Pak. J. Bot., 42(6): 3949-3956, 2010.

ASSESSMENT OF GENETIC DIVERSITY AND GERMINATION PATTERN IN SELECTED COTTON GENOTYPES OF PAKISTAN

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Abstract

The genetic diversity in 18 cotton varieties of Pakistan was assessed using Random Amplified Polymorphic DNA (RAPD). Three RAPD primers: OPO-19, OPQ-14 and OPY-2 revealed amplifications at 470bp, 325bp and 1070bp with a selection efficiency of 27.7, 61.1 and 44.4 % respectively. Marker assisted screening revealed two cotton varieties CIM-240 and CIM-443 to have resistance against *CLCuV*. For all varieties days to germination and germination success were recorded. Based on the data three groups were identified with 13 varieties characterized as slow responding, 3 as good responding and 2 varieties as non-responding. Furthermore plant height and leaf area have been studied as an indicator of biomass production. Based on the pooled data 2 genotypes i.e., CIM240 and CIM443 have been identified as potential genotypes to be used in future cotton breeding.

Introduction

Taxonomically, *Gossypium* is a large genus with more than 50 species (Fryxell, 1971). Most of the species are diploid (2n = 2x = 26) containing a combination of two or more of the seven genomes designated as A, B, C, D, E, F and G. The diploid species having A, B, E, or F genomes are considered African or Asian in origin (Wendel & Cronn, 2003). The species with C, D, E, F or G genomes are Australian and those having the D genome originated in the Western Hemisphere and referred to as New World species (Wendel & Cronn, 2003). In addition, there are 5 allotetraploids (2n = 4x = 52) all of New World origin, 4 of which are indigenous to the continental Americas and one to Hawaii. These allotetraploids contain A and D genomes with 26 large and 26 small chromosomes with some overlap in size among chromosomes.

Gossypium hirsutum L. is an important crop in Asia (Hussain *et al*., 2005). It plays an important role in the economy and provides livelihood to farmers, processors and those fabricating its products (Anon., 2004). Today cotton is grown in more than 80 countries, however, China, USA, India and Pakistan are major cotton producers in the world. In Pakistan cotton is cultivated over 3.07 million hectares with an average yield of 12.7 million bales (Anon., 1992). In recent years, there has been a dramatic decrease in cotton production. Generally, the production is constrained due to soil type, late harvesting of the preceding wheat, excessive rains at the time of sowing, high temperature at flowering stage, leaf curl virus incidences, pest attack and improper production technology. The major production areas in Pakistan i.e., Punjab and Sindh provinces are worse affected by several infectious pests (Younas *et el*., 1980). Among these, the leaf curl virus has been a serious threat to cotton especially in Punjab. The Cotton Leaf Curl Virus (*CLCuV*) was first observed in 1967 on stray plants (Hussain & Ali, 1975) in Pakistan. By 1988 its geographic spread increased (Mahmood, 1999) causing a loss of more than 7.7 million bales (Ahmad *et al*., 2002). Hence, the production (Anon., 1992) has been decreased significantly due to the *CLCuV* infestation. The epidemic of *CLCuV* in Pakistan has been an example of dramatic shift in importance of a previously unimportant endemic disease.

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Development of *CLCuV* resistant cultivars seems the most promising control option (Akhtar *et el*., 2003). However, this requires characterization of cotton germplasm using recent molecular techniques. These methods have been significant in the management and utilization of plant genetic resources (Karp, 2002). The technology not only facilitates the marker assisted selection for breeding but these also help in enriching the gene pool (Fu, 2006). Efforts have been made to characterize cotton germplasm using allozymes, RFLP (Wendel & Brubaker, 1993), RAPD (Multani & Lyon, 1995; Iqbal *et al*., 1997), AFLP (Iqbal *et al*., 2001; Rana *et al*., 2005) and simple sequence repeat (SSR) markers (Liu *et al*., 2000; Reddy *et al*., 2001; Zhang *et al*., 2005; Lacape *et al*., 2007). These characterizations have provided useful information for understanding the genetic diversity and structure of various cotton genotypes found in different geographic regions. The objective of the present study was to investigate genetic diversity in recently developed cotton cultivars released in Pakistan and to develop molecular markers to select genotypes against *CLCuV*.

Materials and Methods

Plant materials: Eighteen cotton cultivars were obtained from Central Cotton Research Institute (CCRI) Multan. The set included known *CLCuV* resistant lines: CIM448, CIM482, and FH900 and a susceptible line: S12 used as reference. The provenance and pedigree information on these lines has been summarized in Table 1.

Sowing conditions, germination pattern and morphological assessment: Two germination trials were performed under glass house conditions. Trial I was conducted at temperature range of 20-30°C and with 12 hours of day length. Trial II was conducted at temperature ranging from 25-40°C and day length of 14 hours. For both the trials 4 seeds each of 18 accessions were sown in the soil with 75% clay and 25% sand. Observations were made for days to germination and germination success. The plant height and leaf size were observed at 15 days after germination. The data thus generated were analyzed to study the genotypic response in both the trials.

DNA extraction: The genomic DNA was extracted using modified CTAB method (Doyle & Doyle, 1990). The leaves weighing 0.2-0.3g were harvested at 2-3 leaf stage, washed and ground in liquid nitrogen and lysed in 1ml of pre-heated 2x CTAB buffer [2% w/v CTAB, 100mM Tris HCl (pH 8.0), 20mM EDTA (pH 8.0), 1.4M NaCl, 2% PVP and 50ml distilled water] with 1% (v/v) β-mercaptoethanol. The samples were incubated at 65°C for 30 minutes, 0.5 ml of chloroform and isomyl-alcohol (24:1) were added, mixed gently and centrifuged at 13,000 rpm for 10 minutes. Avoiding the interface, the supernatant was transferred to a fresh eppendorf, 3M chilled Sodium acetate and 60% isopropanol were added, mixed gently, the precipitating DNA was pelleted by centrifuging at 13,000 rpm for 15 minutes. The DNA pellet so obtained was resuspended in 40µl 1xTE buffer and stored at -20^{o}C for further use.

Primer sequence and reaction conditions for RAPD analysis: Five decamer primers of Operon series (Table 2) were used for the study and obtained from Gene Link Inc., USA. The polymerase chain reaction contained 50ng DNA, 5U *taq* polymerase (Fermentas), 25mM $MgCl_2$, 2.5mM dNTPs, 0.001% gelatin and 15ng of primer, 10x PCR buffer and double distilled water for a final volume of 25µl. The PCR amplifications were carried out at 94°C for 5 min., followed by 40 cycles of 94°C for 1min., 34°C for 1min., and 72°C for 2min and the final extension at 72°C for 10 min. The products were stored for further use at -20°C.

Table 1. The genotypes with pedigrees, their germination pattern and associated morphological and molecular marker data. The abbreviations are as follows: GP=Germination percentage; DTG=Days to germination; LA=Leaf area; PH=Plant height.

Sr. No.	Genotype	Female	Male	GP	DTG	LA (L*W)	PH Avg.	Source	O-19	Q-14	Y-2
11.	CIM-109	NIAB-78	A 89/FM	100	8	19.95	8.25	ND	A	A	A
3.	CIM-240	CIM-70	W-1104	100	8	20	9	ND	A	P	P
9.	CIM-443	CIM-109	LRA-5166	100	7	21	8.25	LRA	P	P	A
8.	CIM-448	W-1104	S-12 x CP-15/21	50	8	26	7.5	CP	P	P	A
14.	CIM-482	CIM-229	CP-15/21	50	10	18	7.5	CP	A	P	A
4.	CIM-496	CIM-425	755-6/93	75	9	18	8	ND	P	P	P
5.	CIM-497 (1282)	CIM-436	840/94 x 1192/543	75	7	21	8	ND	P	P	P
10.	CIM-498/1285	CIM-378	755-6/93	25	10	13.6	6	ND	A	P	A
2.	CIM-506	CIM-360	(CP-15/21) x 460A	75	9	22.4	10.5	CP	P	A	P
7.	CIM-534	34458	CIM-1100	50	9	11	8.5	ND	A	A	P
1.	CIM-541	H-1889	H-1929	75	10	19.6	8.5	ND	A	P	P
6.	CIM-557	CIM-467	1423-4/98	50	10	17	9	ND	A	P	P
13.	CIM-705	CIM-360	738-6/93	100	7	23.6	9.75	ND	A	A	A
12.	CIM-707	CIM-243	738-6/93	75	8	25	11.25	ND	A	A	A
15.	FH-900	FH-672	AET-5 x B-557 x LRA-5166	50	7	23	5.5	LRA	A	P	A
17.	Gomal-93	387F	AC-134	100	9	23	10.5	ND	A	P	A
16.	MNH-786	S-14	(CIM-448) (H-556 x MNH-516)	75	8	30	10	ND	A	A	A
18.	S-12	MNH-93	7203-14-4-Arizona	75	6	20	8.25	SUS	A	A	A

Table 2. Primers employed for amplification.

Primer	Sequence (5'-3')	Annealing
OPH-13	GACGCCACAC	35 C
OPN-14	TCGTGCGGGT	35 C
OPO-19	GGTGCACGTT	34 C
OPQ-14	GGACGCTTCA	36 C
OPY-2	CGTCGCCGCA	34 C

Product resolution and data analysis: The PCR products were resolved on 2% agarose and studied under ultraviolet in a gel documentation system (Bio Rad ®). The data were scored as presence (1) or absence (0) and comparisons were made considering the band sizes in base pairs using the gene ruler^{tm} 50bp DNA ladder (Fermentas). The data were processed in Microsoft excel and analyzed using Popgene32. The Nei's co-efficient was used to calculate the genetic similarity and cluster analyses were performed using UPGMA.

Results and Discussion

The present study with selected cotton genotypes revealed a variable response of germination success under two sets of conditions. In trial I, none of the genotypes revealed 100% germination success while five (27%) genotypes revealed 100% germination in trial II. A number of genotypes revealed 25% improvement in germination success and characterized as slow responding genotypes. For example five (27%) genotypes (CIM448, CIM482, CIM534, CIM557 & FH900) revealed 25% germination in trial I and 50% germination success in trial II. Similarly, six (33%) genotypes (CIM496, CIM497, CIM506, CIM541, MNH786 and S12) showed 50% germination in trial I and 75% in trial II. There were only two (11%) genotypes (CIM109 & CIM240) revealing 75% germination in trial I and 100% in trial II (Table 1). Three (16%) genotypes (CIM443, CIM705 & GOMAL93) revealed 50% germination in trial I and 100% in trial II hence were good respondents. Generally the germination success improves with the temperature in cotton (Casenave & Toselli, 2007). However, there are exceptions to this, for example in the present study two (11%) genotypes (CIM498 & CIM707) were identified that did not show any improvement in germination success. This pattern is rare and unique to cotton. For the present purpose we have characterized these genotypes as non-responding.

For 'days to germination', the varietal response was variable too. Generally in trial I, the genotypes took a minimum of 8 days and a maximum of 14 days to germinate. In trial II, the genotypes took a minimum of 6 and a maximum of 10 days to germinate. Hence germination conditions in trial II were rather favourable compared to those in trial I. In both the trials, the genotype S12 took minimum days to germinate while CIM557 took the maximum (Table 1). Following five genotypes viz., CIM497, CIM557, CIM705, FH900 and MNH786 were unequivocal by showing an early response of three to four days in trial II. However other genotypes especially CIM506 and CIM541 revealed only a little improvement of one day.

Although many important traits for instance seed, fibre and oil traits (Khan *et al*., 2010) may be considered, our focus remained largely on biomass indicators such as plant height and leaf area (Ghaderi & Soltani, 2007). Plant height showed a normal distribution pattern for the genotypes studied. At the seedling stage (15 days after germination), most

of the genotypes achieved 7-10 cm of height. The genotype CIM707 achieved maximum height while FH900 attained minimum height among the lot. Similarly, maximum leaf area was observed in MNH786 ($30cm^2$) followed by CIM707 ($25cm^2$) while minimum leaf area was recorded in the genotype CIM534. Hence the genotype CIM707 with maximum height and better leaf area remains a superior variety.

Genetic diversity based on RAPD markers: Our colleagues have been working in this area and have reported interesting results (Mehmood *et al*., 2010). In RAPD analysis, a total of 139 bands were amplified at 24 loci. Among these five loci were monomorphic while the remaining nineteen were polymorphic. The polymorphic loci revealing difference are helpful in establishing similarity and thus relationship among the genotypes (Hadrys *et al*., 1992). The present study estimated ~79% of the genetic diversity. The data further revealed a maximum of 13 bands in Gomal93 and a minimum of 4 bands in CIM534 and MNH786. The details on individual accessions have been summarized in Table 1. In the previous study, Khan *et al*., (2010) reported 70% polymorphism with a maximum of 18 bands produced. Multani *et al.,* (1995) revealed 92.1-98.9% similarity. Hence, our results also endorsed a similar pattern of genetic similarity among the cotton genotypes.

For each primer, the selection efficiency was different. For example, the OPO19 revealed a total of 19 bands with an average of 1.61 bands per accession. The maximum of 8 bands were amplified in S12 while only 3 bands were found in CIM443. The amplified products ranged in size from 10-1100 bps. Rubeena & Randhawa (1998) found the size range between 201- 2888 bp. Hence the present results explain only a subset of the amplifiable loci in cotton. Altogether, the oligo OPO19 revealed amplification in 7 genotypes with the selection efficiency of 38.8%. The total number of bands amplified by OPQ14 was 61 revealing an average of 3.38 bands per accession. The primer revealed a maximum of 6 bands in CIM240 while only 2 bands were found in CIM534. Altogether, OPQ14 revealed amplification in 11 genotypes; hence its selection efficiency was 61.11%. The oligo OPY2 amplified a total of 49 bands revealing an average of 2.72 bands per accession. A maximum of 7 bands were observed in CIM240 while only one band was observed in CIM443. Altogether, OPY2 was present in 13 genotypes with a selection efficiency of 72.2%.

Cluster analysis: Among 18 cotton accessions, 17 have been successfully assigned to one of the four clusters. At approximately 78% similarity, two genotypes CIM496 and CIM506 cluster together forming cluster A (Fig. 1). The analysis has revealed a minimum of 84% similarity between these genotypes which is the least similarity revealed in this analysis. These results corroborated with the previous studies (Khan *et al.,* 2009) where these genotypes have been placed in distinct subclusters. Similarly at 80% similarity, two other genotypes S12 and Gomal 93 segregated to form cluster B. These 2 genotypes shared ~88% similarity. At ~83% similarity 2 further clusters i.e., C and D may be recognized. The cluster C comprised of 3 genotypes CIM443, CIM448 and CIM497 while cluster D with 10 genotypes remains the biggest cluster within which subclusters may be identified including a subcluster comprised of CIM482 and FH900. Both these genotypes are characterized as resistant while S12 characterized as a susceptible genotype was grouped with Gomal93 and is placed distant from the cluster of resistant varieties.

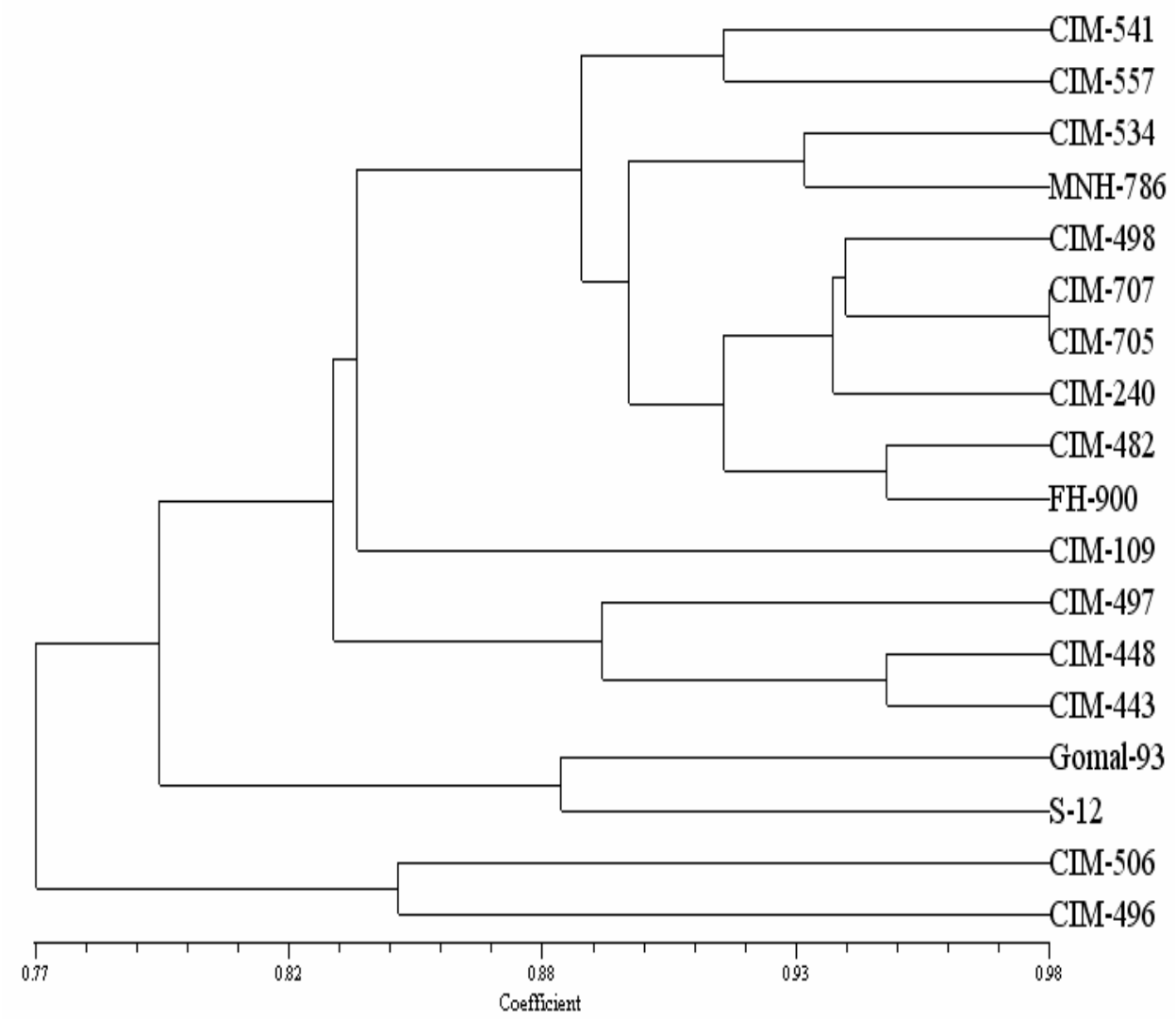

Fig. 1. The UPGMA clustering of cotton varieties based on Nei's coefficient of similarity.

The clustering pattern found in the present study was generally different from that of Khan *et al*., (2009) however distinct pattern found among resistant and susceptible varieties point to their utilization in differentiating among resistant and susceptible genotypes. However the previous studies did not reveal any such capacity, probably due to the narrow genetic base of these varieties causing inconsistencies to arise as highlighted earlier (Meredith, 2000; Iqbal *et al.*, 2001; Zhang *et al.*, 2005). Furthermore, there are only 2 genotypes i.e., CIM705 and CIM707 which showed no dissimilarity. This is attributed to low banding information. Similarly, the genotype CIM109 also remained unresolved. This warrants deploying more oligo-primers to resolve their positions and study diversity pattern.

Acknowledgements

The authors wish to thank Ch. Rehmat Ali, Head Breeding and Genetics Section, CCRI, Multan for generously providing the germplasm for the present study and Higher Education Commission, Islamabad for financial support.

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(Received for publication 16 November 2009)