The Bioscan 9(1): 125-131, 2014
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

REGENERATION MEDIA STANDARDIZATION AND MOLECULAR DIVERSITY ANALYSIS IN COTTON CULTIVARS

A. A. BHAROSE*, S. A.THAKUR AND D. N. DAMSE
College of Agricultural Biotechnology, Marathwada Krishi Vidyapeeth, Latur - 413 512 (M.S.) INDIA.
e-mail: achyut_bharose@rediffmail.com

KEYWORDS
Regeneration media
Standardization
Molecular
Diversity analysis
RAPD analysis

Received on :
06.05.2013

Accepted on :
07.01.2014

***Corresponding author**

ABSTRACT
A direct and indirect shoot regeneration protocol of three cotton cultivars viz. BN-1, PA-255, and PA-08, was optimized. Comparative studies on in vitro regeneration from shoot tip, cotyledonary node, hypocotyls and leaf explants revealed considerable variability in response to plant growth regulators. MS Basal formulation with combination of the cytokinins benzylaminopurine (2mg/l) and kinetin (1mg/l) shown 30-50% maturity response for cotyledonary node and shoot tip explants supplemented with kinetin (0.1mg/l) shown 28-42% matured plants, while callus initiation (35-71%) was observed in medium supplemented with 2, 4-D (0.1mg/l) as auxin source and kinetin (0.5mg/l) as cytokinin source when leaves as explants and hypocotyls shown callusing (35-64%) when KNO_3 (1.9g/l) was used in addition. By second objective 7 genotypes viz. BN-1, PA-255, PA-08, AC-738, PA-402, PA-183 and PA-405 were characterized by ten RAPD primers. Total 67 bands were produced out of which 63 bands were polymorphic while 4 bands were monomorphic. The average genetic similarity coefficient value was 0.392. Dendrogram was developed for RAPD analysis using NTSYS-pc Software. It showed 2 super clusters. Significant similarities were found in the clustering of RAPD Analysis.

INTRODUCTION

Cotton is an excellent source of textile fiber and cultivated in many countries. India is rich in cotton germplasm Dongare *et al*. (2003) and largest producer of cotton in the world after China and USA. Because of its high economic value considerable attention has been paid to improve cotton. However, genetic improvement of cotton through conventional means is limited due to many factors like absence of necessary variation, especially resistance against pests and diseases. Plant tissue culture techniques provide an alternative means of improvement by obtaining somaclones, induced variants and somatic hybridization or by introducing genes of interest against insects and different diseases through genetic engineering Zhang and Zhao *et al*. (1997). *In-vitro* cultures can be utilized for cotton genetic improvement but it requires presence of effective regeneration system which is highly specific in cotton Trolinder *et al*. (1989). The use of biotechnological tools such as biolistics and Agrobacterium - mediated transformation, require as a prerequisite plant regeneration protocols that are genotype-independent, efficient, and which do not yield somaclonal variant Firoozabady *et al*. (1987); McCabe *et al*. (1993). Regeneration of plants has been obtained from explants such as hypocotyls, cotyledon, and root Zang *et al*. (1997). Explants with pre-existing meristems have been used to induce multiple shoots *in vitro* and regenerate whole plants Morre *et al*. (1998); Hazra *et al*. (2000). Early reports of use of hypocotyls explants to induce multiple shoots in cotton were reported by Umbeck *et al*. (1987) and Firoozabady *et al*. (1987). The proliferation of pre-existing meristems from cotyledonary nodes, primary and tertiary leaf nodes, *etc*., into elongated multiple shoots *in vitro* has been used Morre *et al*. (1998); Hazra *et al*. (2000); Caramori *et al*. (2001); Ali *et al*. (2004). A number of regeneration protocols have been developed for cotton using various explants and through manipulation of media composition. However, response was genotype specific and may also produce somaclonal variants where a callus phase is involved. Although efficiency of cotton regeneration has been significantly improved but some difficulties still remain like regeneration in cotton is limited to a few cotton cultivars Trolinder *et al*. (1989). In order to use different techniques of biotechnology, broad range of genotypes along with broad range of explants sources must be responsive to the regeneration. The objective of this study was therefore to optimize the regeneration protocol via direct (Shoot tip and cotyledonary node) and indirect (hypocotyls and leaves) methods on a modified MS basal medium.

For proper utilization of elite germplasm in varietal improvement programs, it must be studied at molecular level. The knowledge of genetic diversity will facilitate aid cotton-breeding strategies and utilization of promising germplasm lines. To have a reliable estimate of genetic diversity and genetic relationships, molecular markers are efficient in assessing polymorphism. DNA markers have proved to be an efficient tool for molecular characterization of plant species Joshi *et al*. (2000). PCR based molecular markers viz. RAPD, ISSR, SSR, STS and AFLP etc. are useful in various applications of plant breeding. RAPD (Random amplified polymorphic DNA) utilize random sequence of 9-14 base oligonucleotide as a primer for PCR amplification of genomic DNA from different species. RAPD method is dominant in nature and hence does not distinguish heterozygous from homozygous.

Reproducible RAPD markers have been used to study genetic variability and evolutionary basis of the wild species of the genus *Gossipium* (Dongare *et al*). It also proved to to be an efficient molecular technique for genetic fingerprinting of the cotton cultivars Multani *et al*. (1995). This technique has also been used for differentiating cultivar identity Singh *et al*. (1998). Taking the advantage of evolutionary background of cotton and the available molecular tools here, an attempt have made to reveal the genetic variability of 7 accessions.

MATERIALS AND METHODS

Experimental material consisted of two intra *hirsutum* hybrids AC-738 (Male parent of cotton variety NHH-44), BN-1, (Female parent of cotton variety NHH-44) and five varieties of *G. arboreum*, PA-183, PA-255, PA-402, PA-405, PA-08. Out of these materials BN-1, PA-255 and PA-08, were used for regeneration on media. The seeds of these varieties were obtained from Cotton Research Station, Maheboob Baug farm (Marathwada Agriculture University).

The fuzzy cotton seeds were first delinted by commercial sulphuric acid. Acid delinted healthy seeds were washed properly and then treated with 0.1% $HgCl_2$ solution for surface sterilization. Out of these 3-4 seeds were inoculated in flasks containing MS basal media (half strength) with meso-inositol 50 mg/l, thiamine 5 mg/l, glucose 15 g/l, and agar 6.5 g/l and transferred to culture room with 16:8 hour photoperiod and 25 ± 2°C temperatures.

In the first set of experiment *in-vitro* germinated shoot tip explants of 0.5 to 1 cm height and cotyledonary node were aseptically isolated and cultured in 10 different 250ml media combination consisting MS basal formulation with vitamins, thiamine and meso-inositol (Table 1). In each flask 5-7 explants were inoculated. All the flasks containing explants were grown in culture room for the period of one month followed by weekly subculture. In second set of experiment the hypocotyls and leaf explants were inoculated in MS medium and sub cultured periodically Murashige, and Skoog, (1962). Medium for regeneration consisted MS basal formulation with meso-inositol 100 mg/L, thiamine 10 mg/L, glucose 30 g/L, and agar 6.5 g/L containing different growth regulator combinations (Table 2). The pH of the media was adjusted to 6.0 before autoclaving. After shooting, individual shoots of 6-7 cm length were separated from explants on media then transferred to MS medium containing meso-inositol 100 mg/L, thiamine 10 mg/L, glucose 30 g/L, NAA, Charcoal 3 gm/l and agar 6.5 g/L. pH 6.0 before autoclaving for rooting (Table.4).

Genomic DNA isolation

Leaves of freshly germinated cotton cultivar seeds were used for genomic DNA extraction by modified CTAB method Saghai-Maroof *et al*. (1984) and Bhat *et al*. (1999). The quantity of DNA was quantified and diluted to a concentration 25 ng/µL by using sterile distilled water.

RAPD analysis

Fifty 10 mer randomly designed primers (Bio Serve Biotechnologies, India Pvt. Ltd. Hyderabad) were screened out of them 10 (OPA-20, OPA-13, OPA-09, OPX-14, OPD-02, OPB-16, OPG-12, OPC-04, OPH-01, OPH-20) were selected for RAPD analysis of genomic DNA of 7 different cultivars. PCR was carried out in 25µL reaction volume containing 10X PCR buffer B 1X, dNTP mix (10 µM) 200 µm, $MgCl_2$ (25 mM) 1.5 µM, Taq polymerase (5U/µL) 1.5U, Primer 20 pm, Template DNA 25 ng, made final volume of 25 µL by adding sterile distilled water. Amplification were carried out in a thermo-cycler (Eppendorf, Germany) programmed for 35 cycles with an initial denaturation at 94°C for 5 min followed by cycling conditions of denaturation at 94°C for 1 min, annealing at 45 seconds at 37°C and extension at 72°C for 1 min. After 45 cycles, there was a final extension step of 10 min at 72°C. The amplicons were analyzed on 1.5% agarose gels and detected by staining with ethidium bromide. UV trans- illuminated gels were photographed with gel documentation system (Alphaimager TM 2200).

Data scoring and analysis

The amplified products of RAPD were scored for presence (1), absence (0), missing and doubtful cases were scored as 9. Band size was determined by comparison with 1 kb DNA ladder (MBT, Fermentas, U.K.) as standard. Data analysis was performed using NTSYS-PC (Numerical Taxonomy System, Version 2.02 Rohlf *et al*. (1993). The SIMQUAL program was used to calculate the Jaccard's coefficient. Dendrogram was constructed using unweighted pair group method for arithmetic mean (UPGMA) based on Jaccard's coefficient. The polymorphic percentage of the obtained bands were calculated by using following formula,

Polymorphic % = (No. of polymorphic bands /total bands) × 100.

RESULTS AND DISCUSSION

During seed germination, in most of the seed lots radical came out within 3-4 days which grown downward penetrating the medium and by 11-17 day of culture sufficient seedlings height was achieved.

Direct shoot organogenesis

Eleven to seventeen days old seedlings used for shoot tip culture. From the media combinations listed in Table 1, it was observed that the treatment T_3 (full MS + Kin (0.1mg/L), T_2 (full MS without growth regulator) and T_1 (half MS without growth regulator) directly regenerated into multiple shoots. Nandeshwar *et al*. (2002) also reported multiple shoots induction from shoots apices of *G. hirsutum* cultivars viz. Bikaneri Nerma, Khandwa-2, on half strength MS medium without growth regulator. Full MS media with kinetin showed highest response to shoot formation (Figure 1). These results were in agreement with Rauf *et al*. (2005); Agrawal *et al*. (1997). As we go on increasing higher concentration of the two cytokinins (BAP + Kin), it shown no response for shoot tip regeneration. Therefore, low concentration of cytokinin/ cytokinin was found to be more effective than high concentration for shoot induction which was in agreement with Satyavathi *et al*. (2002). Cultivar PA-255 responded more to shoot formation than BN-1 and PA-08 (Table 1). The shoot growth was direct and there was no callus formation observed from explants in any of the given media combination.

In second set of experiment cotyledonary node were used for inoculation into the same MS basal formulation as in first experiment. After 30 days of culture, explants shown sprouting of multiple buds from cotyledonary nodal portion. The multiple shoot bud mass was observed to be more in T_7 (full MS + BAP (2.0 mg/L) + Kinetin 1mg/L) as compared to other growth hormone combinations (Table 1). There was little response to multiple shoot induction in T_6 (MS + BAP (1.5) + Kinetin (1.0 mg/L). When kinetin 1mg/L was used alone number of shoots per plant was less while keeping the level of kinetin constant (1mg/L) in combination with BAP (0.5mg/L) has imparted favorable effect on shoot organogenic response. Agrawal *et al*. (1997), cultured cotyledonary nodes of 35-day-old cotton (cv. Anjali-LRK 516) seedlings and they demonstrated multiple shoots on MS medium plus BA and KIN. In another study, Gupta *et al*. (1997), obtained the best regeneration response from 5-10-day-old cotyledonary nodes of 10 different genotypes. Thus, it has marked the necessity of BAP along with kinetin in the medium for cotyledonary node regeneration. The shoots were separated and transferred into the root induction medium.

Indirect regeneration

The MS basal medium supplemented with different hormone concentration (Table 2) was used for callusing of hypocotyl. Callus initiation was shown by almost all treatments supplemented with 2,4-D + kinetin in variable concentrations but with the application of KNO_3 it shown maximum callusing. Thus, treatment MS 2f (MS_{2e} + KNO_3 1.9g/L) showed maximum callusing (Table 2) (Figure-3). It indicates that proliferation of the callus was higher with application of KNO_3 because nitrogen has direct effect on rate of cell growth, differentiation and totipotency Kirby *et al*. (1987) and high amount of total nitrogen shows inhibitory effect which was observed when NH_4NO_3 was added to the medium. This is in agreement with the results of Ikram-ul-Haq *et al*. (2004). Similar results were obtained when leaves were used as explants with same treatments (Figure 2). Maximum callusing was reported by treatment MS 2f (2,4-D 0.1 mg/L + kinetin-0.5 mg/L + KNO_3 (1.9g/L) (Table 2). Similar results reported by Tripathy *et al*. (2002); Kumar *et al*. (1998) described protocol for in *vitro* regeneration of MCU 10 (*G. hirsutum*) important cotton variety of southern India via somatic embryogenesis.

For shooting of calli produced by leaves and hypocotyls treatment MS + 0.25 mg/L kinetin reported 100% shooting and was chosen (Figure 4). This was supported by the findings of Rauf *et al*. (2005), (Table 3) and for rooting NAA 0.5+ Kinetin 0.1mg/l was taken (Table 4). Rooting was induced in R_4 treatment (Table 4) consisting of MS basal composition supplemented with 0.5mg/L NAA and 0.10 mg/L Kinetin and charcoal 3gm/l. Rauf *et al*. (2005), Gupta *et al*. (1997) also observed rooting by culturing isolated shoots on MS basal salts supplemented with NAA in cotton (*Gossypium hirsutum* L. cv. Khandwa-2). Cultivar PA-255 and PA-08 gave better

Table 1: Effect of different growth regulator combinations on shoot tip culture and cotyledonary node

Treatments (medium mg/l)	Varieties	Shoot tip culture				Cotyledonary node			
		No. of explant	No. of plants regenerated	Plants reached to maturity	Maturity (%)	No. of explant	No. of plants responded	Plants reached to maturity	Maturity (%)
T_1- Half MS + no growth regulator	1. BN-1	14	5	2	14.28	10	0	0	0.00
	2. PA-08		2	1	7.14		0	0	0.00
	3. PA-255		6	5	35.71		0	0	0.00
T_2- Full MS + no growth regulator	1. BN-1	14	3	2	14.28	10	0	0	0.00
	2. PA-08		1	1	7.14		0	0	0.00
	3. PA-255		4	4	28.57		0	0	0.00
T_3- Full MS + kinetin (0.1mg/l)	1. BN-1	14	6	5	35.71	10	0	0	0.00
	2. PA-08		5	4	28.57		0	0	0.00
	3. PA-255		9	6	42.85		0	0	0.00
T_4- Full MS + BAP (1.5mg/l)	1. BN-1	14	0	0	0.00	10	0	0	0.00
	2. PA-08		0	0	0.00		1	1	10.00
	3. PA-255		0	0	0.00		0	0	0.00
T_5- Full MS +BAP (2.0mg/l) + kinetin (0.1mg/l)	1. BN-1	14	0	0	0.00	10	1	0	0.00
	2. PA-08		0	0	0.00		1	0	0.00
	3. PA-255		0	0	0.00		1	0	0.00
T_6- Full MS+ BAP (1.5mg/l) + Kinetin (1.0 mg/l)	1. BN-1	14	0	0	0.00	10	2	1	10.00
	2. PA-08		0	0	0.00		1	1	10.00
	3. PA-255		0	0	0.00		3	1	10.00
T_7- Full MS + BAP (2.0mg/l) + kinetin (1.0 mg/l)	1. BN-1	14	0	0	0.00	10	5	3	30.00
	2. PA-08		0	0	0.00		6	3	30.00
	3. PA-255		0	0	0.00		8	5	50.00
T_8- Full MS + BAP (2.5mg/l) + kinetin (1.0mg/l)	1. BN-1	14	0	0	0.00	10	0	0	0.00
	2. PA-08		0	0	0.00		0	0	0.00
	3. PA-255		0	0	0.00		0	0	0.00
T_9- Full MS +BAP (2.0 mg/l)+ kinetin (1.0mg/l)+ NAA(1mg/l)	1. BN-1	14	0	0	0.00	10	0	0	0.00
	2. PA-08		0	0	0.00		0	0	0.00
	3. PA-255		0	0	0.00		0	0	0.00
T_{10}- Full MS + BAP (3mg/l) + kinetin (1.5mg/l)	1. BN-1	14	0	0	0.00	10	0	0	0.00
	2. PA-08		0	0	0.00		0	0	0.00
	3. PA-255		0	0	0.00		0	0	0.00

Table 2: Callus Induction response of leaves and hypocotyl as explants with different combinations of PGRs

Treatments (medium mg/l)	Varieties	Leaves as explants				Hypocotyl as explants			
		No. of explants	No. of calli induced/ explants	Callusing (%)	Average weight of callus (mg)	No. of explants	No. of calli induced/ explant	Callusing (%)	Average weight of callus (mg)
MS 2a: 2,4D-0.1 + kinetin-0.1	1. BN-1	28	4	14.78	15	28	5	17.85	15
	2. PA-08		2	7.14	4		4	14.28	4
	3. PA-255		2	7.14	4		4	14.28	4
MS 2b: 2,4D-0.1 + kinetin-0.2	1. BN-1	28	5	17.85	12	28	5	17.85	12
	2. PA-08		3	10.71	10		3	10.71	10
	3. PA-255		4	14.28	11		4	14.28	11
MS 2c: 2,4D-0.1 + kinetin-0.3	1. BN-1	28	6	21.42	12	28	6	21.42	12
	2. PA-08		5	17.85	8		5	17.85	8
	3. PA-255		8	28.57	10		2	7.14	10
MS 2d: 2,4 D-0.1 + kinetin-0.4	1. BN-1	28	4	14.28	12	28	4	14.28	12
	2. PA-08		5	17.85	14		5	17.85	14
	3. PA-255		7	25	12		7	25	12
MS 2e: 2 ,4 D-0.1 + kinetin-0.5	1. BN-1	28	10	35.71	20	28	8	28.57	20
	2. PA-08		20	71.42	35		7	17.85	35
	3. PA-255		16	57.14	40		7	17.85	40
MS 2f: MS 2e + KNO_3(1.9g/l)	1. BN-1	28	11	39.28	20	28	10	35.71	20
	2. PA-08		15	53.57	34		12	42.85	34
	3. PA-255		17	60.71	55		18	64.28	55
MS 2g: MS 2e + NH_4NO_3 (1.9g/l)	1. BN-1	28	6	21.42	12	28	6	21.42	12
	2. PA-08		9	32.14	14		8	28.57	14
	3. PA-255		12	42.84	18		11	39.28	18

Figure 1: Multiple shoot induction on MS media

Figure 2: Callus from leaves PA-08

Figure 3: Callus from BN-1 hypocotyl

Figure 4: Regeneration of plants from callus

Table 3: Effect of cytokinin on shoot induction after 30 days of culture

TreatmentsMS + kinetin	Varieties	No of shoots evaluated	Response to shooting	Shooting %
MS media(control)	BN-1	4	0	0
	PA-08		0	0
	PA-255		1	25
MS + 0.10mg/l	BN-1	4	4	100
	PA-08		4	100
	PA-255		4	100
MS + 0.25mg/l	BN-1	4	4	100
	PA-08		4	100
	PA-255		4	100
MS + 0.50mg/l	BN-1	4	3	75
	PA-08		3	75
	PA-255		3	75
MS + 1.00mg/l	BN-1	4	1	25
	PA-08		1	25
	PA-255		2	50

Table 4: Effect of different Media on rooting

Treatments	Medium	Growth regulators (mg/l) NAA	Kinetin	Varieties	Number of shoot evaluated for rooting	Number of shoots rooted	Rooting (%)
R_1	MSB	0.00	0.00	BN-1	4	0	0.00
				PA-08	6	0	0.00
				PA-255	8	0	0.00
R_2	MSB	0.5	0.00	BN-1	4	0	0.00
				PA-08	4	0	0.00
				PA-255	4	0	0.00
R_3	MSB	0.0	0.10	BN-1	5	2	40.00
				PA-08	4	2	50.00
				PA-255	2	1	50.00
R_4	MSB	0.5	0.10	BN-1	5	3	60.00
				PA-08	6	4	66.66
				PA-255	7	5	71.42

Table 5: Similarity matrix for Jaccard coefficient of 7 genotypes obtained from RAPD marker analysis

	PA-405	PA-255	PA-402	PA-183	AC-738	BN-1	PA-08
PA-405	1						
PA-255	0.414	1					
PA-402	0.309	0.594	1				
PA-183	0.255	0.431	0.333	1			
AC-738	0.266	0.27	0.318	0.409	1		
BN-1	0.285	0.456	0.454	0.416	0.222	1	
PA-08	0.372	0.451	0.365	0.315	0.351	0.362	1

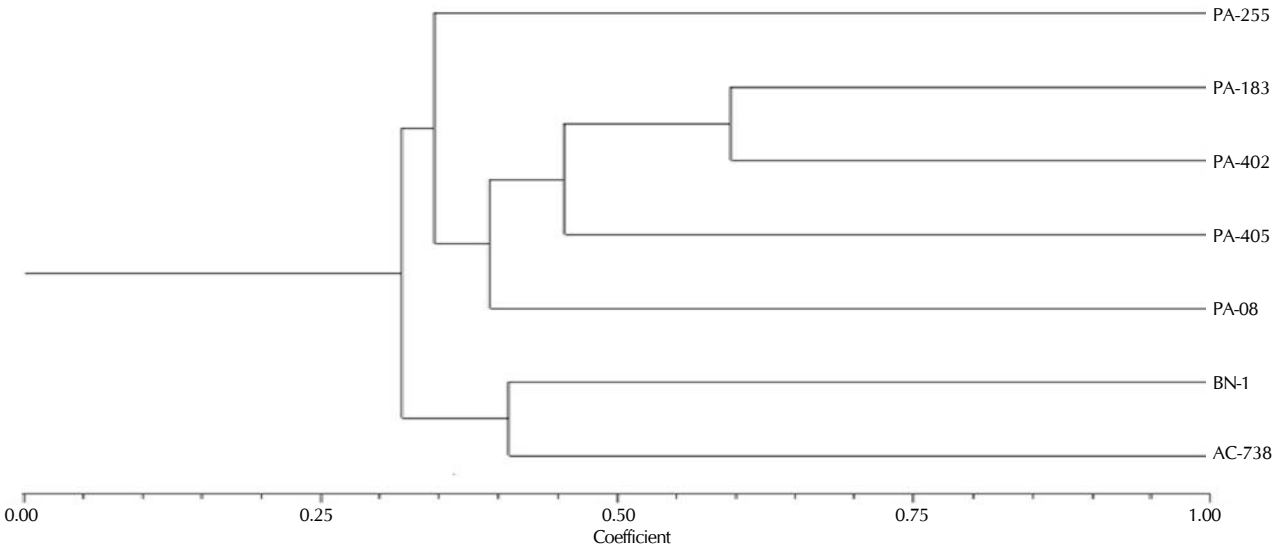
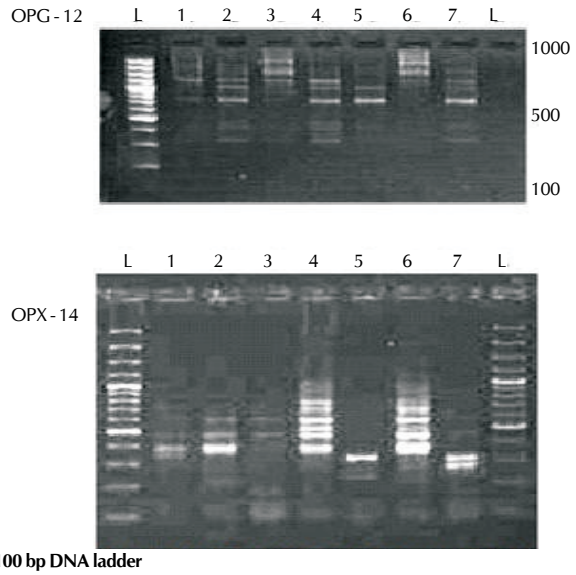
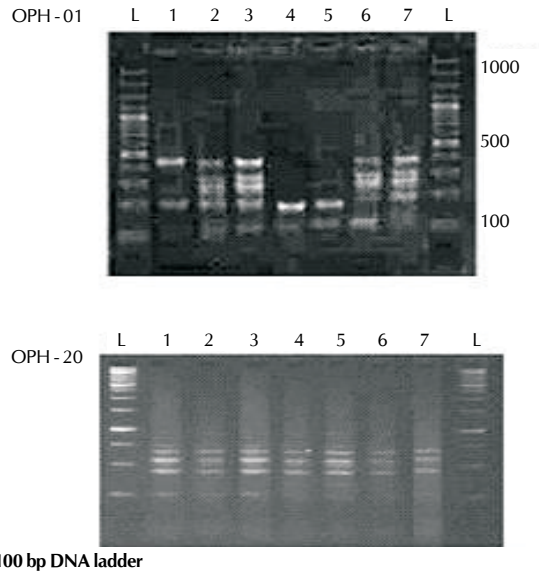


Figure 5: Dendogram generated by UPGMA analysis based on RAPD data showing relationship among 7 cotton genotype



L-100 bp DNA ladder
1. BN-1, 2. PA-402, 3. PA-405, 4. PA-255, 5. AC-738, 6. PA-08, 7. PA-183

Figure 6: RAPD profie of cotton accessions generated by primer OPG-12 and OPX-14



L-100 bp DNA ladder
1. BN-1, 2. PA-402, 3. PA-405, 4. PA-255, 5. AC-738, 6. PA-08, 7. PA-183

Figure 7: RAPD profie of cotton accessions generated by primer OPH-01 and OPX-20

response to rooting. Rooted shoots were taken out, washed with tap water planted in pots having sand. Half strength MS media was applied to moisten sand and covered with polythene bags. Pots were placed in green house for a week. After 3-4 days holes were made in polythene bag to gradually expose external environment. After 10 days plants were transferred to larger pots containing 50% sand and 50% soil.

RAPD analysis

Ten RAPD primers generated 67 amplicons out them 4 bands were monomorphic and 63 were polymorphic among 7 genotypes with average 94.02 per cent polymorphism. Total no of band produced per primers is OPA -20 (8), OPA-13(5), OPA-09 (7), OPC-04 (7), OPD-02 (9), OPB-16 (6), OPG-12 (9), OPX-14 (6), OPH- 01 (6) all these nine primers produced 100% polymorphism and primer OPH 20 (4) shown monomorphic bands. Average 6.3 per cent polymorphic bands per primer were detected. The size of the amplified products ranged from 100 bp (OPA-13) to ~ 2000 bp (OPA - 09). Distinct RAPD profiles were generated by 10 primers (Fig. 6-7) differentiating all 7 varieties.

Dice coefficient study

Genetic relationship between Cotton varieties were determined on the basis of dice's pair wise similarity coefficient values. The value of similarity coefficient ranged from 0 to 1. These genotypes shown Average similarity value as PA-255 (O.436), PA-183 (0.413), PA-402 (0.413), PA-405 (0.316), PA-08 (0.387), BN-1 (0.389), and AC-738 (0.389). Genotype PA-405 represented lowest average similarity coefficient value (0.316) and PA-255 represented highest average similarity coefficient value (0.436). The average genetic similarity coefficient value was 0.392.

Cluster analysis

A dendrogram (Fig. 5) was generated by UPGMA cluster analysis based on dice similarity coefficients (Table 5) Cluster analysis revealed that accessions could be divided into two super clusters 'A' and 'B'. Super cluster 'A' accommodated five genotypes containing *Gossypium arboreum* and super cluster 'B' containing two varieties of *Gossypium hirsutum*. In super cluster 'A' variety PA-183 and PA-402 shown similarity at coefficient 0.60 having similar characteristics of yellow flower with dark yellow anther colour. Genotype PA-405 shown similarity with PA-183 and PA-402 at similarity coefficient 0.47 may be due to the reason that PA-405 is made from CJ-73 × PA-183. Variety PA-08 shown similarity with PA-183, PA- 402 and PA-405 in cluster at coefficient 0.40 may be due to sharing same features of having five medium to deeply serrated leaf lobe, dwarf, sympodial, errect and also have a special feature of big boll size and naked seed. Variety derived from interspecific cross of *G. arboreum* X *G. hirsutum* PA-255 shown similarity at 0.35 to rest of all varieties in cluster A, sharing common features of having sympodial, erect growing, five medium to deeply serrated leaf lobes, yellow flower and anther colour, Bolls medium *round* and tapering at the end. Cluster B having varieties of *G. hirsutum* i.e. BN-1 (female parent of NHH-44) and AC-738 (male parent of NHH-44) shown similarity at coefficient 0.42 having similar feature of number of monopodia 1-2, resistance to sucking pest, days to flowering (48-52) and suitability to rainfed and irrigated areas.

High polymorphic result indicated a wide genetic base in Cotton varieties and this genetic diversity may be due to wide geographical distributions, genotypes of landraces, amplification protocol used (or) selection of suitable primers. The cluster analysis can help to confirm origin information, define relationships that may be assumed through origin data, and assist in defining diverse gene pools for selecting parents in cultivar improvement programs (Zenglu *et al.,* 2001). In present study cluster analysis grouped 7 varieties into two main clusters, indicating that ecological regions or geographical regions play important role in cluster formation (Zenglu *et al.,*

2001). Two main clusters were divided into 5 sub clusters on basis of phenotypic characters like maturity period, anther colour, and parents as distinct characters. It clearly indicates that geographical origin and phenotypic characters play important role in cluster formation and genetic relationships.

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