Assessment of Genetic Stability Among In Vitro Plants of Arachis retusa Using RAPD and AFLP Markers for Germplasm Preservation

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Abstract

Arachis retusa Krapov. et W. C. Gregory et Valls is endemic in the West-central region of Brazil, occurring in areas endangered by human actions. The establishment of in vitro preservation methods for wild species of Arachis is an alternative to seed banks for germplasm storage, multiplication and distribution. The risk of genetic changes induced by tissue culture and the monitoring of the genetic stability of the biological material before, during and after storage must be considered in the context of conservation. Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) fingerprinting were used to evaluate the genetic stability of in vitro plants originated from cotyledons and embryo axes of A. retusa. Cotyledons originated shoots through direct organogenesis and embryo axes displayed multishoot formation induced by 110 mmol/L and 8.8 mmol/L BAP, respectively. Ninety genomic regions (loci) generated from RAPD and 372 from AFLP analyses were evaluated. All amplified fragments detected by both techniques in plants derived from the two explant types were monomorphic. The results indicate that the recovered shoots are genetically stable at the assessed genomic regions.

Key words: amplified fragment length polymorphism; Arachis retusa; in vitro preservation; micropropagation; random amplified polymorphic DNA; somaclonal variation.


Wild species of Arachis are important gene sources for the improvement of groundnut. Arachis retusa is endemic to the West-central region of Brazil (Krapovickas and Gregory 1994) and is restricted to areas endangered by intensive environmental...
dissurbance and human actions. In a recent survey, it was found that 65.5% of its potential habitat is under agricultural land use (Jarvis et al. 2003).

Conservation of Arachis genetic resources in seed banks requires periodical renewal for germplasm maintenance and distribution. However, seed banks frequently face serious drawbacks as a consequence of contamination and loss of viability that lead to losses of entire accessions. Arachis seeds may be classified as sub-orthodox according to the concept applied to seeds that can be stored under the same conditions as true orthodox ones but for shorter periods. This behavior may result from the high fat content of the storage tissues, which can undergo auto-oxidation and originate free radicals that damage proteins and nucleic acids (Benson 1990). In addition, some species characteristically produce low numbers of seeds, which impairs distribution and use.

In vitro preservation techniques are particularly useful for endangered species and to provide large quantities of plant material for germplasm management activities (Ashmore 1997). Arachis seeds have proven to be a suitable explant source for in vitro propagation, even when germinability is lost (Gagliardi et al. 2000). In vitro regeneration of several Arachis species has been achieved from cotyledons, embryo axes and leaflets of non-viable seeds allowing rescue of germplasm and supporting seed bank and field collection.

Nevertheless, phenotypic and genetic variations are reported to occur as a consequence of the propagation process, originating somaclonal variants (Larkin and Scowcroft 1981). The possibility of genetic alterations induced by the processes of tissue culture deserves special consideration when the objective is the multiplication of selected genotypes or germplasm conservation. Thus, in the context of rescuing Arachis germplasm it is particularly important to assess the genetic stability of the primary regenerants.

Molecular markers, which can detect modifications at the DNA level, are increasingly being used to access the fidelity of in vitro propagated plants. Among these, random amplified polymorphic DNA (RAPD)-based fingerprinting is being used by several workers to detect molecular alterations in in vitro regenerated plants, and different rates of variation were reported according to the species and the regeneration system adopted. In Arachis, RAPD has also been used to evaluate genetic diversity (Dwivedi et al. 2001), phylogenetic relationships within wild species (Nobile et al. 2004), interspecific polymorphisms (Lanham et al. 1992) and introgression in A. hypogaea x A. cardenasi hybrids (Garcia et al. 1995).

More recently, the amplified fragment length polymorphism (AFLP) technique has proven to be a highly efficient tool for characterizing somaclonal variation (Carolan et al. 2002; Popescu et al. 2002), in addition to cultivar identification (Ferriol et al. 2003) or redundancy reduction in germplasm collection (van Treuren et al. 2004). In Arachis, this technique was used to detect DNA polymorphism in the cultivated peanut (Herselman 2003) and to establish genetic relationships among species (Gimenes et al. 2002). However, there are still comparatively few reports on the use of AFLP in monitoring the genetic stability of in vitro plants obtained through different regeneration pathways. These studies were performed, for example, in plants of pecan (Vendrame et al. 1999), cork oak (Hornero et al. 2001), Arabidopsis thaliana (Polanco et al. 2002), grapevines (Popescu et al. 2002), and among in vitro cell lines of Papaver bracteatum (Carolan et al. 2002).

The objective of the present work was to evaluate the suitability of both RAPD and AFLP procedures to assess genetic stability of in vitro plants of A. retusa originated from cotyledons through direct organogenesis and from embryo axes through bud multiplication.

**Results**

A. retusa plants were obtained in vitro both from cotyledons and embryo axes (Figure 1). RAPD analysis of the clones resulted in 90 genomic regions (loci), with an average of 18 loci per clone (Table 1, Figure 2). The reproducibility of RAPD bands

![Figure 1. In vitro regeneration of Arachis retusa.](image)

(A) Organogenesis from cotyledon.
(B) Bud multiplication from embryo axis.
(C) Whole plant originated from cotyledon.
(D) Whole plant originated from embryo axis.
Bar = 1 cm
Genetic Stability Among In Vitro Plants of *A. retusa*

was assured by combining a high quality of template DNA preparation, an accurate estimation of DNA concentration and the use of two DNA concentrations in the primer-screening step (Carneiro et al. 2002). For primer selection, 10 primers that were previously employed for establishing phylogenetic relationships in *Arachis* and *Stylosanthes* (Vieira et al. 1997; Subramanian et al. 2000; Dwivedi et al. 2001) were initially screened. Among those, we selected five primers, which showed the highest number of RAPD loci and good reproducibility. No polymorphism among clones obtained both from the two explant types was found with these primers (Figure 2).

For the AFLP analysis, 24 primer combinations were initially screened, leading to the selection of eight combinations that generated a multiloci DNA fingerprinting with 372 fragments (Table 2). The highest number of DNA fragments (55) was obtained with *EACA-MCAT*, whereas the lowest number of bands (31) was produced with *EAAG-MCAC*. The AFLP assays generated 46 bands per primer combination, on average, detecting three times the number of markers as compared to RAPD analyses, and no polymorphism was detected among the 16 clones analyzed (Figure 3, Table 2).

### Discussion

The comparison of DNA patterns among *A. retusa* clones

<table>
<thead>
<tr>
<th>Primer combination</th>
<th>Number of loci</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EACA-MCAT</em></td>
<td>48</td>
</tr>
<tr>
<td><em>EACA-MCAT</em></td>
<td>55</td>
</tr>
<tr>
<td><em>EAAG-MCTC</em></td>
<td>44</td>
</tr>
<tr>
<td><em>EACA-MATT</em></td>
<td>48</td>
</tr>
<tr>
<td><em>EAAG-MCTT</em></td>
<td>48</td>
</tr>
<tr>
<td><em>EAAG-MGCC</em></td>
<td>31</td>
</tr>
<tr>
<td><em>EACT-MCCG</em></td>
<td>44</td>
</tr>
<tr>
<td>Total / Mean</td>
<td>372 / 46</td>
</tr>
</tbody>
</table>

Table 1. Primer codes, nucleotide sequences and number of amplified products (loci) detected in *Arachis retusa* clones

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’→3’)</th>
<th>Number of loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPA 02</td>
<td>TGCCGAGCTG</td>
<td>15</td>
</tr>
<tr>
<td>OPA 07</td>
<td>GAAACGGGTTG</td>
<td>18</td>
</tr>
<tr>
<td>OPG 02</td>
<td>GGCTCGGAG</td>
<td>17</td>
</tr>
<tr>
<td>OPG 03</td>
<td>GAGCCCTCCA</td>
<td>20</td>
</tr>
<tr>
<td>OPJ 17</td>
<td>ACGGCAGTTC</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>90 / 18</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Amplification patterns of in vitro plants of *Arachis retusa*.

DNA from plants regenerated from embryo axes (A, E1-E8) and cotyledons (B, C1-C8) amplified with the oligomer OPA07. Two different concentrations of DNA were used for each sample: 10 ng in even lanes and 20 ng in odd lanes.
M, ladder 100 bp.
obtained both from cotyledons and from embryo axes did not allow the detection of any polymorphism that could be ascribed to the \textit{in vitro} procedure. RAPD and AFLP analyses cover different regions and generate large numbers of bands, thus representing a random-sampling of the genome. While some investigators reported absence of \textit{in vitro}-induced variation in a number of plant species, even in somaclones identified as cytological variants (Vallés et al. 1993; Goto et al. 1998; Rout et al. 1998; Al-Zahim et al. 1999), significant polymorphism rates were found in plants regenerated via somatic embryogenesis (Aronen et al. 1999; Al-Zahim et al. 1999; Vendrame et al. 1999; Hornero et al. 2001; Popescu et al. 2001), calluses (Jazdzewska et al. 2000; Soniya et al. 2001; Polanco and Ruiz 2002) and direct organogenesis (Kumar et al. 1999; Virscek-Marn et al. 1999). The potential reasons for this apparent discrepancy can be related to different factors, including the regeneration procedure, genotype and number of primers used in the amplification reactions.

The absence of polymorphism in a total of 462 loci derived from a combined analysis of both RAPD and AFLP markers indicates that micropropagated \textit{A. retusa} plants obtained through cotyledon-derived direct organogenesis and bud multiplication from embryo-axes are genetically stable in the assessed genome regions. However, no genetic marker can provide absolute evidence that somaclonal variation has not occurred in culture. Therefore, considering the importance of ensuring the genetic stability of \textit{in vitro} plants in conservation programs (Ashmore 1997), it is important to choose regeneration procedures that minimize induced variation. In addition, the use of different strategies for monitoring genetic stability, including cytological analysis and detection of possible DNA-methylation changes using MSAP (methylation sensitive amplified polymorphism), is highly desirable.

**Materials and Methods**

### Plant material

Seeds of \textit{Arachis retusa} Krapov. et W. C. Gregory et Valls (accession 9950) were provided by the seed bank of Embrapa Genetic Resources and Biotechnology (Brasilia/DF, Brasil). Plants were obtained from cotyledons and embryo axes excised from 5–10 seeds that had lost germinative capacity. Cotyledons and embryo axes were cultured on MS medium (Murashige and Skoog 1962) supplemented with 110 and 8.8 mmol/L 6-benzylaminopurine respectively (Gagliardi et al. 2000). Leaves from 16 clones of \textit{in vitro} plants recovered from each explant type from the same seed were used for DNA extraction.

### RAPD assay

The DNA of \textit{A. retusa} plants was extracted and quantified by standard procedures. RAPD reactions were performed in a PTC-100 MJ thermocycler (MJ Research, Inc., Watertown, MA, USA) in a final volume of 25 mL containing 10–20 ng of genomic DNA; 10 mmol/L Tris-HCl pH 8.8; 50 mmol/L KCl; 200 mmol/L dNTP; 50 mmol/L each primer; 4 mmol/L MgCl$_2$ and 2 U Taq polymerase. PCR reagents were purchased from Amersham-Pharmacia-Biotech. The DNA was denatured at 94 °C for 5 min; then 45 cycles of 94 °C for 1 min, 32 °C for 1 min, and 72 °C for 2 min were performed, followed by a final extension step at 72 °C for 6 min. RAPD products were analyzed by electrophoresis (3 V/cm) on 1.4% agarose gels and visualized after ethidium bromide staining. Gels were photographed under ultraviolet light (Geldoc Bio-Rad, USA). Ten primers (Operon Technologies, Alameda, CA, USA) were initially assayed and five of them were selected (Table 1).

### AFLP assay

The AFLP analysis was carried out as described by Vos et al. (1995), with small modifications. Total DNA (250 ng) was double-digested using \textit{EcoRI} and \textit{MseI}. Double-stranded adapters were then ligated to the restriction fragments and the preselective amplification of the template DNA was performed using primers containing one selective nucleotide (E + A; M +
The PCR products were diluted 5-fold in Tris-EDTA buffer and used as template for selective amplification. PCR products were resolved on 6% denaturing polyacrylamide gels with 7.5 mol/L urea at 70 W and 50 °C. The gels were stained with silver nitrate according to Creste et al. (2001).

Data analysis

The size of the amplified products generated by RAPD and AFLP ranged from 50 to 1,800 bp. Due to their dominant inheritance, both marker types were scored for the presence or absence of the bands across the 16 A. retusa clones. Only reliable and repeatable bands were considered.

Acknowledgements


References


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