Molecular markers in clonal fidelity assessment of plants

Potshangbam Nongdam and Thoungamba Amom

Department of Biotechnology, Manipur University, Canchipur, Imphal-795003, Manipur, India

Abstract— ***Somaclonal variation may appear in regenerants due to various factors associated with the in vitro culture conditions. Checking genetic stability of the in vitro clones is obligatory if we are to propagate true- to- type quality planting materials. Different molecular markers have been employed for clonal fidelity testing of in vitro propagated plants. These are DNA markers which are PCR based such random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter- simple sequence repeats (ISSR), simple sequence repeat (SSR) and start codon targeted (SCoT) among others. The present mini review focuses on the description of varied molecular markers, their methodology, strengths and limitations as well as their applications in genetic homogeneity testing of in vitro regenerants belonging to different plant species.***

Keywords: *clonal fidelity, DNA markers, regenerants, somaclonal variation*

I. INTRODUCTION

Plant tissue culture is one of the most important techniques employed to conserve effectively rare and endangered plants by *in vitro* propagating them rapidly in large scale [1]. However, *in vitro* culture conditions might alter the genetic makeup of plant tissue which may lead to somaclonal variation. Appearance of somaclonal variation is a major concern during micropropagation as true-to-type quality planting materials may not be generated as desired. Checking genetic stability of micropropagated plants becomes obligatory and molecular markers play prominent role in detecting genetic variation in clones. Various molecular markers have been employed for detection of genetic variation in micropropagated plants. These DNA markers are generally independent of environmental factors and more numerous than phenotypic characters thereby providing a clear indication of underlying variation in genome of *in vitro* clones. The present mini review highlights some of the important DNA markers which are presently used in clonal fidelity assessment of several plant species.

II. MOLECULAR MARKERS

A. Restriction fragment length polymorphism (RFLP)

This technique is based on generation of different size DNA fragments due to digestion by restriction enzymes. Genomes of individuals belonging to same species will differ in DNA fragment production after restriction digestion as a result of point mutation, insertion/deletion, translocation, inversion and duplication. RFLP steps involve the cutting of genomic DNA by restriction enzyme generating different sized DNA fragments. Restriction enzymes are bacterial enzymes that recognize specific sequences in DNA and cut double stranded DNA at such specific sequences. The greatest resolution in RFLP is obtained by using 4-base pair cutters because such sites are much abundant in the genome. 4 cutters restriction enzymes however produce fragments that are too small to be resolved by agarose gel. DNA fragments after separation are transferred to nitrocellulose membrane by Southern blot technique [2]. Fragments of interest are identified by hybridizing with complementary radioactive labeled probe and specific banding pattern visualized after autoradiography. The results obtained with RFLP technique depend on both restriction enzymes and number of probes. Each enzyme cleaves a segment of genomic DNA at different points and each different probe hybridizes with a different set of genomic DNA fragments [3].

The Southern blot technique involved in RFLP can be replaced by PCR method if the flanking sequences of the target locus are known and specific primers can be designed to amplify RFLP containing region using PCR method. The DNA fragment length polymorphism can be detected through the electrophoresis of PCR products.

RFLP exhibits high reproducibility, codominant inheritance, easy data transferability between laboratories, provides locus specific markers, easy to score due to large size difference. The limitations

of this technique are time consuming, requirement of large amount of high quality DNA, expensive radioactive probes, involvement of tedious Southern blotting method and requirement of prior sequence information for developing radio labeled probe.

B. Random Amplified Polymorphic DNA (RAPD)

Random amplified polymorphic DNA (RAPD) is one of the most important DNA markers used in molecular biology for plant species identification and genetic variability studies. Williams and his co-workers and Welsh and McClelland developed this highly useful molecular technique independently in 1990. This technique involves random PCR amplification of several complementary regions of genomic DNA using single arbitrary primer producing many discrete DNA products. Discrete DNA products which are produced after PCR amplification under appropriate reaction condition are separated on agarose gel. The separated amplification products are visualized as RAPD bands after autoradiography. The sequences of primer are arbitrarily chosen but they should fulfill the two criteria of having a minimum of 40% GC content to withstand 72ºC temperature and absence of palindromic sequence [4]. The single species of primer having length of 10 nucleotide bases anneals to the genomic DNA at two different sites on the opposite strands of DNA template. When the two sites are within an amplifiable distance of less than 4000 bases and are in inverted orientation, PCR can amplify the DNA segment lying between the primer binding sites. The single primer will have several complementary binding sites in the genome which in turn will generate several discrete DNA segments of different sizes as PCR amplification products. The number of amplified product will depend on length and base composition of the primer, genome size and its complexity. Polymorphism generated by RAPD markers is due to changes in nucleotide sequence at the primer binding site or chromosomal changes such as insertion/deletion in the amplified regions which alter the size or prevent the successful amplification of a target DNA. Many factors influencing the reproducibility of RAPD reaction are reported. They are the quality and quantity of DNA template, PCR buffer, concentration of magnesium chloride, primer to template ratio, annealing temperature, Tag DNA polymerase brand or source and thermal cycle brand [5]. Despite concern, the reproducibility of RAPD markers can be achieved through choice of an appropriate DNA extraction protocol to remove contaminants by optimizing parameters [6]. Obtaining reliable results will largely depend on standardizing these conditions or identifying combinations of conditions that give consistent results even when variations in the key variables are encountered. In order to obtain good consistent RAPD profiles, standard primer, nucleotide and magnesium concentration, exact reproduction of temperature, cycling conditions and DNA polymerase type and activity are essential. RAPD markers are dominant markers, highly abundant, randomly distributed throughout the genome, require little amount of DNA and are quick and easy to assay. They are used in determining varietal and taxonomic identity, paternity, kinship relationship and hybrid identification, clonal fidelity assessment, in the study of germplasm diversity, interspecific gene flow, genome mapping, population and evolutionary genetics

C. Inter Simple Sequence Repeat (ISSR)

Zietkiewicz at al. [7] reported this useful technique for the first time and it involved amplification of DNA segments present at an amplifiable distance between two identical microsatellite repeat regions oriented in opposite directions. Microsatellites of di, tri, tetra or penta-nucleotide core sequences are used as primers to amplify mainly inter simple sequence repeats of different sizes. The unanchored or anchored primers can be used for amplification but anchored primers are mostly used with 1 to 4 degenerate bases extending to flanking sequences at either 3´ or 5´[8]. Unanchored primer can anneal anywhere on the repeat regions of template DNA leading to slippage and ultimately smear formation whereas anchored primers can anneal at specific regions on the template DNA and produce clear bands [9]. ISSR primers are longer (15-35 mers) and require higher annealing temperature resulting in higher stringency. But the annealing temperature is dependent on GC content of primer. The PCR amplified products of 200 to 2000bp long are separated by agarose gel electrophoresis and the resulting ISSR banding profile can be visualized through autoradiography. There is no requirement of prior sequence information for template DNA for generating ISSR polymorphism. They are simple, randomly distributed in genome, exhibiting mostly dominant inheritance pattern. The main limitations of ISSR marker are low reproducibility problems and homology of co-migrating amplification products [10].

D. Amplified Fragment Length Polymorphism (AFLP)

This method utilizes both RFLP and PCR by ligating primer recognition sequences to DNA fragments produced through restriction digestion [11].The simultaneous screening of representative DNA regions distributed randomly throughout the genome is the important feature of AFLP. Mutation at restriction site, insertions, duplications or deletions inside amplification fragments and mutations of the sequences flanking the restriction sites may produce polymorphisms of AFLP. The DNA quality can be slightly compromised for AFLP analysis but DNA should be free from restriction enzymes and PCR inhibitors. The genomic DNA might be digested by restriction enzymes which are the combination of rare cutter (Eco RI or Pstl) and frequent cutter (Msel or Taql). The double stranded oligonucleotide adaptors which do not bear initial restriction sites after ligation are developed and ligated to both ends of the fragments to give known sequence for PCR. PCR is first done with primer combinations containing a single base pair extension while final amplification is carried out by using primer pairs with upto 3 base pair extension. AFLP fragments which are produced after gel electrophoresis can be visualized with UV autoradiography. Fragment length differences of less than ten nucleotides are difficult to score on agarose but the maximum resolutions of single-nucleotide length difference are given by polyacrylamide gel electrophoresis. AFLP analysis is tedious process and not so convenient to perform as RAPD but is more effective in polymorphism detection than RAPD and RFLP.

The method is highly reproducible and AFLP markers have high genomic abundance with generation of many informative bands per reaction. The ability of RFLP to determine high polymorphism in a single reaction makes this as one of the most sought after molecular tools for genetic analysis [12]. The drawbacks are the requirement of high molecular weight purified DNAs, possibilities of co-migrating non-homology fragments belonging to different loci. In case of an insertion between two restrictions sites, the amplified DNA fragment results in increased band size which will be interpreted as the loss of a small band and gain of a larger band.

E. Simple Sequence Repeat (SSR)

The simple sequence repeats are present over a hundred repeats of a 1-4 nucleotide sequences in the genome of all eukaryotes. In higher organisms, the simple repetitive DNA sequences are of three types viz., satellite, minisatellite and microsatellites DNAs according to their size [13]. The DNA consisting of long repeats of about 100-1000s are called satellite DNA while tandem repeats having shorter repeat units of 10-100s are described as minisatellite DNA and very short repeat units (1-4) are termed as microsatellite. Primers can be designed for the nucleotide sequences flanking these short repeats and polymorphism can be generated because of the fragment variation in the length of repeat regions present in genome. During DNA synthesis, if slipped-strand mispairing occurs within a microsatellite array, a gain or loss may have happened depending on whether the newly synthesized DNA chains loops out or the template chain loops out, respectively [14]. So, SSR allelic differences are the results of variable number of repeat units within the microsatellite structure. A popular example of a microsatellite is $(CA)_n$, n=10 to 100 and these markers often present in higher level of inter and intra-specific polymorphism particularly when tandem repeats number is ten or greater [15]. Di-nucleotide repeat arrays occur much frequently than tri and tetra-nucleotides so that it is easier to run combinatorial screen for them but gives fewer stutter bands [16]. PCR for SSR runs in the presence of forward and reverse primers that anneal at 5' and 3' ends of DNA templates. The amplification products are either separated by polyacrylamide gels and polymorphism detected with $AgNO_3$ staining or agarose gel electrophoresis through autoradiography. Because of production of high polymorphism even among the genetically much closed lines, SSRs have become markers of choice in most areas of molecular genetics.

They are codominant markers with high genomic abundance and reproducibility, require small amount of DNA, can be easily automated for high through put screening and excellent for studies of population genetics and mapping [17]. The main limitation of SSR marker is the difficulty of cloning and sequencing the flanking regions. The tedious process of developing protocols for cloning and sequencing of SSRs has to be performed for each plant species under study.

F. Start Codon Targeted (SCoT)

This marker system is based on the short conserved regions flanking the ATG start codon in plant genome and using 18-mer primers with annealing temperature at 50°C [18]. The same single primer is used in PCR as forward and reverses primer as in RAPD and ISSR markers. These markers are

generally reproducible but the length and annealing temperature are not the most important factors determining reproducibility [19]. These dominant markers are used for plant genetic analysis, quantitative trait loci (QTL) mapping and bulk segregate analysis[18]. The PCR amplified fragments are subjected to general agarose gel electrophoresis to separate the fragments and bands are visualized through autoradiography. SCoT markers have been used successfully for genetic diversity studies and clonal fidelity assessment in several plants [20]–[22].

III. CLONAL FIDELITY ASSESSMENT

The emergence of somaclonal variants genetically different from elite mother plant is the main limitation in producing quality plants through micropropagation. Soma clones may appear because of genotype, exposure to various growth factors, gene mutation, *in vitro* stress and different culture conditions [23]. Performing genetic fidelity assessment is highly essential to authenticate the quality of *in vitro* regenerated plants for commercial applications. Clonal fidelity assessment had been successfully done in many micropropagated plants using varied DNA markers like AFLP, SCoT, ISSR, RAPD and SARP [8, 24–28].

Ray et al. [29] used RAPD and ISSR markers to reveal three somaclonal variants each from micropropagated banana cultivars Robusta and Giant Governor. 21 RAPD and 12 ISSR primers generated 5330 RAPD and 2741 ISSR fragments respectively in micropropagated plants. The ISSR markers detected more polymorphism than RAPD markers. The percentage of polymorphic loci generated by RAPD and ISSR were 1.75 and 5.08 respectively for Robusta and 0.83 and 5.0 for Giant Governor respectively.

Goyal et al. [26] successfully assessed the clonal fidelity among the *in vitro* regenerated *Dendrocalamus strictus* using RAPD and ISSR markers. The RAPD and ISSR primers produced 58 and 66 amplicons respectively and the banding profiles for the markers were monomorphic indicating genetic similarity between the propagated bamboos. Lakshmanan et al. [30] employed 30 RAPD and 5 ISSR primers to test the genetic stability of micropropagated plants of *Musa acuminate*. The primers of both the markers generated a total of 5088 clear, distinct and reproducible bands with banding pattern of each primer showing similar profile with field grown mother plant.

Lata et al. [31] assessed the genetic stability of Cannabis plants which were *in vitro* propagated from synthetic seeds using ISSR markers. 9 ISSR primers produced 40 distinct and reproducible bands and all the ISSR profiles obtained from the regenerated plants were monomorphic. This confirmed the genetic stability of clones and their similarity to the mother plant. ISSR markers were also used by Wang et al. [32] to evaluate the genetic identity of *in vitro* propagated plants of *Clivia miniata*. 20 ISSR primers generated 137 clearly identifiable bands. All the primers produced monomorphic banding patterns except for primer 7 and 11 which were polymorphic. The similarity coefficient generated from ISSR data analysis of 137 reproducible bands revealed similarity range of 90.5 to 100% between regenerated plants and mother plant. Dendrogram showed the clustering of regenerated plants into one major cluster at 96.5% level.

Muniswamy et al. [28] *in vitro* generated commercial hybrid coffee cultivars of Robusta coffee through somatic embryogenesis. The genetic stability test was conducted for somatic embryogenesis derived plants and mother plant using sequence selected amplified polymorphism (SRAP) markers. The 24 SRAP primers yielded a total of 153 clear, distinct and reproducible bands of variable size. 9 primers produced banding profiles similar to mother and *in vitro* regenerated plants. 95% genetic similarity between the somatic embryogenesis derived plants and mother was disclosed through cluster analysis thus revealing high degree of genetic fidelity.

IIczuk and Jacygard [33] studied the genetic stability of micropropagated *Cornus alba* using ISSR and RAPD markers. The RAPD marker analysis produced a total of 197 and 199 monomorphic bands for *C. alba* cultivars- Aurea and Elegantissima respectively. The ISSR marker on the other hand generated 184 and 187 bands for Aurea and Elegantissima respectively and amplified fragment size produced ranged from 200 to 250bp. RAPD and ISSR profile showed no DNA polymorphism between the mother and *in vitro* propagated plants thus confirming the clonal fidelity of the regenerated plants.

Bhattacharya et al. [34] evaluated the genetic profile of 13 randomly selected *in vitro* clones of *Cymbopogon pendulus* using ISSR markers. Out of the 17 ISSR primers tested, only 6 primers

produced 32 clear and reproducible bands with band size ranging from 200 to 1000bp. Four clones showed minor degree of polymorphism with ISSR profiles of BG-01 and BG-05 primers. The remaining 9 clones showed high degree of genetic similarity with similar ISSR banding profiles. Thus majority of regenerants maintained clonal fidelity except for a few which showed slight variation as depicted from the marker profiles and low similarity coefficient values.

Antony et al. [35] employed targeted region amplification polymorphism (TRAP) and Start codon targeted polymorphism (SCoT) DNA markers to identify somaclonal variation in cryopreserved *in vitro* regenerated propagules of *Dendrobium* Bobby Messina. The TRAP primers produced polymorphic bands in cryopreserved PLBs. Four SCoT primers (S26, S32, S33 and S36) yielded reproducible and clear bands with size ranging from 500 to 3000 bp. The four primers exhibited polymorphism for both the cryopreserved and non-cryopreserved PLBs of *Dendrobium* Bobby Messina. Bhatia et al. [36] tested the genetic fidelity of *in vitro* propagated *Gerbera jamesonii* using RAPD and ISSR markers. 12RAPD and 10 ISSR primers yielded 54 and 55 distinct clear and reproducible bands respectively. The banding profiles generated in micropropagated plants using ISSR and RAPD markers are all monomorphic and similar to those of mother plant. The mother and *in vitro* propagated plants showed closed similarity matrix values indicating 100% similarity thus confirming the true-to –type nature of *in vitro* clones.

Nookaraju and Aggarwal [37] used ISSR and SSR markers to check the genetic homogeneity of the *in vitro* raised grapevine cv. Crimson seedless. 22 ISSR primers produced 134 distinct and band classes with a total of 3216 scorable bands while 5 SSR primers generated 288 scorable bands. The genetic profiles of two marker systems revealed high genetic uniformity between *in vitro* propagated plants and mother plant. Alizadeh and Singh [38] employed RAPD and ISSR markers to examine clonal fidelity of three different grape rootstock genotypes. 12 RAPD and 10 ISSR primers produced a total of 1914 and 1980 scorable bands respectively. RAPD and ISSR primers did not show any polymorphism with the micropropagated plants. The markers profiles of the two marker systems revealed high uniformity and monomorphic patterns. Jaccard's similarity coefficient for both markers in mother and *in vitro* propagated plants were estimated to be 1.00 though three sets of genotypes were clustered into main groups with similarity coefficient of 0.53 (RAPD) and 0.63 (ISSR). The DNA marker analysis showed high genetic uniformity among the micropropagated plants.

CONCLUSION

Micropropagation techniques are one of the best alternatives to effective conservation of rare and endangered plants by *in vitro* propagating them rapidly at large scale. But genetic differences or soma clonal variation may be observed in tissue cultured plants due to host of factors like explant source, genotype, mutations, *in vitro* stress, hormonal effects and other culture conditions. Testing the genetic homogeneity between *in vitro* clones and elite mother plant is highly essential if we are to produce genetically stable high quality planting materials. The DNA markers play an important role as they can be effectively utilized to evaluate successfully the clonal fidelity of different micropropagated plants. The marker technology helps in large scale production and propagation of highly stable and genetically identical clones similar to elite mother plant.

REFERENCES

[1] P. Nongdam and L. Tikendra, "Establishment of an efficient *in vitro* regeneration protocol for rapid and mass propagation of *Dendrobium crysotoxum* Lindl. using seed culture," Article ID740150, The Scientific World Journal, 2014.

[2] E. M. Southern, "Detection of specific sequences among DNA fragments separated by gel electrophoresis," J. Mol. Biol., vol. 98, no. 3, pp. 503–517, Nov. 1975.

[3] M. Pérez de la Vega, "Biochemical characterization of populations," in Plant Breeding, Dordrecht: Springer Netherlands, 1993, pp. 184–200.

[4] J. G. Williams, A. R. Kubelik, K. J. Livak, J. A. Rafalski, and S. V. Tingey, "DNA polymorphisms amplified by arbitrary primers are useful as genetic markers.," Nucleic Acids Res., vol. 18, no. 22, pp. 6531–5, Nov. 1990.

[5] K. Wolff, E. D. Schoen, and J. P.-V. Rijn, "Optimizing the generation of random amplified polymorphic DNAs in chrysanthemum," Theor. Appl. Genet., vol.

86, no. 8, pp. 1033–1037, Sep. 1993.

[6] M. R. Micheli, R. Bova, E. Pascale, and E. D'Ambrosio, "Reproducible DNA fingerprinting with the random amplified polymorphic DNA (RAPD) method," Nucleic Acids Res., vol. 22, no. 10, pp. 1921–2, May 1994.

[7] E. Zietkiewicz, A. Rafalski, and D. Labuda, "Genome Fingerprinting by Simple Sequence Repeat (SSR)-Anchored Polymerase Chain Reaction Amplification," Genomics, vol. 20, no. 2, pp. 176–183, Mar. 1994.

[8] M. Gupta, Y.-S. Chyi, J. Romero-Severson, and J. L. Owen, "Amplification of DNA markers from evolutionarily diverse genomes using single primers of simple-sequence repeats," Theor. Appl. Genet., vol. 89–89, no. 7–8, pp. 998–1006, Dec. 1994.

[9] M. Pradeep Reddy, N. Sarla, and E. A. Siddiq, "Inter simple sequence repeat (ISSR) polymorphism and its application in plant breeding," Euphytica, vol. 128, no. 1, pp. 9–17, 2002.

[10] K. Semagn, Å. Bjørnstad, and M. N. Ndjiondjop, "An overview of molecular marker methods for plants," vol. 5, no. 25, pp. 2540–2568, 2006.

[11] M. Lynch and B. Walsh, "Genetics and Analysis of Quantitative Traits," 1998

[12] P. Vos *et al.*, "AFLP: a new technique for DNA fingerprinting.," Nucleic Acids Res., vol. 23, no. 21, pp. 4407–14, Nov. 1995.

[13] Hancock, "Microsatellites and other simple sequences: genomic context and mutational mechanisms," 1999.

[14] G. Levinson and G. A. Gutman, "Slipped-strand mispairing: a major mechanism for DNA sequence evolution.," Mol. Biol. Evol., vol. 4, no. 3, pp. 203–21, May 1987.

[15] D. C. Queller, J. E. Strassmann, and C. R. Hughes, "Microsatellites and kinship," Trends Ecol. Evol., vol. 8, no. 8, pp. 285–288, Aug. 1993.

[16] N. Diwan and P. B. Cregan, "Automated sizing of fluorescent-labeled simple sequence repeat (SSR) markers to assay genetic variation in soybean," Theor. Appl. Genet., vol. 95, no. 5–6, pp. 723–733, Oct. 1997.

[17] D. B. Goldstein and C. Schlötterer, "Microsatellites : evolution and applications." Oxford University Press, 1999.

[18] B. C. Y. Collard and D. J. Mackill, "Conserved DNA-Derived Polymorphism (CDDP): A Simple and Novel Method for Generating DNA Markers in Plants," Plant Mol. Biol. Report., vol. 27, no. 4, pp. 558–562, Dec. 2009.

[19] A. M. Gorji, P. Poczai, Z. Polgar, and J. Taller, "Efficiency of Arbitrarily Amplified Dominant Markers (SCOT, ISSR and RAPD) for Diagnostic Fingerprinting in Tetraploid Potato," Am. J. Potato Res., vol. 88, no. 3, pp. 226–237, Jun. 2011.

[20] D.-L. Guo, J.-Y. Zhang, and C.-H. Liu, "Genetic diversity in some grape varieties revealed by SCoT analyses," Mol. Biol. Rep., vol. 39, no. 5, pp. 5307–5313, May 2012.

[21] P. Bhattacharyya, S. Kumaria, S. Kumar, and P. Tandon, "Start Codon Targeted (SCoT) marker reveals genetic diversity of Dendrobium nobile Lindl., an endangered medicinal orchid species," Gene, vol. 529, no. 1, pp. 21–26, Oct. 2013.

[22] J. Zhang, X. Zhao, W. Xie, and Y. Wang, "Potential of Start Codon Targeted (SCoT) markers to estimate genetic diversity and relationships among Chinese Elymus sibiricus accessions," Molecules, vol. 20, no. 4, pp. 5987–6001, 2015.

[23] T. P. Devi, D. Kamil, R. Mehndiratta, N. Prabhakaran, and R. S. Toppo, "Molecular and morphological diversity of rhizoctonia bataticola causing dry root rot disease from India Molecular and Morphological Diversity of Rhizoctonia bataticola Causing Dry Root Rot Disease from India," J. Pure Applied Microbio., vol. 10, no. 4, pp. 2735-2745, Dec. 2016.

[24] B. Kour, G. Kour, S. Kaul, and M. K. Dhar, "*In Vitro* Mass Multiplication and Assessment of Genetic Stability of *In Vitro* Raised *Artemisia absinthium* L. Plants Using ISSR and SSAP Molecular Markers," Adv. Bot., vol. 2014, pp. 1–7, Aug. 2014.

[25] S. Thakur *et al.*, "Extensive sequence variation in rice blast resistance gene Pi54 makes it broad spectrum in nature," frontiers in plant sci., May 2015.

[26] A. K. Goyal, S. Pradhan, B. C. Basistha, and A. Sen, "Micropropagation and assessment of genetic fidelity of Dendrocalamus strictus (Roxb.) nees using RAPD and ISSR markers.," 3 Biotech, vol. 5, no. 4, pp. 473–482, Aug. 2015.

[27] A. Ilczuk and E. Jacygrad, "In vitro propagation and assessment of genetic stability of acclimated plantlets of Cornus alba L. using RAPD and ISSR markers.," In Vitro Cell. Dev. Biol. Plant, vol. 52, no. 4,

pp. 379–390, 2016.

[28] B. Muniswamy, B. Kosaraju, M. K. Mishra, and R. Yenugula, "Field Performance and Genetic Fidelity of Micropropagated Plants of Coffea canephora (Pierre ex A. Froehner)," Open Life Sci., vol. 12, no. 1, pp. 1–11, Feb. 2017.

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

[30] V. Lakshmanan, S. R. Venkataramareddy, and B. Neelwarne, "Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR markers," Electron. J. Biotechnol., vol. 10, no. 1, Jan. 2007.

[31] H. Lata, S. Chandra, N. Techen, and M. A. Elsohly, "Molecular analysis of genetic fidelity in *Cannabis sativa L* . plants grown from synthetic (encapsulated) seeds following in vitro storage," Biotechnol Lett., vol. 33, pp 2503-2508, July 2011.

[32] Q.-M. Wang *et al.*, "Regeneration of Clivia miniata and assessment of clonal fidelity of plantlets," Plant Cell, Tissue Organ Cult., vol. 109, no. 2, pp. 191–200, May 2012.

[33] A. Ilczuk and E. Jacygrad, "In vitro propagation and assessment of genetic stability of acclimated plantlets of Cornus alba L . using RAPD and ISSR markers," In Vitro Cell. Dev. Biol., pp. 379–390, 2016.

[34] S. Bhattacharya, T. K. Bandopadhyay, and P. D. Ghosh, "Somatic embryogenesis in Cymbopogon pendulus and evaluation of clonal fidelity of regenerants using ISSR marker," Sci. Hortic. (Amsterdam)., vol. 123, no. 4, pp. 505–513, Feb. 2010.

[35] J. J. J. Antony, R. A. Shamshir, R. Poobathy, B. L. Chew, and S. Subramaniam, "Somaclonal variations were not induced by the cryopreservation: Levels of somaclonal variations of in vitro and thawed protocorms of Dendrobium Bobby Messina analysed by SCoT and TRAP DNA markers," South African J. Bot., vol. 100, pp. 148–157, 2015.

[36] R. Bhatia, K. P. Singh, T. R. Sharma, and T. Jhang, "Evaluation of the genetic fidelity of in vitro-propagated gerbera (Gerbera jamesonii Bolus) using DNA-based markers," Plant Cell, Tissue Organ Cult., vol. 104, no. 1, pp. 131–135, Jan. 2011.

[37] A. Nookaraju and D. C. Agrawal, "Genetic homogeneity of in vitro raised plants of grapevine cv . Crimson Seedless revealed by ISSR and microsatellite markers," South African J. Bot., vol. 78, pp. 302–306, 2012.

[38] M. Alizadeh and S. K. Singh, "Molecular assessment of clonal fidelity in micropropagated grape (*Vitis spp*.) rootstock genotypes using RAPD and ISSR markers," Iranian J. Biotechnol., vol. 7, no. 1, Jan. 2009.