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Molecular Markers in Micropropagation, Tissue Culture and *In Vitro* Plant Research

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ABSTRACT

Molecular marker technologies have become a powerful tool in crop improvement through their use in germplasm characterization, linkage mapping and molecular breeding. *In vitro* plant research has become one of the areas of extensive molecular marker application, as they can be used to monitor the somaclonal variation, verify the genetic fidelity of micropropagated plants and to identify genotypes with desired response to *in vitro* culture conditions. The objective of this paper is to summarize literature concerning application of various molecular markers for genetic fidelity assessment of *in vitro* cultured plants.

Keywords: cryopreservation, genetic stability, *in vitro*, micropropagation, molecular markers, somaclonal variation, tissue culture
Abbreviations: AFLP, amplified fragment length polymorphism; **IRAP**, inter-retrotransposon amplified polymorphism, **ISSR**, inter-simple sequence repeats; **MAS**, marker-assisted selection; **MSAP**, methylation sensitive amplified polymorphism; **QTL**, quantitative trait locus; **RAPD**, random amplified polymorphic DNA; **REMAP**, retrotransposon-microsatellite amplified polymorphism; **RFLP**, restriction fragment length polymorphism; **S-SAP**, sequence-specific amplification polymorphism; **SSR**, simple sequence repeats; **TCL**, thin cell layer

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INTRODUCTION

Molecular markers are specific DNA fragments that can be detected within the whole genome by means of an adequate technique. Several classes of molecular markers, such as RFLP, RAPD, AFLP, SSR, ISSR, have been developed to date, and used for numerous purposes. Recently, *in vitro* plant research has become one of the areas of extensive molecular marker application.

In vitro regeneration and micropropagation form the primary core of many, if not most horticultural plant and agricultural crop studies. A number of well defined problems in physiological, epigenetic and genetic quality are associated with the culture of plant cell, tissue and organs *in vitro*, namely, absence or loss of organogenic potential (recalcitrance), hyperhydricity (vitrification) and somaclonal variation, complex phenomena that are genotype and environment dependent and which affect the practical application of plant tissue culture in plant propagation and genetic manipulation.

In order to ensure the successful use of *in vitro* technology either for breeding programs or market distribution molecular markers are used to monitor the somaclonal variation, verify the genetic fidelity of micropropagated plants

Received: 28 February, 2007. Accepted: 16 April, 2007.

Invited Mini-Review

and to identify genotypes with desired response to *in vitro* culture conditions.

This review investigates how various molecular markers have been used for detection of somaclonal variation in several (but not exhaustively) *in vitro* micropropagation systems, but does not extend towards the stability of transgenes in genetically-modified plants. Application of molecular markers in tissue culture response studies, including identification of QTLs governing the reaction of different explant types to *in vitro* conditions and use of MAS in identification of genotypes with a desired response has been recently reviewed elsewhere (Bolibok and Rakoczy-Trojanowska 2006).

MOLECULAR MARKERS FOR THE ANALYSES OF *IN VITRO* CULTURED PLANTS

The main aim of clonal propagation is to retain the genetic integrity of the propagated plants. Somaclonal variation, although to a certain extent overcomeable by TCLs, a system that has allowed for the standardization of clonal micropropagation of numerous monocotyledonous and dicotyledonous species, allowing for mass-production of morphologically and genetically stable and uniform plants (Teixeira da Silva and Nhut 2003; Nhut *et al*. 2006), remains a factor affecting practical application of micropropagation.

Various molecular techniques are used to check genetic stability and lack of somaclonal variation in tissue culture derived plants, however easy to use, cheap and requiring no previous sequence information RAPD and ISSR markers clearly prevail in the published studies dealing with the subject. Nevertheless, technically more demanding AFLPs and sequence specific, highly polymorphic SSRs are also used relatively frequently.

As some of the somaclonal variation is of epigenetic nature it cannot be detected with conventional structural molecular markers. In those cases markers such as methylation sensitive amplified polymorphism (MSAP) (Jaligot *et al.* 2003; Hao *et al*. 2004), methylation-sensitive RFLPs (Jaligot *et al*. 2002), or a gene expression approach (Morcillo *et al*. 2006) need to be used. Moreover, tissue culture has been shown to induce transposition of transposable elements of plants (Hirochika *et al*. 1996; Courtial *et al*. 2001), and their insertion at novel sites results in somaclonal variation (Wessler *et al.* 1995). Hence, using retrotranspozon-based molecular markers (S-SAP, IRAP, REMAP) in studies of *in vitro* cultured plant seams an interesting option and recently IRAP markers were successfully used for the analysis of banana somaclones (Asif and Othman 2005). Application of RFLP technology with appropriately chosen probes gives the possibility to assess the genetic fidelity of micropropagated plants with respect to their nuclear, mitochondrial and chloroplast genomes (Abe *et al*. 2002; Devarumath *et al.* 2002). Generally, simultaneous application of different DNA fingerprinting techniques is advisable in tissue culture derived plants as it allows for detection of variation in diverse components of the genome (Asif and Othman 2005).

In the following sections we summarize literature concerning application of molecular markers for genetic fidelity assessment of *in vitro* cultured plants.

GENETIC FIDELITY OF MICROPROPAGATED PLANTS

Ornamental plants

If tissue culture is being employed for commercial production of ornamental plants it is important to achieve a rapid and easy method to detect somaclonal variation, not only to make sure that the characteristics of the variety propagated have not been changed, but also, in some cases to use it as a source of variation that may be employed or obtaining new varieties or forms. Somaclonal variation could be quantified in tissue-cultured *Phalaenopsis* (Chen *et al*. 1998), *Pelargonium* (Cassels *et al*. 1997), *Petunia* (Cerny *et al*. 1996), *Saintpaulia* (Paek and Hahn 1999) and *Cymbidium* hybrids (Teixeira da Silva and Tanaka 2006; Teixeira da Silva *et al*. 2006) using RAPD markers. Asiatic *Lilium in vitro*-induced bulblets showed zero RAPD variation (Varshney *et al*. 2001). RAPD has also proved to be an efficient tool for assessment of genetic stability in the tissue culture of *Chrysanthemum* (Martin *et al*. 2002). A more comprehensive analysis of somaclonal variation in ornamental plant tissue culture and its assessment has been covered extensively elsewhere (Teixeira da Silva 2006) and will not be covered here.

Trees

Molecular markers are especially valuable for efficient tree germplasm management due to the high cost of space and maintenance of plants in most clonally-propagated tree species. Moreover out-crossing of many tree species resulting in high levels of polymorphism, thus consequent duplications or mislabeling are avoided. Identifying variants during the early stages of plant development is essential to avoid propagation of mutant plants in species with extensive growing periods.

Tropical and sub-tropical fruit trees

The analysis of somaclonal variation in micropropagated banana, which is widespread, has been recently reviewed (Sahijram *et al*. 2003). RAPDs and SCARs allowed for the successful detection of dwarf off-types in micropropagated cavendish (*Musa* spp. AAA; Umali *et al*. 2002) and bananas (Ramage *et al*. 2004). *In vitro*-micropropagated somaclonal variants could be identified using RAPD profiling of *Musa* (Grajal-Martín *et al*. 1998). No genetic variation was detected in analyses of micropropagated banana plantlets obtained from different cytokinin-treatments. In total 625 reproducible monomorphic bands were obtained with 50 RAPD and 12 ISSR primers (Venkatachalam *et al.* 2006). Micropropagated pineapple (*Ananas comosus*) was shown to be genetically stable using RAPDs and isozymes (Feuser *et al*. 2004). Similarly, no polymorphic marker was found among 669 RAPD and 1307 AFLP markers used for the analysis of micropropagated chestnut rose (*Rosa roxburghii* Tratt; Weng and Deng 2005).

Temperate fruit trees

Modgil *et al*. (2005) showed the genetic fidelity of adventitious bud, clonally propagated apple (*M. pumila*) using RAPD and ISSR, while RAPDs were also used to confirm the genetic stability of leaf-derived apple clones (Virscek-Marn *et al*. 1999), peach somatic embryos (Hashmi *et al*. 1997) or lemon (*Citrus limon*) protoplast regenerants (Deng *et al*. 1995). Hao *et al*. (2004) found that RAPDs were insufficiently sensitive to detect small changes in the DNA of grapefruit callus cultures, but DNA methylation changes could be detected by methylation sensitive amplified polymorphism (MSAP). Eight *Rubus* species could be regenerated on 55 different media, and resulting plants' genetic stability was confirmed by RAPDs. The paternity of embryo culture-derived cherry plants was confirmed with RAPD markers (Hormaza 1999). Repeatable somaclonal variation could only be detected when both RAPD and SSR were used in micropropagated kiwi (Palombi and Damiano 2002).

Nut trees and palms

The clonal identity of 4-year long *in vitro*-propagated chestnut (*Castanea sativa*) trees could be confirmed using 40 arbitrary 10-mer primers, with genetic variants, differing from each other and from the original mother plants (Carvalho *et al*. 2004). In hazelnut RAPD analysis revealed no somaclonal variation between donor plants from which *in vitro* cultures were initiated and micropropagated plants (6-

year cultures), and no somaclonal variation was detected among *in vitro*-propagated plants; polymorphism (15.6%) was detected between the parent plant and its *in vitro*-propagated progenies, however (Nas *et al*. 2004).

Somaclonal variants, which were shown to be age dependent in date palm, were also suitably detected by RAPD analysis in oil palm, *Elaeis guineensis* (Saker *et al*. 2000). However, the occurrence of somaclonal variants (ca 5%) among populations of somatic embryo-derived oil palms currently hampers the scaling-up of clonal plant production. The global methylation in leaf DNA of abnormal regenerants is 0.5-2.5% lower than in their normal counterparts, as shown by methylation-sensitive RFLPs (Jaligot *et al*. 2002).

Forest and timber trees

Molecular markers in forest breeding and biotechnology have been used to elucidate genome mapping, organization and evolution of forest tree species, provided insight into the maternal and paternal mode of organelle inheritance in angiosperm and gymnosperm trees, especially since repetitive DNA sequences are much more frequent than in the genomes of higher plants (Ahuja 2001), and are also widely used for assessing somaclonal variation. SSRs were used to confirm the genetic fidelity of micropropagated trembling aspen (Rahman and Rajora 2001), and of 10 forest tree species (Rani *et al*. 2001). Two of the economically most important and widely planted *Eucalyptus* species, *E. camaldulensis* and *E. tereticornis*, showed uniform tissue culture (axillary branch-derived plantlets) and monomorphic fragments generated from 12 arbitrary 10-mer primers (Rani and Raina 1998). Using 17 RAPD primers Tripathi *et al.* (2006) found a small variation (0.01 and 0.02) in two groups of micropropagated *Eucalyptus* plants. *Melia azedarach*, an important timber species also with therapeutic and insecticidal application, displayed many polymorphisms when *in vitro*-propagated clones were RAPD fingerprinted, the intraclone heterogeneity being attributed to chimerism (Olmos *et al*. 2002). Genetic variation (0.0045%) in *in vitro*-propagated cedar (*Cedrus libani*) could be rapidly detected using 11 arbitrary 10-mer RAPD primers (Piola *et al*. 1999) while *in vitro*-cultured *C. libani* showed reduced methylation as compared to adults (Renau-Morata *et al*. 2005). Long-term cultures (>10 years) of micropropagated shoots of Japanese black pine (*Pinus thunbergii*) showed no RAPD variation (Goto *et al*. 1998) nor was there any somaclonal variation in somatic embryogenic cultures of black spruce (*Picea mariana*; Isabel *et al*. 1993), loblolly pine (*Pinus taeda*; Tang 2002) or Norway spruce (Fourré *et al*. 1997). Both SSR and RAPDs were used to genetically characterize white or pedunculate oak (*Quercus robur*) seedlings, epicormic, crown and micropropagated shoots from mature trees using 9 primers which generated 28 polymorphic bands (Barrett *et al*. 1997). No intraclonal or interclonal polymorphism in *Quercus* was detected between embryogenic lines originating from different types of explant from the same seedling, but every one of the 32 primers detected enough polymorphism among clones originating from different plants to allow these origins to be distinguished. No differences in DNA sequences between regenerated oak plantlets and their somatic embryos of origin were detected using RAPDs, but a nodular callus line that had lost its embryogenic capacity was found to be mutant with respect to three other clones originating from the same plantlet (Sánchez *et al*. 2003). No evidence of genetic variation was found when *Quercus robur* L. plantlets obtained via somatic embryogenesis were analysed with 40 RAPD primers (Valladares *et al.* 2006). Microspore-derived *Q. suber* somatic embryos maintained haploidy over long periods, confirmed by SSR analysis and flow cytometry (Bueno *et al*. 2003). Wilhelm *et al*. (2005) found, however that despite variation in SSR patterns in somatic embryos, regenerated plants showed no polymorphism. Black locust trees (*Robinia pseudoacacia*) multiplied by axillary buds (Shu *et al*. 2003) or terminal shoot buds of 4-year old micropropagated plants (Kanwar and Bindiya 2003) showed no variation in RAPD banding but SSR analyses indicated high levels of mutations in somatic tissues (Lian *et al*. 2004). ISSR analysis of 41 morphologically normal *Robinia ambigua* var. *idahoensis* plants micropropagated by enhanced branching of axillary buds revealed the occurrence of genomic variation, though at a relatively low level (10.62% polymorphic bands; Guo *et al.* 2006). Six of 48 RAPD primers tested were able to detect polymorphism in anther culture derived poplar plants (Kiss *et al*. 2001). To test the genetic fidelity of 23 micropropagated plants of a single *Populus deltoides* clone, eleven 10-mer RAPD primers were used, of which 5 distinguished a total of 13 polymorphisms common across 6 micropropagated plants while the remainder of the amplification products were monomorphic across the micropropagated plants (Rani *et al*. 1995).

Endangered trees

Genetic integrity of introduced rare and endangered species is vital, especially when these are derived from *in vitro* mass propagation. About 97% homology could be detected between the mother plants and micropropagated plants of *Syzygium travancoricum*, a rare and endangered tree (Anand 2003).

Grain, cereal and forage crops, and grasses (*Poaceae*)

Analytical methods have been developed and applied to identify and quantify cereal species in food products and also to fingerprint and identify grain at the genotype and variety levels since the composition of cereal-based foods is a key factor in determining the quality and safety of the final product, while the reliable identification of cereal species and cultivars is essential for the handling, marketing and processing of grain, and for the protection of plant breeders' rights (Terzi *et al*. 2005).

Yang *et al*. (1999) reported more than 50 polymorphic bands using 4 DIG-labelled RAPD primers in regenerated rice plants obtained from callus cultures, while an increase in polymorphism from 12% to 17% and to 26% in 1, 3 and 6 month-old cultures, respectively, could be detected within a single line (Kim *et al*. 2003). Godwin *et al*. (1997) analyzed 8 rice somaclones generated from mature seed-derived callus cultures using RAPDs, and all somaclonal families differed significantly from the original material, indicating genomic alterations in all families, as did Phong *et al*. (2001) in dessication-tolerant-derived plants, but Afza *et al*. (2001) found limited androclonal variation in rice anther cultures. Differences in some somaclonal variants derived from a landrace rice variety, Indrayani, were shown to be high yielding and resistant to multiple diseases, and could be differentiated on the basis of SSRs (Chowdari *et al*. 1998). Alteration in chloroplast DNA (ctDNA) in long-term cultures of calli derived from rice seeds were investigated using a *Bam*HI-1 fragment of rice ctDNA as a probe. Among 10 callus lines cultured for 36 months, two lines (T36-1 and T36-2) were found to have large deletions (76 kb and 63 kb, respectively) in the chloroplast genome, while none of the same callus lines cultured for 6 months showed any ctDNA alteration (Abe *et al*. 2002).

RAPD analysis was performed to assess DNA variation among rye (*Secale cereale*) plants regenerated from immature embryos and inflorescences. From the studied plants, 40% showed at least one variation, and the number of mutations per plant was quite high, ranging from 1 up to 12 (Linacero *et al*. 2000). Similarly, Rakoczy-Trojanowska (2002) found a relatively high proportion of immature embryos derived rye R_1 plants with changed RAPD profiles. In *Triticum* (and also tobacco) protoplast cultures and subsequent regenerants, somaclonal variation was detectable by RAPDs (Brown *et al*. 1993). No restriction fragment length polymorphisms (RFLPs) were detected in the mitochondrial, plastid and nuclear genomes in the *Festuca pratensis* plants

regenerated from suspension cultures (Vallés *et al*. 1993).

Somaclonal variation could be detected using RAPD analysis in tissue-cultured *Lolium* derived from cell suspension-derived protoplasts (Wang *et al*. 1993). The microspore origin of anther-culture-derived plants of flax (*Linum usitatissimum*) was determined using ISSR and RAPD markers (Chen *et al*. 1998). Somaclonal variation in axillary branching propagation and indirect somatic embryogenesis of alfalfa was estimated by RAPD fingerprinting (Piccioni *et al*. 1997), while SSR fingerprinting revealed a reduction in the number of copies of a highly repetitive DNA sequence (Pluhar *et al*. 2001).

Medicinal and aromatic plants

Monitoring genetic fidelity in medicinal plants with the help of molecular markers allows for the formulation of drugs free from batch-to-batch variations (e.g. in garlic, Ipek *et al*. 2003), for effective *ex situ* conservation programs. An overview of the regeneration of medicinal plants by direct and indirect organogenesis and by somatic embryogenesis from various types of explants is presented elsewhere (Rout *et al*. 2000), as is their use with other biotechnological approaches to improve medicinal plants through somaclonal variation, and molecular markers to assess its level.

Jamrosa, a hybrid *Cymbopogon*, is an essential oil-yielding plant containing geraniol, used as a substitute for palmarosa (*C. martinii*), and commercially used in perfumery, pharmaceuticals, soap and cosmetic industries, and reclamation of waste land; Callus-derived *Cymbopogon* somaclones, despite gross differences in RAPD banding patterns displayed uniformity in essential oil yield and quality (Nayak *et al*. 2003). RAPDs and SSRs proved genetic stability in micropropagated *Achillea*, comprising ten taxa of the medicinally important *A. millefolium* group and six related species (Wallner *et al*. 1996). Varietal specific RAPD markers could be detected in tissue-cultured *Piper longum*, a dioecious medicinal herb (Philip *et al*. 2000). *Digitalis obscura* plants produce cardenolides, secondary metabolites of major interest to the pharmaceutical industry. RAPDs provide a simple and effective assay for screening elite cultivars and confirm their genetic stability over long-term cultures (Gavidia *et al*. 1996). Similar RAPD controls for *in vitro*-propagated *Epimedium* (Nakai *et al*. 1996) resulted in standardization of plantlet selection and greater scrutiny of medicinal plants. Guo *et al.* (2006) found genomic instabilities in phenotypically normal regenerated plants of *Codonopsis lanceolata* Benth. *et* Hook. f. using ISSR and RAPD markers as 15.7 and 24.9% of the bands, respectively, were polymorphic. Five arbitrary RAPD primers used to analyze the genetic fidelity of *Chlorophytum arundinaceum* Baker plants regenerated through shoot bud differentiation displayed the same banding profile within all the micropropagated plants and *in vivo* explant donor (Lattoo *et al.* 2006). The cytological and molecular analysis complemented and compared well and showed no genomic alterations in *Chlorophytum arundinaceum* Baker, an endangered medicinal herb regenerated through shoot bud differentiation.

The capacity to detect genetic mono- or polymorphisms in callus-induced plantlets of garlic (*Allium sativum*; Al-Zahim *et al*. 1999), *Panax notoginseng* somatic embryos (Shoyama *et al*. 1997), and turmeric (Salvi *et al*. 2001) allows for elimination of genetic variants for clonal micropropagation, or exploitation of somaclonal variation for the selection of new varieties with novel features, such as increased secondary metabolite yields, but in the case of garlic, morphogenic characters and RAPD banding could not be correlated. In the case of American ginseng, *P. quinquefolium*, long term suspension cultures showed different RAPD banding patterns to young seedling-derived somatic embryos (Punja *et al*. 2004). Gametoclonal variation was detected by novel RAPD bands in onion gynogenesis (Bohanec *et al*. 1995). *Tylophora indica*, a medicinal plant with multifarious uses, once *in vitro*-propagated by 2,4-D- and kinetin-induced callus, produces monomorphic amplification products when 20 arbitrary nucleotide primers are used, indicating the genetic homogeneity and true-to-type nature of regenerants (Jayanthi and Mandal 2001). Devarumath *et al*. (2002) considered RAPD and ISSR 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* spp. *assamica* (Assam-India type). Out of 49 RAPD primers used by Mondal and Chand (2002) to assess genetic instability among micropropagated tea [*Camellia sinensis* (L). O. Kuntze] cultivar 'T-78', 11 generated polymorphism in four out of 17 micropropagated plants and one mother plant, which otherwise were morphologically indistinguishable. Polymorphism among those four plants showed an identical banding pattern suggesting the occurrence of a single mutation. Similar results were obtained by Singh *et al*. (2004) in vegetatively propagated clones. SSRs have been adapted as the standard for cocoa (*Theobroma cacao*) genetic diversity and fingerprinting analysis (Saunders *et al*. 2005), and have been used to detect and quantify *in vitro*-culture induced chimerism (López *et al*. 2005). Somaclonal variability in hop (*Humulus lupulus*) *in vitro* meristem cultures and clones (Patzak 2003) was assessed by RAPDs and ISSRs. Somaclones of *in vitro*-cultured leaf segment-derived shoots of *Lycium chinense*, commercially important as a medicinal plant, and as a beverage in Asia, could be detected by RAPDs (Ahn *et al*. 2004) Mango ginger, *Curcuma amanda*, an Indian medicinal plant showed new polymorphic RAPD bands, i.e. variation, in *in vitro* rhizome-derived, callus-induced shoots (Prakash *et al*. 2004). RAPD bands generated using twenty 10-mer primers used in RAPD were monomorphic in tissue cultured clonal plants of *Plumbago zeylanica*, important for the alkaloid, plumbagin (Rout and Das 2002). In *Angelica*, a local Japanese medicinal plant, RFLP could not distinguish different *in vitro* plants, whereas RAPDs could (Watanabe *et al*. 1998).

Other fruit and vegetable crops

ISSR analysis was effective to eliminate somaclonal variants in *in vitro* leaf-derived horseradish plants (Rostiana *et al*. 1999). Somaclonal variation was detectable (0.05%) in tissue-cultured sugarbeet (*Beta vulgaris*) using *RAPDs* (Munthali *et al*. 1996). *In vitro* propagated cauliflower (*Brassica oleracea*) somatic embryos did not show any variation in ISSR banding (Leroy *et al*. 2000b). Numerous molecular markers are used for checking somaclonal variation in *S. melongena* (eggplant) somatic embryogenesis (Kantharajah and Golegaonkar 2004). Despite the presence of dwarf phenotypes, 157 RAPD markers could not detect intraclonal variation in 77 asparagus plants regenerated from embryogenic lines (Raimondi *et al*. 2001). Taylor *et al*. (1995) reported that RAPDs detected gross genetic changes occurring in sugarcane tissues subjected to prolonged (2 years) *in vitro* culture. Regeneration in sunflower (*Helianthus annuus*) based on successive excision of the apical and axillary shoots originating from pre-existing meristems resulted in no somaclonal variation, as confirmed by AFLP analyses (Hewezi *et al*. 2003).

No polymorphisms could be detected through RAPD analysis in tomato calli or progeny of regenerants, despite morphological differences (Rus-Kortekaas *et al*. 1994), nor using RAPD or ISSR markers (Martins *et al*. 2004).

GENETIC FIDELITY OF PLANTS AFTER CRYOPRESERVATION

Fruit trees

Long-term stored, cryopreserved by vitrification 'Gala' apple (Liu *et al*. 2004), *Amorphophallus* (Zhang *et al*. 2001) or *Prunus* (Helliot *et al*. 2002) shoot tips showed no RAPD polymorphisms, confirming isozyme analyses, showing that RAPDs are useful for discerning variation in this useful

germplasm conservation technique. On the other hand Hao *et al*. (2001) reported that cryopreservation of apple, despite no differences at the cytological and AFLP levels, produced new demethylation sites. No differences were found between the DNA patterns obtained using RAPD from plantlets regenerated from control and plantlets regenerated directly from cryopreserved shoot-tips of four grape (*Vitis vinifera*) cultivars and one kiwi (*Actinidia deliciosa*) cultivar (Zhai *et al.* 2003).

Forest and timber trees

No reproducible variation of the RAPD profiles generated by ten 10-mer primers was observed in cryopreserved tissues of hybrid aspen (*Populus tremula* L. × *Populus tremuloides* Michx.), an economically important woody plant and widely used model system of forest scientists (Jokipii *et al*. 2004). High levels of polymorphism were shown in RAPD analysis of cryopreserved apical meristem-tips of *Melia azedarach* (Scocchi *et al*. 2004). de Verno *et al*. (1999) found large differences in RAPD banding following cryopreservation (2-12 months) of *P. glauca* somatic embryos. In other studies with larch (*Larix* X *eurolepsis*), black spruce (*Picea mariana*; Klimaszewska *et al*. 1992), Norway spruce (*Picea abies*; Nørgaard *et al*. 1993), Douglas fir (*Pseudotsuga menziesii*; Gupta *et al*. 1995), Carribean pine (*Pinus caribaea*; Lainé *et al*. 1992), Scott's pine (*Pinus silvestris*; Häggman *et al*. 1998) and maritime pine (*Pinus pinaster*; Bercetche and Pâques 1995) no genetic changes were found when RAPD analysis was conducted on cryopreserved regenerants.

Medicinal and aromatic plants

Plants derived from cryopreserved shoot tips of *Dioscorea floribunda*, a medicinal species of yam, were genetically stable, with only 1 polymorphic band being obtained from over 5,000 RAPD bands (Ahuja *et al*. 2002).

Tuberous crops

Embryogenic tissues of *Dioscorea bulbifera* (yam) were cryopreserved using encapsulation-dehydration, and the genetic stability of plants regenerated from cryopreserved embryogenic tissues was assessed using RAPDs: 10 primers produced 62 clear reproducible DNA fragment profiles and the amplification products were monomorphic for all the plantlets except one; a total of 4960 DNA fragments were obtained from this study showing no variation in RAPD profiles (Dixit *et al*. 2003).

FUTURE PERSPECTIVES

As the agricultural and horticultural sectors, especially the floricultural sector, move towards a greater efficiency in production, higher stringency in quality control, and greater demand by the consumer, there is a need to accompany the advances in transgenic and molecular biotechnology with an equal focus on tissue culture. More and more plants produced clonally by micropropagation are becoming the source of material for field plantations, which creates a constantly growing demand for sensible, efficient and reliable tools for analysis of the somaclonal variation, or the elimination of genetic variation. Molecular markers are those tools.

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