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Evaluation of genetic fidelity of in vitro-propagated *Aloe vera* plants using DNA-based markers

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ABSTRACT: *Aloe vera* is a world-recognized medicinal herb cultivated for its high value in medicine and the cosmetics industry. Natural multiplication of this plant through suckers is inadequate to meet the demand of high quality planting material for commercial cultivation. Earlier we developed an efficient micropropagation protocol using shoot tip explants of field grown seedlings. Field trials of the micropropagated plants reveal no morphological or growth abnormalities. For commercial utility it is therefore imperative to establish genetic uniformity of micropropagated plants to confirm the true-to-type plantlets. Hence the present investigation assesses the clonal fidelity of the in vitro *A. vera* plants using polymerase chain reaction-based techniques such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR). In this study, we assessed the suitability of 20 RAPD and 12 ISSR primers to observe any polymorphism. Out of these, 12 RAPD and 8 ISSR primers produced resolvable, reproducible and scorable bands. The 12 RAPD primers produced 75 distinct and scorable bands with an average of 6.25 bands, and 8 ISSR primers produced 31 distinct and scorable bands with an average of 3.8 bands per primer. RAPD and ISSR profiles obtained through the amplification of genomic DNA of the in vitro grown *A. vera* plants were similar in all aspects. No RAPD and ISSR polymorphism in the micropropagated plants were detected. This implies that micropropagation through shoot tip explants is a safe method for producing true-to-type plants and could be used for commercial plantation of *A. vera*.

KEYWORDS: micropropagation, molecular marker, PCR

INTRODUCTION

Aloe (*A. vera*) is a recognized medicinal plant that belongs to the family Liliaceae. The plant is mostly used in pharmaceutical and cosmetic industry as well as for fresh consumption as a general health promoting herb. There are more than 40 available aloe-based formulations of medicine and cosmetics in the global market[1]. Due to the wide spectrum of application in human health, the products of *A. vera* have shown a strong demand in both local and international markets, which has prompted industrial and commercial increase in the production of *A. vera* throughout the world. *A. vera* naturally propagated through axillary shoot bud which is very slow, expensive, and low income practice. In addition, high degree of male sterility and low germination rate prevents its propagation through seeds[2]. Industries require constant amount of phytochemicals in raw aloe leaves for their products, which require genetically uniform plant population. Plant tissue culture technique would be a sustainable solution in expanding *A. vera* cultivation through the production of uniform seedlings of desirable genotype. Several attempts have been taken to develop micropropagation of *Aloe* spp[3–10]. However, quality of the seedlings for possible genetic changes was rarely tested.

Micropropagation is the practice of rapidly multiplying stock plant material to produce numerous progeny plants; however, scaling up of any micropropagation protocol is frequently hindered due to incidences of somaclonal variations[11]. Somaclonal variations are induced due to the stress imposed on the plant during propagation and is incorporated in the form of DNA methylation, chromosome rearrangements and point mutations[12]. Both genetic and epigenetic variations are related to the appearance of variation in micropropagation. The first report of somaclonal variants of plants in vitro is sugarcane in 1971[13]. After that, a number of somaclonal variants have occurred in vitro in plants that have been assessed by morphological or molecular markers; for instance sugarcane[14],

kiwifruit[15], and banana (*Musa* spp.)[16]. Owing to this variation, the resulting plant may not possess the same properties as that of the parent plant. It is therefore imperative to establish genetic uniformity of micropropagated plants to confirm the true-to-type plantlets for its commercial utility.

The somaclonal variation present in micropropagated plants can be detected through morphological, physiological, biochemical, or molecular techniques. Of these, molecular techniques are superior to morphological or biochemical techniques. Polymerase chain reaction (PCR)-based techniques such as random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) are immensely useful in establishing the genetic stability of in vitro-regenerated plantlets in many crop species[17–20]. RAPD and ISSR markers are very simple, fast, cost-effective, highly discriminative and reliable. These do not require radioactive probes as in restriction fragment length polymorphism. Thus they are suitable for the assessment of the genetic fidelity of in vitro-raised clones.

Although numerous researchers have worked with micropropagation of *A. vera*, however, few reports are available to assess the clonal fidelity of the clones raised in vitro[21–23]. Many fragments corresponding to multiple loci dispersed throughout the genome are normally amplified, using each single RAPD and ISSR primer. Although most commercially produced primers result in several fragments, some primers may fail to give amplification fragments from some material. It is then necessary to check the suitability of primers for a successful micropropagation project. In our study we tested some new RAPD and ISSR primers not used before for the genome of *A. vera* plants. As previously reported that shoot tip is most widely used explant for micropropagation of *A. vera*, it is necessary to confirm clonal fidelity of micropropagated *Aloe* plants arisen from shoot tip explant. In our lab we have established an efficient in vitro micropropagation protocol of *A. vera* through shoot tip explant. The present investigation was aimed to assess the clonal fidelity of the in vitro raised *A. vera* plants using RAPD and ISSR markers.

MATERIALS AND METHODS

Plant material and in vitro culture conditions

Healthy and diseased free *A. vera* seedlings were obtained from the germplasm collections of the National Institute of Biotechnology. After surface sterilization, shoot tips were used as primary explants. Murashige and Skoog (MS) medium fortified with 3.0 mg/l of BAP and 0.5 mg/l of NAA was used for primary culture establishment. The in vitro raised shoots were multiplied on the same medium and subculture was done after every 5–6 weeks. The in vitro regenerated elongated shoots (3–4 cm) were excised aseptically and implanted on rooting medium for root induction. The rooted plantlets were hardened on garden soil and finally planted in field.

DNA isolation and quantification

To observe possible genetic variation in the micropropagated progeny, 12 in vitro regenerated hardened plants were chosen randomly from the population and compared with the mother plant from which the explants were taken. Total genomic DNA of the mother as well as in vitro clones was extracted from young leaf tissue by using the modified cetyltrimethyl ammonium bromide (CTAB) method. The tissues were homogenized in CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 0.2% (v/v) 2-mercaptoethanol) and incubated at 65 °C for 1 h. Further extraction with carried out with phenol:chloroform:isoamyl alcohol (25:24:1) for twice and followed by chloroform:isoamyl alcohol (24:1) for once. DNA was precipitated by the addition of isopropanol to the solution followed by centrifugation. The resulting pellet was washed with 70% ethanol, allowed to dry and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration of DNA was determined by NanoDrop spectrophotometer (Thermo Scientific) and quality of genomic DNA was checked following electrophoresis on 0.8% agarose gel (data not shown). The DNA samples were then stored at −20 °C until further use. RAPD and ISSR assay were performed using 12 RAPD and 12 ISSR primers (Integrated DNA Technologies, USA) for DNA profiling.

Genetic fidelity analysis by RAPD and ISSR markers

RAPD and ISSR amplification were performed in a volume of 25 µl containing 2 µl of genomic DNA (100 ng), 2 µl of RAPD or ISSR primer (10 pmol/µl) (Integrated DNA Technologies, USA), 12.5 µl of 2× master mix containing 0.1 U/µl *Taq* DNA polymerase, reaction buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (Thermo Fisher Scientific, USA) and 8.5 µl sterile deionized water. DNA amplification was carried out in a DNA thermal cycler (Gradient thermal cycler, Takara Bio Inc, Japan). The PCR program consisted of an initial denaturation for

Fig. 1 (a) Multiple shoots proliferation from shoot tip explant, (b) development of well-root after 28 days of culture on rooting medium, (c) acclimatized plant in soil.

5 min at 95 °C, then 45 cycles of 30 s denaturation at 94 °C, 30 s annealing at annealing temperature (5 °C below the melting temperature T_m) and 1 min extension at 72 °C with final extension at 72 °C for 5 min. The PCR program for ISSR was performed as initial denaturation for 5 min at 95 °C, then 35 cycles of 30 s denaturation at 94 °C, 1 min annealing at annealing temperature (5 °C below T_m) and 1.5 min extension at 72 °C with final extension at 72 °C for 10 min. The amplified products were analysed by electrophoresis in 2% agarose gels using Tris-Borate-Ethylene diamine tetra acetic acid buffer and stained with RedSafe (iNtRON Biotechnology, Korea). The number of amplified products was recorded using a gel imaging system (FluorChem E, Proteinsimple, USA). The sizes of the amplicons were estimated by comparing with 1.5 kb DNA ladder (GeneON, Germany). At least three independent PCR amplifications were performed for each sample with RAPD and ISSR primers. Only reproducible bands were considered for analysis.

RESULTS AND DISCUSSION

Our micropropagation protocol produced morphologically normal seedlings (Fig. 1a–c). However, true-to-type clonal fidelity is one of the most important pre-requisites in the micropropagation of crop species. The occurrence of cryptic genetic defects in the regenerates can seriously limit the extensive utility of the micropropagation system[24]. Hence detection and analysis of genetic variation can help in understanding the molecular basis of various biological phenomena in plants. A number of molecular markers can be used to assess the clonal fidelity of tissue cultured plants. However, a single marker analysis is unable to completely guaranteed the clonal fidelity of the regenerated plants[25]. The effectiveness of more than one DNA marker analysis for detecting genetic fidelity or variability of micropropagated plants is also reported[26]. Hence we used both RAPD and ISSR marker for detection of genetic diversity of in vitro propagated *A. vera* plants. RAPD and ISSR markers have the advantage of being technically simple, quick to perform, and require only small amounts of DNA[27]. In our study, the large numbers of amplicons amplified from distinct regions of the genome may suffice to detect somaclonal variations.

Under the optimized PCR conditions, all the tested primers produced resolvable, reproducible, and distinctly scorable bands. Representative pictures of PCR amplification obtained with RAPD and ISSR primers from in vitro propagated plants and their mother are shown in Fig. 2a–d. For RAPD analysis, 12 primers were used for amplification.

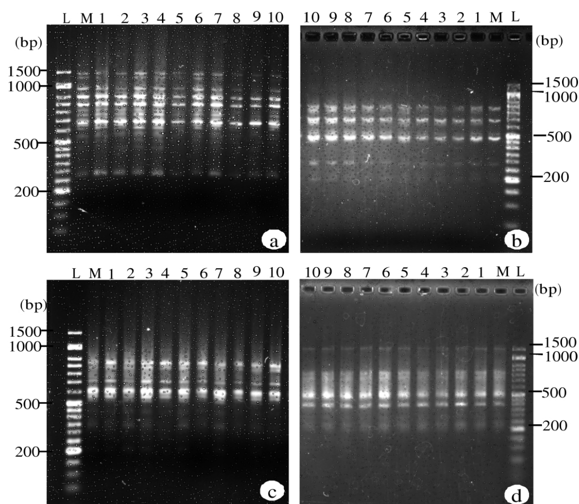

Fig. 2 Representative picture of polymerase chain reaction (PCR) amplification obtained with random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) primers from in vitro propagated plants and their mother. Primers used: (a) RAPD-OPC06, (b) RAPD-OPC02, (c) ISSR-UBC845, and (d) UM18C1. Lane L represents 1.5-kb ladder, lane M represents the mother plant, and lanes 1–10 represent in vitro-raised clones derived from shoot tip explant of *Aloe vera*.

Table 1 List of random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) primers used for assessment of genetic fidelity of in vitro raised *A. vera* with mother plants, their sequences, number, and size of amplified fragments generated.

Primer code	Sequence (5′ − 3′)	Sb/p†	T_m (°C)	Range
RAPD				
OPA-01	CAGGCCCTT	2	36	500–1000
OPA-02	TGCCGAGCTG	8	41	400–2000
OPA-13	CAGCACCCA	6	38	500–900
OPA-16	AGCCAGCGA	4	38	400–1000
OPC-02	GTGAGGCGTC	8	38	350–1500
OPC-6	GAACGCGAC	10	32	300–1500
OPC-06	GGGGGTCTTTT	3	33	300–1000
OPC-12	TGTCATCCCC	7	33	600–1000
OPE-12	TTATCGCCCC	6	34	350–900
OPE-15	ACGCACAAC	8	37	350–900
OPD-07	TTGGCACGGG	7	41	350–1000
OPD-10	TGTCTGGGTG	6	33	250–900
ISSR				
UBC-822	TCTCTCTCTCTCTCTCA	2	45	300–700
UBC-823	TCTCTCTCTCTCTCTCC	2	47	400–700
UBC-835	AGAGAGAGAGAGAGAGYC	4	48	100–1000
UBC-845	CTCTCTCTCTCTCTCTRG	4	48	350–900
UM18-C1	CTCTCTCTCTCTCTCTAC	5	48	300–9000
UM14-T4	CACACACACACAGT	4	37	100–1000
UM14-C6	CACACACACACAGC	4	40	100–700
UM11-C9	CACCACCACGC	6	38	400–1000

† Sb/p = scorable bands per primer.

The number of scorable bands for each RAPD primer varied from 2 (OPA-01) to 10 (OPC-06) (Table 1). The 12 primers produced 75 distinct and scorable bands, with an average of 6.25 bands per primer. Each primer generated a unique set of amplification products ranging in size from 200 bp (OPD-10) to 1500 bp (OPC-06 and OPA-02). Similarly, for ISSR analysis 8 ISSR primers were used for amplification. The number of scorable bands for each ISSR primer varied from 2 (UBC-822) to 6 (UM11-C9) (Table 1). The 8 ISSR primers produced 31 distinct and scorable bands, with an average of 3.8 bands per primer. Each primer generated a unique set of amplification products ranging in size from 100 bp (UBC-835) to 1000 bp (UM11-C9). RAPD and ISSR profile obtained through amplification of genomic DNA of the in vitro grown *A. vera* plants were similar in all aspects. All the 12 RAPD and 8 ISSR primers produced monomorphic bands confirming the genetic homogeneity of the in vitro-raised plants (Fig. 2).

There are a number of studies in the literature which report similar results for the detection of genetic fidelity using RAPD and ISSR markers in different crops such as *Chlorophytum arundinaceum*[17], *Dictyospermum*[18], *Arnebia*[20], banana[27], and *Gerbera*[28]. Our study demonstrates that micropropagation through shoot tip explant is a safe method for producing true-to-type plants at least from the 10th subculture. Hence a combination of plant tissue culture technique followed by clonal fidelity checking would be very useful for establishing a micropropagation system of *A. vera* for commercial plantation.

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REFERENCES

1. Kumar M, Singh S, Singh S (2011) In vitro morphogenesis of a medicinal plant—*Aloe vera* L. *Asian J Plant Sci Res* **1**, 31–40.
2. Food and Agriculture Organization of the United Nations (2007) *Non-Wood News* no. 14, FAO, Rome.
3. Singh BCS, Adugna AD, Rani AR (2017) A method of rapid in vitro proliferation and morphological characterization of the medicinal plant *Aloe vera* L. *Afr J Biotechnol* **16**, 2201–14.
4. Amoo SO, Aremu AO, van Staden J (2012) In vitro plant regeneration, secondary metabolite production and antioxidant activity of micropropagated *Aloe arborescens* Mill. *Plant Cell Tissue Organ Cult* **111**, 345–58.
5. Das A, Mukherjee P, Jha TB (2010) High frequency micropropagation of *Aloe vera* L. Burm. f. as a low cost option towards commercialization. *Plant Tissue Cult Biotechnol* **20**, 29–35.
6. Singh M, Rathore MS, Panwar D, Rathore JS, Dagla HR, Shekhawat NS (2009) Micropropagation of selected genotype of *Aloe vera* L.—an ancient plant for modern industry. *J Sustain Forest* **28**, 935–50.
7. Hashemabadi D, Kaviani B (2008) Rapid micropropagation of *Aloe vera* L. via shoot multilication. *Afr J Biotechnol* **7**, 1899–902.
8. Kumar M, Kumar B, Kumari C, Kumar N (2017) In vitro morphogenesis of *Aloe vera*: the medicinal wonder plant. *J Med Plants Stud* **5**, 130–4.
9. Aggarwal D, Barna KS (2004) Tissue culture propagation of elite plant of *Aloe vera* Linn. *J Plant Biochem Biotechnol* **13**, 77–9.
10. Abrie AL, van Staden J (2001) Micropropagation of the endangered *Aloe polyphylla*. *Plant Growth Regul* **33**, 19–23.
11. Larkin PJ, Scowcroft WR (1981) Somaclonal variation—a novel source of variability from cell cultures for plant improvement. *Theor Appl Genet* **60**, 197–214.
12. Phillips RL, Kaeppler SM, Olhoft P (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. *Proc Natl Acad Sci USA* **91**, 5222–6.

13. Heinze DJ, Mee GWP (1971) Morphologic, cytogenetic and enzymatic variation in *Saccharum* species hybrid clones derived from callus culture. *Am J Bot* **58**, 257–62.
14. Oropeza M, Guevara P, de García E, Ramírez JL (1995) Identification of somaclonal variants of sugarcane (*Saccharum* spp.) resistant to sugarcane mosaic virus via RAPD markers. *Plant Mol Biol Rep* **13**, 182–91.
15. Palombi MA, Damiano C (2002) Comparison between RAPD and SSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev). *Plant Cell Rep* **20**, 1061–6.
16. Sahijram L, Soneji JR, Bollamma K (2003) Analyzing somaclonal variation in micropropagated bananas (*Musa* spp.). *In Vitro Cell Dev Biol Plant* **39**, 551–6.
17. Lattoo SK, Bamotra S,Sapru Dhar R, Khan S, Dhar AK (2006) Rapid plant regeneration and analysis of genetic fidelity of in vitro derived plants of *Chlorophytum arundinaceum* Baker—an endangered medicinal herb. *Plant Cell Rep* **25**, 499–506.
18. Chandrika M, Thoyajaksha, Ravishankar Rai V, Ramachandra Kini K (2008) Assessment of genetic stability of in vitro grown *Dictyospermum ovalifolium*. *Biol Plant* **52**, 735–9.
19. Kumar S, Mangal M, Dhawan AK, Singh N (2011) Assessment of genetic fidelity of micropropagated plants of *Simmondsia chinensis* (Link) Schneider using RAPD and ISSR markers. *Acta Physiol Plant* **33**, 2541–5.
20. Phulwaria M, Rai MK, Shekhawat NS (2013) An improved micropropagation of *Arnebia hispidissima* (Lehm.) DC. and assessment of genetic fidelity of micropropagated plants using DNA-based molecular markers. *Appl Biochem Biotechnol* **170**, 1163–73.
21. Sahoo S, Rout GR (2014) Plant regeneration from leaf explants of *Aloe barbadensis* Mill. and genetic fidelity assessment through DNA markers. *Physiol Mol Biol Plant* **20**, 235–40.
22. Haque SM, Ghosh B (2013) High frequency microcloning of *Aloe vera* and their true-to-type conformity by molecular cytogenetic assessment of two years old field growing regenerated plants. *Bot Stud* **54**, 46.
23. Rathore MS, Chikara J, Mastan SG, Rahman H, Anand KG, Shekhawat NS (2011) Assessment of genetic stability and instability of tissue culture-propagated plantlets of *Aloe vera* L. by RAPD and ISSR Markers. *Appl Biochem Biotechnol* **165**, 1356–65.
24. Salvi ND, George L, Eapen S (2001) Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell Tissue Organ Cult* **66**, 113–9.
25. Mallón R, Rodríguez-Oubiña J, González ML (2011) Shoot regeneration from in vitro-derived leaf and root explants of *Centaurea ultreiae*. *Plant Cell Tissue Organ Cult* **106**, 523–30.
26. Ceasar SA, Maxwell SL, Prasad KB, Karthigan M, Ignacimuthu S (2010) Highly efficient shoot regeneration of *Bacopa monnieri* (L.) using a two-stage culture procedure and assessment of genetic integrity of micropropagated plants by RAPD. *Acta Physiol Plant* **32**, 443–52.
27. Venkatachalam L, Sreedhar RV, Bhagyalakshmi N (2007) Genetic analyses of micropropagated and regenerated plantlets of banana as assessed by RAPD and ISSR markers. *In Vitro Cell Dev Biol Plant* **43**, 267–74.
28. Bhatia R, Singh KP, Sharma TR, Jhang T (2011) Evaluation of the genetic fidelity of in vitro-propagated gerbera (*Gerbera jamesonii* Bolus) using DNA-based markers. *Plant Cell Tissue Organ Cult* **104**, 131–5.