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A Novel Strategy for *in vitro* Conservation of *Aloe vera* L. through Long Term Shoot Culture

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Abstract: Aloe (*Aloe vera* L., Family Liliaceae), with proven multiple medicinal values finds favour readily in *Ayurevdic* applications, is facing a serious threat to its population as well as biodiversity due to its popularly harvested aloe leaves by the local communities and herbal medicine vendors. To succeed in dealing with the specified problem along with the facilitation of the mass production of commercial level, constant supply of quality propagules can be possible through conservation of propagules *in vitro*. The present study is thus concerned with *in vitro* conservation of multiple shoot culture of aloe to achieve unbroken supply of propagules maintaining their genetic purity. Rhizomatous stem explants result multiple bud break in MS plus 0.25 mg L^{-1} of NAA and 1.5 mg L^{-1} of BAP. Separated shoot buds further result in shoot multiplication and proliferation on MS with 2.5 mg L^{-1} of BAP. *In vitro* generated multiple shoots were split into individual shoot and subcultured for further multiplication in a sustainable. Five subcultures were performed at an extended 5 month interval over a period of 25 months in the same medium. Plantlets regenerated after 1st subculture and plantlets from 5th subculture showed no significant difference in the phenotypic response. Genetic integrity of *in vitro* clones was tested using ISSR primers. All monomorphic bands in the ISSR assay, both for primary culture as well long term culture, ascertained their genetic integrity to a great extent.

Key words: Aloe, clonal fidelity, ISSR, micropropagation, multiple shoot, sustained culture

INTRODUCTION

Aloe (*Aloe vera* L.) is a versatile plant of the family Liliaceae having wonderful healing and softening properties. It works immediately and effectively to heal and soothe skin injuries due to minor wounds and burns (Davis *et al*., 1994). It is now being marketed as a remedy for coughs, wounds, stomach and mouth ulcers, gastritis, diabetes, cancer, headaches, arthritis, immune-system deficiencies, and many other conditions (Leon, 2003). Presently it is used as an important ingredient in different cosmetic formularies (Ahmed *et al*., 2007). But, the uncontrolled collection and sale of aloe leaves result in destruction of the plants. Everybody, from the local communities to the herbal medicine vendors, popularly harvest aloe leaves, causing a serious problem to its population as well as biodiversity (Supe, 2007). Hence, it is utmost necessary to conserve this plant species for its sustainable use in future. With the aid of *in vitro* biotechnology, the additional conservation strategies are now being developed for several medicinal plants (Chaudhuri and Jha, 2004).

Long term conservation *in vitro*, is an important aspect of plant tissue culture. Elite germplasm of various rare and endangered species have been conserved *in vitro* (Martin and Pradeep, 2003; Tyagi *et al*., 2004; Islam *et al*., 2005; González *et al*., 2006). There are several methods available for *in vitro* conservation of germplasm for virtually unlimited time span in limited space (Bhojwani and Razdan, 1996); as for example, cryopreservation, alginate encapsulation, use of *in vitro* minimum growth (Sarkar *et al*., 2005; Goncalves and Romano, 2007) and through extension of subculture period (Martin and Pradeep, 2003) but no such intervention reported yet in *Aloe vera* L. Moreover, establishment of aseptic *in vitro* culture and genetic stability of the individual species are the most important prerequisite to conserve the cultures for a long period of

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time. The present study is concerned with the conservation of aloe *in vitro* through a simple and efficient protocol maintaining the genetic integrity of the germplasm.

MATERIALS AND METHODS

Collection of explants and *in vitro* propagation: The whole experiment was carried out in the Green House and Plant Tissue Culture Laboratory of Biotechnology Department, Bidhan Chandra Krishi Viswavidyalaya, W.B., India during 2006-2009. Surface sterilized mature plants which are regenerated through suckers were grown under greenhouse condition. Forty five days old rhizomatous stems were collected, trimmed to 2 cm, surface sterilized and inoculated in artificial growth medium under laminar air flow. For establishment of explants, MS medium was supplemented with 0.25 mg L^{-1} of NAA and 1.5 mg L^{-1} of BAP. Induced buds were excised and inoculated on MS, fortified with 2.5 mg L^{-1} of BAP only, for multiple shoot proliferation as described by Gantait (2009).

Physical condition of *in vitro* culture: For the present study MS (Murashige and Skoog, 1962) basal medium (consisted of salts, vitamins and 3% sucrose) was used after solidifying with 0.7% (w/v) agar. Different growth regulators like α-naphthalene acetic acid (NAA) and N^6-benzylamino purine (BAP) were added in variable concentrations to MS before adjusting pH to 5.7 and autoclaved at 1.06 kg cm^{-2}, 121°C for 15 min. MS basal salts, agar, vitamins and growth regulators were obtained from SRL, India. The *in vitro* cultures in all growth stages were accomplished at 25±2°C temperature, 60% RH and 16 h photoperiod (using white fluorescent tubes) under a photosynthetic photon lux density (PPFD) of 30 $\mu mol/m^2/sec^2$.

***In vitro* maintenance of sustained culture:** *In vitro* generated shootlets were subcultured for further multiple shoot proliferation in a sustainable manner. For sustained multiple shoot proliferation, the identified best performing multiple shooting medium i.e., MS supplemented with 2.5 mg L^{-1} of BAP was used. Five subcultures were performed at a 5 month interval over a period of 25 months in the same medium formulation. The performance of these subcultures was assessed on the basis of response, number and length of multiple shoots. Randomly selected propagules were assessed for clonally fidelity through ISSR.

Genomic DNA extraction and PCR amplification: Fresh young leaf samples were collected from three different sources such as mother plant; rhizomatous stem derived shootlets (considered as primary cycle of regeneration) and from the shootlets regenerated through fifth subculture (referred to as secondary explants) after 25 months. Genomic DNA was extracted from 80 mg tender leaves from each sample under study, according to the procedure described by Bhattacharyya and Kanta (1999). Extracted DNA samples were subjected to PCR (Polymerase Chain Reaction) amplification using 10 selected ISSR primers (Gantait *et al*., 2010). The 25 μL PCR mixture contained 40 ng DNA, 2.5 μL 10X Taq polymerase assay buffer, 3.5 μL 2.5 mM dNTPs, 0.5 U Taq DNA polymerase (all from Chromous Biotech Pvt. Ltd., India) and 200 ng of primer (Bangalore Genei Pvt. Ltd., India). PCR consisted of an initial denaturation at 94°C for 5 min followed by 35 cycles of 45 sec at 94°C, 45 sec at annealing temperature 90 sec at 72°C and final extension at 72°C for 7 min, 4°C for 5 min was done using Gene Amp PCR system 2400 (Applied Biosystems, USA). The annealing temperature was adjusted according to the Tm of the primer being used in the reaction. The amplified PCR products, along with 50 bp DNA ladder were resolved by electrophoresis on 1.5% agarose (SRL) gel in 1X TBE buffer stained with ethidium bromide (10 μg L^{-1} TBE buffer). Bands were scored and photographed on Gel Logic 200 trans-illuminator system (Kodak).

Statistical analysis: The potentiality of producing multiple shoots was the criteria to assess the difference between plantlets regenerated from primary explants and plantlets regenerated from secondary explants (without any reference to explants of origin) after 25 months. Then plantlets of both phases were screened through ISSR assay which revealed the genetic clonality of all the propagules. Complete Randomized Design (CRD) was followed for the *in vitro* culture experiments. The experiments were carried out in six replications including 20 explants in each and standard deviation was calculated. For data scoring the inoculant response to multiple shoot proliferation, number of shoot and shoot growth were considered as morphological competence. Data were subjected to Analysis of Variance (ANOVA). Significant difference among the treatments were tested by Duncan's multiple range test (Duncan, 1955) at 5% level using WINDOWSTAT 7.5 (Indostat services, Hyderabad, India) (Gantait *et al*., 2010) software package. For ISSR profiles, the well-resolved and consistently reproducible amplified DNA fragments were scored in terms of their presence or absence. To detect the genetic purity, the resulting banding patterns were compared between DNA samples for each ISSR primer.

RESULTS AND DISCUSSION

Assessment of regeneration efficiency of long term culture: Morphological attributes: Successful bud induction (Fig. 1a) and shoot multiplication (Fig. 1b) was achieved within 50 days of culture. A novel attempt was made to favour mid-term conservation through sustained *in vitro* culture of multiple shoot of aloe. *In vitro* conservation as well as constant supply of quality propagules as and when necessary, was achieved through extending the subculture interval up to 5 months. There are two distinct advantages to maintain a steady supply of propagules from regenerated shootlets under prescribed MS media and acclimatization process as described. First, there was no need to start with a fresh explant. As a result time lag for culture establishment would be restrain. Moreover, each subculture resulted in an exponential increase in number of shoots (Ray *et al*., 2006). Finally, unbroken propagule supply can possibly be sustained over a period of time without genetic damage and so reduction of pressure on its natural population of concerned plant species can be achieved.

It was observed from the present data that plantlets regenerated after 1st subculture and plantlets from 5th subculture showed no significant difference in the response to shoot multiplication according to Duncan's multiple range test (Duncan, 1955) as it was around 19-20 out of 20 inoculants in both cases (Table 1). The uniformity in number and length of multiple shoots was also confirmed by DMRT where these were more than

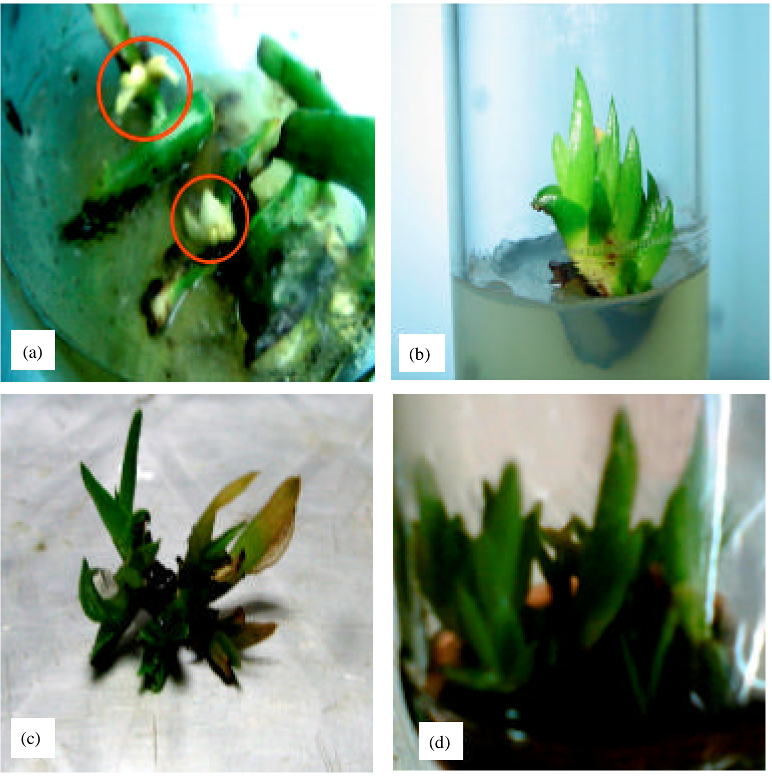

Fig. 1: *In vitro* culture of *Aloe vera* L. (a) Bud break, (b) Multiple shoot induction, (c) Shoot proliferation and (d) Sustained culture for *in vitro* conservation

Table 1: Morphological performance of sustained *in vitro* multiple shoot culture of aloe

Treatments*	No. of buds showing shoot multiplication (out of 20)	No. of shoots	Shoot length (cm)	Remarks
Subculture I	19.67±0.52[a]	9.33±1.21[ab]	3.88±0.34[a]	Healthy, stout, green
Subculture II	19.67±0.52[a]	10.00±1.67[ab]	4.08±0.40[a]	Healthy, stout, green
Subculture III	20.00±0.00[a]	9.50±1.64[ab]	4.25±0.42[a]	Healthy, stout, green
Subculture IV	19.50±0.84[a]	11.17±0.75[a]	4.20±0.36[a]	Healthy, stout, green
Subculture V	19.67±0.52[a]	10.17±1.47[ab]	4.10±0.33[a]	Healthy, stout, green
Statistical analysis				
Mean	19.72	10.03	4.10	
SE (±)	0.2759	0.5686	0.1521	
CD at 5%	0.761	1.656	0.443	

*Each MS medium was supplemented with 2.5 mg L^{-1} BAP. Data represent Mean±SD of 20 replicants per treatment in three repeated experiments. Means within columns separated by Duncan's Multiple Range Test p = 0.05; Duncan, 1955

9.33-11.17 (Fig. 1c) and around 4 cm (Fig. 1d), respectively. No apparent variation was detected between *in vitro* generated clones and they were as good as their mother plant. The morphological efficiency was not hampered even after long-term sustained culture of 25 months. From the primary and secondary explants a similar multiple shoot proliferation was maintained. The sustained unaltered efficiency of *in vitro* multiple shoot culture of aloe supports the earlier report of Joshee *et al*. (2007) who observed the uniform regenerability of embryogenic calli for more than one year with regular subculture. Present study also resulted the production of a large number of multiple shoots through recycling the secondary cultures in 5 cycles for 25 months without losing regeneration efficiency.

Confirmation of genetic stability: ISSR assay: In the present study, 10 ISSR primers were used for the verification of the fidelity of *in vitro* generated clones among which IS-9, IS-10, IS-11 and IS-12 did not react with aloe DNA. Between remaining primers, IS-61, IS-63 and IS-65 displayed a positive interaction but failed to reproduce any major scorable band, whereas IS-6 (Fig. 2a), IS-7 (Fig. 2b) and IS-8 (Fig. 2c) showed positive reproducible bands (Table 2). Each of these three primers generated a unique set of amplified products with the size range of 250 bp in IS-8 to 1.95 kb in IS-6. The variation of the number of bands from each of these successfully used primers was from 4 (in IS-7) to 8 (in IS-8) per sample (Table 2). With an average of 5.6 bands per primer a total number of 102 reproducible monomorphic bands were scored from the clones including their mother. None of the primers showed any difference in banding pattern (Fig. 2).

In the present study, di-nucleotide SSRs motifs AG, GA, GT, TG, CT and CA were used among which three positive and reproducible primers (based on GA, GT and AG motif) amplified distinct scorable number of bands (Table 2). Interestingly, IS-8 based on AG motif amplified more number of bands thus resulting in more coverage of the genome. Similar results have been reported in other

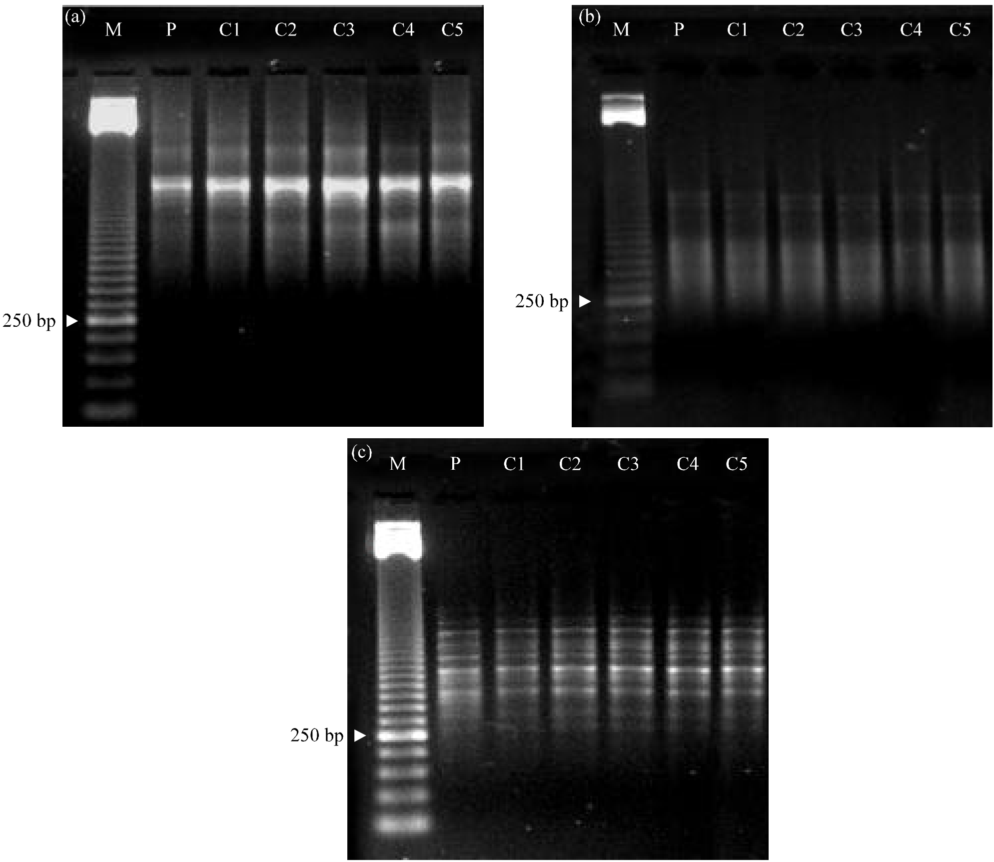

Fig. 2: Agarose gel electrophoresis of ISSR fragments of *in vitro* regenerated clones (C1-C2 from primary culture, C3-C5 from secondary culture) with their mother (P) showing monomorphic bands generated by (a) primer IS-6, (b) primer IS-7 and (c) primer IS-8. Lane M-50 bp ladder

Table 2: List of primers with their sequences, anchoring, annealing temperature, mode of reaction, number and size of amplified fragments

Oligo-name	Temperature (°C)	Sequences (5'-3')	Anchoring	Reaction to aloe DNA	No. of scorable bands per primer	Total No. of scorable bands	Size range (bp)
IS-6	52	$(GA)_8C$	3′ anchor	Positive, reproducible and monomorphic	5	30	700-1950
IS-7	50	$(GT)_8A$	3′ anchor	Positive, reproducible and monomorphic	4	24	300-850
IS-8	52	$(AG)_8C$	3′ anchor	Positive, reproducible and monomorphic	8	48	250-950
IS-9	46	$(TG)_7TA$	3′ anchor	Negative	-	-	-
IS-10	52	$C(GA)_8$	5′ anchor	Negative	-	-	-
IS-11	52	$(CA)_8G$	3′ anchor	Negative	-	-	-
IS-12	52	$(GT)_8C$	3′ anchor	Negative	-	-	-
IS-61	50	$(GA)_8T$	3′ anchor	Positive but not reproducible	-	-	-
IS-63	52	$(AG)_8C$	3′ anchor	Positive but not reproducible	-	-	-
IS-65	50	$(AG)_8T$	3′ anchor	Positive but not reproducible	-	-	-
Total					17	102	250-1950

medicinal plants like *Swertia chirayita* (Joshi and Dhawan, 2007) and *Allium ampeloprasum* (Gantait *et al*., 2010). Aloe being a diploid with 2n = 14, it can be assumed that the limited number of bands which are produced by these ISSR primers would partially cover the genome. However, none of the primers showed any difference in the banding pattern. Considering the detected uniform morphological competence of the primary and long-term secondary culture *in vitro* along with the displayed monomorphic banding pattern, it can be suggested that *in vitro* regenerated clones maintained their genetic integrity.

CONCLUSION

The results revealed the significance of the protocol reported in this study. Shoot tip explants maintained their regeneration frequency with morphogenetic competence even after prolonged *in vitro* culture. So, this protocol proves it potentiality to save this species from extinction and facilitate germplasm conservation. Considering these characteristics, we suggest that the developed strategy can be adopted for other endangered species also.

REFERENCES

Ahmed, S., A.H. Kabir, M.B. Ahmed, M.A. Razvy and S. Ganesan, 2007. Development of rapid micropropagation method of *Aloe vera* L. Sjemenarstvo, 24: 121-128.

Bhattacharyya, S. and M.R. Kanta, 1999. Identification of molecular marker in septumless bold pod in *Brassica campestris*. J. Plant Biochem. Biotechnol., 8: 93-99.

Bhojwani, S.S. and M.K. Razdan, 1996. Plant Tissue Culture: theory and Practice. A Revised Edn. Elsevier, Armsterdam-Oxford, Tokyo.

Chaudhuri, R.K. and T.B. Jha, 2004. Conservation and production of Ipecac (*Cephaelis ipecacuanha* Rich.) plants from long term shoot cultures. Plant Tissue Cult. Biotechnol., 18: 157-164.

Davis, R.H., J.J. Donato, G.M. Hartman and R.C. Haas, 1994. Anti-inflammatory and wound healing activity of a growth substance in *Aloe vera*. J. Am. Podiatr. Med. Assoc., 84: 77-81.

Duncan, D.B., 1955. Multiple range and multiple F-tests. Biometrics, 11: 1-42.

Gantait, S., 2009. Accelerated cloning *in vitro* and induction of variation in six plants having medicinal, aromatic or aesthetic importance. Ph.D. thesis, Bidhan, Chandra Krishi Viswavidyalaya, pp: 84.

Gantait, S., N. Mandal, S. Bhattacharyya and P.K. Das, 2010. Determination of genetic integrity in long-term micropropagated plantlets of *Allium ampeloprassum* L. using ISSR markers. Biotechnology, 9: 218-223.

Goncalves, S. and A. Romano, 2007. *In vitro* minimum growth for conservation of *Drosophyllum lusitanicum*. Biol. Plant., 51: 795-798.

González, M.L., R. Mallón, J. Reinoso and J. Rodríguez-Oubiña, 2006. *In vitro* micropropagation and long-term conservation of the endangered moss *Splachnum ampullaceum*. Biol. Plant., 50: 339-345.

Islam, M.T., D.P. Dembele and E.R. Keller, 2005. Influence of explant, temperature and different culture vessels on *in vitro* culture for germplasm maintenance of four mint accessions. Plant Cell Tissue Organ Cult., 81: 123-130.

Joshee, N., B.K. Biswas and A.K. Yadav, 2007. Somatic embryogenesis and plant development in *Centella asiatica* L., a highly prized medicinal plant of the tropics. HortScience, 42: 633-637.

Joshi, P. and V. Dhawan, 2007. Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay. Biol. Plantarum, 51: 22-26.

Leon, L., 2003. Aloe as a medicinal plant. Ganzheitliche Tiermedizin, 17: 138-143.

Martin, K.P. and A.K. Pradeep, 2003. Simple strategy for the *in vitro* conservation of *Ipsea malabarica* an endemic and endangered orchid of the Western Ghats of Kerala, India. Plant Cell Tissue Organ Cult., 74: 197-200.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant., 15: 473-497.

Ray, T., I. Dutta, P. Saha, S. Das and S.C. Roy, 2006. Genetic stability of tree economically important micropropagated banana (*Musa* spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. Plant Cell Tissue Organ Cult., 85: 11-21.

Sarkar, D., S. Pandey and A. Chanemougasoundharam, 2005. the role of calcium nutrition in potato (*Solanum tuberosum*) microplants in relation to minimal growth over prolonged storage *in vitro*. Plant Cell Tissue Organ Cult., 81: 221-227.

Supe, J.U., 2007. *In vitro* regeneration of *Aloe barbadensis*. Biotechnology, 6: 601-603.

Tyagi, R.K., A. Yuasf, P. Dua and A. Agrawal, 2004. *In vitro* plant regeneration and genotype conservation of eight wild species of *Curcuma*. Biol. Plant, 48: 129-132.