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In vitro regeneration and PCR-RAPD based detection of somaclonal variation in kenaf (*Hibiscus cannabinus*)

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Abstract

Though direct systems of regeneration through culture of organized meristems usually produce true-to-type plants, variations in the progenies have widely been reported. Fiber producing kenaf plants (*Hibiscus cannabinus* L.) were regenerated from petiole, hypocotyls and cotyledonous petiole explants on MS medium containing BAP (benzyl amino purine) and NAA (α-napthaleneacetic acid) followed by assessment of regenerants by RAPD markers to detect somaclonal variation among them. Genomic DNA from twenty seven plants [three mother plants and two clones (clone 1 and 2) from each mother plant with three replications] was subjected to random amplified polymorphic DNA (RAPD) analysis. Fifteen polymorphic loci amplified by three decamer random primers were used to estimate genetic diversity and relatedness in mother plants and their regenerated plantlets. The results showed some degree of polymorphism between mother plants and their regenerated plantlets as well as between regenerated plantlets indicating somaclonal variation among the regenerants. These suggest that the RAPD technique could effectively be used to detect somaclonal variation in *H. cannabinus* and could be promising for the detection of markers associated with desirable traits.

Key words: *Hibiscus cannabinus*, *in vitro* regeneration, RAPD, somaclonal variation, enetic diversity, polymorphism

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Introduction

Kenaf (*Hibiscus cannabinus* L., *Malvaceae*) is a worldwide adapted potential fiber and biomass producing crop mostly used as a jute substitute. Next to cotton and jute, kenaf is an important natural fiber. The traditional uses of kenaf have mainly focused on it as a source of fiber for making ropes, sacks, canvas, and carpets (Dempsey 1975; Li 1980). More applications of kenaf have been reported, for example, pulping and papermaking, oil absorption and bio-remediation, board and filtration media making, and animal feed (AKS 2000; JKA 2000). Kenaf is grown for nutritional, medicinal, and industrial purposes (Cisse *et al.,* 2009).

One of the major constrains to increase the kenaf productivity is the non-availability of modern varieties, as well as infectitous diseases caused by fungi, bacteria, virus, nematode and many other environmental factors. Genetic transformation is one possible way to introduce resistant genes against insects and fungal diseases into kenaf cultivars. The pre-requisite for the genetic transformation in kenaf is to establish an efficient plant regeneration system from explants to matured fertile plants.

Genetic variation in regenerated plants results from pre-existing genetic variation in the cells of mother explants and genetic variation induced by *in vitro* culture, particularly in the callus phase (Maharana *et al*., 2012). Regenerants often display altered phenotypes, termed as somaclonal variation (Larkin and Scowcroft, 1981). The exploitation of heritable somaclonal variants has been used in various plant improvement strategies (Phillips *et al.,* 1994) but success has been sporadic. Somaclonal variation is genetic and phenotypic variations among clonally propagated plants of a single donor clone (Olhoft & Philips, 1999; Kaeppler *et al.,* 2000). The mechanism of somaclonal variation involves extensive genomic flux e.g., repeated DNA sequences can rapidly be amplified or reduced in copy number, altered methylation patterns can be inherited, DNA replication is disturbed by altered nucleotide pools, and active genes can be silenced or silenced gene may be activated by mutations in associated non-coding regions (Scowcroft *et al.,* 1985). Epigenetic factors are also involved making it more complex (Kaeppler *et al.,* 2000). Somaclonal variation has been related to growth regulators, cultivar variability, cultivars age in culture, ploidy level, explant source and other culture conditions (Skirvin *et al.,* 1994). Larkin & Scowcroft (1981) suggested that somaclonal variation is a useful source of novel variation for plant improvement.

Methods for detection of somaclonal variation have been explored for many years (Noval, 1980). To detect somaclonal variation at DNA level, different molecular markers have been used. Among them, RAPD markers are technically simple, quick to perform with small amount of DNA and do not require radioactive labeling (Michelmore *et al.,* 1991). RAPD primers have been used for the germplasm characterization of kenaf (Cheng *et al*., 2000), jute (Hossain et al., 2002, 2003), roselle (Hanboonsong *et al*., 2000) and many other plants and animals (Bala *et al*., 2017; Saclain *et al*., 2016; Mollah *et al*., 2009; Rahman *et al*., 2007). In the present study, we report the induction of somaclonal variation caused by hormone treatments in kenaf callus and its detection using PCR-RAPD.

Materials and Methods

Sample collection*:* Seeds of three varieties of kenaf viz., HC-2, HC-3 and HC-95 were collected from Bangladesh Jute Research Institute (BJRI). Among them, HC-2 (1977) and HC-95 (1995) are high yielding varieties and resistant to nematode (Khatun, 2009). *In vitro* grown plants from seeds were used as the source of plant material.

Hormone treatment and plantlet regeneration from callus*:* Approximately 8-10 mm^2 segments of hypocotyls, petiole and cotyledonous petiole from *in vitro* grown plants on MS (Murashige and Skoog, 1962) medium were excised and placed on callus production medium (MS medium containing NAA, BAP). Two treatments were applied to produce somaclones. Treatment 1 refers to the use of NAA and BAP at a concentration of 2.00 and 0.50 mgl^{-1}, respectively whereas treatment 2 comprised of the use of NAA and BAP at a concentration of 1.00 and 1.00 mgl^{-1} respectively (Table 1). Somaclones produced by treatment-1 were considered as clone-1, whereas somaclones produced by treatment-2 were considered as clone-2. All cultures were kept in a culture room at a 16h photoperiod (2000-3000 lux illumination) and 25°C controlled temperature. After 3-4 weeks, calli were initiated. Three segments of calli (approximately 8-10 mm^2 each) were sub-cultured on the same medium at an interval of 2 weeks. After 6-7 weeks, shoots and roots were initiated from the calli. Finally, a total of twenty seven individuals [three mother plants and two clones (clone 1 and 2) from each mother plant with three replications] were subjected to RAPD analysis to detect somaclonal variation.

Genomic DNA extraction*:* Genomic DNA was isolated from leave tissues of twenty seven individuals using phonol, chloroform and isoamylalcohol extraction and ethanol precipitation method (Yasmin *et al*., 2006; Saclain *et al*., 2016). In brief, approximately

50 mg of fresh, vigorous, young actively growing leaf tissues were taken into a microcentrifuge tube, cut into small pieces, homogenized and digested with RNAse in extraction buffer (50 mM Tris-HCl, 25 mM EDTA, 300 mM NaCl, pH=8.0 and 1% SDS). DNA was purified by successive extraction with 600 µl of phenol: chloroform: isoamylalcohol (V: V: V = 25: 24: 1). DNA was precipitated first using about 0.1 volume of sodium acetate with 2.5 volumes of absolute ethanol and peletted by centrifugation. DNA was reprecipitated by adding two volumes of 70% ethanol and peletted by centrifugation. The pellets were then air-dried and resuspended in an appropriate volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH= 8.0). DNA quality was checked by electrophoresis and quantified using a spectrophotometer (Spectronic^{R} Genesis^{TM}).

Primer selection*:* Initially, twenty five decamer primers (Merck, India) of random sequence were screened on a sub sample of three randomly chosen individuals from each variety, to test their suitability for amplifying kenaf RAPDs that could be accurately scored. Primers were evaluated on the basis of intensity or resolution of bands repeatability of markers and consistency within individual and potential to different cultivars (polymorphism). Finally, three primers (Table. 2) were selected for the analysis of the whole sample set of the three varieties.

PCR amplification and agarose gel electrophoresis*:* PCR amplification was done following the procedure (Williams *et al.,* 1990) with some modificatoins. PCR reactions were performed on each DNA sample in a 10µl reaction mix containing: 1µl of 10x Ampli *Taq* polymerase buffer, 1µl of 2.5 mM dNTPs, 0.6 µl of 50 mM $MgCl_2$, 1 unit of Ampli *Taq* DNA polymerase (Merck, India), 50 ng template DNA, 2 µl of 10 µM primer and a suitable amount of sterile deionized water. DNA was amplified in a thermal cycler (Eppendorf Mastercycler Gradient). The reaction mix was preheated at 94ºC for 3 min followed by 40 cycles of 1 min denaturation at 94ºC, 1 min annealing at 36ºC band elongation at 72ºC for 2 min. After the last cycle, a final extension of 7 min at 72ºC was added to allow complete extension of all amplified fragments. The amplified product from each sample was separated electrophoretically on 1.4% agarose gel in a 1XTBE buffer at 120V for 1½ h. Two molecular weight markers (1 kb and 20 bp DNA markers) were run alongside the RAPD reactions. The gels were stained with ethidium bromide and bands were observed and photographed by a gel documentation system (Bio-Rad).

RAPD data analysis*:* Fragments were scored as 1 if present and 0 if absent. The scores were then pooled for constructing a single data matrix. This was used for comparing the frequencies of all polymorphic RAPD markers and estimating Nei's (1973) gene diversity (h), gene flow (N_m), coefficient of gene differentiation (G_{ST}), genetic distance (D) and constructing a UPGMA (Unweighted Pair Group Method of Arithmetic Means) dendrogram among populations using POPGENE (version 1.31) (Yeh *et al.,* 1999) computer program. The similarity index values (SI) between the RAPD profiles of any two individuals were calculated from RAPD markers according to the formula: Similarity index (SI) = $2\ N_{xy}/N_x+N_y$, Where, N_{xy} is the number of RAPD bands shared by individuals X and Y respectively, and N_x and N_y are the number of bands in individual x and y, respectively (Lynch, 1990).

Results and Discussion

The process of callus formation and organogenesis differed with the variety (Table. 1). Out of three varieties, HC-95 produced the highest percentage of callus (95.00%), shoots per explant (2.333) and roots per explant (16.00) on MS medium supplemented with NAA (1.00 mgl^{-1}) and BAP (1.00 mgl^{-1}) compared to other varieties. Likewise, McLean *et al.,* (1992) studied *in vitro* kenaf plants regeneration using intermodal stem explants and found that the most abundant callus production was occurred at 1.0 mgl^{-1} NAA and mgl^{-1} BAP combinations. However, the HC-95 variety produced the lowest percentage of callus (76.67%), lower number of shoot per explants (1.67) and root per

explant (10.67) on MS medium supplemented with NAA (2.00 mgl^{-1}) and BAP (0.50 mgl^{-1}) compared to other varieties. The results show that different combinations of kenaf variety and concentrations of plant growth regulators have a great influence in response of organogenesis. The variation in performance was further determined through genetic analysis.

Three RAPD primers amplified different loci or fragments resulting in different banding patterns and the number of fragments amplified per primer varied (Table 2). Primer S1063 yielded the highest number of bands whereas the OPP13 and OPAB10 produced the least number of bands. The banding patterns of three kenaf cultivars amplified by the three primers are shown in Figure 1. The three primers yielded a total of 22 distinct bands of which 15 (68.18%) were considered polymorphic.

Table 1. Organogenesis from different explants of three kenaf varieties

Variety	Hormone concentration (mgl^{-1})		% callus regeneration	Days for shoot regeneration	Number of shoots per explant	Days for root regeneration	Number of roots per explant
	NAA	BAP					
HC-2	2.00	0.50	90.00	29.22	1.333	32.00	15.33
	1.00	1.00	93.33	24.00	1.667	26.00	14.33
HC-3	2.00	0.50	90.00	30.00	1.333	30.33	12.33
	1.00	1.00	83.33	21.33	1.333	26.67	13.33
HC-95	2.00	0.50	76.67	20.00	1.667	30.33	10.67
	1.00	1.00	95.00	15.67	2.333	25.67	16.00

Table 2. RAPD primers with corresponding total and polymorphic bands observed in three kenaf cultivars

Primers codes	Sequence of primers (5´ to 3´)	Number of bands	Number of polymorphic bands	Polymorphic loci (%)
S1063	GGTCCTACCA	10	7	70.00
OPP13	TTCCCTCCCA	6	3	50.00
OPAB10	GGAGTGCCTC	6	5	83.33
Overall	-	22	15	68.18

(((Figure 1)))))

The result of the RAPD analysis demonstrates that the RAPD method is capable of revealing nuclear DNA variation in kenaf cultivars. Cheng *et al.,* (2002) studied genetic diversity of 23 accessions of kenaf from 14 countries and found 76.9 % polymorphism. The differences in polymorphism in different studies might be due to differences in experimental conditions including use of different kenaf varieties, primers and geographical differentiation.

The percentage of polymorphic loci was found to be the highest (36.36%) in the HC-3 clone 2 population followed by other cloned populations whereas no polymorphic loci was found for mother plants (Table. 3). The Nei's (1973) gene diversity (h) and Shannon's information index (i) were also higher in the HC-3 clone 2 population compared with others (Table. 3). Estimates of the proportion of polymorphic loci, Nei's gene diversity (h), Shannon's Information index (i) indicate that relatively higher level of genetic variation exists in the HC-3 clone 2, HC-3 clone 1 and HC-2 clone 2 populations. This also reveals that treatment-2 induces more variation in the clones. With the decrease of NAA concentration and increase of BAP

concentration there is an increased trend of the percentage of polymorphic loci and gene diversity in HC-3 clone 2 population. The overall Nei's gene diversity and gene flow values were 0.206 and 0.179 reflecting that there is a some degree of genetic variation between mother plants and clones.

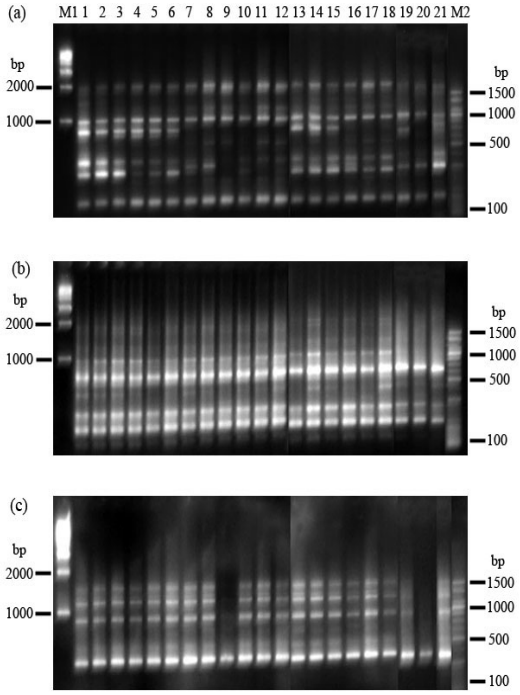

Figure 1. RAPD profiles of three different kenaf clones and their mother plants using primers S-1063 (a), OPP-13 (b) and OPAB-10 (c). Lane 1-3: HC-2 clone 1, lane 4-6: HC-2 clone 2, lane 6-9: HC-3 clone 1, lane 10-12: HC-3 clone 2, lane 13-15: HC-95 clone 1 and lane 16-18: HC-95 clone 2. Lane 19: HC-2 mother plant, lane 20: HC-3 mother plant, lane 21: HC-95 mother plant. M1 and M2: 1 kb and 20 bp molecular markers, respectively.

Frequency of polymorphic loci is shown in Table 4. One marker (OPAB10-2) was found at a frequency of 0.423 in the HC-2 clone 2 population while absent in other populations. Another marker OPP13-3 was observed in the HC-2 clone 2 and HC-3 clone 2 populations at a frequency of 0.184 and 0.423, respectively. Moreover, marker S1063-8 was observed in the HC-3 clone 1 and HC-3 clone 2 populations at a frequency of 0.184 and 0.423, respectively while absent in other populations (Table 4). The highest level of genetic similarity (93.73%) was found between HC-2 clone 1 and HC-2 clone 2 and the least similarity (56.11) was found between HC-3 mother plant and clone 2 (Table 5).

The higher genetic distance (0.490) was found between HC-3 mother plant and HC-95 clone 2 whereas the lower genetic distance (0.046) was found between HC-3 clone 2 and HC-95 clone 1 (Table. 6). The result indicated that kenaf mother plants and their clones are genetically different from each other.

The values of pair-wise comparisons of Nei's unbiased genetic distance (D) between populations, computed from combined data for the three primers, ranged from 0.046 to 0.490 . The dendrogram grouped the populations into two main clusters. The HC-3 mother plant was the most genetically distinct population, which was segregated from the HC-95 Clone 2 population with the D value of 0.490.

Table 3. Population wise genetic variation: polymorphic loci, Nei's gene diversity and Shannon's Information index

Population	Number of polymorphic loci	Percentage of polymorphic loci	Nei's gene diversity (h)	Shanon's Information index (i)
HC-2 Mother plant	0	0.000	0.000	0.000
HC-2 clone 1	1	4.550	0.014	0.022
HC-2 clone 2	5	22.730	0.085	0.127
HC-3 Mother plant	0	0.000	0.000	0.000
HC-3 clone 1	7	31.820	0.121	0.180
HC-3 clone 2	8	36.360	0.152	0.220
HC-95 Mother plant	0	0.000	0.000	0.000
HC-95 clone 1	4	18.180	0.080	0.115
HC-95 clone 2	2	9.090	0.036	0.053

Table 4. Frequency of polymorphic loci in different populations of kenaf

Gene frequency	HC-2 MP	HC-2 C1	HC-2 C2	HC-3 MP	HC-3 C1	HC-3 C2	HC-95 MP	HC-95 C1	HC-95 C2
S1063-2	0.000	0.000	0.184	0.000	0.184	0.184	0.000	0.423	1.000
S1063-4	0.000	1.000	1.000	0.000	0.423	0.184	1.000	0.000	0.000
S1063-5	1.000	0.184	0.423	0.000	0.423	1.000	0.000	0.000	0.000
S1063-6	1.000	0.000	0.000	0.000	0.184	0.184	0.000	0.423	1.000
S1063-7	1.000	1.000	1.000	0.000	1.000	0.423	1.000	0.423	1.000
S1063-8	0.000	0.000	0.000	0.000	0.184	0.423	0.000	0.000	0.000
S1063-9	1.000	1.000	1.000	1.000	0.423	0.423	1.000	0.184	1.000
OPP13-1	1.000	1.000	1.000	0.000	1.000	1.000	0.000	1.000	1.000
OPP13-3	0.000	0.000	0.184	0.000	0.000	0.423	0.000	0.000	0.184
OPP13-4	1.000	1.000	0.184	1.000	0.184	0.000	1.000	0.000	0.423
OPAB10-1	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000
OPAB10-2	0.000	0.000	0.423	0.000	0.000	0.000	0.000	0.000	0.000
OPAB10-3	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000
OPAB10-4	1.000	1.000	1.000	0.000	1.000	1.000	1.000	1.000	1.000
OPAB10-5	1.000	1.000	1.000	0.000	1.000	0.423	0.000	1.000	1.000

Table 5. Between population similarity indices in kenaf

Population pair	S1063	OPP13	OPAB10	Average
HC-2 Mother plant and clone 1	76.92	100	100	92.31
HC-2 Mother plant and clone 2	85.71	88.89	100	91.53
HC-2 clone 1and HC-2 clone 2	92.31	88.89	100	93.73
HC-3 Mother plant and clone 1	72.72	75.00	33.33	60.35
HC-3 Mother plant and clone 2	60.00	75.00	33.33	56.11
HC-3 clone 1 and HC-3 clone 2	46.15	100	100	82.05
HC-95 Mother plant and clone 1	54.55	75.00	88.89	72.81
HC-95 Mother plant and clone 2	76.92	88.89	88.89	84.90
HC-95clone 1 and HC-95 clone 2	83.33	88.89	100	90.74
Overall	72.07	86.73	82.72	80.50

Table 6. Summary of Nei's (1972) genetic distance values between 12 samples

Variety	HC-2 mother plant	HC-2 clone 1	HC-2 clone 2	HC-3 mother plant	HC-3 clone 1	HC-3 clone 2	HC-95 mother plant	HC-95 clone 1
HC-2 mother plant								
HC-2 clone 1	0.130							
HC-2 clone 2	0.166	0.047						
HC-3 mother plant	0.452	0.388	0.484					
HC-3 clone 1	0.113	0.074	0.049	0.446				
HC-3 clone 2	0.221	0.165	0.127	0.357	0.064			
HC-95 mother plant	0.258	0.098	0.158	0.258	0.190	0.212		
HC-95 clone 1	0.181	0.176	0.141	0.416	0.048	0.046	0.292	
HC-95 clone 2	0.116	0.173	0.161	0.490	0.111	0.148	0.285	0.095

The HC-3 Clone 2 and HC-95 Clone 1 seperated from each other with the lowest genetic distance (D=0.046) (Figure. 2). The results indicated that treatment 2 induces more somaclonal variation among HC-3 cultivars. It is clear that the variation was cultivar dependent. Similar result was reported by Khatun *et al.* (2003) while working with kenaf genotypes for organogenesis. Somaclonal variation in regenerated plants of garlic has been found in our previous study (Parvin *et al*., 2008).

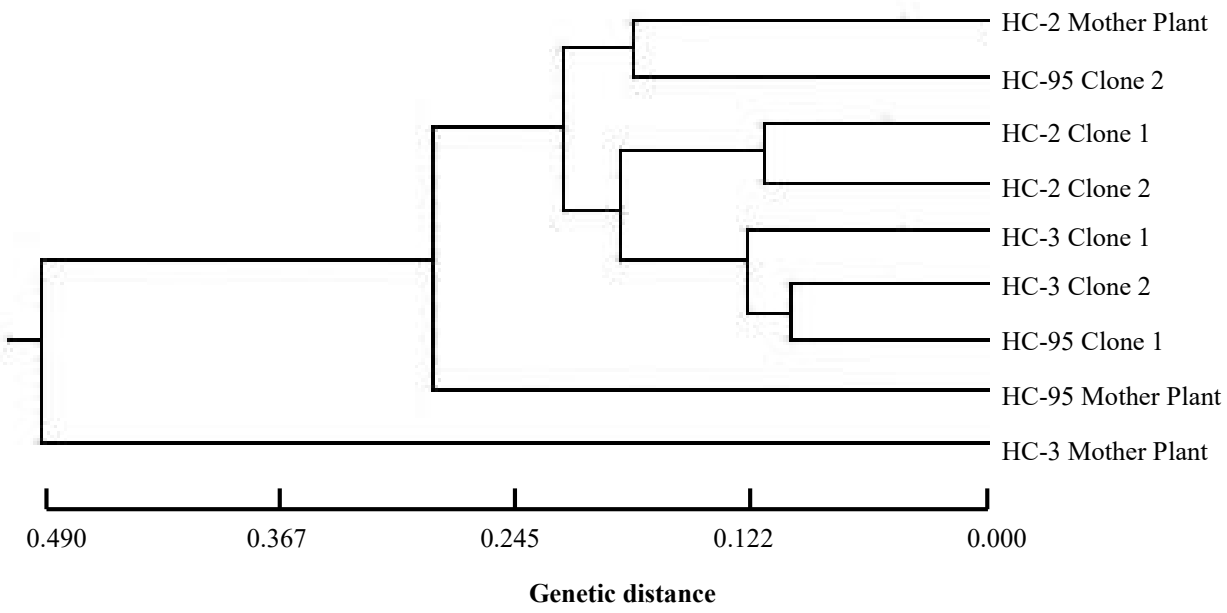

Figure 2. UPGMA dendogram based on Nei's genetic distance, summarizing the data on differentiation between 9 kenaf plants, according to RAPD analysis.

Conclusion

No information on *in vitro* induced genetic variation of the kenaf cultivars is available in Bangladesh. RAPD variations were detected among mother plant and somaclones, suggesting that tissue culture is an important way to create genetic variation in kenaf varieties. We have also developed a method based on RAPD–PCR as a molecular marker for detection of somaclonal variation caused by hormone treatment of kenaf callus and it might be helpful to identify clones, which are superior.

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