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# Evaluation of somaclonal variation in almond using RAPD and ISSR markers

**D. Sarmento*, M. Martins*,** and M.M. Oliveira*,****
*ITQB/IBET, Quinta do Marquês, 2784-505, Oeiras, Portugal
**Dep. Biologia Vegetal, Fac. Ciências Lisboa, Campo Grande 1749-016, Lisbon, Portugal
mmolive@itqb.unl.pt

**SUMMARY –** Two PCR-based techniques, RAPD (Randomly Amplified Polymorphic DNA) and ISSR (Inter-Simple Sequence Repeat), were used for analysis of somaclonal variation in almond shoots regenerated from *in vitro* cultures. No polymorphic band classes were generated when analysing 20 axillary branching regenerated shoots with 64 RAPD and 10 ISSR primers. This result allowed us to use these shoots (mother-plants) as internal controls when analysing somaclonal variation associated with shoots recovered from adventitious regeneration, meristem culture protocols, virus elimination programs and genetic engineering. No polymorphic bands were generated when 15 randomly chosen shoots, regenerated from the previously mentioned techniques, were tested with 10 ISSR and 40 (of the previous 64) RAPD primers. These results allow us to conclude that the studied regenerants possess high genetic stability and that the protocols used seem to be suitable for maintaining the integrity of the genome.

**Key words:** RAPD, ISSR, somaclonal variation, *in vitro* culture.

***RESUME –** "Analyse de la variation somaclonale chez l'amandier en utilisant des marqueurs RAPD et ISSR". Deux techniques de PCR, RAPD (Randomly Amplified Polymorphic DNA) et ISSR (Inter-Simple Sequence Repeat) ont été employées pour l'analyse de variation somaclonale chez les pousses régénérées d'amandier provenant de cultures* in vitro*. Aucune classe de bande polymorphe n'a été produite en analysant 20 plants régénérés à partir de ramifications axillaires avec 64 primers de RAPD et 10 primers d'ISSR. Ce résultat nous a permis d'employer ces regenerants (plantes-mére) en tant que témoin interne pour l'analyse de variation somaclonale chez les pousses de réegénération adventice, de culture de méristèmes, de programmes d'élimination virale et de manipulations génétiques. Aucune bande polymorphe n'a eté produite quand 15 pousses régénérées, aléatoirement choisis parmi les techniques préalablement mentionnées, ont été examinés avec 10 primers de ISSR et 40 primers (des 64 préalablement testés) de RAPD. Ces résultats nous permettent de conclure que les pousses régénérées possèdent une bonne stabilité génétique et que les protocoles utilisés semblent être ajustés pour le maintien de l'intégrité du génome.*

***Mots-clés :** RAPD, ISSR, variation somaclonale, cultures* in vitro*.*

## Introduction

Almond [*Prunus dulcis* (Miller) D.A. Webb.] is an important crop, with a growing number of applications for its kernel and oil. Biotechnology, using *in vitro* cultures, is indisputably one of the main responsible fields for the increase of several crops productivity, as some plants, mainly woody plants like almond, are very difficult to propagate using conventional propagation techniques.

In the case of almond, several studies were conducted, aiming for the increase of the propagation rate, using biotechnological techniques such as axillary branching, adventitious regeneration (Miguel *et al*, 1996) or plant regeneration from isolated meristems (Santos, unpublished). Some sanitation work was also developed in the scope of the production of virus-free plants (Gonçalves, 1998), and genetic transformation protocols were tested in order to create new and improved cultivars (Miguel and Oliveira, 1999).

However, associated with *in vitro* culture, is a phenomenon termed somaclonal variation, responsible for several phenotypic, genotypic or cytological changes that may arise amongst subclones of one parental line (Larkin and Scowcroft, 1981). A growing number of publications and reviews confirm those variations among several plants species (Karp, 1991; Peschke and Phillips, 1992).

In order to verify the presence of somaclonal variation among almond shoots regenerated from several *in vitro* biotechnological procedures, two PCR-based methods were used: the Randomly Amplified Polymorphic DNA (RAPD) (Williams *et al.*, 1990) and the Inter-Simple Sequence Repeat (ISSR) (Zietkiewicz *et al.*, 1994). Molecular biology techniques are fundamental in this kind of study, as almond's long generation cycle could cause a problem if those variations did arise, since they would only be detected in late development stages, or even in its offspring.

This work is, to our knowledge, the first work developed in almond correlating the *in vitro* regeneration protocols, the virus-elimination program and the genetic transformation events with the incidence of somaclonal variation.

## Materials and methods

### Plant material

*Prunus dulcis* shoots analysed for somaclonal variation were derived from one single genotype, the Portuguese almond cultivar 'Boa Casta', established in culture from a selected seedling (Miguel *et al.*, 1996) and denominated "Clone VII". This strategy allowed the comparison of the influence of several protocols in the genetic stability of the plantlets.

#### *Axillary branching*

Shoots from the "Clone VII" were excised and transferred to MJ medium every 21 days and kept in culture conditions described in Miguel *et al.* (1996). Unless otherwise stated, all plantlets were kept under those conditions and shoots used in other protocols came from axillary branching plantlets (mother-plants). Twenty randomly chosen plantlets ($AB_{01}$ to $AB_{20}$), were used to evaluate the genetic stability of the shoots obtained through axillary branching.

#### *Adventitious regeneration*

Several almond shoots were recovered after inducing a dedifferentiation stage in wounded leaves into an intermediate callus phase using the protocol developed by Miguel *et al.* (1996). Two plantlets ($AR_{01}$ and $AR_{02}$) were tested. Two other adventitiously regenerated plants (Santos, unpublished) and cultured in $MS_4$ supplemented with 100 µM and 200µM acetosyringone (respectively, $AR_{100}$ and $AR_{200}$), were also tested.

#### *Culture of isolated meristems*

Apical meristems from plantlets of "Clone VII" were isolated in aseptic conditions and put to grow in induction medium (MD). After 21 days in darkness, the explants were transferred to elongation medium (EM) and 16 h of light (Santos, unpublished). Two plantlets ($IM_{01}$ and $IM_{02}$) regenerated from this protocol were tested.

#### *Virus elimination programs*

Consisted in the elimination of endogenous virus using chemotherapy alone or in association with thermotherapy (Gonçalves, 1998). Eight plantlets were used as follows: 2 plantlets chemotherapeutically treated ($CT_{01}$; $CT_{02}$), 4 plantlets treated with thermotherapy and chemotherapy ($CT_{03}$; $CT_{04}$; $CT_{05}$; $CT_{06}$) and 2 plantlets for internal control ($CT_{07}$; $CT_{08}$).

#### Agrobacterium*-mediated transformation*

*Agrobacterium tumefaciens* was used in order to introduce a genetic construct in *Prunus dulcis* (Miguel and Oliveira, 1999). From the several plantlets transformed (positive scoring with Southern Blotting), only one was evaluated for somaclonal variation ($GT_{01}$).

## DNA extraction and PCR amplification conditions

Total DNA was extracted from plantlets grown *in vitro*, following the method described by Martins *et al.* (2003). RADP analysis was performed as described by Martins *et al.* (2003), 100 RAPD primers (Kit A, B, C, D and E) were tested. ISSR analysis was performed as described by Martins (2003), 10 ISSR primers were used ($IS_{01}$, $IS_{03}$, $IS_{06}$, $IS_{07}$, $IS_{10}$, $IS_{12}$, $IS_{16}$, $IS_{17}$, $IS_{18}$ and $IS_{19}$). At least two PCR amplifications were performed for each sample with RAPD and ISSR primers.

## Data analysis

This experiment was subdivided in two distinct phases: the first phase consisted in the estimation of the genetic stability of shoots obtained through axillary branching, in order to appreciate the consistency of this technique in the maintenance of the genetic fidelity of the propagated plants (mother-plants); the second phase was intended for the evaluation of the effect of different protocols (adventitious regeneration, plant regeneration from isolated meristems, virus-disease sanitation and genetic transformation events) in the genetic stability of shoots, from comparison with axillary branching regenerated plantlets.

Only distinct, reproducible, well-resolved fragments, in the size range of 200 bp to 2.8 kb (for RAPD and ISSR) were scored as present or absent for each of the RAPD and ISSR markers.

# Results and discussion

From the total number of RAPD primers tested (100) for the axillary branching regenerated plantlets, only 64 produced scorable bands, while all 10 ISSR primers tested produced scorable bands. A total of 326 band classes (266 RAPD and 60 ISSR bands) were generated, and no polymorphism could be found among them. This result allowed us to select these shoots (mother-plants), to use as internal controls when analysing somaclonal variation associated to adventitious regeneration, regeneration from isolated meristems, chemical treatments, thermotherapy or genetic engineering.

In the second phase, only 40 RAPD primers (of the previous 64 with scorable bands) and the same 10 ISSR primers were used. The RAPD and ISSR primers generated a total of 248 band classes (188 RAPD and 60 ISSR), but none of them gave rise to polymorphic bands. The obtained RAPD and ISSR profiles were identical to the ones obtained with axillary regenerated plantlets (Fig. 1).

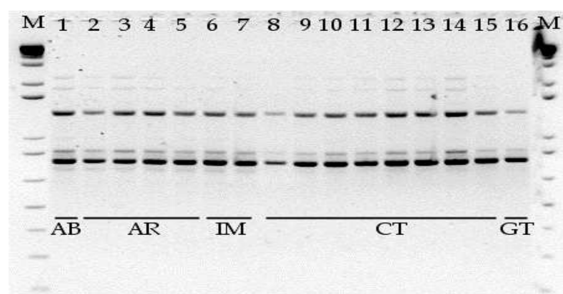


Fig. 1. Plantlets tested with RAPD primer B06: 1: Axillary Branching (AB); 2-5: Adventitious Regeneration (AR); 6-7: Isolated Meristems (IM); 8-15: Viral disease sanitation (CT); 16: Genetic Transformation (GT). M: GeneRuler$^{TM}$ ladder.

Axillary branching regenerated plantlets recorded no variation. This result was somewhat expected, since several authors referred it as being one of the *in vitro* culture systems with the lowest risks of somaclonal variation (Rout *et al.*, 1998). This stability is frequently associated to the presence of organised meristems, less susceptible to genetic variations that might occur during cell division or

differentiation under *in vitro* conditions (Shenoy and Vasil, 1992). The same reason justifies the absence of somaclonal variation among plantlets regenerated from isolated meristems.

Although no somaclonal variation was found among adventitiously regenerated almond shoots, plants regenerated through this technique are usually considered to be more susceptible to somaclonal variation, since it involves the presence of an intermediate callus phase (Soniya *et al.*, 2001). It has been assumed that certain growth regulators are mutagenic, although Bayliss (1980) refers that at concentrations used in tissue culture (similar to those used in almond *in vitro* tissue culture), these usually have no direct mutagenic effect. It seems that none of the growth regulators used in almond tissue culture (2,4-D or BAP) induced genetic instability. The acetosyringone treated plants revealed no polymorphisms as well, and although it is a phenolic compound that activates virulence genes from *Agrobacterium sp.* (Vanhala *et al.*, 1995) and that is widely used during transformation protocols, there are no references to its effect in plant genome stability.

Almond plantlets recovered from transformation events did not verify the presence of somaclonal variation. Cervera *et al.* (2000), also reported the absence of somaclonal variation among transgenic *Citrus* plants using isoenzimatic markers.

No somaclonal variation could be detected among plantlets submitted to virus elimination programs. One could expect to find a certain degree of variation, since almond plantlets were submitted to extreme chemical and thermal conditions, and those stressful conditions could have induced variation. Besides, trifluorothymidine, used in the chemotherapy treatments and known to interfere in the Herpes Simplex (HSV) viral DNA synthesis (La Lau *et al.*, 1982), could have had a certain degree of influence in the plant DNA synthesis. However, no bibliographic data could be found relating the influence of trifluorothymidine in plant DNA synthesis.

The results allow us to infer that all tested protocols seem to be suitable in maintaining almond's genome integrity. This result is of outmost importance for our laboratory, where a high genetic stability is necessary to validate the developed work, as we must certify that the regenerated plants are copies of the mother explant.

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