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Assessment of Genetic Stability and Instability of Tissue Culture-Propagated Plantlets of *Aloe vera* L. by RAPD and ISSR Markers

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Abstract Efficient plantlet regeneration with and without intermediate callus phase was achieved for a selected genotype of *Aloe vera* L. which is sweet in test and used as a vegetable and source of food. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) marker assays were employed to evaluate genetic stability of plantlets and validate the most reliable method for true-to-type propagation of sweet aloe, among two regeneration systems developed so far. Despite phenotypic similarities in plantlets produced through both regeneration systems, the differences in genomic constituents of plantlets produced through intermediate callus phase using soft base of inflorescence have been effectively distinguished by RAPD and ISSR markers. No polymorphism was observed in regenerants produced following direct regeneration of axillary buds, whereas 80% and 73.3% of polymorphism were observed in RAPD and ISSR, respectively, in the regenerants produced indirectly from base of the inflorescence axis via an intermediate callus phase. Overall, 86.6% of variations were observed in the plantlets produced via an intermediate callus phase. The occurrence of genetic polymorphism is associated with choice of explants and method used for plantlet regeneration. This confirms that clonal propagation of sweet aloe using axillary shoot buds can be used for commercial exploitation of the selected genotype where a high degree of fidelity is an essential prerequisite. On the other hand, a high degree of variations were observed in plantlets obtained through indirect regeneration and thus cannot be used for the mass multiplication of the genotype; however, it can be used for crop improvement through induction of somaclonal variations and genetic manipulations.

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Introduction

Aloe (*Aloe vera* L.) is one of the economically important crops of the Liliaceae and is being used worldwide in pharmaceutical, food, and cosmetic industries or in traditional medicine, due to the plethora of biological activities of some of its primary and secondary metabolites [1]. Pharmaceutical and cosmetic industries have a high demand for *A. vera* material. Although this plant is used in the herbal industry, some of the genotypes of *A. vera* (sweet aloes) are sweet and edible and are consumed as a vegetable and to make curry and desserts. Due to wide use of aloe products as alternative medicines and dietary supplements and with rapid expansion of the aloe product industries, there is an increased demand for its leaves as raw material. Current production of leaves is insufficient to meet the industry demand. Thus, there is a need to undertake large-scale cultivation of selected genotype(s). Propagation of aloe by conventional methods or by means of offshoots is slow; a single plant produces three to four offshoots every year, which is not sufficient for commercial plantation. The presence of male sterility is also a barrier in rapid propagation. Tissue culture and in vitro plantlet regeneration systems as a non-genetically modified biotechnology provide alternative production systems for mass propagation of the desired plant genotype to supply the standard clonal propagules for commercial planting of a selected genotype.

Aloe species has been cultured in vitro by various workers [2–9]. Efficient plantlet regeneration for a selected genotype of *A. vera* L. sweet aloe was achieved through direct shoot bud proliferation using axillary shoot buds [10] and indirect shoot bud regeneration via an intervening callus phase using inflorescence axis as explants [11]. Plant tissue culture techniques are of extensive uses where quality material of a selected genotype is required at mass scale, for commercial cultivation and the natural propagation do not meet the required demands. The sustainability of the regeneration systems depends upon the maintenance of the genetic integrity of micropropagules. However, in vitro techniques are known to induce somaclonal variations. Further, the frequency of these variations varies with the source of explants and their regeneration pattern, media composition, and cultural conditions [12]. Since the first observation and report of somaclonal variation by Braun [13], these variations remain one of the major problems of many tissue-cultured plants [14]. The occurrences of uncontrolled and random spontaneous variations during culture process are undesirable and seriously limit the utility of micropropagation system. On the other hand, variations are sought for crop improvement through applications of advanced biotechnological tools by creation of novel variants and are also well documented [14]. Also, induced variations can be used for genetic manipulation of crops with phylogenic traits [15, 16]. Literature reports on the occurrence of tissue culture-induced variations warrant the need to test and verify the genetic stability of regenerated plantlets and assess the reliability and commercial suitability of the regeneration systems.

Tissue culture-induced variations can be determined at the morphological, cytological, biochemical, and molecular levels with several techniques. At present, molecular markers are powerful tools used in the analysis of genetic fidelity of in vitro propagated plantlets. These are not influenced by environmental factors and generate reliable and reproducible results. Randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism, inter simple sequence repeats (ISSR), and simple sequence repeats (SSR)

have been widely used to evaluate, detect, and identify changes in the DNA sequence caused by somaclonal variations [17–20]. Among multilocus markers, RAPD and ISSR techniques are simple, fast, and cost-effective methods of testing the genetic integrity of plants. Use of more than one DNA amplification technique has been suggested as advantageous in evaluating somaclonal variations [21]. Use of simple and cost-effective molecular techniques does not add much to the cost of plant production; therefore, these were methods of choice for present investigation. The present investigation was aimed to test the genetic purity of in vitro regenerated plantlets using RAPD and ISSR profiles to evaluate and confirm the most reliable method of mass multiplication of an elite and selected genotype (sweet aloe) of *A. vera* that maintains genetic integrity of genotype among two regeneration protocols developed so far using two different explants and regeneration patterns. To our knowledge, this is the first report on assessment of genetic stability and instability in tissue-cultured plants of the genus *Aloe*.

Material and Methods

In Vitro Regeneration of *A. vera* L. and Plant Material for Genetic Analysis

A sweet genotype of aloe maintained in the greenhouse in Department of Botany, J.N. Vyas University, Jodhpur served as source of explants. Efficient plantlet regeneration both through direct shoot bud proliferation without callus phase (protocol 1) using axillary shoot buds [10] and indirect shoot bud proliferation via intermediate callus phase (protocol 2) using soft base of inflorescence axis as an explant [11] was achieved. Figure 1 shows a schematic representation of different steps of protocols and Murashige and Skoog's basal medium [22] with plant growth regulators used for plantlet regeneration. The in vitro regenerated plantlets were hardened in a greenhouse and transferred to soil with 100% survival rate. More than 2,500 (600 plantlets produced through indirect and 1,900 plantlets produced through direct regeneration) tissue-cultured propagated plantlets were successfully transferred in fields under the environmental conditions of Western Rajasthan at various sites namely Rampura Agriculture Research Station, Garden of Botanical Survey of India, and Field of Anuraj Farms, Jodhpur. A total 30 plantlets, 15 plantlets produced through axillary bud regenerated (AR) by protocol 1 (sample AR 1–15) and 15 plantlets through callus mediated regeneration (CR) by protocol 2 (sample CR 1–15), were selected randomly from field and used for genetic analysis. These plantlets (AR 1–15 and CR 1–15) together with original mother plant (sample P) were used for DNA extraction and genetic analysis through RAPD and ISSR markers.

Genomic DNA Extraction and PCR Amplifications

Genomic DNA was extracted using cetrimonium bromide (CTAB) protocol [23] with slight modifications. Pure and analytical grade chemicals of Sisco Research Laboratories, Pvt. Ltd., Mumbai, India and Sigma-Aldrich Chemical Company, USA were used during present course of study for extraction of DNA and reagent preparations. Approximately 1 g of leaf tissue (apical portions) was taken from samples and subsequently grinded in liquid nitrogen using mortar and pestle along with a pinch of polyvinylpyrrolidone (PVP; MW 29,000). To the grinded samples, 5 ml of extraction buffer [2% CTAB, 100 mM Tris–HCl, 3.5 M sodium chloride (NaCl), 20 mM ethylenediaminetetraacetic acid (EDTA), 0.2 M β-mercaptoethanol, 2% PVP, pH 8.0] was added, and these were incubated at 65 °C for

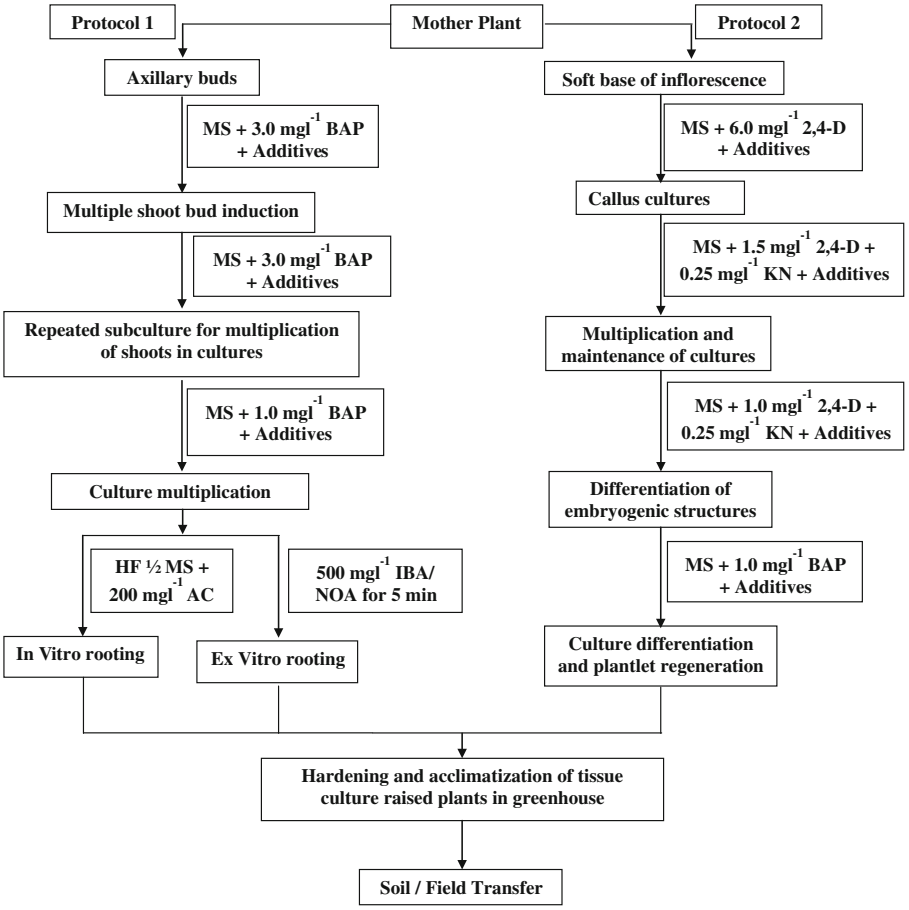

Fig. 1 Outline showing summary of tissue culture processes (protocols 1 and 2) for aloe plantlet regeneration

90 min. The above samples were extracted with equal volume of chloroform and isoamyl alcohol (24:1), and the supernatant was transferred to a new tube following centrifugation at 10,000×*g* for 10 min. The samples were treated with 20 μl of 10 mg ml^{-1} of RNase. These were extracted with Tris-saturated phenol (pH 8.0) and precipitated with 80% of ethanol and 3 M sodium acetate (pH 5.2) at −20 °C. The DNA pellet was air dried and dissolved in 100 μl of sterile Milli Q water. Total genomic DNA was quantified spectrophotometrically (Analytical spectrophotometer, UK), and aliquots were diluted to the final concentration of 10–15 ng/μl.

PCR amplifications for RAPD and ISSR analysis were performed in thermal cycler (Master cycle epgradient S, Eppendorf, Germany). Amplification of RAPD fragments was performed according to [24] using decamer arbitrary primers (Operon Technologies Inc, USA; IDT, USA). The reaction was carried out in a volume of 15 μl of reaction mixture containing 25 ng template DNA, 1× PCR buffer (Fermentas, USA), 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 0.4 μM primer, and 1 U Taq DNA polymerase (Fermentas, USA). The amplification reaction was carried out using the program of initial denaturation at 94 °C for 3 min followed by 42 cycles of denaturation at 94 °C for 30 s, primer annealing at 32 °C for 1 min, extension at 72 °C for 2.5 min, and final extension at 72 °C for 4 min.

For ISSR analysis, PCR amplifications were carried out in a volume of 15 μl of reaction mixture containing 1× PCR buffer (Fermentas, USA), 0.2 mM each dNTP, 3.0 mM $MgCl_2$, 0.4 μM primer (Sigma-Aldrich, USA), 25 ng template, and 1 U Taq DNA polymerase (Fermentas, USA). Fifteen ISSR primers were used for standardization of optimum annealing temperature. The conditions for PCR cycle consisted of an initial denaturation step at 94 °C for 5 min, 35 cycles comprising denaturation at 94 °C for 30 s, optimum annealing temperature for particular ISSR primer for 30 s, and extension at 72 °C for 1 min and a final extension step at 72 °C for 7 min. RAPD and ISSR amplification products were electrophoresed in 1.5% agarose in 1× TBE (90 mM Tris–borate, 2 mM EDTA, pH 8.0). The gels were stained with ethidium bromide and documented using gel documentation system (Syngene, UK).

Data Analysis

RAPD and ISSR fingerprints generated were individually scored and statistically analyzed considering fragment size at a locus as biallelic (present=1, absent=0), and the binary matrix was created. RAPD and ISSR profiles were compared for 30 in vitro regenerated plantlets (AR 1–15 and CR 1–15) and mother plant (P) with each primer. RAPD and ISSR data were pooled separately for protocol 1 and protocol 2 for genetic similarity calculation and dendrogram construction. Genetic similarity based on Jaccard's coefficient was calculated separately for both protocol 1 and protocol 2 among all possible pairs of the 16 samples using SimQual module and arranged into a similarity matrix. A dendrogram was constructed using NTSYS version 2.20 software package (Exeter software) by following UPGMA option of SAHN module. The percentage of polymorphism was calculated as the ratio of total number of polymorphic bands to the total number of bands and expressed as percentage.

Results and Discussion

There has been an increasing interest in in vitro regeneration of medicinal plants especially rare, endangered and slow-propagating and economically important plants. The application of plant tissue culture and biotechnology for the improvement of *Aloe* species has received little attentions from researchers. A successful crop improvement program requires the development of an efficient and comprehensive in vitro culture system so that explants can be mass produced. Many factors including plant genotype, medium, explant type, plant growth regulator, culture conditions, and mutagens can affect plantlet regeneration. Two efficient and reliable in vitro plantlet regeneration systems for a selected genotype of aloe (sweet *A. vera* genotype) were developed. Use of commercial grade sugar cubes and agar-agar for multiplication on a semi-solid medium, the omission of agar-agar in case of multiplication of shoots in liquid medium, higher rate of shoot multiplication, and the single-step ex vitro rooting and hardening in the greenhouse are the advantageous features of regeneration systems. The regeneration system involving indirect shoot bud proliferation could be a useful tool to carry out somaclonal variations and for genetic engineering experiments to improve of plant species. These regeneration protocols can be of greater use for promotion of cultivation of desired genotype(s) of this species. Literature reports on somaclonal variations warrant the need to test the sustainability of the regeneration systems, and here, we report on analysis of genetic stability and instability in plantlets of aloe produced through these regenerations systems.

In RAPD, out of the 25 primers that were initially screened, 15 primers produced reproducible and intense banding pattern and were used for data analysis. A total of 119 RAPD bands, each ranging from 323 to 3,052 bp in size, were produced in PCR amplification. The number of bands scored in each primer ranged from 4 to 14 with an average of 7.93 bands per primer. The number and percentage of monomorphic bands in RAPD differed with the protocol used for regeneration. These varied from 119% and 100%, respectively, in protocol 1 to 107% and 89.915% in protocol 2. RAPD profile of the samples (AR 1–15) obtained through protocol 1 and that of the mother plant (P) was similar in all respects; however, the occasional appearance of different bands was not stable across replicates. Out of 15, only five RAPD primers, namely OPL-7, OPN-3, OPN-12, OPQ 15, and OPR-18 (Fig. 2a), produced monomorphic bands in samples obtained from protocol 2 while the other 10 primers produced polymorphic banding pattern (Table 1).

Maximum polymorphism (i.e., 25%) was observed in the amplification pattern of the primer OPO-15 and OPQ-12, and these were followed by OPL-1 with 22.22% polymorphism. OPL-4 and OPO-7 showed 12.5% polymorphism while OPL-14, OPL-18 and OPQ-16 (Fig. 2b) showed 20% polymorphism in plantlets produced through protocol 2. Least polymorphism (i.e., 10%) was observed with OPL-5.

RAPD technique has been extensively used to assess genetic variations generated by in vitro techniques [25]; however, the successful assessment of RAPD profiles generated requires validation through repeated experiments. Use of one technique cannot guarantee the genetic purity of tissue-cultured plants. Thus, if RAPD analysis showed no genetic alterations, as in the present study (in plantlets produced through protocol 1), this does not necessarily mean that there are none [26]. In fact, sometimes RAPD fail to reveal changes in the repetitive regions of the genome of some species [21]. Therefore, use of more than one DNA amplification technique has been suggested advantageous. In the present study, ISSR has been employed to assess the reliability of results of RAPD profiling. However, the

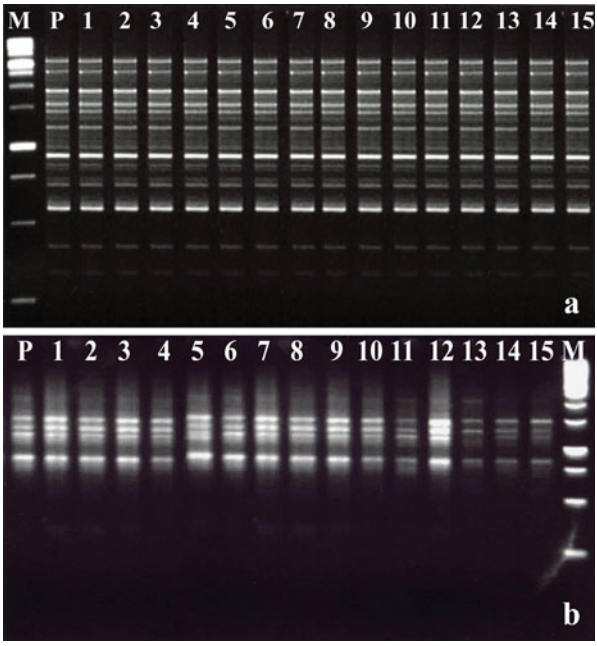

Fig. 2 RAPD fingerprints of plantlets produced by protocol 1 with OPL 7 (**a**) and by protocol 2 with OPQ-16 (**b**). *Lane M*: 1.0 kb DNA ladder; *lane P*: mother plant; and *lanes 1–15*: AR 1–15 in **a** and CR 1–15 in figure **b**

Table 1 Total numbers and range of molecular weights of the amplified fragments generated as products of PCR amplification with 15 RAPD (sequence no. 1–15) and 10 ISSR (sequence no.16–25) primers and DNA from parent (P) plant and callus regenerated plantlets (CR 1–15) of aloe

Sequence number	Primer	Sequence (5′-3′)	Size (bp)	Scorable bands	Profiling of callus regenerated plantlets (CR)	
					Monomorphic bands	Polymorphic bands
RAPD fingerprinting						
1	OPL-1	GGCATGACCT	637–1,926	9	7	2
2	OPL-4	GACTGCACAC	478–3,052	8	7	1
3	OPL-5	ACGCAGGCAC	622–2,807	10	9	1
4	OPL-7	AGGCGGGAAC	323–2,317	14	14	0
5	OPL-14	GTGACAGGCT	516–2,274	5	4	1
6	OPL-18	ACCACCCACC	994–2,284	5	4	1
7	OPO-7	CAGCACTCAC	517–2,784	8	7	1
8	OPO-15	TGGCGTCCTT	731–2,956	4	3	1
9	OPQ-11	TCTCCGCAAC	400–2,049	7	6	1
10	OPQ-12	AGTAGGGCAC	693–1,753	8	6	2
11	OPQ-15	GGGTAACGTG	419–1,479	7	7	0
12	OPQ-16	AGTGCAGCCA	866–1,500	5	4	1
13	OPN-3	GGTGCACGTT	622–1,553	8	8	0
14	OPN-12	CACAGACACC	323–1,884	11	11	0
15	OPR-18	CCCGTTGCCT	410–2,784	10	10	0
ISSR fingerprinting						
16	ISSR-A	$(CA)_6GG$	366–2,057	10	8	2
17	ISSR-C	$(CA)_6AG$	373–963	9	9	0
18	ISSR-D	$(CA)_6GT$	842–2,213	6	5	1
19	ISSR-E	$(GA)_6GG$	411–1,075	6	6	0
20	ISSR-F	$(GA)_6CC$	358–1,341	10	10	0
21	ISSR-G	$(GT)_6CC$	451–1,042	4	2	2
22	ISSR-I	$(AG)_8C$	411–1,086	8	7	1
23	ISSR-K	$(AC)_8G$	328–576	4	3	1
24	ISSR-M	$(AC)_8T$	494–1,073	8	6	2
25	ISSR-O	$(AG)_8T$	511–1,286	8	6	2

method can be used for rapid evaluation of somaclonal variability in tissue-cultured plants, by fast scanning of the whole genome.

In the present study, dinucleotide SSRs motifs CA, GA, GT, AG, and AC were used for screening the samples. Of the 20 ISSR primers initially screened, ten produced reproducible and scorable banding patterns. The optimum annealing temperature for primers varied from 37.4 to 54.9 °C. Primer ISSR-A and ISSR-F amplified more number of bands, thus revealed more coverage of the genome, while on the other hand ISSR-G and ISSR-K amplified least number of bands. Overall, in the present study, ten ISSR primers generated 73 scorable ISSR bands ranging in size range from 328 to 2,213 bp. The number of bands for each primer was 4 to 10 with an average of 7.3 bands per ISSR primer. Overall, only three ISSR primers, namely C (Fig. 3a), E, and F, produced monomorphic bands throughout the samples (AR 1–15 and CR 1–15) irrespective of protocol used for plantlet regeneration. None of the ISSR primers used in present study revealed polymorphism (i.e., 100% genetic

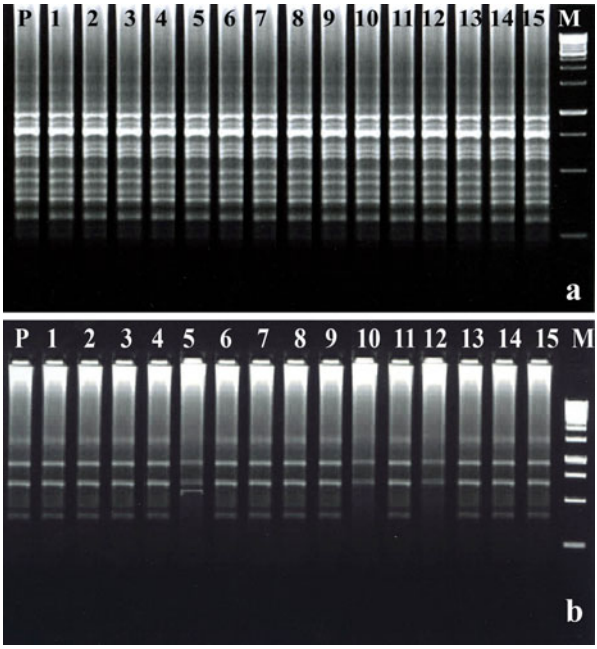

Fig. 3 ISSR fingerprints of plantlets produced by protocol 1 with ISSR-C (**a**) and by protocol 2 with ISSR-G (**b**). *Lane M*: 1.0 kb DNA ladder; *lane P*: mother plant; and *lanes 1–15*: AR 1–15 in **a** and CR 1–15 in **b**

similarity) in the plantlets (AR 1–15) regenerated with protocol 1. This confirms the reliability of results obtained with RAPD profiling for plantlets (AR 1–15) regenerated directly using axillary meristem by protocol 1. In ISSR profiling, 11 polymorphic bands were observed with seven out of ten ISSR primers, in the plantlets (CR 1–15) obtained from protocol 2, showing 15.06% polymorphism. Maximum polymorphism (i.e., 50%) was observed with primer ISSR-G (Table 1; Fig. 3b), and it was followed by ISSR-K, ISSR-M, and ISSR-O primers which showed 25% polymorphism. From this observation, it can be concluded that use of the two PCR based assays to screen the genome of regenerants was found to be effective in detecting polymorphism in tissue-cultured plants of aloe [27].

On the basis of individual analysis of RAPD and ISSR profiles, the plantlet CR 14 was found different between RAPD and ISSR; it clustered with mother plant in ISSR dendrogram and found to be unclustered in RAPD dendrogram (data not given). Jaccard's genetic similarity coefficient calculated using pooled data of RAPD and ISSR profiles was found 1.0 for plantlets (AR 1–15) of protocol 1, showing genetic identicalness to mother plant (P). Similar results were obtained in dendrogram for plantlets of protocol 1, as all plantlets clustered with mother plant in the dendrogram. Overall, there were no genetic variations in these plantlets (AR 1–15) and they were true-to-type to the mother plant (P). The genetic similarity coefficient ranged from 0.93 to 1.0 for plantlets (CR 1–15) of protocol 2 (Table 2). In dendrogram generated on the basis of pooled data, two major clusters were obtained. Plantlets CR 2, 7, 12, and 14 formed a small cluster and remained separate from mother plant, while the rest of the CR samples grouped with mother plant; however, in larger cluster, four small sub-clusters were formed (Fig. 4). The percentage of polymorphism in RAPD varied with either of the protocols from 0% in samples (AR 1–15) obtained from protocol 1 to 10.08% in the samples (CR 1–15) of protocol 2. Similarly, in ISSR, this varied from 0% in samples of protocol 1 to 15.06% in the samples of protocol 2.

Table 2 Genetic similarity matrix generated on the basis pooled data of RAPD and ISSR fingerprints for plantlets (CR1–15) regenerated with protocol 2

	P	CR 1	CR 2	CR 3	CR 4	CR 5	CR 6	CR 7	CR 8	CR 9	CR 10	CR 11	CR 12	CR 13	CR 14	CR 15
P	1.00															
CR 1	0.98	1.00														
CR 2	0.96	0.95	1.00													
CR 3	1.00	0.98	0.96	1.00												
CR 4	0.97	0.96	0.94	0.97	1.00											
CR 5	0.97	0.96	0.94	0.97	0.95	1.00										
CR 6	0.98	0.99	0.94	0.98	0.95	0.95	1.00									
CR 7	0.97	0.95	0.99	0.97	0.94	0.94	0.95	1.00								
CR 8	1.00	0.98	0.96	1.00	0.97	0.97	0.98	0.97	1.00							
CR 9	0.97	0.96	0.93	0.97	1.00	0.95	0.95	0.94	0.97	1.00						
CR 10	0.98	0.96	0.94	0.98	0.95	0.99	0.96	0.95	0.98	0.95	1.00					
CR 11	0.98	1.00	0.95	0.98	0.96	0.96	0.99	0.95	0.98	0.96	0.96	1.00				
CR 12	0.96	0.94	0.98	0.96	0.93	0.95	0.94	0.99	0.96	0.93	0.96	0.94	1.00			
CR 13	0.98	0.99	0.94	0.98	0.95	0.95	0.99	0.95	0.98	0.95	0.96	0.99	0.93	1.00		
CR 14	0.96	0.94	0.96	0.96	0.95	0.93	0.94	0.97	0.96	0.95	0.94	0.94	0.96	0.94	1.00	
CR 15	0.99	0.97	0.95	0.99	0.96	0.96	0.97	0.96	0.99	0.96	0.97	0.98	0.95	0.97	0.95	1.00

Overall, none of RAPD and ISSR primers showed genetic variation in samples of protocol 1, while 11.97% polymorphism (calculated on the basis of pooled data of RAPD and ISSR) was observed in samples of protocol 2.

It is obvious from the results that all the plantlets generated following protocol 1 were genetically stable and true-to-type clones to the mother plant. This data concurs with reports of Martins et al. [20] and Salvi et al. [12], who suggested that use of organized meristems generally maintains genetic integrity of the plantlets obtained through tissue culture with a

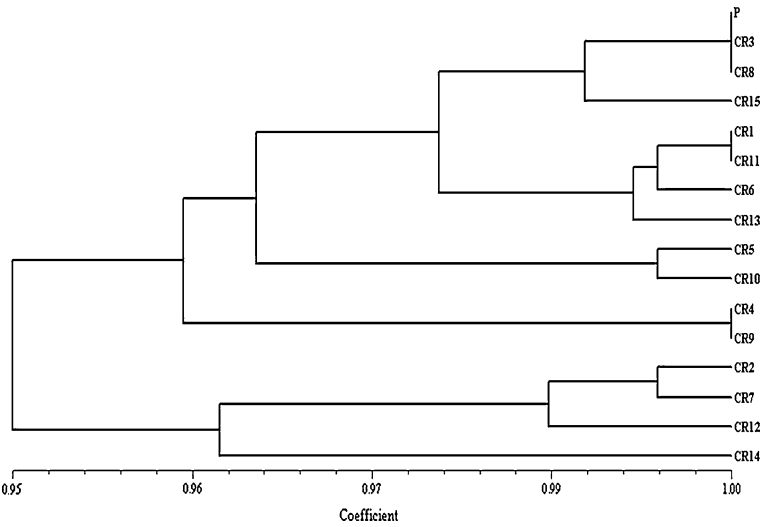

Fig. 4 Dendrogram generated for callus regenerated plantlets (CR 1–15). Sample *P*: mother plant; *CR 1–15*: regenerates produced through protocol 2

least risk of producing somaclonal variants. On the other hand, plantlets generated through an intermediate callus phase, following protocol 2, have shown a genetic variation frequency of 73.3% and 80% within the set in ISSR and RAPD, respectively, whereas, the overall frequency calculated on the basis of pooled data was found 86.6%. This high frequency of somaclonal variations observed in plantlets regenerated from soft base of inflorescence through an intermediate callus phase is therefore not desirable for generating true-to-type plants. Similar higher frequency of somaclonal variations were observed in garlic plants regenerated from callus tissue [28] and in turmeric [12]. However, these can be used for biotechnological improvement of the plant species.

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References

1. Campestrini, L. H., Kuhnen, S., Lemos, P. M. M., Bach, D. B., Dias, P. F., & Maraschin, M. (2006). *Journal of Technology Management Innovation, 1*, 77–79.
2. Roy, S. C., & Sarkar, A. (1991). *Scientia Horticulturae, 47*, 107–113.
3. Meyer, H. J., & van Staden, J. (1991). *Plant Cell Tissue and Organ Culture, 26*(3), 167–171. doi:10.1007/BF00039939.
4. Richwine, A. M., Tipton, J. L., & Thompson, A. (1995). *Horticulturea Science, 30*, 1443–1444.
5. Abrie, A. L., & van Staden, J. (2001). *Plant Growth Regulation, 33*, 19–23. doi:10.1023/A:1010725901900.
6. Liao, Z., Chen, M., Tan, F., Sun, X., & Tang, K. (2004). *Plant Cell Tissue and Organ Culture, 76*(1), 83–86. doi:10.1023/A:1025868515705.
7. Aggarwal, D., & Barna, K. S. (2004). *Journal of Plant Biochemistry and Biotechnology, 13*, 77–79.
8. Velcheva, M., Faltin, Z., Vardi, A., Eshdat, Y., & Perl, A. (2005). *Plant Cell Tissue and Organ Culture, 83*(3), 293–301. doi:10.1007/s11240-005-7192-1.
9. Bairu, M. W., Stirk, W. A., Dolezal, K., & van Staden, J. (2007). *Plant Cell Tissue and Organ Culture, 90*, 15–23. doi:10.1007/s11240-007-9233-4.
10. Singh, M., Rathore, M. S., Panwar, D., Rathore, J. S., Dagla, H. R., & Shekhawat, N. S. (2009). *Journal of Sustainable Forestry, 28*, 935–950. doi:10.1080/10549810903344660.
11. Rathore, M. S., Chikara, J., & Shekhawat, N. S. (2011). *Applied Biochemistry and Biotechnology, 163*, 860–868. doi:10.1007/s12010-010-9090-1.
12. Salvi, N. D., George, L., & Eapen, S. (2001). *Plant Cell Tissue and Organ Culture, 66*, 113–119.
13. Braun, A. C. (1959). *Proceedings of National Academy of Sciences USA, 45*, 932–938.
14. Bairu, M. W., Aremu, A. O., & Van Staden, J. (2011). *Plant Growth Regulation, 63*, 147–173.
15. Brar, D. S., & Jain, S. M. (1998). In S. M. Jain, D. S. Brar, & B. S. Ahloowalia (Eds.), *Somaclonal variation and induced mutations in crop improvement* (pp. 15–37). Dordrecht: Kluwer Academic.
16. Jain, S. M. (2001). *Euphytica, 118*, 153–166.
17. Bhatia, R., Singh, K. P., Jhang, T., & Sharma, T. R. (2009). *Scientia Horticulturae, 119*, 208–211.
18. Lu, G., Zhang, X., Zou, Y., Zou, Q., Xiang, X., & Cao, J. (2007). *Plant Cell Tissue and Organ Culture, 88*, 319–327.
19. Smykal, P., Valledor, L., Rodriguez, R., & Griga, M. (2007). *Plant Cell Reports, 26*, 1985–1998.
20. Martin, M., Sarmento, D., & Oliveira, M. M. (2004). *Plant Cell Reports, 23*, 492–496.
21. Palombi, M. A., & Damiano, C. (2002). *Plant Cell Reports, 20*, 1061–1066.
22. Murashige, T., & Skoog, F. (1962). *Physiologia Plantarum, 15*, 473–479. doi:10.1111/j.1399-3054.1962.tb08052.x.
23. Doyle, J. J., & Doyle, J. L. (1990). *Focus, 12*, 13–15.
24. Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). *Nucleic Acids Research, 18*, 6531–6535.
25. Goto, S., Thakur, R. C., & Ishii, K. (1998). *Plant Cell Reports, 8*, 193–197.
26. Mallon, R., Rodrıguez-Oubina, J., & Gonzalez, M. L. (2010). *Plant Cell Tissue and Organ Culture, 101*, 31–39.
27. Ray, T., Dutta, I., Saha, P., Das, S., & Roy, S. C. (2006). *Plant Cell Tissue and Organ Culture, 85*, 11–21.
28. Al-Zahim, M. A., Ford-Lloyd, B. V., & Newbury, H. J. (1999). *Plant Cell Reports, 18*, 473–477.