

Genetic Stability Assessment of *in Vitro* Plants by Molecular Markers

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SUMMARY. Genetic stability of *in vitro* plants should be assessed in order to develop proper programs of conservation. Such conservation programs could be developed only after evaluation of genetic variability in the natural populations and the genetic stability or somaclonal variability after conservation. In case of valuable economic plants as fruit trees, vegetables, medicinal plants, the natural variability from populations should be preserved as sources of new cultivars or variations. In order to preserve endangered, vulnerable or endemic plants it is very important to evaluate the genetic variability in natural populations and to preserve this variability as well as individuals or habitats. Genetic variability or stability after plant conservation is usually assessed by DNA-based molecular markers as RAPD, SSR, ISSR, SRAP, RFLP and AFLP.

Keywords: conservation, genetic fidelity, *in vitro* plants, micropropagation, molecular markers.

Introduction

Plant cell and tissue culture became a versatile tool for rapid propagation and biomass production of valuable species. *In vitro* culture of plant cell, tissue and organ is associated with differences in physiological, epigenetic and genetic quality, namely, absence or lack of organogenic potential (recalcitrance), hyperhydricity (vitrification) and somaclonal variation. All of these phenomena are dependent of genotype and culture conditions and affect the practical application of tissue culture in plant propagation and genetic manipulation (Teixeira da Silva *et al.*, 2007).

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Usually by *in vitro* culture a huge number of somaclone could be obtained in short time and space. Multiplication of elite genotypes follows the preservation of the valuable genotype, thus the somaclonal variation is not a desired event. Despite of this, in some instances, somaclonal variation could be a valuable source of variability in order to select new genotypes. Genetic variations occur in undifferentiated cells, isolated protoplasts or calli. Recent studies have revealed that cell or tissue cultures undergo frequent genetic changes (polyploidy, aneuploidy, chromosomal breakage, deletion, translocation, gene amplifications and mutations) and these are also expressed at biochemical or molecular levels (Teixeira da Silva *et al.*, 2007).

Somaclonal variation in regenerated plants is generated during dedifferentiation and is uncontrollable and unpredictable in nature and most variation is of no apparent use. The occurrence of cryptic genetic defects arising *via* somaclonal variation in the regenerants can seriously limit the broader utility of micropropagation systems.

Clonal propagation and preservation of elite genotypes, selected by their superior characteristics, require high degree of genetic uniformity among the regenerated plants. The occurrence of somaclonal variation is a disadvantage for both *in vitro* cloning as well as germplasm preservation method, therefore, the investigation of genetic variability/stability of *in vitro* plants is extremely important. There are several strategies to ascertain the genetic variability or stability, each of them having merits and limitations (Alizadeh *et al.*, 2015). Techniques based on morphophysiological, biochemical and cytological approaches are mainly based on characters which can be affected by the *in vitro* manipulation, environment, and types of plant tissue, thus the differentiation of somaclonal variation is difficult to achieve. Despite of this, DNA-based molecular markers are a versatile tool in various fields of biology. The major advantage offered by DNA molecular markers is objective analysis, thus the results could be easily repeated and shared between laboratories. Molecular markers are used to monitor somaclonal variation, verify the genetic fidelity of micropropagated plants and to identify genotypes with the desired response to *in vitro* culture conditions.

In this paper we summarize the most used DNA-based molecular markers for assessment of stability of *in vitro* plants.

Analysis of genetic stability of in vitro plants

Various molecular techniques are used to check genetic stability and the lack of somaclonal variation in tissue derived plants as Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeat (SSR), Inter Simple Sequence Repeat (ISSR) and Sequence-related Amplified Polymorphism (SRAP).

Molecular markers as RAPD and ISSR are easy to use, cheap and requiring no previous sequence information, thus most of the studies showing the genetic stability of *in vitro* plants used these markers. SSR requires previous information about region containing repeated sequences, but they are also used in many studies because they are cheap.

Nevertheless, technically more demanding AFLP are also used relatively frequently (Teixeira da Silva *et al.*, 2007). Application of RFLP markers with appropriately chosen probes gives the possibility to assess the genetic fidelity of micropropagated plants (Abe *et al.*, 2002; Devarumath *et al.*, 2002).

Several somaclonal variations is of epigenetic nature and it could not be detected with conventional structural molecular markers. In this cases, markers as Methylation Sensitive Amplified Polymorphism (MSAP) (Jaligot *et al.*, 2003; Hao *et al.*, 2004), methylation-sensitive RFLP (Jaligot *et al.*, 2002) or gene expression approaches (Morcillo *et al.*, 2006) need to be used. It has been shown that tissue culture induces transposition of several transposable elements as well (Courtial *et al.*, 2001).

Among the numerous molecular markers available, in the last years the most used molecular markers for different applications including assessment of *in vitro* plants fidelity are RAPD, SSR and ISSR markers because they are cost effective and require low amounts of DNA (Zietkiewicz *et al.*, 1994). These type of markers were suitable for establishing genetic stability of several micropropagated plants in crops such as wheat (Ateş Sönmezoğlu, 2012). In this study several wheat accessions were characterized, which in particular are not morphologically identifiable. In potato, RAPD, ISSR, SSR and AFLP markers proved that *in vitro* culture is a safe method for conservation of potato microtubers to produce true-to-type plants (Tiwari *et al.*, 2013). RAPD markers were also used for assessment of olive *in vitro* micropropagated plants (Peyvandi *et al.*, 2013).

SSR markers were applied in tree analysis as well, as for *in vitro* plants characterization of *Populus tremuloides* (Rahman and Rajora, 2001) and *Pinus pinea* (Cuesta *et al.*, 2010). In case of white or pedunculated oak (*Quercus robur*) seedlings, epicormic, crown and micropropagated shoots from mature trees were analysed by SSR and RAPD markers and no intraclonal or interclonal polymorphism was detected (Barrett *et al.*, 1997). *In vitro* plants of *Robinia pseudacaccia* multiplied by axillary buds showed no variations in RAPD banding but SSR markers showed high level of mutations in somatic tissues (Shu *et al.*, 2003). Two species of *Albizia in vitro* plants were analysed by RAPD markers regarding their genetic uniformity in comparison with mother plants (Tudor, 2012). In case of jojoba, a slow growing plant, vegetative propagation is the alternative for multiplication of this species. Genetic stability of *in vitro* regenerated plants was assessed by RAPD and ISSR markers (Bekheet *et al.*, 2015).

Several medicinal and aromatic plants were also analysed by molecular markers. RAPD and SSR markers proved the genetic stability in micropropagated *Achillea millefolium* group and other 6 related species (Wallner *et al.*, 1996). The genetic stability of the *in vitro* plants of *Artemisia absinthium* was assessed using ISSR and SSAP molecular markers. Both markers were able to detect the somaclonal variations in the callus regenerated plants, while no variation was detected in the plants regenerated from the nodal explants. SSAP has been found to be more useful in detection of variability as compared to ISSR (Kour *et al.*, 2014).

ISSR analysis was effective to eliminate the somaclonal variant in *in vitro* leaf-derived horseradish plants (Rostiana *et al.*, 1999). Somaclonal variation was detected in tissue culture of sugarbeet by RAPD markers (Munthali *et al.*, 1996), but in cauliflower *in vitro* plants obtained by somatic embryogenesis, no somaclonal variation was detected by ISSR banding (Leroy *et al.*, 2000). Numerous molecular markers were used for detection of somaclonal variation in eggplant obtained by somatic embryogenesis (Kantharajah and Golegaonkar, 2004). AFLP analysis detected no variation in sunflower plants obtained by regeneration from apical or axillary shoots originating from pre-existing meristems (Hewezi *et al.*, 2003).

Molecular markers are also valuable tools for characterization of *in vitro* plants of fruit trees. Thus, RAPD and ISSR markers were used to prove the genetic fidelity of clonally propagated apple from adventitious buds (Modgil *et al.*, 2005). RAPD markers were also used to prove the genetic stability of somatic embryos of peach (Hashmi *et al.*, 1997), or lemon (Deng *et al.*, 1995). The paternity of embryo culture-derived cherry plants was confirmed with RAPD markers (Hormaza, 1999). RAPD and SSR markers detected repeatable somaclonal variation in micropropagated kiwi plants (Palombi and Damiano, 2002). *In vitro* micropropagated plants of *Morus alba* were analysed by RAPD and ISSR markers for genetic stability assessment (Saha *et al.*, 2016).

Analysis of genetic stability of cryopreserved plants

In vitro plants of medicinal plants species *Dioscorea floribunda* were genetically stable, only 1 polymorphism band was obtained from over 5000 RAPD bands (Aruja *et al.*, 2002).

Genetic stability of several fruit tree plants cryopreserved was also proved by molecular markers. Thus, 15 cryopreserved plants of *Pyrus pyraster* derived from single buds were used for genetic analysis with RAPD and SSR and no polymorphism was detected between cryopreserved plants and the original genotype (Condello *et al.*, 2009). ISSR markers were used to prove genetic stability in apple (Yi *et al.*, 2015). In apricot, genetic stability of cryopreserved shoot tips was demonstrated by RAPD markers (Soliman, 2013).

Analysis of genetic stability of endangered and endemic plants preserved by in vitro culture

Molecular markers were used for analysis of genetic stability of several endangered or rare species. Thus, in *Zingiber rubens* the genetic uniformity of all regenerants was demonstrated by RAPD and ISSR markers (Mohanty *et al.*, 2011). *Swertia chirayita* a medicinal herb was multiplied for a span of three and a half years and the genetic fidelity of *in vitro* plants was analysed by ISSR and SSR markers (Joshi and Dhawan, 2007). These markers showed the homogenous amplification patterns in all regenerants. In *Dictyospermum ovalifolium* ISSR markers showed the genetic similarity of over 1650 plants obtained *in vitro* (Chandrika *et al.*, 2008). RAPD markers were also used for investigation of stability *in vitro* plants of endangered *Dendrobium nobile* orchid species (Bhattacharyya *et al.*, 2014).

Several endangered or rare species of *Caryophyllaceae* were preserved by *in vitro* culture. Genetic stability after *in vitro* conservation and cryopreservation was shown by SSR and ISSR markers in *Dianthus giganteus* subsp. *banaticus* (Jarda *et al.*, 2014) and *D. spiculifolius* (Cristea *et al.*, 2014).

RAPD markers were used to assess the genetic variability of critically endangered *Draba dorneri* for their conservation (Catană *et al.*, 2013).

Conclusions

Genetic fidelity or variability of *in vitro* plants is extremely important to evaluate in order to develop proper conservation programs.

The most valuable tools for analysis of *in vitro* plants are DNA-based molecular markers. Among these, RAPD, SSR and ISSR markers are cheap and easy to use.

RAPD and ISSR makers do not need previous information about the targeted sequence, the primers could be used in different plants species. Despite of these, SSR markers need previous information about repeated sequences targeted in this analysis.

For genetic variability and fidelity assessment it is strongly recommended to use different types of molecular markers.

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