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Assessment of clonal fidelity in micropropagated horticultural plants

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ABSTRACT

With the advancements made in plant tissue culture techniques, it has now become possible to regenerate various horticultural species in vitro as micropropagation protocols for commercial scale multiplication are available for a wide range of crops. Clonal propagation and preservation of elite genotypes, selected for their superior characteristics, require high degree of genetic uniformity amongst the regenerated plants. The occurrence of subtle somaclonal variation is a drawback for both in vitro cloning as well as germplasm preservation. Therefore, it is of immense significance to assure the genetic uniformity of in vitro raised plants at an early stage. Several strategies have been followed to ascertain the genetic fidelity of the in vitro raised progenies comprising morpho-physiological, biochemical and cytological approaches. However, each tool has its own merits and limitations. These approaches are mainly based on characters, which can be affected by the in vitro *manipulation, environment and types of plant tissue; hence it is no easy to differentiate clonal fidelity with a high probability. Presently, DNA-based molecular markers have acted as versatile tools in various fields of biology. Application of such DNA-based markers offers several advantages over traditional aforementioned markers, as they provide data that can be analysed objectively. In the present paper, strategies to ascertain and confirm genetic fidelity in a variety of in vitro raised plantlets are reviewed.*

Keywords: micropropagation, somaclonal variation, Clonal fidelity, molecular markers.

INTRODUCTION

The goal of vegetative propagation is to reproduce progeny plants identical in genotype to a single source plant. The biological process is known as "cloning" and the resulting population of plants is called a "clone". The importance of clones to horticulture and other aspects of agriculture can hardly be overemphasized. This is not only because of benefits but also because of the problems that must be controlled to make the process successful. The *in vivo* clonal propagation of plants is often difficult, expensive and even unsuccessful. Tissue culture methods offer an alternative means of plant vegetative propagation. Clonal propagation through tissue culture (popularly known as micropropagation) can be achieved in a short time and space [79]. The uniformity of individual plants within a clone population is a major advantage of clonal cultivars in commercial production. However, it is well known now that genetic variations occur in undifferentiated cells, isolated protoplasts, calli, tissues and morphological traits of

regenerated plants. Recent advances have revealed that cell or tissue cultures undergo frequent genetic changes (polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplifications and mutations) and that these are also expressed at biochemical or molecular levels. Variants selected in tissue cultures have been referred to as "calliclones"(from callus cultures)[100] or "protoclones"(from protoplast cultures)[97].In 1981, Larkin and Scowkraft coined a general term "somaclonal variation" for plant variants derived from any form of cell or tissue cultures. Somaclonal variation in regenerated plants is generated during *in vitro* culture stage and particularly during de-differentiation. This is accompanied by increased frequency of chromosomal abnormalities with time in culture. Somaclonal variation is uncontrollable and unpredictable in nature and most variation is of no apparent use.
The commercialization of micropropagation technology in horticultural crops began with orchids in 1970s, which was later witnessed in many ornamentals, fruits, spices and plantation crops as well. At present, micropropagated plants, in various crops such as strawberry, papaya, banana, grapes, pineapple, tomato, cucumber, watermelon, rhododendron etc., are preferred over plants propagated through conventional means. However, ever since the first formal report of morphological variants in sugarcane plants produced *in vitro* in 1971, several instances of somaclonal variations have been reported in different horticultural crops. The notable example could be banana in which occurrence of off-types from tissue-cultured plantlets ranged from 6 to 38% in Cavendish

cultivars [87]; however, it could be as high as 90%[102]. This has presented a grave crisis for micropropagation programs, where production of true-to-type plant material is of utmost importance. Hence, in commercial micropropagation, it is mandatory to regularly check the clonal fidelity or genetic uniformity of the micropropagated plantlets to confirm their quality (true-to-the-type), (Fig. 1) in order to avoid variations of any kind, which if induced may multiply very fast and lead to loss of the chief characteristics of the parent genotypes [2]. Of late, somaclonal variations are getting proper attention as they are serious threat to the genetic integrity of regenerated plants. The lull in noticing such variations may be owed to three factors like, i) unreasonably fervent belief in the remote chance or non-existence of the occurrence of genetic variability in micropropagation systems; ii) absence of molecular tools to ascertain variations precisely and rapidly and iii) delayed observation of a large number of field-grown plants of species, which come in flowering 5±20 yr after transplantation to the field [76].Somaclonal variation can be identified employing an array of techniques with their own strengths and limitations. Therefore, the choice of detection method depends largely on the task at hand.

GENETIC FIDELITY AND ITS SIGNIFICANCE IN PLANT TISSUE CULTURE

Genetic fidelity is the maintenance of the genetic constitution of a particular clone through its life span [14].The occurrence of cryptic genetic defects arising *via* somaclonal variation in the regenerants can seriously limit the broader utility of micropropagation systems [76].

Clonal propagation and preservation of elite genotypes, selected by their superior characteristics, require high degree of genetic uniformity amongst the regenerated plants. The occurrence of somaclonal variation is a drawback for both *in vitro* cloning as well as germplasm preservation method. Therefore, it is of immense significance to assure the genetic uniformity of *in vitro* raised plants at an early stage. Many phenotypic variations reported in the regenerated fruit crop plants were extensively reviewed by Hammerschlag (1992) [32]. Important changes include growth rate and reproductive apparatus modification (sterility, precocious flowering and flower abnormalities, internodal length), and leaf (variegation, albino, chlorotic, etc.), thornlessness, isoenzymatic activity changes, and increased salt resistance, fruit color, etc. An increased ploidy level has been reported in kiwi subcultures and in grape.

IDENTIFICATION OF VARIATIONS IN TISSUE CULTURE

Both genetic and epigenetic alterations are associated with *in vitro* propagation, which may have phenotypic consequences, and are collectively called somaclonal variation [29].A wide variety of tests/tools are available for the detection and characterization of somaclonal variants which are primarily based on the differences in morphological traits, cytogenetical analysis for the determination of numerical and structural variation in the chromosomes, biochemical and molecular DNA markers. The best test for assessing somaclonal variation is to fruit out the plants and conduct an extensive horticultural evaluation, which is unfortunately a long-term endeavor with woody fruit crops, particularly [28].Every tool has its own advantages and disadvantages in assessment of the variations (Table 1), which govern their use for limited or large scale application. The choice of technique for any given application depends upon the material used and the nature of the question being addressed [42].

MORPHOLOGICAL OBSERVATIONS

This is one of the oldest and most extensively used methods to detect variants in *in vitro* culture. Somaclonal variants can effortlessly be distinguished on the basis of characters like variation in plant height, canopy structure, leaf morphology, pigmentation abnormality etc[35]. However, morphological traits used for phenotypic characterization are limited in number, and are developmentally regulated and very often affected by environmental factors, which in many instances may not reflect the true genetic composition of a plant [16]. In addition, the detection by phenotypic identification of off-types among micropropagated plants is time-consuming, and is of less significance to perennial crops, where many observations are recorded until maturity. Besides, all the genetic changes may not be reflected in the observed phenotypic changes. And sometimes, the observed variations may lead to improper interpretation as well.

Variations observed could also be due to physiological changes induced by *in vitro* culture environment. Such variations are temporary and may disappear once the culture conditions are withdrawn. However, in some instances, the altered phenotype may continue over a longer duration and may be passed from one cell generation to another. Such variations, which are also ultimately reversible, and are not sexually transmitted, are caused by epigenetic changes. Epigenetic control of gene expression can be defined as somatically or meiotically heritable changes in gene expression, which is liable to be reversible and is not due to sequence modification. Consequently, epigenetic aspects of somaclonal variation involve mechanisms of gene silencing or gene activation, which are not attributed to chromosomal aberrations or sequence change. Epigenetic changes brought about by *in vitro* propagation could be exhibited as a result of activation of quiescent loci or as epimutation of loci sensitive to chromatin-level control of expression. Genetic and epigenetic changes in tissue culture can be distinguished from each other, at least, on four counts such as frequency of occurrence, nature of change, Stability of change in somatic lineages and sexual transmission of the change [9].

Kosky *et al.* (2006) [44]. observed that banana hybrid 'FHIA-18' (AAAB) regenerated from somatic embryos showed similar characteristics to plants propagated from shoot tip cultures both in the acclimatization stage and in field as well. Both groups of plants obtained from in vitro cultures were significantly different to the plants obtained from suckers during the flowering period of the mother plants, which was shortened by two months. The greater plant height and diameter of the pseudostem in the plants coming from somatic embryos and shoot tip was due to the effect of in vitro culture. During the second cycle of evaluation, the plants coming from the three propagation methods had similar growth habits without significant differences in the majority of the morphological parameters evaluated. These results confirm that the difference obtained during the first cycle between the distinct populations is attributed to temporary changes. The original characteristics of the cultivar were evident from the second cycle of culture.

In many instances, it has been stated that the regenerated plants did not present somaclonal variations. This also could be improper owing to fact that the recessive mutations are not expressed in the R_0 plants (plants regenerated from tissue culture), which are generally heterozygous for the mutation. Therefore, plants which look normal could segregate abnormal plants in the R_1 or R_2 generations (successive sexual generations of R_0) depending upon the autogamous or allogamous nature of the species.

Examples of application of such traits in different horticultural crops are presented in Table 2.

Sometimes to improve efficiency of detection of variants morphologically, physiological characters are also taken in to account. Recently, Perez *et al.* (2009)[73] recorded physiological parameters to distinguish between two pineapple somaclones (P3R5 and Dwarf) derived from in vitro culture of the donor cv. Red Spanish Pinar. The stoma diameter, number of stomata per square millimiter, diameter of leaf vascular tissue, thickness of the leaf aquiferous parenchyma, and thickness of the leaf photosynthetic parenchyma were measured. The photosynthetic rate, the transpiration rate, the water use efficiency, the internal leaf CO_2 concentration, and the chlorophyll pigment contents were recorded as well.

CYTOLOGICAL ANALYSIS

Genetic composition of an organism changes with the numerical/structural chromosomal variations and with the changes in content of RNA/DNA. Analyses of chromosomes as well as other nuclear components variations have been used by many workers to determine variations in *in vitro* regenerants [25].Cytological analysis based on observation of conventionally stained, condensed somatic chromosomal aberration using light microscopy, oil

immersion or other complex microscopy techniques have been used by several workers [64,86], however, it has severe limitations such as time-consuming and often cumbersome particularly when chromosome number is high or difficult to observe due to their small size[76]. Presently, the conventional method of counting and examining chromosomes has been replaced by precise and sensitive, advanced technology, flow cytometry [19],which involves preparation of aqueous suspensions of intact nuclei whose DNA is stained using a DNA fluorochrome, followed by suspending them in a stream of fluid and passing them by an electronic detection apparatus. This technique has been employed successfully for the detection of somaclonal variants in strawberry[69]. At present, flow cytometric analysis has increasingly been the favoured method for the determination of DNA ploidy and nuclear DNA content in plants [1].Another technique, cytophotometric analysis is used for the estimation of the 4C nuclear DNA content. This involves collection of tissue sample followed by fixing over night in propionic acid/ethanol (1:3). This treatment is followed by hydrolysis in 1 N HCl (v/v) at 60 ^{0}C. The tissues are then washed in distilled water, stained in Schiff's reagent and squashed with 45% acetic acid. The DNA content of nuclei is measured with micro spectrophotometer at 550 nm. This technique has been used for the detection of variants in *Curcuma aromatica* [64] and turmeric (*Curcuma longa* L.) [68].

Hoa and Deng (2002) [39] noticed significant chromosomal variations in embryogenic callus of Anliucheng sweet orange (*Citrus sinensis* Osbeck) when subcultured and preserved for a long time. Cytological observation revealed a variety of mitotic irregularities underlying the occurrence of chromosomal variations. Randomly amplified polymorphic DNA (RAPD) analysis was also carried out to detect DNA sequence variation in regenerated plants derived from the embryogenic callus. However, the RAPD technique failed to detect these chromosomal variations. The frequency of other genome rearrangements, if any, was too low to detect using RAPD analysis. This instance gives the credibility to cytological techniques. On the contrary, some workers have other story to narrate. In their study, Fiuk *et al.* (2010) [25]. used cytometric as well as molecular techniques to verify genetic uniformity among somatic embryo-derived plantlets of *Gentiana pannonica* Scop. Cytometric analysis of regenerants revealed absence of chromosomal changes and alterations in ploidy. However, reverse phase high pressure liquid chromatography detected higher levels of methylation in regenerated plants than those of control plants. These changes were further investigated using a quantitative molecular marker-based approach. This revealed that numerous tissue culture-induced variations, (epi)mutations, were observed, including sequence variation and changes in methylation patterns.

Absence of any morphological and cytological variations among *in vitro* raised plants does not necessarily implies that (epi)genetic differences among them do not exist. Moreover, the most probable changes may result from point mutations, small indeletions or alterations in methylation patterns; therefore, more appropriate molecular analytical tools are required to delve into complicated aspects of tissue culture-derived variation [109].

ISOZYME MARKERS

It is well known that morphological variation is a result of biochemical variation which is expressed as variation among proteins. Among protein (direct products of genes)-based markers, isozyme electrophoresis has been recognized as a promising technique to determine the genetic variation, if any, among in vitro-derived plants. Isozymes or isoenzymes are protein markers, which differ in amino acid sequence but catalyze the same chemical reaction. The technique is based on the principal that allelic variation exists from many different proteins. Therefore, the proteins/isozymes (product of genes) from two different alleles of the same gene would not migrate to the same location in a polyacrylamide gel due to difference in their electrophoretic mobility. As a result, the discriminating property of proteins and isozymes is a function of the number of polymorphic loci that can be identified and genetically characterized in an organism [38].Lassner and Orton (1983) [51] proposed the use of isozyme markers for studying somaclonal variation. Since then, proteins and isozymes such as peroxidase, malate dehydrogenase and superoxide dismutase have been extensively used to study variation in different horticultural crops [7].This technique is useful for detecting differences among individuals hence changes of isoenzyme patterns could reflect gene expression, or even gene changes [114]. Variations in somaclones can be detected by analysing clones for protein and enzyme polymorphism. The variation characterized has been summarized into three categories: (i) altered electrophoretic mobility; (ii) loss/gain of protein bands; and (iii) altered level of specific protein. To identify isozymes, a crude protein extract is made by grinding plant tissue with an extraction buffer, and the components of extract are separated according to their charge by gel electrophoresis. All the proteins from the tissue are present in the gel, so that individual enzymes must be identified using an assay that links their function to a staining reaction. Isozymes were found to be useful markers for somaclonal variation among regenerants from apple rootstocks [57].

and date palm [6].Isozyme polymorphism was observed among regenerants. Based on banding patterns, rootstocks and regenerants could be distinguished in apples [57].

Mandal *et al.* (2001) [56]. also reported the limitation of salt soluble peptide as molecular markers for varietal identification of banana cultivars. As a result, at present, this technique is no longer considered as a reliable tool to detect variants and it has now been replaced largely by other more sensitive and precise alternative techniques like molecular markers.

Feuser *et al.* (2003) [23]. evaluated pineapple plantlets (cv. 'Amarelinho') micropropagated by stationary and temporary immersion systems in terms of genotypic fidelity by isozyme and RAPD markers. Neither isozymes (average 0.67%) nor RAPDs (average 7.5%) alone detected significant differences between the two micropropagated systems. However, when combined isozymes and RAPDs data more somaclonal variants were detected in stationary than temporary immersion, with RAPDs revealing more variation than isozymes. This particular example suggests the importance of isozymes as supplementary to DNA markers for better detection of variants in *in vitro* regenerants rather abandoning it completely.

MOLECULAR DNA MARKERS

Molecular assessment of clonal fidelity of in vitro raised plantlets

Morphological characters, chemical composition and cytological information have been used over the years for classification of plants. These techniques have certain limitations as they could be influenced by environmental and developmental effects. The presence of a low level of polymorphism prompted workers to rely more on DNA markers [46]. Molecular markers have widespread applications in management of genetic resources and in crop improvement. They can be used for i) Characterization of germplasms, ii) Assessment of genetic diversity, iii)Validation of genetic relationships, iv) Marker-assisted selection (MAS), v) Varietal identification and clonal fidelity testing [4].

Random Amplified Polymorphic DNA (RAPD)

Since first reported, RAPD markers [117,118]. have been used in numerous scientific studies in plants and animals [17].Arbitrarily Primed PCR (AP-PCR) and RAPD are essentially the same technique. Regardless of the acronym used, the techniques are similar in that single primers, of a known sequence, are used in a polymerase chain reaction to amplify random segments of genomic DNA.

The ability of RAPDs to produce multiple bands using a single primer means that a relatively small number of primers can be used to generate a very large number of fragments. These fragments are usually generated from different regions of the genome and hence multiple loci may be examined very quickly [20].This marker system was used in many different applications involving the detection of DNA sequence polymorphisms, mapping different types of populations, isolation of markers linked to various traits or specific targeted intervals and other applications such as variety identification and analysis of parentage[27]. The RAPD technology however, has some limitations. RAPD markers are in general dominant thereby they have lower information content than codominant markers in the linkage analysis of F_2 populations [118].

RAPD-PCR was chosen as a method to screen grapevine protoclones (regenerants from protoplast) for somaclonal variation [92]. Determination of genetic stability of micropropagated plants of ginger (*Zingiber officinale*) using RAPD markers reported by Rout *et al.* (1998). [85] There are limited reports available to assess the genetic stability of tree species. For example, the genetic stability of *in vitro* propagated pines has been very sparsely studied through isozyme and RAPD markers [34]. Rani *et al.* (1995) [77] reported the usefulness of RAPD markers for genetic analysis in micropropagated plants of *Populus deltoids* Marsh. RAPD markers have been used successfully to assess genetic stability among somatic embryos in spruce species. Similarly this technology has also been used for detection of somaclonal variations in tissue cultured dried date palm plants [88]. Rani *et al.* (2001) [78] studied RAPD finger-printing diagnostics for genetic integrity of enhanced axillary branching derived plants of 10 forest tree species.

Palombi and Damiano (2001) [71] used different molecular markers; RAPDs and SSRs to investigate clonal stability in micropropagated Kiwifruit (*Actinidia deliciosa* A. Chev.). The results indicated that when tissue culture procedure induces genetic variability its detection depends on the choice of tools for DNA analysis. It is in relation with different polymorphism capability detectable by molecular markers.

Devarmath *et al.* (2007) [18] considered RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* (Assam, India type). Molecular analysis of genetic stability in micropropagated apple rootstock MM 106 has been demonstrated by Modgil *et al.* (2005). [62] Singh *et al.* (2005) [98] studied genetic uniformity of micropropagated Pusa Urvashi plantlets, a newly released grape cultivar employing RAPD analysis.

To verify genetic stability Martins *et al.* (2004) [59] compared RAPD and ISSR patterns of almond plantlets obtained after 4 and 6 years of *in vitro* multiplication. Their results suggest that the culture conditions used for axillary branching proliferation are appropriate for clonal propagation of almond clone VII, as they do not seem to interfere with the integrity of the regenerated plantlets. Khawale *et al.* (2006) [50] reported the application of RAPD analysis using 30 decamer primers for adjudging clonal fidelity in the *in vitro* propagated grape cv. Perlette plants. Long-term micropropagated shoots of *Pinus thunbergii* Parl. and banana [50] have been subjected to RAPD and ISSR analyses. In both reports, the authors concluded that micropropagated plants were genetically stable. The typical significant RAPD studies associated with clonal fidelity are shown in Table 3.

Inter simple sequence repeat (ISSR)

Inter simple sequence repeat technique is a PCR-based method, which involves amplification of DNA segment present at an amplifiable distance in between two identical microsatellite repeat regions oriented in opposite direction. The technique uses microsatellites, usually 16 to 25 bp long, as primers in a single primer PCR reaction targeting multiple genomic loci to amplify mainly the inter-SSR sequences of different sizes. The microsatellite repeats used as primers can be di-nucleotide, tri-nucleotide, tetranucleotide or penta-nucleotide. The primer used can be either unanchored [30] or more usually enchored at 3' or 5' and with 1 to 4 degenerate bases extended into the flanking sequences [120].

The ISSR technique combines most of the benefits of AFLP and microsatellite analysis with the universality of RAPD. ISSRs have high reproducibility possibly due to the use of longer primers (16- to 25-mers) as compared to RAPD primers (10-mers) which permits the subsequent use of high annealing temperature (45 to 60^0C) leading to higher stringency [80]. ISSRs segregate mostly as dominant markers following simple Mendelian inheritance [30,116]. However, they have also been shown to segregate as co-dominant markers in some cases thus enabling distinction between homozygotes and heterozygotes [116,89]. Inter simple sequence repeat (ISSRs) have emerged as an efficient and effective tool for clonal fidelity and genome mapping.

In order to assess the feasibility of ISSR primers as markers for genomic instability, Leroy and Leon (2000) [52] conducted a study of DNA stability in cauliflower callus. According to these authors, ISSRs can conveniently detect and measure common genetic events underlying plant genomic instability. These include deletions, amplifications, translocations, insertions, recombination or chemical alterations. Their results indicated that instability occurred in an early step in the process of callogenesis.

A comparison between RAPD and ISSR molecular markers in detecting genetic variation in kiwifruit (*Actinidia deliciosa* A. Chev) carried out by Palombi and Damiano (2001). [71] Both DNA-based techniques were able to amplify all of the genotypes, but only SSR markers could detect genetic variation induced in micropropagated plants of cv. Tomuri.

Inter simple sequence repeat (ISSR) marker assay was also employed to validate the genetic fidelity of *Swertia chirayita* plantlets multiplied *in vitro* by axillary multiplication up to 42 passages. The results confirmed the clonal fidelity of the tissue culture raised plantlets and corroborated the fact that axillary multiplication is the safest mode for multiplication of true-to-type plants of *S. chirayita*.

Molecular analysis of genetic stability in long-term micropropagated shoots of banana using RAPD and ISSR carried out by Lakshmanan *et al.* (2007) [50]. A large number of micropropagated plantlets of banana that were developed from axillary shoot bud explants over a 10 years period were screened for their genetic stability. According to these workers, this is the first report on the use of genetic markers to establish genetic fidelity of long-term micropropagated banana using RAPD and ISSR markers. A list of crop in which ISSR markers have been used is presented in Table 4.

Amplified Fragment Length Polymorphism (AFLP)
A recent approach by Vos *et al*.(1995) [113] known as AFLP, is a technique based on the detection of genomic restriction fragments by PCR amplification and can be used for DNAs of any origin or complexity. The fingerprints are produced, without any prior knowledge of sequence, using a limited set of generic primers. The number of fragments detected in a single reaction can be 'tuned' by selection of specific primer sets. AFLP technique is reliable since stringent reaction conditions are used for primer annealing. This technique thus shows an ingenious combination of RFLP and PCR techniques and is extremely useful in detection of polymorphism between closely related genotypes. AFLP, has now become a preferred technique as it combines the reliability of RFLP with the efficiency of RAPD [113].

Owing to its high reliability, RFLP marker has been utilized extensively for ascertaining the clonal fidelity of micropropagated plantlets [96,43].

In a molecular study, aided by basic phytochemical (dominant alkaloid) and cytological data (chromosome counts), Carolan et al., (2002) [13] wished to develop a marker system capable of identifying the genetic constitution of in vitro-generated Papaver plants and assessing the stability and quality of repeatedly subcultured cell lines. Although, regenerated plants exhibited morphological and phytochemical characteristics dissimilar to those of their source material, but the loss in genetic uniformity assessed by AFLP was not due to somaclonal variation occurring during the *in vitro* culture process but it was due to hybrid origin of seeds used for *in vitro* culture initiation. A list of crop in which AFLP markers have been used is presented in Table 5.

Simple sequence repeat (SSR)
Microsatellites (Litt and Luty, 1989) [53], also known as simple sequence repeats (SSRs) [106], short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs) [60], are the smallest class of simple repetitive DNA sequences. SSR allelic differences are, therefore, the results of variable numbers of repeat units within the microsatellite structure. The repeated sequence is often simple, consisting of two, three or four nucleotides (di-, tri-, and tetra-nucleotide repeats, respectively). One common example of a microsatellite is a dinucleotide repeat (CA)n, where n refers to the total number of repeats that ranges between 10 and 100. These markers often present high levels of inter- and intra-specific polymorphism, particularly when tandem repeats number is ten or greater [75]. Microsatellite loci are more common in some organisms than in others, and screening may produce few useful loci in some species[93].The efficiency of microsatellite marker development depends on the abundance of repeats in the target species and the ease with which these repeats can be developed into informative markers. Microsatellites can serve as highly sensitive markers for monitoring genetic variation that may signal potential deleterious mutations during in vitro culture, because they reflect a relatively high rate of mutation and corresponding degree of genetic variability [55].

Microsatelites also proved to be a useful tool for screening somaclonal variation in *Q. suber* somatic embryos and their derived plantlets [55] and for monitoring somatic mutation in long-term storage of silver birch plants [86]

Efficacy of marker system
The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics. The presence of various types of molecular markers, and differences in their principles, methodologies, and applications require careful consideration in choosing one or more of such methods. No molecular markers are available yet that fulfill all requirements needed by researchers. According to the kind of study to be undertaken, one can choose among the variety of molecular techniques, each of which combines at least some desirable properties [93]. DNA-based markers are a more attractive means for examining clonal fidelity of micropropagated plants since they are more informative and are not developmentally regulated, but techniques such as RAPD suffer from a lack of reproducibility [82]. Furthermore, RAPDs are dominant diallelic markers; thus, individual parental alleles cannot usually be differentiated by these markers in diploid organisms. Therefore, dominant markers, including amplified fragment length polymorphisms (AFLPs), are not quite informative enough for examining somaclonal variation. The sensitivity, reproducibility, co-dominance and strong discriminatory power of microsatellite DNA/SSR (simple sequence repeat) markers [80] make them particularly suitable for detecting somaclonal variation, but their application in the study of somaclonal variation has been rather quite limited [116].

The technique which is capable of higher resolution and elimination of faint bands (while scoring), reduces the percentage of false negative but not affects the number of false positives and are thus ideal for determining clonal fidelity. The reliability and efficiency of markers in detecting large genomic rearrangements greatly vary with the kind of marker used.

The variations due to genetic or epi-genetic factors are very likely to be reflected in the banding profiles developed by employing different marker systems[65]. These differences could possibly be due to the high melting temperature for the ISSR primers which permits much more stringent annealing conditions and consequently more specific and reproducible amplification. Devarumath *et al*. (2007) [18] also revealed that ISSR fingerprints detected more polymorphic loci than RAPD fingerprints.

Despite the wide range of methods available, to date none of the techniques can guarantee the identification of a single random mutation/point mutation in the genome, as required for the identification of random mutants and somaclonal variants [42].Approaches aimed to detect differences of this magnitude require specific modifications in the existing techniques. This is important because some of the applied techniques study a random part of the genome (RAPDs, AFLPs, etc.) while others are designed only to check repetitive sequences (SSRs, VNTRs, ribosomal DNA probes, etc.) [108]. The potential application of two recent methods has been discussed by Karp (2000) [42]. These are based on the detection of changes known to be induced at high frequency in tissue culture: AFLPs with methylation-sensitive enzymes and detection transposon insertional polymorphisms. Moreover, it is amply clear that no single technique being ideal or sufficient, taken alone, for the assessment of somaclonal variation; therefore, a combination of several techniques should be used to evaluate the micropropagated plants [72]. A list of crop in which a combination of markers has been used is presented in Table 6.

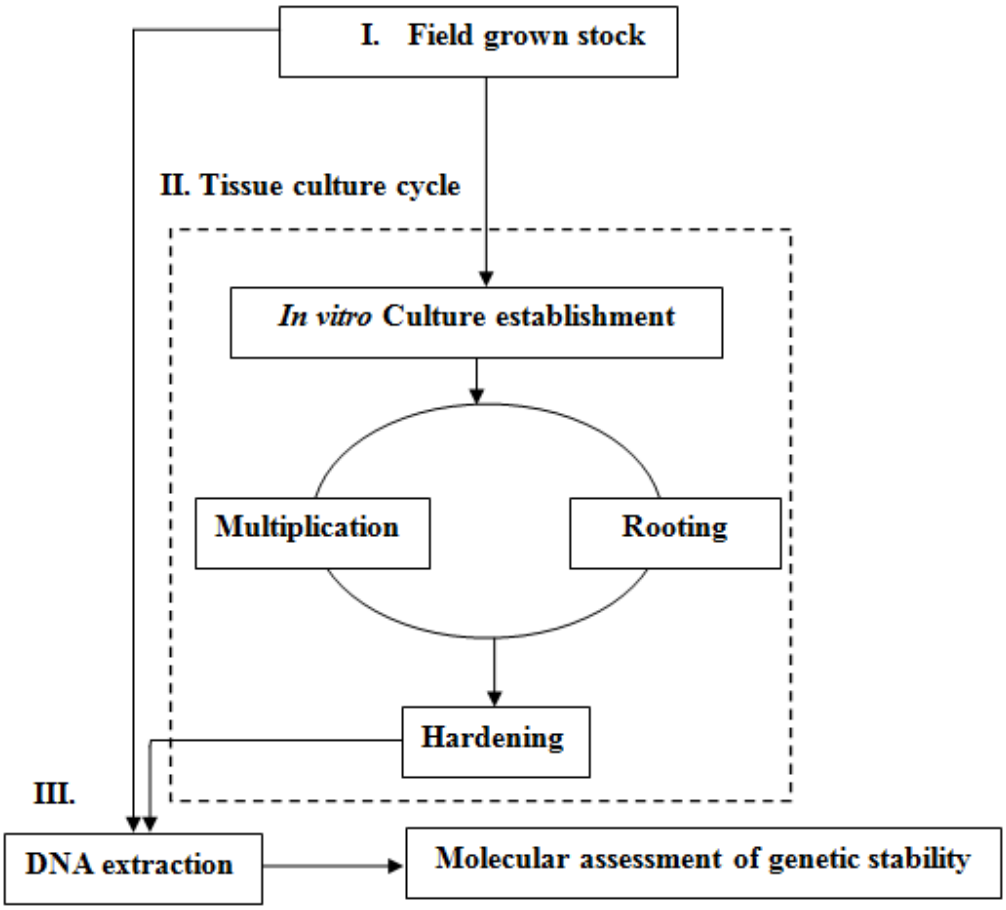

Fig.1. A typical micropropagation protocol and production of true-to-type, highly uniform plants. The stage III actually validates the protocol to be applied in a commercial scale.

Table 1. Strengths and weaknesses of different marker systems for the assessment of clonal fidelity

Advantages	Disadvantages
Morphological traits	
Visual differentiation	Sensitive to ontogenic changes and other environmental factors
Does not require any laboratory facility	Limited in numbers
Suitable for preliminary detection	Time-consuming
Cytological markers (Flow-cytometry)	
Sample preparation and analysis is convenient and rapid in case of in flow-cytometry	Cytosolic compounds may interfere with quantitative DNA staining in flow-cytometry
Rapid and efficient method for routine large-scale studies of ploidy level	Absence of a set of internationally agreed DNA reference standards in case of in flow-cytometry
Unfailing detection of even the smallest modifications in chromosome number	Time consuming chromosome counting
Isozyme markers	
Codominant expression	Sensitive to ontogenic changes and other environmental factors
Ease of performance	Limited in numbers
	Not all of these reagent systems work efficiently with all plant species
	Tissue specific expression
DNA markers	
Codominant expression	RAPD markers are dominant and do not permit the scoring of heterozygous individuals. Besides, they exclusively identify sequence changes.
Any source DNA can be used for the analysis	
Phenotypically neutral	
Not sensitive to ontogenic changes and other environmental factors	
Capability to detect culture-induced variation both at the DNA sequence and methylation pattern levels	

Table 2. Instances of use of morphological traits as one of the tools for the identification of somaclonal variants

Crop	Morphological traits	Reference
Pineapple (*Ananus comosus* (L.) Merr.)	Plant height; the peduncle diameter; the number of shoots, slips and suckers; the fruit mass with crown; the number of eyes in the fruit; the fruit height and diameter; the leaf color; the plant architecture; the length of plant generation cycle; and the fruit color and shape	[73]
Begonia (*Begonia* x *elatior*)	Leaf variegation, non-flowering, dwarfing of plants, and slow growth	[36]
African violet (*Saintpaulia ionantha* L.)	Height of plants at flowering, number of flowers per plant, and flower size	[37]
Tea (*Camellia sinensis* L.)	Leaf characteristics such as colour, texture, venation, serration, leaf tip and angle	[106]
Coffee (*Coffea arabica* L.)	Height, morphology, leaf shape, productivity, fruit shape, leaf density, stomatal density and guard cell chloroplast number	[22]
Strawberry (*Fragaria* L.)	Fruit shape, fruit texture, leaves color, leaf shape	[11]
Statice (*Limonium perezii* Hubbard)	Leaf shape index (length/width of the leaf blade), length of the petiole of fully expanded leaves, width and length of the flower stalk, diameter, length and color of the calyx, and number of petals and stamens	[49]
Plantain banana (*Musa* spp.)	Pseudostem height and diameter and number of functional leaves and suckers	[45]
Plantain banana (*Musa* spp.)	Shoot height, shoot diameter at leaf base, number of leaves and roots per shoot and fresh weight	[83]
Geranium (*Pelargonium graveolens*)	Plant height, herb yield, canopy size and number of branches per plant	[90]
Olive (*Olea europea* L.)	Plant height, canopy dimensions, leaf, inflorescence and fruit dimensions	[8]
Papaya (*Carica papaya* L.)	Plant height, stem and petiole color/pigmentation, leaf shape, female flower size and color, fruit shape and skin color	[40]

Table 3. Molecular assessment of clonal fidelity of micropropagated plantlets using RAPD marker

Crop	Variation detected	Reference(s)
Apple (MM106 rootstock)	Three off-type plants were detected	[62]
Apple (EMLA rootstock)	Nil	[31]
Banana	Dwarf off-types were detected	[115]
Banana	Somaclonal variants were detected	[12]
Banana	Somaclonal variants were detected	[112]
Banana	Nil	[112]
Betula pendula	Nil	[86]
Cedrus libani; *C.atlantica*	*C. libani* tissue cultured progenies being genetically more stable than those of *C. atlantica*	[79]
Chrysanthemum (*Dendranthema grandiflora*)	One out of 20 cryopreserved regenerants showed a different banding pattern	[58]
Clerodendrum serratum	Nil	[94]
Date palm	Yes	[88]
Date palm	Variations detected among callus derived plantlets	[41]
Dioscorea bulbifera L.	Nil	[67]
Ginger	Nil	[85]
Grapevine	Protoclones were screened	[92]
Grapevine	Nil	[98]
Grapevine	Nil	[2]
Hagenia abyssinica	Yes	[24]
Hop	No genetic variation was detected but epigenetic variation was detected, when field and *in vitro* samples were compared.	[72]
Kiwifruit	Yes	[71]
Lemon	Nil	[70]
Mentha arvensis	99.9 % homogeneity	[95]
Ornamental strawberry *(Fragaria* x *Potentilla)*	Nil	[105]
Papaya	Varying levels of genomic DNA modifications (0–10.07%) following cryopreservation were detected using different molecular markers	[40]
Pineapple	*In vitro* induced variant regenerants were successfully analyzed.	[103]
Pineapple	Yes, (Using RAPD combined with isozymes more variations were detected)	[23]
Platanus occidentalis	Lower than 0.0104% polymorphism	[104]
Pyrus	Nil	[91]
Robinia pseudoacacia L.	32% polymorphism	[10]
Sugarcane	Nil	[18]

Table 4. Molecular assessment of clonal fidelity of micropropagated plantlets using ISSR marker

Crop	Variation detected	Reference(s)
Banana	Nil	[110]
Banana	Few plants showed variation at the DNA level in "Grand Naine"variety	[84]
Gerbera	Variation in a leaf-derived clone	[8]
Grapevine	Nil	[3]
Hydrangea macrophylla	Thirty-two analyzed regenerants did not deviate at all from the parental genotype.	[54]
Platanus acerifolia	2.88 % polymorphism	[33]

Table 5. Molecular assessment of clonal fidelity of micropropagated plantlets using AFLP marker

Crop	Variation detected	Reference(s)
Echinacea purpurea	Out of 40 regenerants only 2 were found to be similar to their donor plants and rest of them were detected as genetic variants	[15]
Eucalyptus globulus	32 (66.7 %) of the 48 analyzed regenerated plants showed at least one polymorphic AFLP marker,compared with plants obtained from the same callus.	[61]
Kiwifruit	Regeneration was achieved from leaf explants of adult male kiwifruit plants and genetic variation among field-grown plants and tissue culture-derived regenerants was observed	[74]
Papaver	Variation detected but it was not due to somaclonal variations (see text)	[13]
Pineapple	Phenotypic variations were detected among *in vitro* derived plants. The genetic variation of these plants was further confirmed by AFLP.	[73]

Table 6. Molecular assessment of clonal fidelity of micropropagated plantlets using more than one marker system

Crop	Technique	Variation detected	Reference(s)
Banana	RAPD+Flow cytometry	Characterization of a banana somaclonal variant (CIEN BTA-03) was undertaken	[26]
Banana	-do-	Nil	[110]
Banana	-do-	Nil	[110]
Citrus madurensis Lour.		Genetic instability induced by diphenylurea was detected	[99]
Date palm	-do-	Nil	[47]
Ginger	-do-	Nil	[63]
Grapevine	-do-	Nil	[2]
Lemon	RAPD+ Flow cytometery	Five different populations of lemon plants obtained from undeveloped ovules were examined. Among all tested plants, genetic variation was detected only within the group of plants recovered from irradiated embryogenic calli. Rest of the plants was genetically identical.	[70]
Pineapple	RAPD+Isozymes	Two micropropagation systems were compared for occurrence of genetic instability. Temporary immersion in comparison with the stationary system resulted in the lowest proportion of somaclonal variants.	[23]
Saussurea involucrate	RAPD + ISSR	The percentages of polymorphic bands in the RAPD and ISSR analysis were 35% and 33%, respectively.	[119]

CONCLUSION

It is well accepted that somaclonal variations arising out of unique tissue culture environment are very often noticed phenomenon in clonally propagated plants, which can advantageously be utilized as a source of new variation in horticultural crops [41]. However, suitable tools for detection, evaluation, identification and improvement of resistant clones should be designed in order to realize the benefits of such variations [87].A number of cultivars have been developed through somaclonal variations in different horticultural crops [41]. Though, on one hand, tissue culture induced variations provide a tool of improvement to plant breeders, particularly for the improvement of crops with narrow genetic base; on the other hand, they pose a major threat to the genomic integrity of regenerated plants. Several strategies have been followed to ascertain the genetic fidelity of the *in vitro* raised progenies in view of the fact that the sustainability of the micropropagation technique is reliant upon the continuance of genetic fidelity of the regenerated plants. Therefore, a thorough assessment of micropropagated plants becomes very crucial, especially, for perennial crops such as fruit species, which have long pre-bearing gestation period. The efficiency of new molecular tools in terms of their sensitivity has enabled us to detect somaclonal variation at an early stage. These tools have become very useful for the rapid detection and accurate identification of variants. Nevertheless, the morphological and cytological assays should continue to remain as the primary and essential assay for the sustained success of fidelity tests associated with production of clonal plants. In view the an array of genomic aberration taking place at cellular and molecular levels, which in turn manifested in the form of somaclonal variations *in vitro*[76], it would be worthy to ascertain the genetic integrity of tissue culture raised plants exploiting a combination of the aforementioned techniques [108]. Hence to achieve the desideratum so as to make a complete characterization of tissue culture derived plants, a multidisciplinary approach (involving horticulture, biochemistry, physiology, cytology and molecular biology) with all our previous knowledge and experience towards the assessment of clonal fidelity in micropropagation programme is the need of the time.

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