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Detection of somaclonal variation in micropropagated *Hibiscus sabdariffa* L. using RAPD markers

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Abstract

The main objective of micropropagation is to produce clones i.e. plants which are phenotypically and genetically identical to the mother plants. The culture of

organized meristems usually guarantees the production of true-to-type plants but variations in the progenies have been widely reported. *Hibiscus sabdariffa* L. plants were regenerated on MS (Murashige and Skoog) medium containing BAP (Benzyl amino purine) and IBA (Indole 3 butyric acid) and were propagated *in vitro* on hormone-free MS medium. The aim of this study was to detect variation in micropropagated plantlets of *Hibiscus sabdariffa* using RAPD amplification. DNA extraction from *Hibiscus sabdariffa* L. plants was optimized using CTAB buffer supplemented with 5M NaCl to eliminate polysaccharides and the isolated DNA proved amenable to PCR amplification. RAPD analysis was carried out on DNA samples to compare the mother plant with 10 randomly selected regenerated plants. Out of 30 primers screened, primers OPB-01, OPX-06 and DK-02 produced polymorphic bands. These results show that RAPD is a suitable technique which can be used to detect genetic change caused by somaclonal variation and could be promising for the selection of desirable traits or transformation systems.

Keywords: *Hibiscus sabdariffa* L. *In vitro* culture. RAPD, Somaclonal Variation

**For correspondences and reprints*

1. **INTRODUCTION**

Micropropagation also known as tissue culture or *in vitro* culture involves the multiplication of plants through the culture of plant parts on sterilized media containing nutrients, growth promoters and plant growth regulators. The use of tissue culture techniques offers several advantages such as production of disease-free plants, propagation of seedless plants and rapid multiplication rates. The objective of micropropagation is to produce 'clones' i.e. exact copies of the mother plants. The common methods *in-vitro* propagation involving organized meristems are considered as the "safest" methods in terms of maintaining genetic stability (Rani and Raina, 2000). However, genetic and phenotypic variation among clonally propagated plants from a single donor plant can sometimes be observed (Kaeppler *et al*., 2000) and is termed 'somaclonal variation' (Larkin and Scowcroft, 1981). Somaclonal variation has been reported in numerous studies, such as cabbage (Leroy *et al.* 2000), tomato (Soniya *et al*., 2001), rice (Abeyaratne *et al*., 2004), kiwifruit (Palombi and Damiano, 2002) and pineapple (Santos *et al*., 2008). Somaclonal variation is normally associated with such systems as protoplast culture, regeneration through the formation of adventitious meristems arising after a phase of disorganized callus or cell suspension growth. The extent of somaclonal variation depends on several factors namely genotype, ploidy level, source and explant age, species, length of culture and the presence of plant hormones such as synthetic auxins at high concentrations (Jain, 1997). Detection of variants based on phenotypic observations may lead to erroneous conclusions hence validating the need for detection at the genome level (Ahmad *et al*., 2004; Dhanaraj *et al*., 2002).

Various methods can be used for the detection of somaclonal variation and these include numerical and structural chromosomal changes (Obute and Aziagba, 2007), Restriction Fragment Length Polymorphism (RFLPs) of nuclear and organellar genomes (Rani and Raina, 2000), random oligonucleotide fingerprinting patterns (Inter Simple Sequence Repeats (ISSR) (Martins et al., 2004), and Random Amplified Polymorphic DNA (RAPD) (Saker *et al*., 2000; Santos *et al*., 2008). RAPD marker technology has been considered as a simple molecular tool (Saker *et al*., 2000), dominantly inherited (Elmeer *et al*., 2009) and as elaborated by Williams *et al.*, 1990, it is a technique that requires only a few nanograms of DNA to obtain polymorphism. RAPD is not only a commonly used molecular technique among plants but also other organisms. Martin et al. (1993) and Godwin et al. (2001) reported that RAPD has several advantages over other polymorphic DNA detecting techniques such as RFLP, in terms of quickness, small amount of template DNA required and no need of DNA sequence information. RAPD technique has been appraised for its suitability, and applied in germplasm characterization studies such as genotyping of Taro (Godwin *et al*., 2001), potato (Mc Gregor *et al*., 2000), Brassica (Geraci *et al*., 2001) and cassava collections (Zacaria *et al*., 2004). RAPD has also been used as an effective molecular tool in the detection of somaclonal variation in date palm plants (Saker *et al*., 2000), micropropagated propagules of ornamental pineapple (Santos *et al*., 2008) and in cucumber plants derived from somatic embryos (Elmeer *et al*., 2009).

Hibiscus sabdariffa L. (Malvaceae), an annual shrub, commonly known as 'Roselle' grows in the tropical and subtropical regions. Throughout the world, the fleshy calyces are used for wine making, juice, jam, jelly, syrup, gelatin, pudding, ice cream, flavours(Vaidya, 2000). The crop is also used in the folk medicine of many countries including India (Yadong *et al.*, 2005). With the drop in sugar prices in Mauritius, Roselle is now regarded as a new crop with promising potential for intensive cropping systems owing to its multifunctional attributes. *Hibiscus sabdariffa* L. is conventionally propagated by seeds but owing to their limited viability and storage time, occurrence of seed-borne pathogens and the heterozygous nature of some seeds, (Vasil and Thorpe, 1994), vegetative methods of propagation are proposed. Tissue culture represents a means of producing true-to type plantlets; eliminating pathogens (through meristem culture) and can be considered as an effective tool for mass propagation towards the commercial production of Roselle. However, this objective can be achieved, provided the genetic integrity of micropropagated plants is guaranteed, hence the need for the early detection of variation.

2. METHODOLOGY

2.1 Plant Material

In-vitro Roselle plants were regenerated on 0.1-2.0 mg/L BAP and kinetin and rooted on 1.5-2.5 mg/L IBA (Govinden Soulange *et al.*, 2009) and the sterile plantlets were maintained by monthly subculture of sterile single nodes of *Hibiscus sadbariffa* L. on MS (1962) medium basal medium with 40g/ L sucrose. The pH of medium was adjusted to 5.7 with 1M NaOH. Jellifying agent (6g/ L Oxoid Number 3 Agar) was dissolved in microwave and 20 ml of medium was placed in jars and sterilized by autoclaving at 121ºC and 105kPa for 20 minutes. The cultures were maintained at 25 ºC and a 16/8 h photoperiod with a light intensity of 25 $\mu molm^{-2}sec^{-1}$.10-15 plantlets obtained by single node maintained on MS for a period of 5 months were used for analysis of genetic stability.

2.2 DNA extraction & RAPD

Total DNA was extracted from 10-15 plantlets grown *in-vitro* by using a modified CTAB method (Govinden-Soulange *et al.*, 2007) to increase yield and purity of DNA. 0.5g fresh leaf tissue was ground in a spot plate with 5ml hot 60 °C CTAB buffer. 0.2% mercaptoethanol and 2% PVP was added. The grindate was transferred to a 15 ml corning tube. The leaf tissue was suspended evenly in buffer and placed in a 60°C water bath for 25-30 minutes with occasional swirling (approximately every 10 minutes). After incubation, the corning tube was removed from the water bath and 2/3 volume of chloroform:isoamyl alcohol (24:1) was added. The tubes were closed and inverted several times. The tubes were spin in a microcentrifuge at 10,000 rpm for 10 minutes. The aqueous layer was removed with a wide-bore pipette and placed in clean 15 ml tube. 200µl of 5M NaCl to eliminate polysaccharide contamination and to allow a higher amount of DNA recovery was added followed by 2/3 volume of ice-cold isopropanol. The tubes were then left overnight in -20 ºC freezer to allow further precipitation of DNA.

The tube was spin for 30 minutes in the microcentrifuge at maximum rpm. The supernatant was poured off. The pellet was washed twice using 95% ethanol. It was then air dried for 10-15 minutes. The pellet was re-suspended in 100 µl sterile distilled water.

RNA elimination was carried out by incubating the tube at 37 ºC for 30 minutes to dissolve the DNA followed by addition of RNase. The DNA was stored at -20 ºC until use. 8µl of DNA stained with 2µl bromophenol blue dye was used to check for purity on 1.5 % agarose gel in TBE buffer and visualized by ethidium bromide staining under UV light. Purified total DNA was quantified and its quality verified by spectrophotometry using a UV-VIS Spectronic Genesys 5 (Milton Roy) spectrophotometer at 260 nm.

RAPD amplification was carried out in a total volume of 25 µl containing 2.5 µl PCR buffer, 2.0 mM $MgCl_2$ (Bioline), 200 µM of each of dATP, dCTP, dGTP, dTTP (Bioline), 20 pmol primer (Oligonucleotides primers (5 µM), available commercially from Operon Technologies), 1 U Bioline *Taq* DNA polymerase and 100 ng DNA. PCR was performed in a thermal cycler (Biorad thermal cycler) with the following cycling conditions: 2 minute at 94°C, 1 minute at 35°C and 1 minute at 72°C, for 40 cycles; followed by a further extension at 72°C for 10 minutes. Amplicons were separated on 1.5% agarose gel in Tris Base buffer and visualized by ethidium bromide staining under UV light; their sizes were estimated using hyper ladderII (Fermentas).

2.3 Data Analysis

Only consistent, reproducible, well-resolved fragments, in the size range of 300 to 1000 bp were scored as present or absent for RAPD markers in each *in vitro* regenerated plantlet and weak bands were excluded. Using this approach, the possibility of losing more than one useful information was not left out but the goal was to obtain reproducible and clear data. Furthermore, data analysis was conducted only on products that were reproducible over two amplifications.

3. RESULTS

3.1 Micropropagation of Hibiscus sabdariffa L.

Shoot growth and root initiation were visible within 1-2 weeks following transfer of single nodes on MS (1962) basal medium. Normal growth with vigorous stem and extensive rooting were observed after 6 weeks in healthy plants which demonstrated signs of adaptation (Fig. 1a). However in 75% of cases, regenerated plantlets showed symptoms of yellowing and a lack of chlorophyll development ranging from partial to complete chlorosis after a period of 4 weeks (Fig. 1b). Chlorosis was identified by a pale colouration of interveinal leaf tissue from yellowish green to pale yellow. The network of veins remained green. Chlorotic plantlets had stunted growth, dwarf leaves with angular brown spots which eventually curled and dropped prematurely.

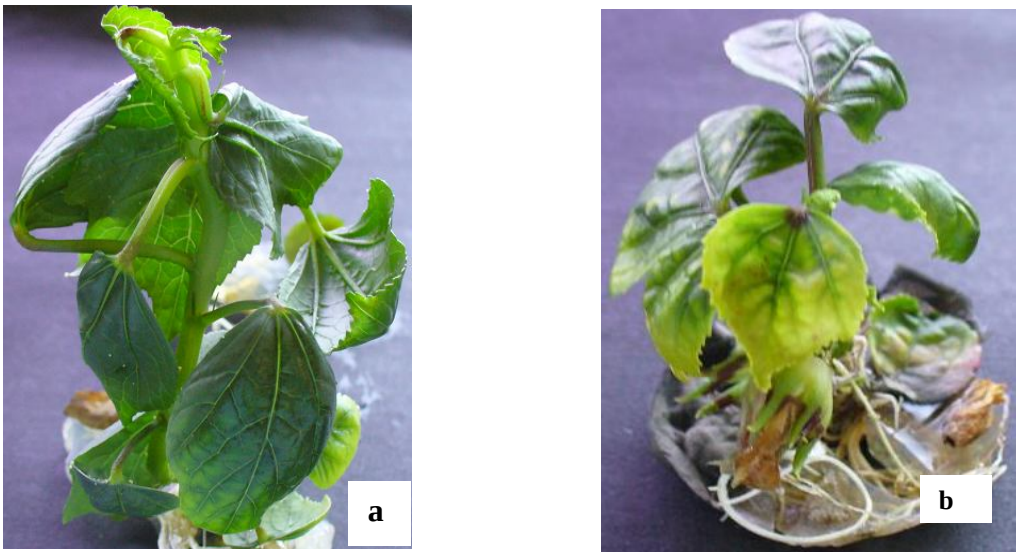

Figure 1. Micropropagation of *Hibiscus sabdariffa* L. (a) Extensive rooting and vigorous stem showing adaptation, 6 weeks after subculture (on MS medium). (b) 6-week old Chlorotic plantlets.

3.2 DNA extraction and RAPDs

Initially the DNA contained large amounts of RNA, polysaccharides, and proteins. Phenol and chloroform were used to denature and precipitate the proteins from the sample (Zidani *et al.*, 2005). 5M NaCl was used to remove polysaccharides. Genomic DNA of *Hibiscus sabdariffa* L. *in-vitro* regenerated plants were analysed based on RAPD markers using arbitrarily chosen oligonucleotide primers. Out of 30 primers screened, polymorphism was obtained using OPB-01, OPX-06, DK-02. DNA fragments ranging in the size of 300 to 1000 bp were observed. The representative profiles of the 10 *in-vitro* raised plants are illustrated in Figure 2. Bands for each primer varied from 4 to 5 with an average of 4.5 bands per RAPD primer (Table 1).

Primer OPB-01 produced DNA fragments of 600 bp and 1000 bp common to all 10 micropropagated plants. However, a 350 bp DNA fragment was revealed in samples 4 and 8. Furthermore, one specific fragment of 300 bp from sample 7 was observed. As for primer OPX-06, DNA fragments of 900 and 550 bp were amplified in all clones. A DNA fragment of 500 bp was amplified in samples 4 and 8 was detected. Moreover, a fragment of 450 bp was noted in sample 7. Primer DK-02 gave rise to DNA fragments of 600 to 1000 bp similar in all samples. DNA segments of 700 and 1000 bp were similar in almost all clones. However, polymorphic non parental bands were observed. Samples 5, 7, 9 and 10 produced a fragment of 700 bp. Furthermore, small variations of 650 bp in clones 7 and 9 and a fragment of 600 bp in sample 8 were observed. It was noted that changes expressed in samples 4, 7 and 8 were detected similarly in all three primers. Primer

DK -02 yielded the most polymorphism where small genetic changes could be observed in the other samples.

Primer No.	Nucleotide sequence (5' - 3')	Number of scorable bands	Size range (bp)
OPB-01	GTTTCGCTCC	4	300 to 1000
OPX-06	ACGCCAGAGG	4	500 to 900
DK-02	CGACCGCAGT	5	600 to 1000

Table 1. List of primers that produced polymorphic bands, their sequence and size of the amplified fragments generated by RAPD

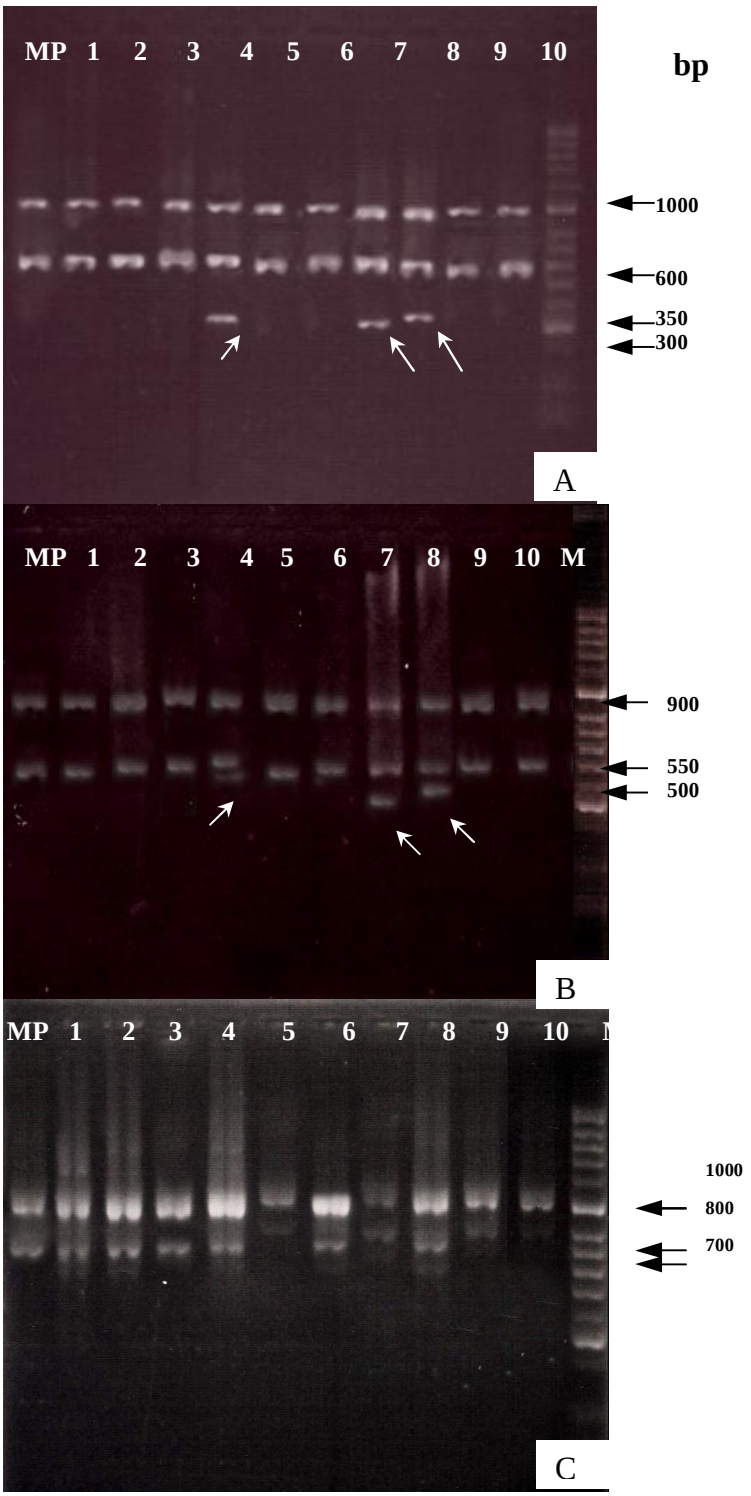

Figure 2. A-C RAPD profiles generated by primer OPB-01 (A), OPX-06 (B) and DK-02. RAPD bands of motherplant are indicated by M in lane 1. Lanes 2 to 11 are RAPD profiles of clones. Band size of fragments as compared with markers is indicated. White arrows show variations.

4. DISCUSSION

In this study, although, single node explants were used for the micropropagation of *Hibiscus sabdariffa*, symptoms of chlorosis have been observed in regenerated plantlets. These phenotypic variations seem be due to a mineral imbalance affecting chlorophyll formation (Tsipouridis *et al.*, 2005) and have been reported in *in vitro* propagated *Hibiscus rosa-sinensis* (Christensen *et al.*, 2008) and in the endemic plant *Scrophularia takesimensis* Nakai (Sivanesan *et al.*, 2008). As the main objective of micropropagation is to produce clones, the occurrence of any type of variation in regenerated plantlets needs to be closely investigated as it could be heritable.

Several strategies have been adopted to detect somaclonal variation *in vitro*. These include phenotypic observation in tulips (Podwyszyńska, 2005); karyological analysis in banana (Obute *et al.*, 2007); isozyme markers in sugar beet somaclones (Levall *et al.*, 1994); polymerase chain reaction (PCR) with short primers of arbitrary sequence; random amplified polymorphic DNA in tomato (Soniya *et al.*, 2001), single sequence repeats in kiwi (Palombi and Damiano, 2002); AFLP markers in asparagus (Pontaroli *et al.*, 2005). It has been reported that RAPD molecular markers are rapid, easy and less expensive than AFLPs and RFLPs (Palombi and Damiano, 2002). In this work, RAPD markers have been used complementarily along with traditional methods of assessing somaclonal variation in micropropagated *H. Sabdariffa.*

RAPD markers as a means of molecular analysis of *in-vitro* regenerated plants have been very well documented (Al-Zahim *et al.*, 1999; Dhanaraj *et al*., 2002; Isabel *et al.*, 1995; Martins *et al.*, 2004; Palombi and Damiano, 2002;). RAPDs have been efficient in the detection of somaclonal variation in tomato (Soniya *et al*., 2001); white spruce (Isabel *et al*., 1995), garlic (Al-Zahim *et al.*, 1999) and pineapple (Santos *et al.*, 2008). The use of RAPDS to detect somaclonal variation in micropropagated plantlets has been widely reported and clones have been assessed using RAPD profiles and in most cases no genomic alterations have been revealed (Gaafar and Saker, 2006; Gómez-Leyva ,2008; Lattoo *et al*., 2006; Martins *et al.*, 2004). However, in this study, probable genomic alterations due to somaclonal variation have been observed. Polymorphism obtained with RAPD primers could be explained by *in-vitro* culture time, genotype or explant source or three-way interactions between initial explants, the culture conditions and the genotype of mother plants (Rani and Raina, 2000;Vencatachalam et al., 2007). The results obtained from this piece of investigation are promising and suggest that RAPD markers can be utilized as a simple molecular tool to assess the genetic integrity of plants derived *in-vitro* on a commercial scale or integrated in a crop improvement program.

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