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Analysis of Genetic Fidelity of Wild Type and in Vitro Regenerated Aloe Vera Plants Through RAPD and ISSR Molecular Markers

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Abstract

Random Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeats (ISSR) marker assays were employed to investigate polymorphism level of conventionally grown wild type and in vitro propagated Aloe vera plants. Despite having phenotypic similarities in the plantlets, variation in the genomic constituents has been effectively established through RAPD and ISSR markers showing 33% and 25% polymorphism respectively

Keywords: Aloe Vera, Wild Type, in Vitro Regenerated, Genetic Fidelity,RAPD, ISSR

Introduction:

Plant tissue culture techniques are known to induce somaclonal variations. Frequency of these variations differ with the source of explants, their regeneration methods, composition of culture medium and cultural conditions. The first observation of somaclonal variations was reported by Brown in 1984. Advances in molecular biology have revolutionized every field of biological sciences such as, DNA based markers have been used for individual identification, genome mapping, pedigree and phylogenetic diversity analysis in numerous taxa and in selecting somaclonal variations. Molecular biological tools can accelerate artificial breeding processes and clarify the genetic mechanisms that cannot be easily detected with plant breeding techniques (Gupta et al., 2010). Molecular tools can also give important information about the genetic distances between species (Mihalte et al., 2011).

The molecular marker technique efficiency is based on the amount of polymorphism, it can detect in the given accessions (Tharachand et al., 2012). Molecular marker studies on Aloe vera have been published by using the following molecular techniques such as: AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplified Polymorphic DNA), and ISSR (Inter Simple Sequence Repeats), (Alagukannan & Ganesh, 2016; Kumar et al.,2016).

The increasing development and generalized use of a large number of methodologies during the last years, requires comparative studies in order to choose the best DNA marker technology to be used in fingerprinting and in diversity studies, considering reproducibility, costs, sensibility and level of polymorphisms detection. Molecular technique comparisons have become important because, depending on the objective of the study, one technique can be more appropriate than another, as well as different techniques being informative at different taxonomic levels. There is a range of molecular marker types available, but the choice of an appropriate genetic marker depends on the experience and competence of the researchers, also on laboratory facilities. Several works report comparable results among different markers while others show considerable variations (Goulao & Oliveira, 2001).

Random Amplified Polymorphic DNA (RAPD)

Molecular markers have overcome limitations of morphological and biochemical markers due to the influence of environment on the performance of genotypes. A wide range of molecular markers have been used to assess genetic diversity of Aloe vera (Nayanakantha et al., 2010; Alagukannan & Ganesh, 2016). Random Amplified Polymorphic DNA (RAPD) is powerful technique considered as a useful tool, it need tiny amounts of DNA to give rapid and accurate identification of alien species especially in the developing countries, where DNA based methods are unavailable due to their high cost, the requirement for complex equipments and expertise (El-Mergawy et al., 2011). Among molecular markers RAPDs are the most widely applied most probably because they do not require the knowledge of genomic sequences and also the protocol is relatively simple, rapid and cost effective (Bornet et al., 2002).

Inter Simple Sequence Repeats (ISSR)

The Inter Simple Sequence Repeats (ISSR) was first developed by Zietkiewicz et al., (1994) to rapidly differentiate between closely related individuals. This technique is known with other names such as, MP-PCR (microsatellite-primed PCR) or ISA (Inter-SSR Amplification) or RAMP (Randomly Amplified Microsatellite Amplification) marker system. ISSR technique is also a very simple, fast, cost-effective, highly discriminative and reliable (Kumar et al., 2016).

The ISSR combines the advantages of AFLP markers and SSR with the convenience of RAPD. It requires very small amount of template DNA and is convenient in result recording and highly reproducible. ISSR markers are known to be more reproducible than RAPD markers and they have been successfully applied to the study of genetic diversity in plants (Bornet et al., 2002). First, it permits detection of polymorphisms in microsatellites and intermicrosatellites loci without previous knowledge of the DNA sequence. Microsatellite regions are abundant throughout the eukaryotic genome, which are highly polymorphic in length and are interspersed. Secondly, ISSR is informative about many loci and are suitable to discriminate closely related genotype variants. And lastly, ISSR markers constitute discrete markers suitable in the DNA fingerprinting (El-Azeem et al., 2016; Wang et al., 2017).

Genetic diversity is the basis of plant breeding, so understanding and assessing it is important for crop management, crop improvement by selection, use of crop germplasm, detection of genome structure, and transfer of desirable traits to other plants (Sohrabi et al., 2012, Williams et al., 1990, Welsh & McClelland, 1990).

Materials And Methods

Molecular studies using RAPD and ISSR were carried out for the assessment of genetic stability and variability of in vivo mother plant and in vitro regenerated plantlets of Aloe vera L.

Plant Material

The leaves of conventionally grown Aloe vera plants were collected from nursery of H.E.J Research Institute of Chemistry and leaves of in vitro propagated plants were taken from the growth room of Plant Tissue Culture and Biotechnology Wing.

DNA extraction

The Aloe vera plant DNA was extracted using (CTAB) method. Plant material was grinded using liquid nitrogen. Liquid nitrogen is frequently used in DNA extraction protocols as it facilitates grinding of the tissue by turning it into solid form and has an additional advantage of maintaining low temperature. Many small laboratories of developing countries faced the problem of unavailability of liquid nitrogen. Storage and maintenance of liquid nitrogen is also difficult. The highly versatile Cetyl trimethyl ammonium bromide (CTAB) method (Sambrok, et al., 1989) is a standard method for the extraction of DNA from various plant materials. The availability of high-quality genomic DNA is a crucial prerequisite for molecular genetic analysis of crops. There are three main contaminants associated with plant DNA that can cause considerable difficulties when conducting PCR experiments: polyphenolic compounds, polysaccharides and RNA. Extraction of intact, high molecular-weight DNA that can support PCR, genomic blot analysis, fingerprinting and other molecular analysis is a challenge when the plant tissue is rich in polysaccharides, secondary metabolites or polyphenolics.

It is necessary to isolate good quality DNA that is relatively free from many contaminants found in plant cells. Many plant species contain characteristically high amounts of proteins, polysaccharides, polyphenols (Angeles et al., 2005) and other secondary metabolites, substance known for binding firmly to nucleic acids during DNA extraction and interfering with subsequent reactions (Ribeiro and Lovato, 2007).

Total cellular/genomic DNA isolation was performed by classical cetyltrimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1987) with some modifications. To obtain good quality DNA, the utilization of fresh and young leaf tissue is ideal (Ribeiro and Lovato, 2007). Latex gloves were worn constantly during the DNA extraction to minimize the risk of contamination of samples with nucleases from the skin and to protect the skin from hazardous chemicals such as phenol, chloroform and liquid nitrogen. Goggles were worn during the procedure to protect eyes especially during grinding. About 1.5 g of Aloe vera leaf samples were cut into small pieces and ground to fine powder in pre-chilled pestle and mortar. Fine powder was suspended/homogenized into 2 mL preheated (65°C) CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0 and 2% mercaptoethanol), kept on hot plate at 600C for 40 minutes to homogenize and poured into 15 mL falcon tubes. The homogenate was incubated at 65°C for 1 hour in shaking water bath after then 2 ml of fresh chloroform: iso-amyl alcohol (24:1, v/v) was added and gently mixed by inverting the tubes for 50 times until there was no interface. After then mixture was centrifuged (Eppendorf® Centrifuge 5804R) at 10,000 rpm for 10 min at 4^0C to separate the phases. The supernatant/aqueous phase was collected in other falcon tubes and 1:1 ratio of ice cold isopropanol was added to precipitate the DNA. Isopropanol was mixed by shaking the tube, when precipitation was not obvious the samples were kept at -20^0C for an hour in freezer. After 1 hour the DNA was spooled with the help of wire loop. In case, if DNA was not spool able then it was again centrifuged at 10,000 rpm for 23 min at 4^0C. The DNA made

pellet at the bottom of the tube. The spooled DNA was put into 5 mL washing buffer for 20 min, DNA was collected from washing buffer, air dried and then resuspended in TE buffer into labeled microfuge tubes. The DNA dissolved/eluted into 1 mL TE buffer, vortexed and left for overnight. Upon extraction of total genomic DNA of each cultivar was given an extraction code and transferred to eppendroff tubes.

Next day DNA samples were vortexed, added 10 µl RNase A and incubated at 370C for 20 min. After incubation the samples were centrifuged (Biofuge pico Heraeus) at 13,000 rpm for 10 min. The supernatant was extracted; finally DNA quantification was detected on 0.8% agarose gel (Wang et al., 2012) and by UV spectrophotometer. All the coded eppendorff tubes were kept in the freezer at – 20,. at Molecular Biology and Quality Control Laboratory, Plant Tissue Culture and Biotechnology Wing, International Center for Chemical and Biological Sciences, University of Karachi-Karachi-75270, Pakistan. The remaining leaf samples were kept in -200C freezer.

DNA quantification

The nucleic acid concentration was determined using a spectrophotometer (Schimadzu, 2000). Silica (Quartz) Ultra Micro Spectrophotometer cuvette was used for holding the samples and T.E buffer (10 mM Tris Hcl (pH 7.5), 1 mM EDTA) solution was used as a standard/blank for calibrations of the spectrophotometer at 260 nm and 280 nm. The DNA concentrations were measured in spectrophotometer using 1:1000 dilutions. Readings were recorded for each DNA sample at wavelength of 260 nm and 280 nm. An O.D. of 1 corresponded to approximately 50µg ml-1 double stranded DNA. The ratio of the readings was noted at 260 nm and 280 nm provided an estimation of the purity of nucleic acids. The readings at 260 nm were used to calculate the DNA concentration in the samples, by using the formula:

DNA concentration (µg/ml) = A260 x 50 x dilution factor (100)

An optical density value 1.0 corresponds to approximately 50 µg/mL for double stranded DNA. Pure samples of DNA have ratios of 1.8 to 2.0 at O.D. 260/O.D. 280 and values of less than this for DNA samples of lower purity. If there is contamination of protein or phenols the ratio will be significantly lower than 1.8 and higher ratio signify contamination by RNA (Sambrook et al., 1989). Besides this conventional method of DNA estimation was performed by gel on 0.8% with λ DNA. The amount of plant genomic DNA was estimated by visual comparison of band intensity between λ DNA and plant DNA under following ethidium bromide staining. DNA samples were diluted in T.E buffer to a working concentration of approximately 10 ng μl^{-1}.

Primers

The sequences of the different primers RAPD (octamer oligonucleotide) used in this study are given in Tables 10b.

Molecular Marker

Molecular size of PCR amplified products were estimated by using 1kb (Invitrogen) and 100 bp (Fermentas) DNA ladder.

DNA Amplification by RAPD-PCR

PCR amplification was carried out in Hi-Temp 96 wells Thermal cycler (Master Cycler, Eppendorf, Germany). The following concentration of PCR reagents (Fermentas, USA) were used for 25 µl final reaction volume (Table 1 a). The cycling parameters consisted of 30 min denaturation at 94^{0}C, followed by 45 cycles of 30 sec at 94^{0}C, 1 min at 32^{0}C, and 2 min at 72^{0}C (denaturation, annealing and extension).

Table 1 a: Composition of master mix for RAPD-PCR reaction

Reagents	Concentration	Volume
PCR Mastermix	2x	12.5uL
MgCl	25mM	1.5 µL
Primer	4 µM	2.0 µL
DNA template	10ng/ µl	2.5 µL
ddH_2O	-----	6.5uL
Total volume		**25.0 µl**

The reaction was finally incubated at 72^{0}C for 4 min, followed by soaking period at 4^{0}C until recovery. The lid temperature was kept constant at 109^{0}C.

Table 1 b: Primer sequence used for the RAPD-PCR reactions

No.	Primer Name	Primer Base Sequence
1.	LC-76	GTGACGTAGG
2.	LC-77	GGGTAACGCC
3.	LC-81	AGCCAGCGAA
4.	LC-83	AGCCAGCGAA
5.	LC-87	AGGTGACCGT
6.	LC-90	GTGAGGCGTC
7.	OPL-1	GGCATGACCT
6.	OPQ-12	AGTAGGGCAC

Agarose Gel Electrophoresis

Gel agarose was prepared in order to verify the presence and the size of PCR products (amplicons) and 1 Kb and 100 bp markers were used to calculate the molecular weight of the amplified PCR products. The procedure was performed as following:

Melting of the Agar

Molecular Biology grade agarose (Gene-Link and Scharlu) was used at 1 % for the separation of amplified PCR products. The agarose was suspended in the 1x TBE (Tris Borate EDTA) buffer pH 8.0 in a flask. The flask was covered with another small mouth flask as the vapors can come back again in the same flask of agarose. The solution was heated in microwave oven (Sharp 1000W/R-2197), till the agarose dissolved completely and solution become clear. During boiling the solution was gently swirled to re-suspend any settled agarose. The flask was removed carefully using thick hand gloves/protector. Then gels were allowed to cool at about 600C, as the flask can be handled comfortably. 5 µl ethidium bromide (0.05 µg/mL) was mixed properly in the agarose solution.

Pouring the Gel

The electrophoresis gel tray/plate was prepared and a comb was placed in it to make loading wells. The cooled agarose gel was poured/casted into gel tray containing a comb. The agarose should come at least half way up the comb teeth. The agarose gels were allowed to solidify for 40 min after than comb was removed.

Setting up the Gel Tank

After solidification gel was placed in the appropriate electrophoresis tank, the wells were placed to negative electrode and covered with 1X TBE running buffer. The DNA samples/ PCR products were mixed with gel loading dye (0.25% (w/v) Bromophenol Blue, 40% (w/v) sucrose, 0.1mM tris, and 0.05 mM EDTA) in a 1:4 ratio. The bromophenol blue dye give density to PCR product as the sample can sink to the bottom of the well properly and PCR product 8 µl plus 2 µl of loading dye mixed properly and loaded into each well created by comb. One (1) Kb ladder (Life TechnologiesTM) used as molecular size marker or gene ruler on each gel.

Running and Analyzing the Gel

The lid was placed on the tank and the electrode leads were connected to the power supply. The electrophoresis was generally conducted by using large submarine units (Thermo EC-320) at 60V for 2hr (Thermo EC, EC-250-90). Then gels were removed from the electrophoresis tank and placed in gel documentation system (UV TechTM, UK), images of DNA bands obtained and photographed. The photos were saved in specific file till to be used. The PCR reactions were repeated twice for the confirmation of produced results.

Results And Discussion

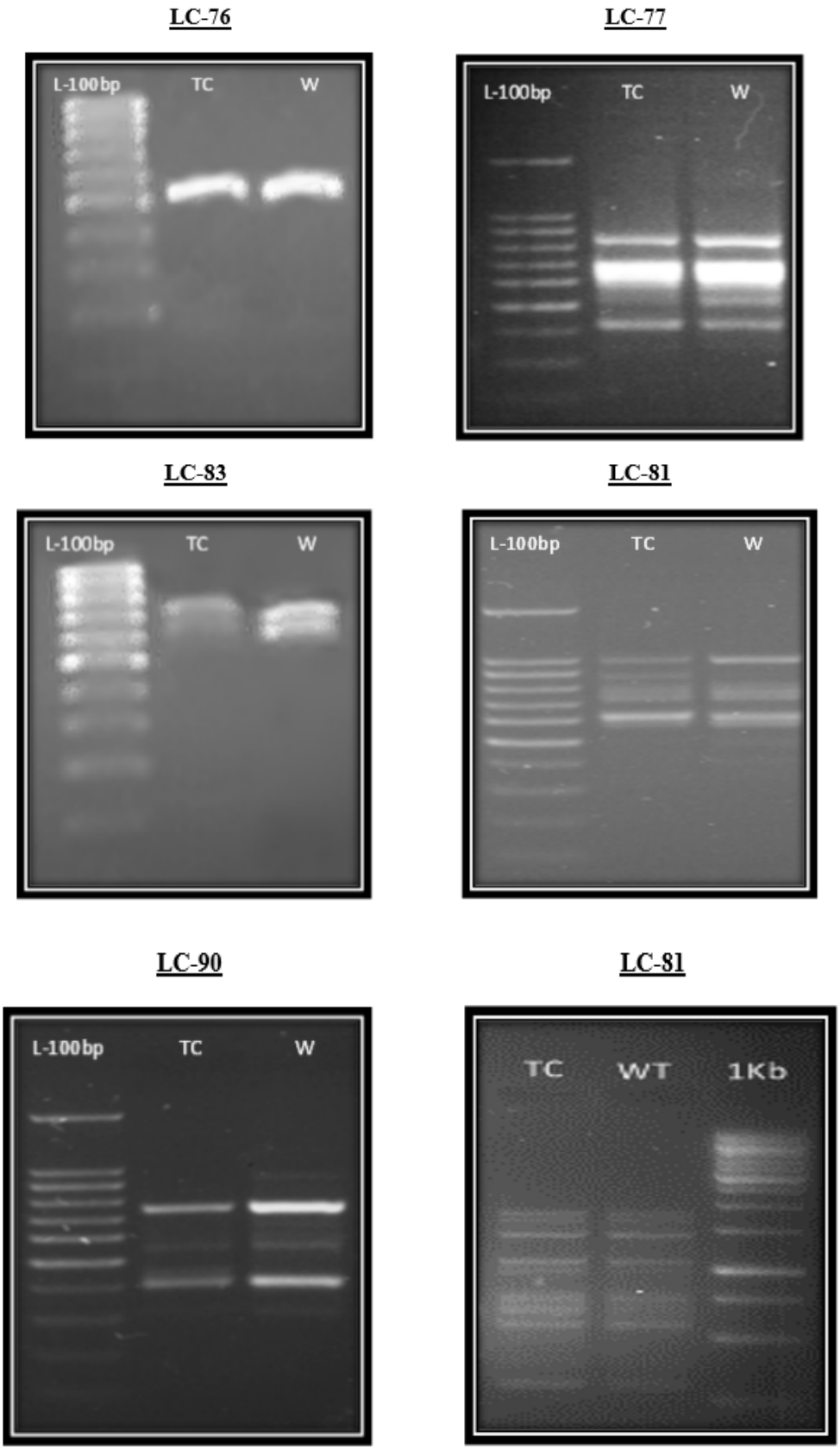
Evaluation of Genetic Stability of Aloe vera plants by RAPD Marker Assay Among multilocus markers RAPD and ISSR techniques have been widely used to detect, evaluate and identify changes in the DNA sequence caused by somaclonal variation in Aloe (Alagukannan & Ganesh, 2016; Kumar et al., 2016). Molecular characterization using PCR based technique Random Amplified polymorphic DNA (RAPD) was conducted on conventionally grown (wild type) and tissue cultured Aloe vera plants to compare their genetic similarity/diversity. Eight primers were selected for the analysis as they were reported to produce polymorphic amplicons as shown in Figure 1 and Table 2a.

The primer LC-76 generated total 2 monomorphic bands with DNA templates of both conventional and tissue cultured plants and showed 0% polymorphism. Both Primers LC-77 and LC-83 produced total 4 bands with 3 monomorphic and 1 polymorphic band, each exhibiting 25% polymorphism. Primer LC-81 produced all 4 monomorphic bands and no polymorphism was seen. LC-87 produced total 5 band with 3 monomorphic and 2 polymorphic amplicons, showing 40% polymorphism. While LC-90 produced total 5 amplicons with 4 monomorphic and 1 polymorphic bands giving 20% polymorphism. OPL-1 generated 5 monomorphic bands with no polymorphism. OPQ-12 produced highest number of bands (9), of which 6 were monomorphic and 3 polymorphic exhibiting 66.6% polymorphism. Thus with both types of Aloe vera DNA templates, all the eight RAPD primers generated 4.75 amplicons on the average, of which 3.75 were monomorphic and 1 polymorphic, showing 22% polymorphism.

The DNA bands produced by individual wild type and in vitro cultured plants with all the eight bands was also calculated (Table 2a). With all the eight primers tested, conventionally grown Aloe vera plants produced total 29 band, showing minimum 1 and maximum 6 bands. However DNA templates of tissue cultured plants generated 32 bands with all the primers giving minimum 1 and maximum 9 bands.

Rathore and his coworkers in 2011, published the first report on the comparison of genetic stability/instability of tissue-culture Aloe vera plants exploring two regeneration systems. No polymorphism was observed in regenerates produced from direct regeneration system from axillary buds, whereas 80% polymorphism was observed in plants produced through indirect regeneration via intermediate callus phase. Our results are in harmony with the previous findings (Das et al., 2016), as our Aloe vera plant regenerants produced through indirect regeneration via callus phase, have exhibited polymorphism in a range of 20-33% (Table 2b).

Figure 1: RAPD profiles of Aloe vera wild type soil grown and tissue cultured plants with primers LC-76, LC-77, LC-81, LC-83, LC-87, LC-90, OPL-1 and OPQ-12.



OPQ-12 **OPL-1**

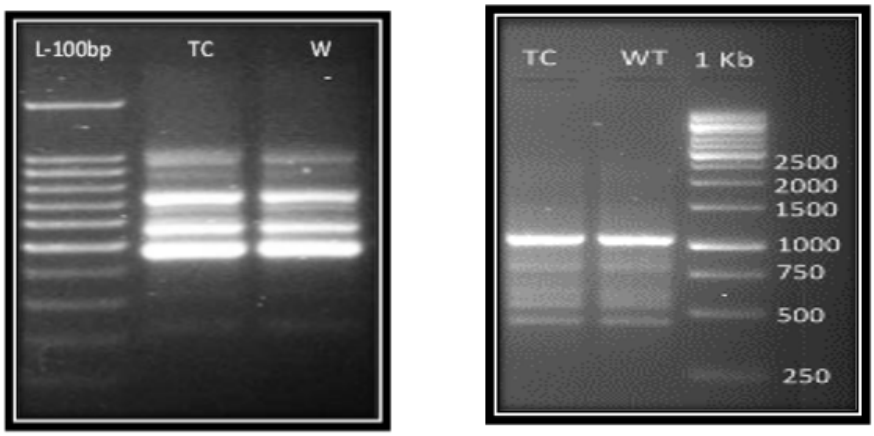


Table 2a: Total number of bands calculated from tissue cultured and wild type Aloe vera plant with eight RAPD primers

Primers	Tissue Culture	Wild type	Total Bands
LC-76	01	01	02
LC-77	03	04	07
LC-81	02	02	04
LC-83	04	03	07
LC-87	03	04	07
LC-90	05	04	09
OPL-1	05	05	10
OPQ-12	09	06	15
Total	32	29	59

Table 2b: Polymorphism percentage between tissue cultured and soil grown Aloe vera plant by using 8 RAPD primers

Sequence of primer 5'----------3'	Annealing temperature	MW (µg/µmole)	GC Contents %	Total bands	Monomorphic bands	Polymorphic bands	Polymorphism %
LC-76- GTGACGTAGG	41°C	3,108.09	60	02	02	0	0
LC-77- GGGTAACGCC	41°C	3,053.05	70	04	03	01	25
LC-81- AGCCAGCGAA	41°C	3,046.07	60	04	04	0	0
LC-83- AGCCAGCGAA	41°C	3,046.07	60	04	03	01	25
LC-87- AGGTGACCGT	41°C	3,068.06	60	05	03	02	40
LC-90- GTGAGGCGTC	41°C	3,084.06	70	05	04	01	20
OPL-1-GGCATGACCT	32°C	3,028.03	60	05	05	0	0
OPQ-12-AGTAGGGCAC	32°C	3,077.08	60	09	06	03	33.3
Total				**38**	**30**	**8**	-
Average				**4.75**	**3.75**	**1**	**18**
Range				**02-09**	**02-06**	**01-03**	**20-33.3**

Evaluation of Genetic Stability of Aloe vera plants by ISSR Marker Assay

During the last decade new development in the PCR based techniques on DNA markers are used as powerful tools to validate the genetic fidelity of in vitro plants (Butiuc-Keul et al., 2016; Shahzad et al., 2017). The present study was aimed at analyzing the genetic similarity and diversity in wild type field gown and tissue cultured Aloe vera plants by using ISSR markers. ISSR is a type of molecular markers based on inter-tandem repeats of short DNA sequences. These inter repeats are highly polymorphic, even among closely related genotypes, due to the lack of functional constraints in these nonfunctioning regions. ISSR markers are simple, more reliable and proved highly efficient in analysis of genetic diversity studies (Nookaraju and Agrawal, 2012; Das et al., 2016). The analysis was carried out with 4 inter simple sequence repeat (ISSR) markers, which were screened out for their effectiveness and reproducibility. The primers successfully produced clear bands with all the DNA templates of Aloe vera (Table 3a and Figure 2) as shown below.

Figure 2: ISSR profiles of Aloe vera wild type soil grown and tissue cultured plants with primers ISSR-A, ISSR-G, ISSR-M and ISSR-O.

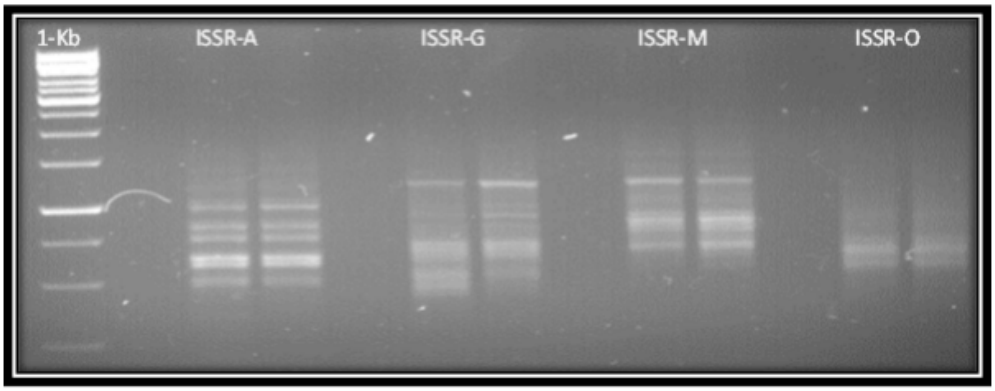


Table 3a: Total number of bands calculated from Tissue culture and Wild type Aloe vera plant with four ISSR primers

Primers	Tissue Culture	Wild Type	Total Bands
ISSR-A	06	06	**12**
ISSR-G	04	03	**07**
ISSR-M	04	04	**08**
ISSR-O	04	04	**08**
Total	**18**	**17**	**35**

Tissue cultured plants produced total 18 bands with all the primers and 17 bands of wild type plants were produced. Of all the primers tested only primer ISSR-G exhibited 25% polymorphism with 3 monomorphic and 1 polymorphic band. The other primers did not produce any polymorphic band. On the average 4.25 monomorphic band were generated with all the four markers with a range of minimum 3 and maximum 6 amplicons (Table 3b).

Combined Analysis of RAPD and ISSR Markers

In the present study polymorphism level of conventionally grown wild type and in vitro propagated Aloe vera plants was investigated using RAPD and ISSR based molecular markers (Table 4). Tissue cultured plants produced maximum number of bands (32) with all RAPD primers and 18 bands with ISSR primers. Wild type field grown Aloe vera plants produced 29 DNA bands with all primers of RAPD and 17 bands with ISSR primers. Thus total 50 DNA bands of tissue culture plants were generated from all the 12 primers of both molecular markers, whereas total 46 bands of conventionally grown plants were generated. In sum up of all the two molecular markers with both Aloe vera plants total 94 bands were produced

Table 3b: Polymorphism percentage between Tissue culture and Wild type Aloe vera plant by using 4 ISSR primers

Sequence of primer 5'---------- --3'	Annealing temperature	Total bands	Monomorphic bands	Polymorphic bands	Polymorphism %
ISSR-A- (CA)6GG	37.4^{0}C	06	06	0	0
ISSR-G -(GT)6CC	37.4^{0}C	04	03	01	25
ISSR-M- (AC)8T	37.4^{0}C	04	04	0	0
ISSR-O -(AG)8T	37.4^{0}C	04	04	0	0
Total		**18**	**17**	**01**	**25**
Average		**4.5**	**4.25**	**0.25**	**6.25**
Range		**04-06**	**03-06**	**01**	**25**

Table 4: Total number of scorable amplicons from RAPD and ISSR analysis

Molecular Markers	Tissue culture	Wild	Total
RAPD	32	29	59
ISSR	18	17	35
Total	**50**	**46**	**94**

REFERENCES

1. Alagukannan, G., & Ganesh, S. (2016). Genetic diversity in Aloe and its breeding strategies. International Journal of Farm Sciences, 6(2), 312-326.

2. Angeles, J. G. C., Laurena, A. C., & Tecson-Mendoza, E. M. (2005). Extraction of genomic DNA from the lipid-, polysaccharide-, and polyphenol-rich coconut (Cocos nucifera L.). Plant Molecular Biology Reporter, 23(3), 297-298.

3.Bornet, B., Goraguer, F., Joly, G., & Branchard, M. (2002). Genetic diversity in European and Argentinian cultivated potatoes (Solanum tuberosum subsp. tuberosum) detected by inter-simple sequence repeats (ISSRs). Genome, 45(3), 481-484.

4.Brown, D., Thorpe, T. (1984). Organization of a plant tissue culture laboratory.In: (ed.) Vasil I. Cell culture and somatic cell genetics of plants. Laboratory procedures and their applications. New York: Acad. Press; p1-12

5. Butiuc-Keul, A., Farkas, A., & Cristea, V. (2016). Genetic Stability Assessment of in Vitro Plants by Molecular Markers. Studia Universitatis Babes-Bolyai, Biologia, 61(1).

6.Das, A., Haque, S. M., Ghosh, B., Nandagopal, K., & Jha, T. B. (2016). Morphological and Genetic Characterization of Micropropagated Field Grown Plants of Aloe vera L. Plant Tissue Culture and Biotechnology, 25(2), 231-246.

7. Doyle J.J. and J.L. Doyle. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull 19:11–15.

8. El-Azeem, R. M. A., Hashem, M., & Hemeida, A. (2016). Identification and genetic similarity analysis of Date Palm (Phoenix dactylifera L.) collected from different regions in Siwa Oasis using morphological traits and molecular markers. Egyptian Journal of Genetics and Cytology, 40(2).

9. El-Mergawy, R. A. A. M., Al-Ajlan, A. M., Abdallah, N. A., Nasr, M. I., & Silvain, J. F. (2011). Determination of different geographical populations of Rhynchophorus ferrugineus (Olivier)(Coleoptera: Curculionidae) using RAPD-PCR. International Journal of Agriculture and Biology, 13(2), 227-232.

10. Goulao, L., & Oliveira, C. M. (2001). Molecular characterisation of cultivars of apple (Malus× domestica Borkh.) using microsatellite (SSR and ISSR) markers. Euphytica, 122(1), 81-89.

11. Gupta, P. K., Kumar, J., Mir, R. R., & Kumar, A. (2010). 4 Marker-Assisted Selection as a Component of Conventional Plant Breeding. Plant breeding reviews, 33, 145.

12.Kumar, R., Naik, P. K., Kumar, A., Aggarwal, H., Kumar, A.,

& Chhokar, V. (2016). A combined approach using RAPD, ISSR and bioactive compound for the assessment of genetic diversity in Aloe vera (L.) Burm. f.

13- Mihalte, L., Sestras, R. E., Feszt, G., & Tamas, E. (2011). Assessment of Genetic Variation on Four Genera of'Cactaceae'Using Taxonomic, Cytological and Molecular Markers Methods. Plant omics, 4(3), 142.

14. Nayanakantha, N. M. C., Singh, B. R., & Gupta, A. K. (2010). Assessment of genetic diversity in Aloe germplasm accessions from India using RAPD and morphological markers. Ceylon Journal of Science (Biological Sciences), 39(1).

15. Nookaraju, A., & Agrawal, D. C. (2012). Genetic homogeneity of in vitro raised plants of grapevine cv. Crimson Seedless revealed by ISSR and microsatellite markers. South Afri. J. of Bot., 78: 302-306.

16. Rathore, M. S., Chikara, J., Mastan, S. G., Rahman, H., Anand, K. G. V., & Shekhawat, N. S. (2011). Assessment of genetic stability and instability of tissue culture-propagated plantlets of Aloe vera L. by RAPD and ISSR markers. Appl. Biochem. and Biotec., 165 (56): 1356-1365.

17. Ribeiro, R. A., & Lovato, M. B. (2007). Comparative analysis of different DNA extraction protocols in fresh and herbarium specimens of the genus Dalbergia. Genet. Mol. Res, 6(1), 173-187.

18. Sambrook J, Fritsch EF and Maniatis T (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Nova York.

19. Shahzad, A., Parveen, S., Sharma, S., Shaheen, A., Saeed, T., Yadav, V & Upadhyay, A. (2017). Plant Tissue Culture: Applications in Plant Improvement and Conservation. In Plant Biotechnology: Principles and Applications (pp. 37-72). Springer Singapore.

20. Sohrabi, M., Rafii, M. Y. Hanafi, M. M., Akmar, A. S. N. and Latif, M. A. (2012). Genetic Diversity of Upland Rice Germplasm in Malaysia Based on Quantitative Traits. The Sci. World J., Article ID 416291, 9 pages.

21. Tharachand, C., Selvaraj, I., & Mythili, M. N. (2012). Molecular markers in characterization of medicinal plants: An overview. Research in Plant Biology, 2(2).

22. Tran, T. M. H. 2005. Genetic variation in cultivated coffee (Coffea arabica L.) accessions in northern New South Wales, Australia. M.Sc. Thesis. Southern Cross University. pp.19.

23. Wang, B.S., Huang, G.J., Tai, H.M., Huang, M.H., (2012). Antioxidant and antiinflammatory activities of aqueous extracts of Schizonepeta tenuifolia Briq. Food Chem. Toxicol. 50, 526–531.

24. Wang, A., Zhou, G., Lin, C., Wang, B., Huang, X., deng, Y., & Zhang, R. (2017). Genetic diversity study of Brassica campestris L. ssp. chinensis Makino based on ISSR markers. Caryologia, 70(1), 48-54.

25. Welsh, J. and M. McClelland. (1990). Fingerprinting genome using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.

26.Williams, J. G., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acids Res., 18(22): 6531-6535.

27- Zietkiewicz, E., Rafalski, A., & Labuda, D. (1994). Genome fingerprinting by simple sequence repeat (SSR)-anchored polymerase chain reaction amplification. Gen., 20(2): 176-183.