from different pear growing areas of Punjab (India) using Nei's genetic diversity formulae (Nei, 1973).

MATERIALS AND METHODS

A total of 13 *Botryodiplodia theobromae* isolates representing populations from three diverse pear growing areas of Punjab were collected. The isolates were B-PL_1 (from pear cv. Patharnakh), B-PL_2 (from pear cv. Baggugosha), B-PL_3 (from pear cv. Punjab Beauty), B-PL_4 (from pear cv. Kiefer), B-PL_5 (from pear cv. Smith), B-PL_6 (from pear cv. LeConte) and B-ML (from mango cv. Dashehri) from PAU, district Ludhiana, B-PA_1 (from pear cv. Patharnakh) , B-PA_2 (from pear cv. Baggugosha) and B-PA_3 (from pear cv. Punjab Beauty) from Manawala, district Amritsar; and B-PH_1 (from pear cv. Patharnakh), B-PH_2 (from pear cv. Baggugosha) and B-MH (from mango cv. Dashehri) from district Hoshiarpur. Each isolate was grown on PDA medium, purified by single spore technique and maintained on PDA medium at 4°C.

Genetic diversity and gene flow

The total genomic DNA of each *B. theobromae* isolate was extracted following the procedures of Saghai-Maroof *et al.* (1984) and Shah *et al.* (2011). A set of 18 randomly amplified polymorphic DNA (RAPD) primers [Integrated DNA Technology, Inc., USA] (Table 1) were used for PCR amplification. The amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume containing 2.0 µl of 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), 6 µl primer (0.1 µM), 0.3 µl of *Taq* polymerase (Bangalore Genei India; 5 U μl^{-1}), 2 µl of DNA template (25 ng ml^{-1}) and 5.7 µl of sterilized distilled water.

Reaction mixture was vortexed and centrifuged in a micro-centrifuge (Bangalore Genei, India). Amplifications were performed using thermal cycler (MJ research PTC200 or Eppendorf master cycler) programmed for initial denaturation at 94°C for 5 min. followed by 45 cycles, each cycle programmed at 94°C for 1 min. denaturation, annealing of primers at 37°C for 1 min., extension at 72°C for 2 min. and a final extension at 72°C for 5 min.

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/*EcoR I-Hind III* double digest (MBI Fermentas) were used as markers. The gels were run at 5V cm^{-1} for 2-3 h using Bangalore Genei power system. The gels were stained with ethidium bromide (0.5 µg ml^{-1}). The gels were viewed and images captured using UVP gel documentation system (FOTODYNE) with Foto/Analyst®PC Image software programme.

Data analysis

Fifty one polymorphic bands were scored for presence or absence of a band and interpreted as the two alleles at a locus (Williams *et al.*, 1993). Allele frequency (X_{ij}) at each locus was calculated. Genetic diversity within (H_S, Eq. 1) and among populations (G_{ST}, Eq. 3) was calculated using the following Nei's formulae of diversity (Nei, 1973; Nei and Li, 1989) in *POPGENE version 1.31*).

$$H_s = 1 - \sum_{i=1}^{h} x_{ij}^2, \qquad (1)$$

$$H_T = 1 - \sum_{i=1}^{k} x_{ij}^{-2}, \qquad (2)$$

$$G_{ST} = \frac{H_T - H_S^-}{H_T}, \qquad (3)$$

Where h is the number of alleles and X_{ij} is the frequency of ith allele in population j, H_T (Eq. (2) is the total genetic diversity among all populations where k is the number of populations and X_{ij}^- is the frequency of allele i averaged over 3 populations. H_s is the average genetic diversity of all populations.

Gene flow (Nm) estimates was calculated using the programme POPGENE (Yeh *et al.*, 1999). Heterogeneity in allele frequencies among samples was tested using the likelihood ratio Chi-square statistic (G^2) (Michelmore and Hulbert, 1987; Hamelin *et al.*, 1994; Brown 1996). Genetic differentiation (G_{ST}) among sub-populations was estimated using Nei's coefficient of differentiation (Nei, 1973; Nei and Li, 1989).

DNA fingerprint data generated by RAPD primers was converted into binary matrix. The presence (1) and absence (0) of each DNA band of a specific molecular weight was recorded for each gel (Fig. 1). The binary matrix obtained from individual gel and data was analyzed using DICE coefficient and UPGMA method with NTSYS 2.02e computer software (Rohlf, 1997) and dendrogram was constructed.

RESULTS AND DISCUSSION

Genetic diversity and gene flow

The *Botryodiplodia theobromae* isolates comprising of 13 isolates constituted 3 populations (Table 1). The isolates were fingerprinted (Fig. 1) using RAPD primers for their gene flow (N_m) genetic diversity within the population (H_S), among the populations (G_{ST}) and overall population (H_T) at all the loci irrespective of geographic distances. Of the 18 RAPD markers, only 7 were produced polymorphism. The RADP primers used produced 51 polymorphic loci. There were 49 alleles (48 polymorphic) among the population from Ludhiana, 34 alleles (20 polymorphic) in Amritsar population and 40 alleles (29 polymorphic) in Hoshiarpur population. There were 4 unique alleles in Ludhiana population (*S111*, *S116* and *S1110*) and 1 locus each in Amritsar (*S116*) and Hoshiapur (*S1109*) population (Table 2).

Allele frequencies (X_{ij}) varied significantly among the 3 sub-populations at all the 51 loci ranging from 0.0 to 1.0 (Table 2). The mean genetic diversities within population (H_S) also varied in all the 3 locations ranging 0.25 to 0.80 indicating high genetic diversity within each population as low H_S values mean high diversity.

Total genetic diversity across all the populations (H_T) ranged from 0.0 to 0.5 [mean 0.39] and differed significantly from mean H_S (0.27). Diversity indices of RAPD loci varied from very low (H_S <0.09) to very high (H_S >0.50) value across all the populations (G_{ST}) of *B. theobromae* in Punjab with a mean G_{ST} of 0.30 (Table 3). The lower G_{ST} values of 0.019, 0.036, 0.052, 0.074 and 0.097 were observed at S116, S1109, S1120, S1118 and S1110 loci, respectively, showing high genetic diversity among all the isolates of *B. theobromae*. However, at S111 locus the G_{ST} value observed was 0.80 indicating low diversity in the population at this locus indicating a high genetic differentiation within

the population. The remaining 18 loci showed *Nm* higher than 1 indicating the frequent existence of gene flow across the populations of *B. theobromae*.

Table 1. Randomly amplified polymorphic DNA (RAPD) markers and their primer sequences for PCR analysis of *Botryodiplodia theobromae* populations

S. No.	Primer number	Primer sequence
1	S 103	5'-AGACGTCCAC-3'
2	S 108	5'-GAAACACCCC-3'
3	S 110	5'-CCTACGTCAG-3'
4	S 111*	5'-CTTTCCGCAGT-3'
5	S 114	5'-ACCAGGTTGG-3'
6	S 115	5'-AATGGCGCAG-3'
7	S 116*	5'-TCTCAGCTGG-3'
8	S 1101	5'-TCACGTACGG-3'
9	S 1103	5'-CTTCCCTGTGT-3'
10	S 1104*	5'-GAGGGACCTC-3'
11	S 1105	5'-GGGCTATGCC-3'
12	S 1109*	5'-ACCACGAGTG-3'
13	S 1110*	5'-CAGACCGACC-3'
14	S 1111	5'-AGATGCGCGG-3'
15	S 1113	5'-CACGGCACAA-3'
16	S 1114	5'-TGGTTGCGGA-3'
17	S1118*	5'-ACGGGACTCT-3'
18	S 1120*	5'-ACCAACCAGG-3'

* Polymorphic markers

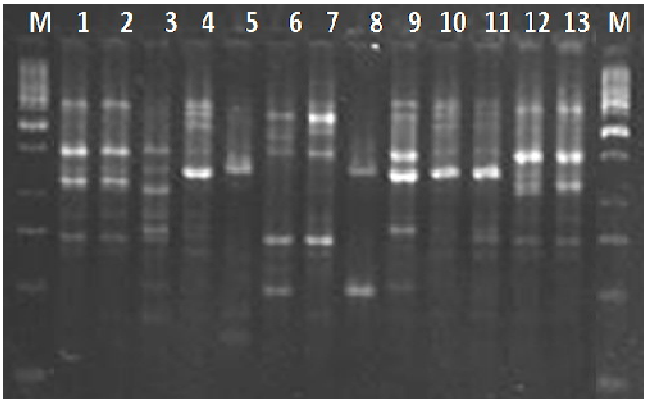

Fig. 1: Ethidium bromide stained DNA amplification profile of 13 isolates of *Botryodiplodia theobromae* using RAPD markers; M is the marker, Lane 1-13 are 13 isolates

In order to obtain detailed information on the movement of loci across the populations, gene flow (*Nm*) at all the 51 loci was determined (Table 3). Out of 51 loci, 33 loci showed gene flow (*Nm*) below 1 indicating a high genetic differentiation.

To obtain information regarding diversity within population, a pair-wise comparison (G_{ST}) values among all the loci were calculated. The values obtained between a pair of population ranged from 0.22 to 0.35 [only between Amritsar and Ludhiana (0.22), between Ludhiana and Hosiarpur (0.25), and between Amritsar and Hoshiarpur (0.35)], which indicate a low genetic differentiation among the populations of *B. theobromae*.

The high genetic diversity (H_S) within population was observed in the present study suggesting that *B. theobromae* possessed high variability in Punjab. The diversity indices may be attributed to the asexual reproduction or homothallic asexual reproduction (Coppin *et al*., 1997; Turgeon, 1998; Mohali *et al*., 2005; Shah *et al*., 2010) and isolation resulting from geographic distances.

Looking at pair-wise comparison (G_{ST}) among the populations, low difference was observed thereby indicating low diversity. Further, out of 51 loci, only 33 loci showed *Nm* more than 1 indicating frequent gene flow among the populations. This observed linkage of alleles between different loci in all the populations suggests a predominantly clonal mode of reproduction of the fungus along with the frequent gene flow among the populations. Frequent gene flow among different populations of *Lasiodiplodia theobromae* and *B. theobromae* had been reported from time to time (Mohali *et al.*, 2005; Shah *et al*., 2011).

Data set of DNA markers obtained from all the isolates with RAPD markers was analyzed to construct dendrogram (Fig. 2). All the isolates were divided into 3 major groups. Group I consisted of 6 isolates (B-PL_1, B-PL_2, B-PA_1, B-PA_2, B-PH_1 and B-PH_2) with 40% genetic similarity, though collected from different districts of Punjab. Group II comprising of 5 isolates (B-PL_3, B-PA_3, B-PL_6, B-PL_5 and B-PL_4) exhibited genetic similarity of 50%. All these isolates were collected from different pear cultivars from Ludhiana district, except B-PA_3 which was collected from Amritsar district of Punjab. Group III comprised of 2 isolates (B-ML and B-MH) having genetic similarity of 66%. At 61% of genetic similarity coefficient, Group I was divided into three sub-groups with 2 isolates in each subgroup i.e. Ia

Table 2: Allele frequency and genetic diversity within three populations of *B. theobromae* using RAPD markers

Locus	Ludhiana		Amritsar		Hoshiarpur	
	X_{ij}	H_s	X_{ij}	H_s	X_{ij}	H_s
S111	0.4286	0.4898	1.0000	0.0000	0.3333	0.4444
S111	0.5714	0.4898	0.0000	0.0000	1.0000	0.0000
S111	0.7143	0.4082	1.0000	0.0000	0.3333	0.4444
S111	0.2857	0.4082	0.0000	0.0000	0.0000	0.0000
S111	0.5714	0.4898	0.0000	0.0000	0.6667	0.4444
S111	0.5714	0.4898	1.0000	0.0000	0.0000	0.0000
S111	0.8571	0.2449	0.0000	0.0000	0.0000	0.0000
S111	0.7143	0.4082	0.0000	0.0000	0.3333	0.4444
S111	0.4286	0.4898	1.0000	0.0000	0.3333	0.4444
S111	0.5714	0.4898	1.0000	0.0000	0.0000	0.0000
S111	0.5714	0.4898	1.0000	0.0000	0.3333	0.4444
S111	0.7143	0.4082	1.0000	0.0000	0.6667	0.4444
S111	0.5714	0.4898	0.0000	0.0000	0.3333	0.4444
S111	0.5000	0.5000	0.0000	0.0000	1.0000	0.0000
S116	0.2857	0.4082	0.3333	0.4444	0.0000	0.0000
S116	0.4286	0.4898	0.3333	0.4444	0.5000	0.5000
S116	0.2857	0.4082	0.6667	0.4444	0.6667	0.4444
S116	0.0000	0.0000	0.3333	0.4444	0.0000	0.0000
S116	0.5714	0.4898	0.0000	0.0000	0.0000	0.0000
S1104	0.4286	0.4898	0.0000	0.0000	0.6667	0.4444
S1104	0.7143	0.4082	1.0000	0.0000	1.0000	0.0000
S1104	0.1429	0.2449	0.0000	0.0000	0.3333	0.4444
S1109	0.0000	0.0000	0.0000	0.0000	0.6667	0.4444
S1109	0.1429	0.2449	0.5000	0.5000	0.6667	0.4444
S1109	0.7143	0.4082	0.5000	0.5000	0.6667	0.4444
S1109	0.7143	0.4082	1.0000	0.0000	1.0000	0.0000
S1109	0.5714	0.4898	0.5000	0.5000	1.0000	0.0000
S1110	0.1429	0.2449	0.0000	0.0000	0.0000	0.0000
S1110	0.5714	0.4898	0.0000	0.0000	0.6667	0.4444
S1110	0.2857	0.4082	0.5000	0.5000	0.6667	0.4444
S1110	0.5714	0.4898	0.0000	0.0000	0.3333	0.4444
S1110	0.7143	0.4082	1.0000	0.0000	0.6667	0.4444
S1110	0.1429	0.2449	0.0000	0.0000	0.3333	0.4444
S1110	0.2857	0.4082	0.0000	0.0000	0.3333	0.4444
S1110	0.5714	0.4898	1.0000	0.0000	1.0000	0.0000
S1110	0.7143	0.4082	1.0000	0.0000	1.0000	0.0000
S1110	0.4286	0.4898	0.0000	0.0000	0.3333	0.4444
S1118	0.6667	0.4444	0.3333	0.4444	0.0000	0.0000
S1118	1.0000	0.0000	1.0000	0.0000	0.3333	0.4444
S1118	0.3333	0.4444	0.3333	0.4444	0.6667	0.4444
S1118	0.8333	0.2778	0.6667	0.4444	0.3333	0.4444
S1118	0.5000	0.5000	0.3333	0.4444	0.6667	0.4444
S1118	0.1667	0.2778	0.3333	0.4444	1.0000	0.0000
S1118	0.8333	0.2778	1.0000	0.0000	0.6667	0.4444
S1120	0.2857	0.4082	0.6667	0.4444	0.6667	0.4444
S1120	0.8571	0.2449	0.3333	0.4444	1.0000	0.0000
S1120	0.5714	0.4898	0.3333	0.4444	0.3333	0.4444
S1120	0.7143	0.4082	0.3333	0.4444	1.0000	0.0000
S1120	0.7143	0.4082	0.3333	0.4444	0.0000	0.0000
S1120	0.8571	0.2449	0.6667	0.4444	1.0000	0.0000
S1120	0.5714	0.4898	0.3333	0.4444	0.0000	0.0000
Mean	-	0.3879	-	0.2541	-	0.3630

*Allele frequency (X_{ij}) and genetic diversity within populations (H_S)

Table 3: Genetic diversity, gene flow and heterogeneity among the three populations of *B. theobromae* in Punjab

Locus	H_T	H_S	G_{ST}	Gene flow	G^2
S111	0.4848	0.3114	0.3576	0.8983	1.79
S111	0.4989	0.1633	0.6727	0.2432	4.86
S111	0.4334	0.2842	0.3442	0.9527	2.23
S111	0.1723	0.1361	0.2105	1.8750	2.06
S111	0.4848	0.3114	0.3576	0.8983	1.78
S111	0.4989	0.1633	0.6727	0.2432	5.60
S111	0.4082	0.0816	0.8000	0.1250	9.42
S111	0.4545	0.2842	0.3747	0.8343	2.96
S111	0.4848	0.3114	0.3576	0.8983	1.78
S111	0.4989	0.1633	0.6727	0.2432	5.60
S111	0.4636	0.3114	0.3283	1.0232	1.78
S111	0.3275	0.2842	0.1323	3.2791	0.70
S111	0.4213	0.3114	0.2608	1.4174	1.78
S111	0.5000	0.1667	0.6667	0.2500	5.14
S116	0.3275	0.2842	0.1323	3.2791	1.30
S116	0.4874	0.4781	0.0191	25.6419	0.15
S116	0.4969	0.4324	0.1298	3.3516	1.93
S116	0.1975	0.1481	0.2500	1.5000	3.23
S116	0.3084	0.1633	0.4706	0.5625	6.49
S1104	0.4636	0.3114	0.3283	1.0232	3.94
S1104	0.1723	0.1361	0.2105	1.8750	2.79
S1104	0.2671	0.2298	0.1396	3.0811	1.60
S1109	0.3457	0.1481	0.5714	0.3750	6.99
S1109	0.4919	0.3964	0.1941	2.0759	2.94
S1109	0.4678	0.4509	0.0361	13.3545	0.31
S1109	0.1723	0.1361	0.2105	1.8750	2.44
S1109	0.4274	0.3299	0.2281	1.6919	2.94
S1110	0.0907	0.0816	0.1000	4.5000	1.14
S1110	0.4848	0.3114	0.3576	0.8983	3.26
S1110	0.4995	0.4509	0.0974	4.6360	1.33
S1110	0.4213	0.3114	0.2608	1.4174	2.92
S1110	0.3275	0.2842	0.1323	3.2791	1.30
S1110	0.2671	0.2298	0.1396	3.0811	1.25
S1110	0.3275	0.2842	0.1323	3.2791	1.30
S1110	0.2449	0.1633	0.3333	1.0000	3.94
S1110	0.1723	0.1361	0.2105	1.8750	2.44
S1110	0.3789	0.3114	0.1782	2.3060	1.90
S1118	0.4444	0.2963	0.3333	1.0000	4.84
S1118	0.3457	0.1481	0.5714	0.3750	6.99
S1118	0.4938	0.4444	0.1000	4.5000	1.02
S1118	0.4753	0.3889	0.1818	2.2500	2.23
S1118	0.5000	0.4630	0.0741	6.2500	0.68
S1118	0.5000	0.2407	0.5185	0.4643	7.07
S1118	0.2778	0.2407	0.1333	3.2500	1.59
S1120	0.4969	0.4324	0.1298	3.3516	1.93
S1120	0.3941	0.2298	0.4169	0.6994	4.48
S1120	0.4848	0.4596	0.0520	9.1200	0.75
S1120	0.4334	0.2842	0.3442	0.9527	3.85
S1120	0.4545	0.2842	0.3747	0.8343	5.75
S1120	0.2671	0.2298	0.1396	3.0811	1.60
S1120	0.4213	0.3114	0.2608	1.4174	3.94
Mean	0.3914	0.2735	0.3013	1.1594	2.98

*Total genetic diversity (H_T), genetic diversity within (H_S) & among populations (G_{ST}), gene flow (Nm) & heterogeneity (G^2)

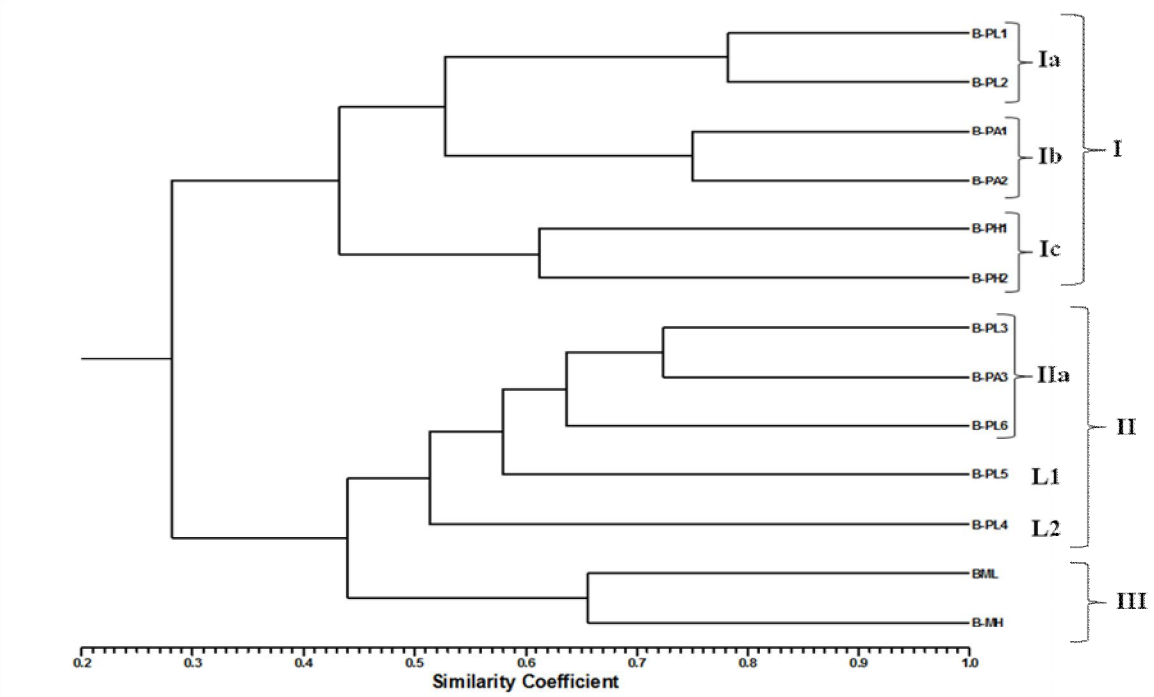

Fig. 2: Dendrogram obtained from the data set of 13 *Botryodiplodia theobromae* isolates using RAPD markers

($B\text{-}PL_1$, $B\text{-}PL_2$), Ib ($B\text{-}PA_1$, $B\text{-}PA_2$) and Ic ($B\text{-}PH_1$, B-PH2) while Group II was divided into subgroup IIIa 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 66% similarity coefficient. In general, 20-25% similarity was observed in all the isolates of *B. theobromae* in Punjab.

REFERENCES

Anderson, H.W. 1956. *Diseases of Fruit Crops.* McGraw Hill Book Co., New York, USA.

Anonymous. 2004. *Statistical Database of Crops.* Food and Agriculture Organization of United Nations, Rome, Italy.

Barnes, I., Gaur, A., Burgess, T., Roux, J, Wingfield, B.D. and Wingfield, M.J. 2001. Microsatellite markers reflect intra-specific relationship between isolates of the vascular wilt pathogen *Ceratocystis fimbriata. Molecular Plant Pathology,* **2**: 319-325.

Brown, J.K.M. 1996. The choice of molecular marker methods for population genetic studies of plant pathogens. *New Phytology,* **133**: 183-195.

Burgess, T., Wingfield, M.J. and Wingfield, B.D. 1984. Development and characterization of microsatellite loci for the tropical tree pathogen *Botryosphaeria rhodina. Molecular and Ecological Notes,* **3**: 91-94.

Coppin, E., Debuchy, R., Arnaise, S. and Pichard, M. 1997. Mating type and sexual development in filamentous ascomycetes. *Microbiology and Molecular Biology Review,* **61**: 411-428.

Hamelin, R.C., Doudrick, R.L. and Nance, W.L. 1994. Genetic diversity in *Cronartium quercuum* f. sp. *fusiformae* on locally pines in South U.S. *Current Genetics,* **26**: 359-363.

Michelmore, R.W. and Hulbert, S.H. 1987. Molecular markers for genetic analysis of phytopathogenic fungi. *Annual Review Phytopathology,* **25**: 385-404.

Mohali, S., Burgess, T. and Wingfield, M.J. 2005. Diversity and host association of the tropical tree endophyte *Lasiodiplodia theobromae* revealed using simple sequence repeat markers. *Forest Pathology,* **35**: 385-396.

Mottos, L. and Ames, T. 1986. *BotryodipIodia theobromae* pathogenic on apple. *Fitopathologia,* **12**: 26-32.

Nei, M. and Li, W. 1989. Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of National Academy Science USA,* **76**: 5269-5273.

Nei, M. 1973. Analysis of gene diversity in subdivision populations. *Proceedings of National Academy Science USA,* **70**: 3321-3323.

Punithalingam, E. 1976. *Botryodiplodia theobromae. CMI Description of Pathogenic Fungi and Bacteria. No. 519*. Commonwealth Mycological Institute, Kew, Surrey, UK.

Rathore, D.S. 1991. Pears. pp. 123-178. In: *Temperate Fruits* (eds. S.K. Mitra, T.K. Bose and D.S. Rathore). Horticultural and Allied Publishers, Calcutta, India.

Rohlf, F.J. 1997. *NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System). Ver 2.02e.* Applied Biostatic Inc., New York, USA.

Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. and Anard, R.W. 1984. Ribosomal DNA spacer length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. *Proceedings of National Academy Science USA,* **81**: 8014-8018.

Shah, M.D., Verma, K.S. and Dhillon, W.S. 2007. Severity and management of die-back and bark canker (*Botryodiplodia theobromae*) of pear in Punjab. *Journal of Research PAU,* **44**: 216-218.

Shah, M.D., Verma, K.S., Singh, K. and Kaur, R. 2010. Morphological, pathological and molecular variability in *Botryodiplodia theobromae* (Botryosphaeriaceae) isolates associated with die-back and bark canker of pear trees in Punjab, India. *Genetics and Molecular Research,* **9**: 1217-1228.

Shah, M.D., Verma, K.S., Singh, K. and Kaur, R. 2011. Genetic diversity and gene flow estimates among three populations of *Botryodiplodia theobromae* causing die-back and bark canker of pear in Punjab. *Archives of Phytopathology and Plant Protection,* **44**: 951-960.

Slippers, B., Burgess, T., Wingfield, B.D., Crous, P.W., Coutinho, T.A. and Wingfield, M.J. 2004. Development of simple sequence repeat markers for *Botryosphaeria* species with *Fusicocum* anamorphs. *Molecular and Ecological Notes,* **4**: 675-677.

Turgeon, B.G. 1998. Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology,* **36**: 115-137.

Verma, K.S. and Cheema, S.S. 1984. *Botryodiplodia theobromae* - the cause of die-back and bark canker of pear in Punjab. *Indian Phytopathology,* **37**: 325-27.

Williams, T.G.K., Hanafey, M.K., Rafalski, J.A. and Tingey, S.V. 1993. Genetic analysis using random amplified polymorphic DNA markers. *Methods in Enzymology,* **218**: 704–740.

Yeh FC, Yang RC, Boyle T. 1999. POPGENE Version 1.31. Microsoft windows-based freeware for population genetic analysis. University of Alberta, Alberta. http:/www.ualberta.ca/fyeh.

Zhou, X.D., Burgess, T., De-Beer, Z.W., Wingfield, B.D. and Wingfield, M.J. 2000. Development of polymorphic microsatellite markers for the tree pathogen and sap stain agent, *Ophiostoma ips*. *Molecular and Ecological Notes,* **2**: 309-312.