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Somaclonal variation on *in vitro* callus culture potato cultivars

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ABSTRACT

Synthetic seeds can be an alternative for those species in which botanical seeds are not viable. One of the major problems of *in vitro* plant cultivation is the high level of somaclonal variation. The most common factors affecting somaclonal variation are genotype, explant source, *in vitro* period and cultivation conditions in which the culture is established. In this work, calli were induced using leaf and stem explants of the commercial potato cultivars Achat, Baraka, Baronesa, Bintje, and Contenda in MS culture media supplemented with 1.65 mM of picloram and 11.5 mM of 2,4-D. Seventy and 90 days after induction, DNA samples of 40 calli were compared concerning the effects of the two explant (leaf and stem) and two growth regulator sources on five potatoes cultivars. A total of 20 arbitrary sequence primers were evaluated. The RAPD pattern generated by these primers suggested a high percentage of polymorphic fragments among the five genotypes, indicating a high level of genetic variation among cultivars. Cultivar Baronesa showed the highest number of polymorphic fragments for all treatments. The cultivar Contenda showed the smallest somaclonal variation, for most of the treatments, except for the treatment which consisted of stem explants, picloram (1.65 mM) application, and a 70-day period of callus formation. 'Contenda' is, therefore, the most suitable cultivar for synthetic seed production.

Keywords: *Solanum tuberosum* L., synthetic seed, genetic variation, 2,4–dichloro phenoxyacetic acid (2,4-D), picloram.

RESUMO

Variação somaclonal *in vitro* em cultura de calos de cultivares de batata

A produção de sementes sintéticas pode ser uma altenativa para culturas, cuja produção de sementes botânicas não é viável. Um dos principais problemas do cultivo de planta *in vitro* é o alto nível de variação somaclonal resultante. Os fatores mais comuns que afetam a variação somaclonal são genótipo, fonte de explante e duração e condições de cultivo. Neste trabalho, calos foram induzidos utilizando explantes de folha e caule das cultivares comerciais de batata Achat, Baraka, Baronesa, Bintje e Contenda em meio de cultura MS, suplementado com 1.65 µM de picloram ou 11.5 µM de 2,4-D. Após 70 e 90 dias de indução, amostras de DNA de 40 calos foram analisadas por meio de RAPD, em um estudo comparativo entre os efeitos das duas fontes de explante e os dois reguladores de crescimento nas 5 cultivares. Para este propósito, 20 primers de seqüência arbitrária foram testados. No padrão RAPD gerado por estes primers observou-se alta porcentagem de fragmentos polimóficos entre os 5 genótipos, indicando alto nível de variação genética entre as cultivares. A cultivar Baronesa apresentou o mais alto número de fragmentos polimórficos para os tratamentos. A cultivar Contenda apresentou a menor variação somaclonal genética entre os genótipos e tratamentos utilizados, exceto para o tratamento que consistiu de caule como explante, uso de picloram (1.65 mM) e 70 dias para formação de calos. Portanto, 'Contenda' é a cultivar mais adequada para a produção de sementes sintéticas.

Palavras-chave: *Solanum tuberosum* L., semente sintética, variação genética, ácido 2,4–dicloro fenoxiacético (2,4-D), picloram.

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Cultivated potato (*Solanum tuberosum* L.) is one of the most important vegetable crop of Brazil and the world's fourth most important crop (Solomon-Blackbourn & Barker, 2001). In 2002, Brazil produced 3,126,411 t of potato with an average yield of 19,4 kg/ha (IBGE, 2002).

Potato is a tetraploid species and the use of its botanical seeds in commercial cultivation is precluded by low germinability and large variability in the segregant generations (Gardner & Snustad, 1986). Synthetic seed is one of the alternatives to solve this problem. The original definition of synthetic seeds, "a single encapsulated somatic embryo", was given by Murashige (1978). In this concept, the synthetic seed is a clonal product that can be handled and used as a real seed for transport, storage and *ex vitro* as well as *in vitro* planting. Currently, the most accepted definition of synthetic seed is that of Aitken-Christe *et al.* (1994): "artificially encapsulated somatic embryos, sprouts and other tissues that can be used for *in vitro* or *ex vitro* plantings". This definition extends the concept of synthetic seeds to all types of plant propagules, as structures that can be used similarly to botanical seeds. (Standardi & Piccioni, 1998).

Somatic embryogenesis produces somatic embryos and can be used for the production of synthetic seeds. However, somaclonal variation may be associated with somatic embryogenesis. Somaclonal variation, a common phenomenon in plant cell cultures, includes all types of variations among plants or cells and derives from all kinds of tissue cultures (Skirvin *et al.*, 1993). Somaclonal variation is also called tissue or culture-induced variation. (Kaeppler, *et al.*, 2000). Because the goal of synthetic seed production is to obtain clonal identity, controlling the somaclonal variation is a challenge (Amirato, 1991).

Many causes have been identified or proposed for each type of variation; these, however, may vary from species to species and determining the genetic nature of the observed variation is difficult (Maraschin *et al.*, 2002). These variation causes include: changes in the

structure and/or chromosome number, noticeable point mutations, changes in the expression of a gene as a result of structural changes in the chromosome (heterochromatin and effects of position) or activation of transposable elements, chromatin loss, DNA amplification, somatic crossing over, somatic reduction and structural changes in the cytoplasmatic organelle DNA (Rao *et al*., 1992; Kaeppler, *et al*., 2000). Evans & Sharp (1988) reported four critical variables for somaclonal variation: genotype, explant origin, cultivation period and the cultural condition in which the culture is made.

Plant genotype may have important effects on somaclone regeneration and frequency. These effects are very evident on potatoes: differences are observed in the number of regenerated plants of distinct cultivars grown under identical conditions (Gunn & Shepard, 1981). It is possible to identify cultivars prone to somaclonal variation, which suggests the involvement of a genetic component on the susceptibility to somaclonal variation (Karp & Bright, 1985).

Explant source is considered the most frequent critical variable for somaclonal variation. Since explants may present dissimilar regeneration rates, selection procedures can differ among different explants types. Plants regenerated from chrysanthemum petal epidermis-induced calli showed greater somaclonal variation than those from apex-induced calli (De Jong & Custers, 1986).

The correlation between the culture time-length and the accumulation of chromosome variations was first documented in *Daucus carota* (Smith & Street, 1974). Also, Chaturvedi *et al*. (2001) reported a shift in the morphogenetic pattern of differentiation from shoot bud to embryoid regeneration during the long-term culture of callus of *Citrus grandis*. Hirochika *et al*. (1996) reported an increase in the copy number of transposon *Tos 17* in rice, when submitted to long periods of incubation.

Addition of growth regulators to culture medium is known to have influence on the frequency of the karyotype alterations in cell cultures. Frequently, the auxin 2.4-D (2.4–Dichloro-phenoxyacetic Acid) is considered to be responsible for the chromosome variation (Singh *et al*., 1975). Using 2,4-D to substitute ANA (naphtalenoacetic acid) in potato culture medium increases the frequency of abnormal plants (Shepard, 1981). Increasing amounts of 2,4-D enhances methylation levels in carrot cultures (Kaeppler *et al*., 2000).

Many strategies can be used to evaluate plant genetic structure from *in vitro* derived plant clones, but most of them have limitations. Karyology analysis cannot reveal alteration in specific genes or in small chromosome arrangements. Isoenzyme markers provide an appropriate method to detect genetic changes. However, these markers are susceptible to ontogenetic variation and are limited in number, and only DNA segments coding for soluble proteins can be sampled. RFLP (Restriction Fragment Length Polymorphism) markers are reliable for sampling various genome regions and are potentially unlimited in number. However, this technique is slow, expensive, and requires large quantities of tissue.

RAPD (Random Amplified Polymorphic DNA) analysis using PCR in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals (Xena de Enrech, 2000). The advantages of this technique are: a) a large number of samples can be quickly and economically analyzed using only micro-quantities of material; b) the DNA amplicons are independent from the ontogenetic expression; and c) many genomic regions can be sampled with a potentially unlimited number of markers (Isabel *et al*., 1993).

This work had two objectives: 1) identify the method that produces the larger quantity of somaclones with less somaclonal variation for the cultivars analysed, and 2) determine which *Solanum tuberosum* L. cultivar is the most adequate for synthetic seed production.

MATERIAL AND METHODS

Potato cultivars and experimental treatments

The plant material consisted of five *in vitro* potato commercial cultivars (*Solanum tuberosum* L.) Achat, Baraka, Baronesa, Bintje and Contenda obtained from Embrapa Hortaliças. Nodal segments with at least 2 buds were used as plant propagules in MS medium (Murashige & Skoog, 1962) supplemented with agarose 3%, 0.6 mmol/L myo-inositol, 83.3 µmol/L cisteine, 26.6 µmol/L glycine, 2.9 µmol/L thiamine-HCl, 2.4 µmol/L pyridoxine, 4.0 µmol/L nicotinic acid, 1.4 µmol/L giberelic acid, and pH adjuted to 5.7 (Buso *et al*, 1989). Plantlets were kept under 1000 lux with 16 hours photoperiod at 25ºC. The medium described previously was supplemented with picloram (1.65 µmol/L) or 2,4-D (11.5 µmol/L) and used to induce callus formation under dark conditions at 25ºC. Stem and leaf explant sizes were approximately 1 cm long and 1 cm^2, respectively.

Each of the five cultivars was submitted to eight treatments of callus induction. In terms of callus cultivation, treatments 1 to 4, and 5 to 8 consisted of 70 and 90 days culture time length, respectively. Considering traits explant and growth regulators, treatments were set as follows: 1 and 5, leaf and 2,4-D (11.5 µmol/L); 2 and 6, stem and 2,4-D (11.5 µmol/L); 3 and 7, leaf and picloram (1.65 µmol/L); 4 and 8, stem and picloram (1.65 µmol/L), in that order. The control source plant is represented by treatment 9.

Isolating the genomic DNA for RAPD analysis and amplification reactions

Genomic DNA of plants and calli were extracted using a modified protocol from Doyle & Doyle (1990). The RAPD analyses were carried out as described by Williams *et al*. (1990). Twenty primers (Operon Technologies Inc., Alameda, CA, USA), that were selected due to their efficiency for DNA amplification of all cultivars are presented in Table 1.

The amplification reactions were performed in a Perkin-Elmer 9600 thermocycler (Norwalk, CT USA), programmed for 40 cycles of 15 seconds at 94°C, 30 seconds at 35°C, and 1 minute at 72°C. The amplification products were analyzed by electrophoresis in 1.2% agarose gel and visualized under UV light.

Table 1. Primers used in RADP analysis. Viçosa, UFV, 1996.

Primer code	Sequence 5' → 3'	Primer code	Sequence 5' → 3'
OPA-09	GGGTAACGCC	OPQ-17	GAAGCCCTTG
OPA-10	GTGATCGCAG	OPR-11	GTAGCCGTCT
OPA-11	CAATCGCCGT	OPR-12	ACAGGTGCGT
OPB-07	GGTGACGCAG	OPS-03	CTACTGCGCT
OPB-08	GTCCACACGG	OPS-05	TTTGGGGCCT
OPC-09	CTCACCGTCC	OPS-11	AGTCGGGTGG
OPD-01	ACCGCGAAGG	OPS-13	GTCGTTCCTG
OPD-04	TCTGGTGAGG	OPS-14	AAAGGGGTCC
OPQ-11	TCTCCGCAAC	OPT-14	AATGCCGCAG
OPQ-14	GGACGCTTCA	OPT-17	CCAACGTCGT

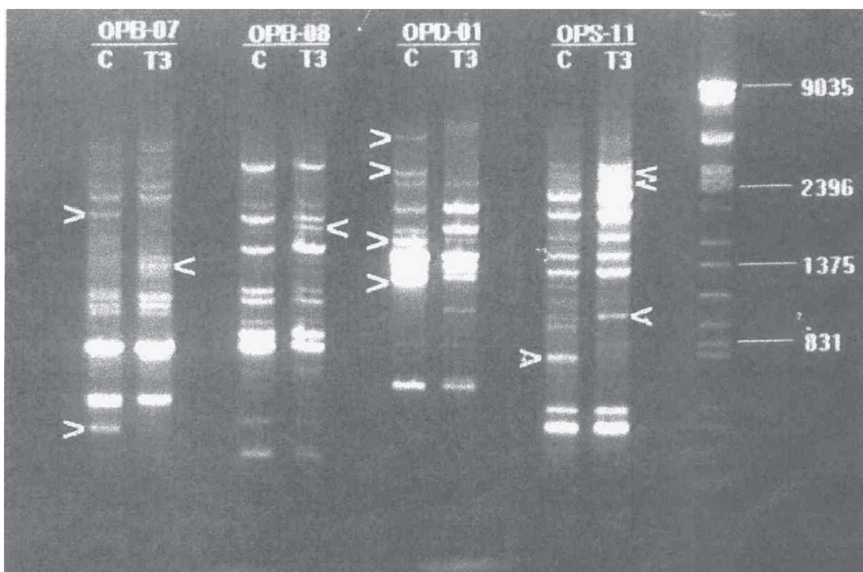

Figure 1. RAPD treatment 3 polymorphism detection for the Contenda cultivar. The genomic DNA of the Contenda cultivar (C-source plant) and the treatment 3 (T3) induced calli were amplified using the primers indicated on the top of the Figure. The analyzed variables in the referred treatment were leaf explant callus, 70 days of callus formation and Picloram as growth regulator. The electrophoretic migration position of the DNA patterns in base pairs is indicated at the right hand side of the picture. The arrows indicate the polymorphic bands. Viçosa, UFV, 1996.

Statistical analysis

Cluster analysis of individuals within each potato cultivar was performed adopting as measure of dissimilarity the arithmetic complement of the Jaccard index, which is given by: $J=a/a+b+c$, where,

a=1-1: number of coincidence of the type "1" and "1";

b=1-0: number of non-coincidence of the type "1" and "0"; and;

c=0-1: number of non coincidence of the type "0" and "1";

and

1= presence of band; and,

0= lack of band.

The arithmetic complement expressed by DJ is given by $DJ=1-J$.

The cluster technique used was based on the method of Tocher (Cruz & Regazzi, 1997).

RESULTS AND DISCUSSION

Eighteen out of the 20 primers used in this research presented polymorphic bands in the calli DNA amplification process after the 8 treatments. The number of primers that presented polymorphism to at least one of the treatments for each cultivar was: 5 to Baraka, 4 to Bintje, 14 to Contenda, 7 to Baronesa, and 4 to Achat. The present analysis suggested that the RAPD methodology was efficient in detecting somaclonal variation in potatoes.

The method of Tocher allowed the construction of two clusters for the Baraka cultivar. Cluster A was formed by calli-derived treatments 1, 2, 3, 4, 5, 6, 8 and 9, and the cluster B was formed by the calli from treatment 7. The method showed that treatment 7 (leaf, picloram and 90 days of callus formation) was the most effective in the induction of somaclonal variation, suggesting an interaction between the explant, the growth regulator, and long periods of cultivation that increased variability. Varying the cluster routine (i.e. Nearest Neighbor Analysis) had no effect on the grouping of treatments for this cultivar, or the other tested cultivars.

Three distinct clusters were obtained for the Bintje cultivar by the method of Tocher: cluster A was formed by calli from treatments 2, 3, 4, 5, 6, 7 and 8; cluster B was formed by the control source plant (treatment 9); and cluster C, by calli from treatment 1, which also generated the most genetically-distant individuals. Treatment 1 involved picloram, leaf explants, and 70 days of calli formation; it induced more somaclonal variation than the others. An unexpected result occurred in this case because a greater number of polymorphic fragments was expected for the same treatment condition after 90 days of callus formation than after 70. The premise that prolonged periods of *in vitro* cultivation result in increased frequency of chromosome aberrations was not supported by the results (Bayliss, 1980; Larkin, 1987; Karp & Bright, 1985).

Fourteen, out of the 20 primers tested in the Contenda cultivar, showed polymorphic fragments from leaf-derived calli (treatment 3 - leaf explant, picloram (1.65 mM) and 70 days of callus formation). The cluster formation confirmed this result. This significant effect of the picloram treatment conflicts with reports in literature that describe

2,4-D as the most efficient growth regulator for promoting somaclonal variation. Figure 1 shows the results of the amplification products of Contenda cultivar leaf-derived calli from treatment 3 by comparing polymorphic fragments with the control plant.

Two clusters were obtained for the Baronesa cultivar. Cluster A contained calli from all the treatments while cluster B contained the control source plant (treatment 9). The RAPD analysis showed that, among the genotypes analyzed, the Baronesa cultivar presented the highest number of polymorphic fragments for all treatments. This shows that susceptibility to somaclonal variation is related to genotype, as described by Roest *et al*. (1981). They found 43% variability in one *Begonia hiemalis* cultivar and only 7% in another. Likewise, differences in somaclonal variation incidence in three potato cultivars were observed: Russet Burbank cultivar somaclones showed higher variability than those of the Kennebec and Superior cultivars (Rietveld *et al*., 1993).

The method of Tocher divided the Achat cultivar treatments in two clusters. Calli stemming from treatments 6 and 8 were in cluster B; the others were in cluster A. Results showed the joint effect of time of callus formation and growth regulators on promoting greater somaclonal variation than other treatments. Likewise, Mangolin *et al.* (2002) found a high genetic variability in callus tissue of *Cereus peruvianus* maintained with 4 mg/L 2,4-D and 4 mg/L or 8 mg/L kinetin. Larkin (1987) reported that longer period of *in vitro* cultivation seemed to increase somaclonal variations, whereas a shorter callus phase should reduce them.

Our results showed that RAPD analysis allows discrimination of the treatments for induction of somaclonal variation. This conclusion is similar to the results obtained by Mangolin *et al.* (2002) regarding analysis of genetic variation in callus of *Ceres peruvianus*. Thus, this strategy can be used to evaluate integrity of *in vitro* propagated genetic material. Because only micro quantities of material are needed and also because the protocol for DNA isolation from calli and leaves is relatively simple, this methodology can be used to evaluate each step of the *in vitro* culture, until the regenerated plant is obtained.

Except for treatment 3, which consisted of leaf as explant source, picloram (1.65 mM) and 70 days of callus formation, all treatments using the Contenda cultivar showed the smallest genetic variation among the five tested cultivars for *in vitro* cultures. Contenda was, therefore, the most appropriate cultivar to be used for the production of synthetic seeds. An alternative would be the Achat cultivar, because the combination of 2,4-D or picloran leaf explant, and periods no longer than 70 days did not present somaclonal variation.

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