Journal of Integrative Plant Biology 2007, **49** (3): 307–312

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

Rachel Fatima Gagliardi[1*†], Luiz Ricardo Hanai[2†], Georgia Pacheco[1], Carlos Alberto Oliveira[2], Leonardo Alves Carneiro, José Francisco Montenegro Valls[3], Elisabeth Mansur[1] and Maria Lucia Carneiro Vieira[2]

([1]*Laboratório de Micropropagação e Transformação de Plantas, Universidade do Estado do Rio de Janeiro,* postal code 20550-013 Maracanã, Rio de Janeiro, Brazil;
[2]*Departamento de Genética - Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo,* postal code 13418-900 Piracicaba, Brazil;
[3]*Embrapa Recursos Genéticos e Biotecnologia,* postal code 70770-901 Brasília, Brazil)

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.

Gagliardi RF, Hanai LR, Pacheco G, Oliveira CA, Carneiro LA, Valls JFM, Mansur E, Vieira MLC (2007). Assessment of genetic stability among *in vitro* plants of *Arachis retusa* using RAPD and AFLP markers for germplasm preservation. *J. Integr. Plant Biol.* **49**(3), 307–312.

Available online at www.blackwell-synergy.com/links/toc/jipb, www.jipb.net

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

Received 30 Mar. 2006 Accepted 5 Jul. 2006

Supported by FAPERJ (Research Foundation of the State of Rio de Janeiro) and CNPq (National Council for Scientific and Technological Development Research Fellowships).

†These authors contributed equally to this work.

Publication of this paper is supported by the National Natural Science Foundation of China (30624808) and Science Publication Foundation of the Chinese Academy of Sciences.

*Author for correspondence.

Tel (Fax): +55 21 2587 7663;

E-mail: <gagliard@gmail.com>.

doi: 10.1111/j.1672-9072.2007.00402.x

disturbance 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-ortodox according to the concept applied to seeds that can be stored under the same conditions as true ortodox 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 (Nóbile et al. 2004), interspecific polymorphisms (Lanham et al. 1992) and introgression in *A. hypogaea* x *A. cardenasii* 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

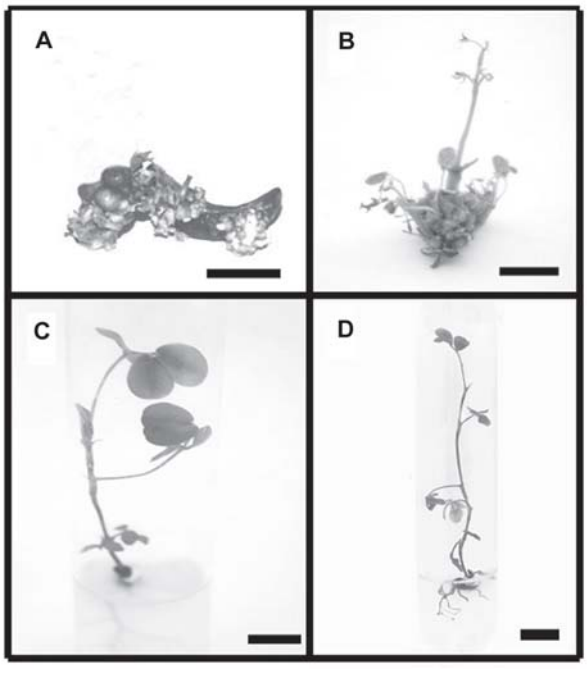

Figure 1. *In vitro* regeneration of *Arachis retusa.*

(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

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

Primer	Sequence (5'→3')	Number of loci
OPA 02	TGCCGAGCTG	15
OPA 07	GAAACGGGTG	18
OPG 02	GGCACTGAGG	17
OPG 03	GAGCCCTCCA	20
OPJ 17	ACGCCAGTTC	20
Total / Mean	90 / 18	

Table 2. AFLP-primer combinations and number of amplified products (loci) detected in *Arachis retusa* clones

Primer combination	Number of loci
E_{AAC}-M_{CAT}	48
E_{ACA}-M_{CAT}	55
E_{AAA}-M_{CTC}	44
E_{AAA}-M_{CTT}	48
E_{AAC}-M_{CTT}	48
E_{AAG}-M_{CTT}	54
E_{AAG}-M_{CAC}	31
E_{ACT}-M_{CGC}	44
Total / Mean	372 / 46

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 E_{ACA}-M_{CAT}, whereas the lowest number of bands (31) was produced with E_{AAG}-M_{CAC}. 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

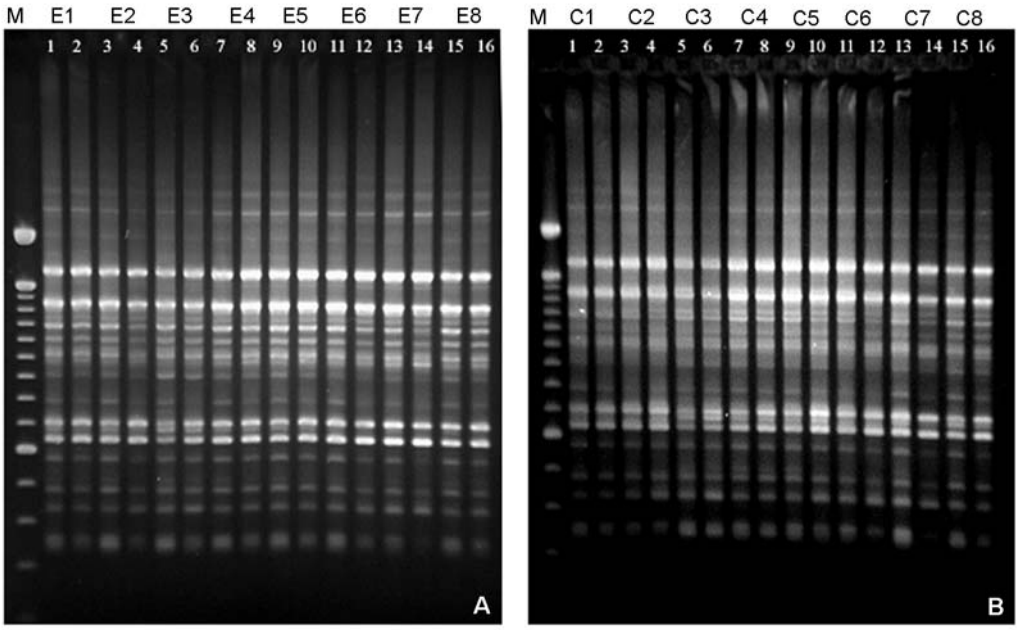

Figure 2. Amplification patterns of *in vitro* plants of *Arachis retusa*.

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.

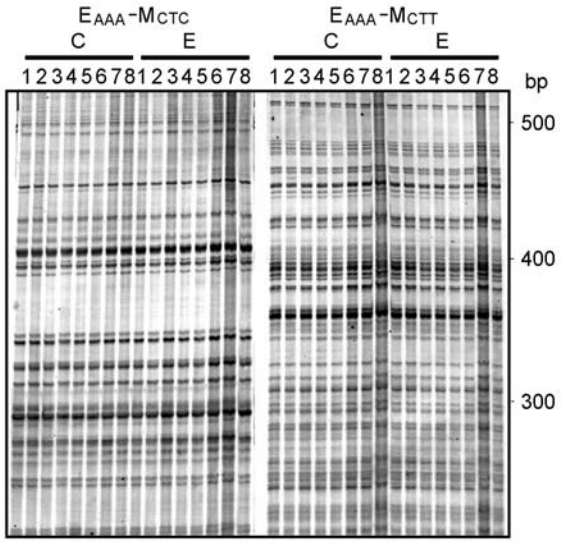

Figure 3. DNA profiles from cotyledon (C) and embryo-axis (E)-derived plants of *Arachis retusa* using two amplified fragment length polymorphism (AFLP)-primer combinations. Identical patterns were observed for the 16 plants analyzed (C1–C8 and E1–E8). On the right, fragment sizes in bp.

obtained both from cotyledons and from embryo axes did not allow the detection of any polymorphism that could by ascribed to the *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 *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 *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 *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 (methlylation sensitive amplified polimorphism), is highly desirable.

Materials and Methods

Plant material

Seeds of *Arachis retusa* Krapov. et W. C. Gregory et Valls (accession 9950) were provided by the seed bank of Embrapa Genetic Resources and Biotechnology (Brasília/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 *in vitro* plants recovered from each explant type from the same seed were used for DNA extraction.

RAPD assay

The DNA of *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 *Eco*RI and *Mse* I. 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 +

C). 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

E. Mansur, J. F. M. Valls and M. L. C. Vieira are recipients of National Council for Scientific and Technological Development Research Fellowships. G. Pacheco is indebted to Research Foundation of the State of Rio de Janeiro for a doctoral fellowship.

References

Al-Zahim MA, Ford-Lloyd BV, Newbury HJ (1999). Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. *Plant Cell Rep*. **18**, 473–477.

Aronen TS, Krajnakova J, Haggman HM, Ryynanen LA (1999). Genetic fidelity of cryopreserved embryogenic cultures of open-pollinated *Abies cephalonica*. *Plant Sci*. **142**, 163–172.

Ashmore SE (1997). *Status Report on the Development and Application of In Vitro Techniques for the Conservation and Use of Plant Genetic Resources.* IPGRI, Rome, Italy.

Benson EE (1990). *Free Radical Damage in Stored Plant Germplasm.* IBPGR, Rome, Italy.

Carneiro MS, Camargo LEA, Coelho ASG, Vencovsky R, Leite RP, Stenzel NMC et al. (2002). RAPD-based genetic linkage maps of yellow passion fruit (*Passiflora edulis* Sims. f. *flavicarpa* Deg.). *Genome* **45**, 670–678.

Carolan JC, Hook ILI, Walsh JJ, Hodkinson TR (2002). Using AFLP markers for species differentiation and assessment of genetic variability of *in vitro* cultured *Papaver bracteatum* (Section *Oxytona*). *In Vitro Cell Dev*. *Biol*. *Plant* **38**, 300–307.

Creste S, Tulmann Neto A, Figueira A (2001). Detection of single sequence repeat polymorphism in denaturing polyacrylamide sequencing gels by silver staining. *Plant Mol*. *Biol*. *Rep*. **19**, 299–306.

Dwivedi SL, Gurtu S, Chandra S, Yuejin W, Nigam N (2001). Assessment of genetic diversity among selected groundnut germplasm. I: RAPD analysis. *Plant Breeding* **120**, 345–349.

Ferriol M, Pico B, Cordova PF, Nuez F (2003). Molecular diversity of a germplasm collection of squash (*Cucurbita moschata*) determined by SRAP and AFLP markers. *Crop Sci*. **44**, 653–664.

Gagliardi RF, Pacheco GP, Coculilo SP, Valls JFM, Mansur E (2000). *In vitro* plant regeneration from seed explants of wild groundnut species (Genus *Arachis*, Section *Extranervosae*). *Biodiv*. *Conserv*. **9**, 943–951.

Garcia GM, Stalker HT, Kochert G (1995). Introgression analysis of an interspecific hybrid population in peanuts (*Arachis hypogaea* L.) using RFLP and RAPD markers. *Genome* **38**, 166–176.

Gimenes MA, Lopes CR, Valls JFM (2002). Genetic relationships among *Arachis* species based on AFLP. *Genet*. *Mol*. *Biol*. **25**, 349–353.

Goto S, Thakur RC, Ishii K (1998). Determination of genetic stability in long-term micropropagated shoots of *Pinus thunbergii* Parl. using RAPD markers. *Plant Cell Rep*. **18**, 193–197.

Herselman L (2003). Genetic variation among Southern African cultivated peanut (*Arachis hypogaea* L.) genotypes as revealed by AFLP analysis. *Euphytica* **133**, 319–327.

Hornero J, Martinez J, Celestino C, Gallego FJ, Torres V, Toribio M (2001). Early checking of genetic stability of cork oak somatic embryos by AFLP analysis. *Int*. *J*. *Plant Sci*. **162**, 827–833.

Jarvis A, Fergurson MA, Williams DE, Guarino L, Jones PG, Stalker HT et al. (2003). Biogeography of wild *Arachis*: Assessing conservation status and setting future priorities. *Crop Sci*. **43**, 1100–1108.

Jazdzewska E, Sadoch Z, Niklas A, Majewska-Sawka A (2000). Plant regeneration from sugar beet leaf protoplasts: Analysis of shoots by DNA fingerprinting and restriction fragment length polymorphism. *Can*. *J*. *Bot*. **78**, 10–18.

Krapovickas A, Gregory WC (1994). Taxonomia del género *Arachis* (*Leguminosae*). *Bomplandia* **8**, 1–186.

Kumar MB, Barker RE, Reed BM (1999). Morphological and molecular analysis of genetic stability in micropropagated *Fragaria* x *Ananassa* cv. Pocahontas. *In Vitro Cell Dev*. *Biol*. *Plant* **35**, 254–258.

Lanham PG, Fennell S, Moss JP, Powell W (1992). Detection of polymorphic loci in *Arachis* germplasm using random amplified polymorphic DNAs. *Genome* **35**, 885–889.

Larkin PJ, Scowcroft WR (1981). Somaclonal variation – a novel source of variability from cell cultures for plant improvement. *Theor*. *Appl*. *Genet*. **60**, 197–214.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bio-assay with tobacco tissue cultures. *Physiol*. *Plant.* **15**, 437–497.

Nóbile PM, Gimenes MA, Valls JFM, Lopes CR (2004). Genetic variation within and among species of genus *Arachis*, section *Rhizomatosae*. *Genet*. *Resour*. *Crop Evol*. **51**, 299–307.

Polanco C, Ruiz ML (2002). AFLP analysis of somaclonal variation in *Arabidopsis thaliana* regenerated plants. *Plant Sci*. **162**, 817–824.

Popescu CF, Falk A, Glimelius K (2002). Application of AFLPs to characterize somaclonal variation in anther-derived grapevines.

Vitis **41**, 177–182.

Rout GR, Das P, Goel S, Raina SN (1998). Determination of genetic stability of micropropagated plants of ginger using random amplified polymorphic DNA (RAPD) markers. *Bot*. *Bull*. *Acad*. *Sin*. **39**, 23–27.

Soniya EV, Banerjee NS, Das MR (2001). Genetic analysis of somaclonal variation among callus-derived plants of tomato. *Curr*. *Sci*. **80**, 1213–1215.

Subramanian V, Gurtu S, Nageswara RC, Nigam SN (2000). Identification of DNA polymorphism in cultivated groundnut using random amplified polymorphic DNA (RAPD) assay. *Genome* **43**, 456–660.

Vallés MP, Wang ZY, Montavon P, Potrykus I, Spangenberg G (1993). Analysis of genetic stability of plants regenerated from suspension cultures and protoplasts of meadow fescue (*Fetusca pratensis* Huds.). *Plant Cell Rep*. **12**, 101–106.

Van Treuren R, Magda A, Hoekstra R, van Hintum TJL (2004). Genetic and economic aspects of marker-assisted reduction of redundancy from a wild potato germplasm collection. *Genet*. *Resour*. *Crop Evol*. **51**, 277–290.

Vendrame WA, Kochert G, Wetzstein HY (1999). AFLP analysis of variation in pecan somatic embryos. *Plant Cell Rep*. **18**, 853–857.

Vieira MLC, Fungaro MHP, Jubier MF, Lejeune B (1997). Determination of taxonomic relationships among Brazilian taxa of *Stylosanthes* S.W., *Leguminosae*, using RAPD markers. *Pesq*. *Agropec*. *Bras*. **32**, 305–310.

Virscek-Marn M, Bohanec B, Javornik B (1999). Adventitious shoot regeneration from apple leaves — optimization of the protocol and assessment of genetic variation among regenerants. *Phyton* **39**, 61–70.

Vos P, Hogers R, Bleeker M, Rejans M, Lee T, Hornes M et al. (1995). AFLP: A new technique for DNA fingerprinting. *Nucleic Acids Res*. **23**, 4407–4414.

(Handling editor: Song Ge)