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CRYOPRESERVATION OF *Arachis pintoi* (LEGUMINOSAE) SOMATIC EMBRYOS

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Abstract

In this study, we successfully cryopreserved cotyledonary somatic embryos of diploid and triploid *Arachis pintoi* cytotypes using the encapsulation-dehydration technique. The highest survival rates were obtained when somatic embryos were encapsulated in calcium alginate beads and precultured in agitated (80 rpm) liquid establishment medium (EM) with daily increasing sucrose concentration (0.50, 0.75, and 1.0 M). The encapsulated somatic embryos were then dehydrated with silica gel for 5 h to 20% moisture content (fresh weight basis) and cooled either rapidly (direct immersion in liquid nitrogen, LN) or slowly (1ºC min^{-1} from 25ºC to -30ºC followed by immersion in LN). Beads were kept in LN for a minimum of 1 h and then were rapidly rewarmed in a 30ºC water-bath for 2 min. Finally, encapsulated somatic embryos were post-cultured in agitated (80 rpm) liquid EM with daily decreasing sucrose concentration (0.75 and 0.5 M) and transferred to solidified EM. Using this protocol, we obtained 26% and 30% plant regeneration from cryopreserved somatic embryos of diploid and triploid cytotypes. No morphological abnormalities were observed in any of the plants regenerated from cryopreserved embryos and their genetic stability was confirmed with 10 isozyme systems and nine RAPD profiles.

Key words: somatic embryo, cryopreservation, encapsulation-dehydration, genetic stability.

Abbreviations: BAP: 6-benzylaminopurine; EM: establishment medium: MS supplemented with 0.05 µM BAP and 0.05 µM NAA; LN: liquid nitrogen; MS: Murashige & Skoog salts and vitamins with 3% sucrose; MC: moisture content (fresh weight basis); NAA: 1-naphthaleneacetic acid; OTU: operational taxonomy unit; PIC: 4 amino-3,5,6-trichloropicolinic acid; PPFD: photosynthetic photon flux density; r: cophenetic correlation coefficient; SE: standard error; UPGMA: unweighted pair-group method using arithmetic averages.

INTRODUCTION

The genus *Arachis* is a member of the Leguminosae family. Currently, the genus consists of 80 described species grouped into nine taxonomic sections. All of them originated in South America (23, 48, 24). The cultivated peanut (*A. hypogaea*) is economically the most important species because of the value of its seeds. It is currently the fourth most important oilseed worldwide (14) and plays a prominent role in subsistence agriculture in several countries (44). Some of the other species are used for human consumption such as *A. stenosperma* and *A. villosulicarpa*, which are cultivated by Brazilian Indian populations (7), or used in cultivated pastures, such as *A. glabrata* (1), *A. repens* (50) and *A. pintoi,* mostly in tropical and subtropical areas of the world (22, 32).

Many wild species of the genus *Arachis* are in danger of extinction due to environmental causes and human actions. These species have been recognized as very important for genetic improvement of cultivated peanut. Conservation of *Arachis* germplasm is mostly carried out in seed banks (10ºC and 45% relative humidity) or in field collections. The seeds of *Arachis* species are characterized by their high lipid content as well as by their fragile tegument. They are very susceptible to oxidative damage (31) and have a sub-orthodox storage behavior (49). For these reasons, their conservation in seed banks is limited by the fact that their viability decreases markedly with time. In some cases, seeds do not germinate after 1-3 years in storage. This behavior requires a constant renewal of stored seeds (9, 17).

The conservation of *A. pintoi* germplasm presents additional difficulties. The diploid (2n = 2x = 20) cytotype (15) produces few seeds, which rapidly lose their viability after a few months in storage. On the other hand, the triploid (2n = 3x = 30) cytotype (34) does not produce seeds and it is an obligate vegetatively propagated plant. Like for other *Arachis* species, *in vitro* culture (9, 18, 28, 30, 31) and cryopreservation (3, 4, 19, 20, 29) techniques appear as complementary procedures for the conservation of *A. pintoi* germplasm. *In vitro* cultured shoot tips and nodal segments have been stored for one year under slow growth conditions (37). More recently, shoot tips and apical meristems were successfully cryopreserved using encapsulation-dehydration (40) and seeds of the diploid cytotype were cryopreserved using vitrification (39). The use of somatic embryos is a very suitable alternative for cryopreservation (6, 13) because their bipolar structure allows direct and quick regeneration of whole plants (27). Furthermore, somatic embryos have been cited as organized explants that can ensure the genetic stability of the cryopreserved material (41).

In the present work, we report the first successful cryopreservation of somatic embryos of both diploid and triploid *A. pintoi* cytotypes using the encapsulation-dehydration technique. We also tested the genetic stability of plants regenerated from cryopreserved somatic embryos using isozyme and RAPD markers.

MATERIALS AND METHODS

Plant material

Plants of the diploid (2n = 2x = 20) cytotype of *A. pintoi* Krapov & W.C. Gregory were obtained from seeds collected by A. Krapovickas and W. Gregory in Cruz das Almas, Brazil (a herbarium specimen is deposited in CTES as Gregory and Krapovickas 12787) and germinated in a soil and sand (1:1) potting mixture. Plants of the triploid (2n = 3x = 30) cytotype were kindly supplied by Francisco Valls from Empresa Brasileira de Pesquisa Agropecuária / Centro Nacional de Recursos Geneticos e Biotecnologia (EMBRAPA/CENARGEN), Brasilia, Brazil (a herbarium specimen is deposited in CTES as Lavia 90). Plants of both cytotypes were cultivated under greenhouse conditions. Shoot tips (2 to 4 mm in length) of 9-month old plants were excised and surface-sterilized by immersion in

70% ethanol for 30 s followed by immersion in a solution of commercial bleach (final concentration of 0.9% sodium hypochlorite and 0.1% Tween 20®) for 12 min, then rinsed three times with sterile distilled water. Shoot tips were then individually cultured in 11 cm^3 glass tubes containing 3 cm^3 solidified (0.65% agar Sigma A-1296) establishment medium (EM). The pH of the medium was adjusted to 5.7 with KOH or HCl prior to adding agar and autoclaved at 1.46 kg cm^{-2} for 20 min. The tubes with the explants were then sealed with Resinite AF-50® film (Casco S.A.C. Buenos Aires, Argentina) and incubated in a growth room at 27±2°C with a 14 h light/10 h dark photoperiod (irradiance of 116 µmol m^{-2} s^{-1} PPFD provided by cool white fluorescent lamps). After 60 days of culture, plantlets were ready to be used as source of explants.

For somatic embryo induction (36, 38), immature leaves (2-4 mm in length) from *in vitro* stock plants were dissected and cultured in 11 cm^3 glass tubes containing 3 cm^3 MS (26) medium supplemented with 41.4 µM PIC and 0.044 µM BAP and solidified with 0.65% agar. The pH of the medium was adjusted to 5.7 with KOH or HCl prior to adding agar and autoclaved at 1.46 kg cm^{-2} for 20 min. Tubes containing one immature leaf were sealed with Resinite AF-50® film and incubated in a growth room at 27±2°C with a 14 h light/10 h dark photoperiod (irradiance of 116 µmol m^{-2} s^{-1} PPFD). After 40 days of culture, the somatic embryos obtained were subcultured on MS (26) medium + 0.054 µM NAA + 0.044 µM BAP and maintained in the environmental conditions described above. After 50 days of incubation, somatic embryos at the cotyledonary stage were ready to be used for cryopreservation experiments.

Cryopreservation of somatic embryos

Twenty treatments were tested for cryopreservation of diploid and triploid cytotypes of *A. pintoi* using the encapsulation-dehydration technique (Table 1). For encapsulation, cotyledonary somatic embryos were suspended in 3% sodium alginate solution and poured in a drop wise manner in a 0.1 M calcium chloride solution, thus forming calcium alginate beads of 4-5 mm diameter (Fig. 1). In some treatment (T 2, 3, 5, 6, 7, 8, 17, 18, 19, and 20), beads were precultured in agitated (80 rpm) liquid EM with daily increased sucrose concentration (0.50, 0.75, and 1.0 M).

Encapsulated somatic embryos with or without sucrose preculture were desiccated for 0 to 6 h on aluminum nets 15 mm above 30 g silica gel in hermetically closed sterile plastic containers. Bead moisture content (MC, fresh weight basis) was measured gravimetrically after each desiccation period by drying the beads in an oven at 100ºC until two successive weightings gave the same value. MC was calculated using the equation [fresh weight (FW)-dry weight (DW)/FW] x 100.

After preculture and/or desiccation, beads were placed in 5.0 mL sterile propylene cryotubes (10 beads per tube), which were either directly immersed in LN (rapid cooling) or submitted to a two-step cooling protocol (slow cooling) consisting of slow precooling at 1ºC min^{-1} from 25ºC to -30ºC using a Controlled Rate Freezing System Model 9000 (Gordinier Electronics Inc., USA) followed by immersion of the cryotubes in LN. Cryotubes were kept in LN for a minimum of 1 h, and then they were rapidly rewarmed in a 30ºC water-bath for 2 min. In some treatments (T 3, 6, 8, 10, 12, 14, 16, 18, and 20), encapsulated somatic embryos were postcultured in agitated (80 rpm) liquid EM with daily decreased sucrose concentration (0.75 and 0.5 M). After postculture, the beads were transferred to solidified EM and incubated in a growth room in the environmental conditions described previously to achieve conversion of somatic embryos into plants. In treatments without postculture (T2, 4, 5, 7, 9, 11, 13, 15, 17, and 19) the beads were transferred directly to solidified EM. Non-encapsulated somatic embryos (T1) were cultured on solidified EM as control treatment.

Table 1. Treatments experimented for cryopreservation of somatic embryos of diploid and triploid *Arachis pintoi* cytotypes. Without encapsulation (×); with encapsulation (✓).

Treatment	Encapsulation	Preculture with sucrose	Dehydration	Slow cooling	Rapid cooling	Postculture with sucrose
T 1	×					
T 2	✓	+				
T 3	✓	+				+
T 4	✓					
T 5	✓	+		+		
T 6	✓	+		+		+
T 7	✓	+			+	
T 8	✓	+			+	+
T 9	✓		+		+	
T 10	✓		+		+	+
T 11	✓		+	+		
T 12	✓		+	+		+
T 13	✓				+	
T 14	✓				+	+
T 15	✓			+		
T 16	✓			+		+
T 17	✓	+	+		+	
T 18	✓	+	+		+	+
T 19	✓	+	+	+		
T 20	✓	+	+	+		+

Once the plantlets obtained had elongated sufficiently (after approximately 3 months), they were transferred to the greenhouse, planted in a soil and sand (1:1) mixture and covered with plastic bags for 2 weeks to prevent desiccation and to allow acclimatization. In these conditions they developed into healthy plants (Fig. 1).

Genetic stability tests

Isozyme and RAPD analyses were carried out to test genetic stability of plants derived from non-cryopreserved and cryopreserved somatic embryos. Enzyme extracts and genomic DNA were isolated from leaf tissue of *in vitro* plantlets of diploid and triploid cytotypes. Leaves were sampled from 10 control plants (T1, Table 1), 10 plants cryopreserved using rapid cooling (T18) and 10 plants cryopreserved using slow cooling (T20).

Ten isozyme systems were assayed on 3%-7.5% discontinuous native polyacrylamide gels (PAGE), according to Laemmli (25): acid phosphatase (ACP), diaphorase (DIA), esterase (EST), leucine aminopeptidase (LAP), malic enzyme (ME), peroxidase (PER), phosphogluconate dehydrogenase (PGD), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), and shikimate dehydrogenase (SKDH).

Protein extracts were obtained by mechanically grinding 0.1 g of the first four full-expanded leaves with 1 mL buffer Tris - HCl 0.1 M pH = 6.8 + glycerol 2% + β-mercaptoethanol 0.1% + bromophenol blue 0.01%. The homogenates were centrifuged at 14,000 rpm for 10 min and supernatants were immediately used for isozyme analyses or stored at -70ºC.

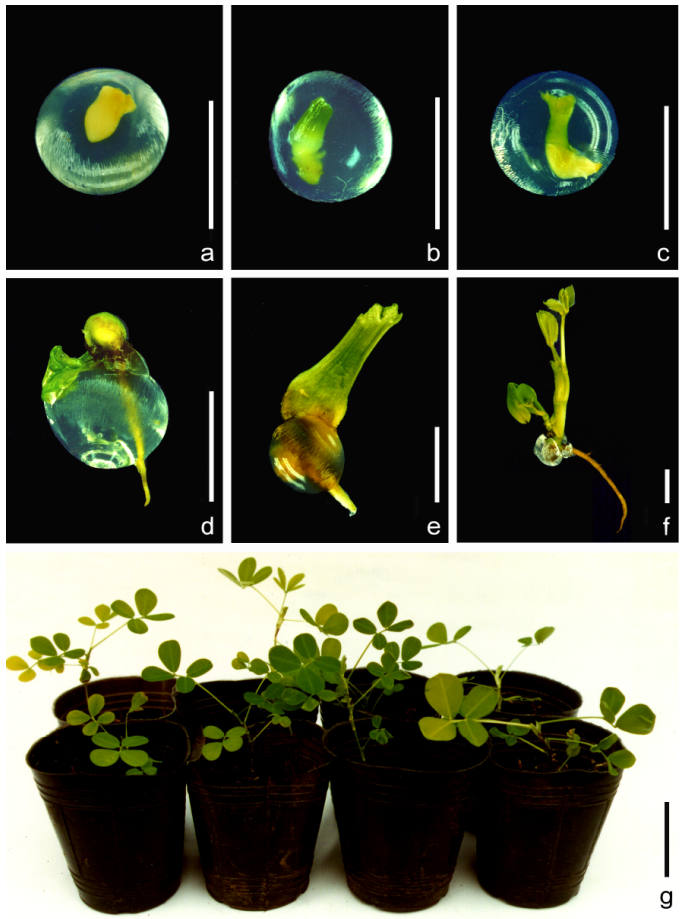

Figure 1. Cryopreservation of *Arachis pintoi* (2n=3x=30) somatic embryos and plant regeneration from. **a.** somatic embryo (SE) in alginate bead at 7 days; **b.** 14 days; **c.** 21 days; **d.** germinated SE at 30 days; **e.** germinated SE at 60 days; **f.** regenerated plant at 90 days; **g.** regenerated plants growing in pots 60 days after acclimatization. Vertical bars: 5 mm in a, b, c, d and f; 4 cm in g.

Since the enzymatic systems may have different ranges of activity, protein extract samples were differentially loaded on the gel as follows: 10 µl for PER, 15 µl for DIA, EST, PGM and SKDH, and 20 µl for ACP, LAP, ME, PGI and PGM. Electrophoresis was performed at 4ºC under a constant 1.2 mA cm^{-1} current. Staining of gels was carried out using redox dye for DIA and PER; diazonium system for ACP, EST and LAP, and tetrazolium system for ME, PGD, PGI, PGM and SKDH. The optimized protocols described by Arulsekar and Parfitt (2), Cardy *et al*. (5), and Soltis *et al*. (45) were followed. The relative electrophoretic mobility (Rf) was calculated for each isozyme band.

DNA samples were extracted according to Doyle and Doyle (8). The standard protocol for Random Amplified Polymorphic DNA (52) was followed. RAPD profiles were generated using nine arbitrary 10-mers from Operon Technologies, Alameda, California, USA (OPG-02, 08, 10; OPP-01, 02, 04, 06, 07 y 08). The reaction mixture for PCR contained 50 ng genomic DNA, 0.2 M primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs Promega and 2 U GoTaq® DNA polymerase Promega, in 25 µL buffer reaction. The reaction was carried out in a Biometra DNA Thermal Cycler, which was programmed for 45 cycles of 94ºC for 1 min, 35ºC for 1 min and 72ºC for 2 min. Amplified products were separated on 1.5% agarose gels, stained with ethidium bromide and examined under UV light.

Cluster analysis was used for testing genetic stability between control and cryopreserved plants of the diploid and triploid cytotypes. RAPD fragments were scored for each of 30 Operational Taxonomy Units (OTU). Each character was scored for presence [1] or absence

[0]. The resulting OTU x OTU matrix served as input in the calculation of a phenogram by the unweighted pair-group method using arithmetic averages (UPGMA). On this matrix, the Dice similarity coefficient was applied to construct a similarity matrix. The phenogram distortion was measured by computing the cophenetic correlation coefficient (r). The computational work was done using the NTSYS-pc (Numerical Taxonomy of Multivariate Analysis System) software package version 2.11W (42).

Statistical analyses

The experiment was arranged in a completely randomized design for each cytotype analyzed. Each treatment consisted of three replicates with 10 explants per replicate, making 30 explants per treatment (n=30). When studying the effect of bead MC on survival of somatic embryos (Fig. 2), survival was determined as the percentage of embryos remaining green after 7 days of culture. In all others experiments, survival was determined as the percentage of embryos forming plants after 30 days of culture. All data were subjected to analysis of variance (ANOVA) and comparisons of means were made with Tukey's Multiple Comparison Test ($p<0.05$).

RESULTS

Encapsulation-dehydration and cryopreservation of somatic embryos

MC of beads containing somatic embryos of *A. pintoi* diploid cytotype decreased from an initial 78.9% without desiccation to 11.1% after 6 h desiccation (Fig. 2a). Survival of control somatic embryos was marginally affected during the first 5 h of dehydration, remaining between 90.0 and 96.6%, and then dropped to 33% after 6 h. After LN exposure, survival was nil for up to 2 h dehydration; it increased progressively to 70% (rapid cooling) and 76.6% (slow cooling) after 5 h dehydration, then decreased rapidly to 3.3-16.6% after 6 h. Similar results were obtained with somatic embryos of the triploid cytotype (Fig. 2b).

Table 2 shows the results obtained with somatic embryos submitted to treatments T1 to T20. In general, the results achieved with both cytotypes were similar. After cryopreservation, survival was obtained only when embryos were precultured with sucrose and desiccated to approximately 20% MC (T17-20). Sucrose postculture (T18 and 20) significantly improved plant formation after cryopreservation, resulting in 27% and 30% plant formation for the diploid and triploid cytotype, respectively. In these cases, no significant differences were noted after slow and rapid cooling. When cryopreserved embryos of both cytotypes were not submitted to sucrose postculture (T17 and 19), the percentage of embryos forming plants decreased compared to non-cryopreserved controls (T1). LN exposure was lethal for non-encapsulated embryos (data not shown) as well as for non-dehydrated embryos (T5-8 and T13-16), or dehydrated embryos without sucrose preculture (T9-12). Surviving embryos started growing rapidly after plating on EM. They pierced the alginate beads and gave rise to *in vitro* plantlets, which developed into phenotypically normal plants after transfer to the greenhouse (Fig. 1).

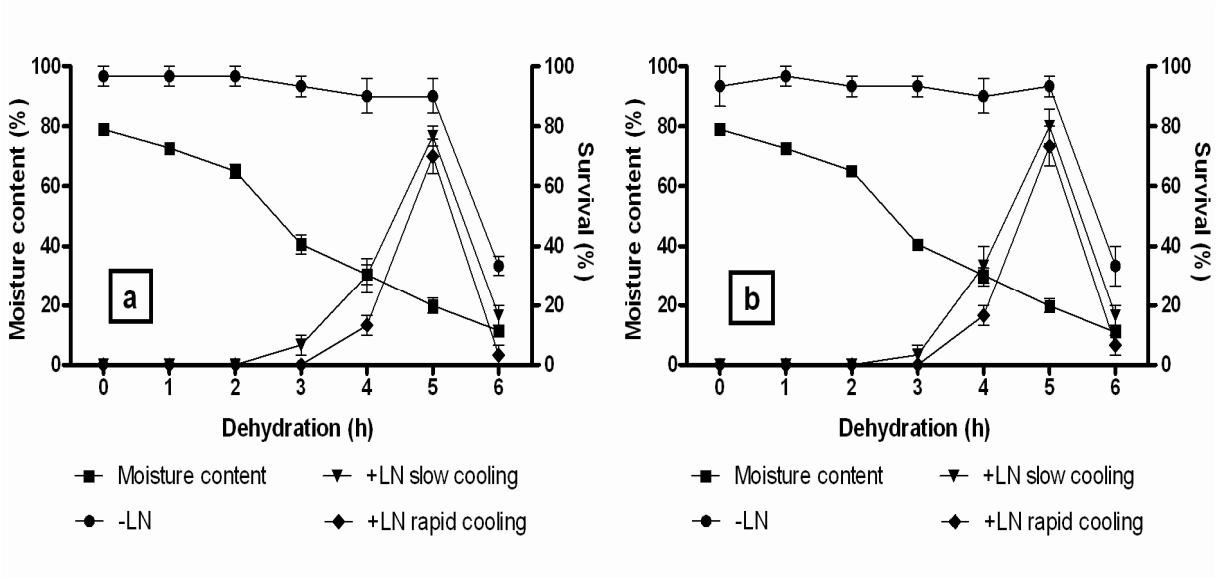

Figure 2. Moisture content (% fresh weight) and survival (% at 7 days of culture) of encapsulated *Arachis pintoi* somatic embryos of diploid (a) and triploid (b) cytotypes after desiccation (-LN) and after desiccation followed by slow or rapid cooling in LN (+LN).

Table 2. Effect of treatment (see Table 1 for treatment details) on plant formation (%) from *A. pintoi* diploid and triploid cytotype somatic embryos cryopreserved using the encapsulation-dehydration technique. Data were recorded at 30 days of culture. In columns, different letters indicate significant differences between treatments ($p<0.05$).

Treatment	Somatic embryos forming plants (% ± SE)	
	Diploid cytotype	Triploid cytotype
T 1	30.0 ± 5.8^{cd}	36.7 ± 3.3^{c}
T 2	10.0 ± 5.8^{abc}	13.3 ± 3.3^{ab}
T 3	30.0 ± 5.8^{cd}	26.7 ± 3.3^{bc}
T 4	50.0 ± 5.8^{d}	40.0 ± 5.8^{c}
T 5-16	0	0
T 17	3.3 ± 3.3^{ab}	3.3 ± 3.3^{a}
T 18	26.7 ± 3.3^{bcd}	30.0 ± 5.8^{bc}
T 19	10.0 ± 5.8^{abc}	10.0 ± 0.0^{ab}
T 20	26.7 ± 3.3^{bcd}	30.0 ± 5.8^{bc}

Genetic stability tests

1) *Isozyme analysis*

Ten isozyme systems were analyzed and good quality zymograms with reproducible band patterns were obtained using young leaves of *in vitro* plantlets. Most isozyme systems assayed were monomorphic for plants derived from control and cryopreserved somatic

embryos of both cytotypes. Different isozyme patterns were found in ACP and PER for the diploid cytotype and in ACP, EST, PER and SKDH for the triploid cytotype; however, these different profiles appeared regardless of the treatments tested. These patterns were related to the genotype of the plants used. Figure 3 shows the isozyme pattern for ACP of diploid and triploid cytotypes. Comparison of the zymograms of 10 isozymes revealed no sign of genetic change resulting from LN exposure.

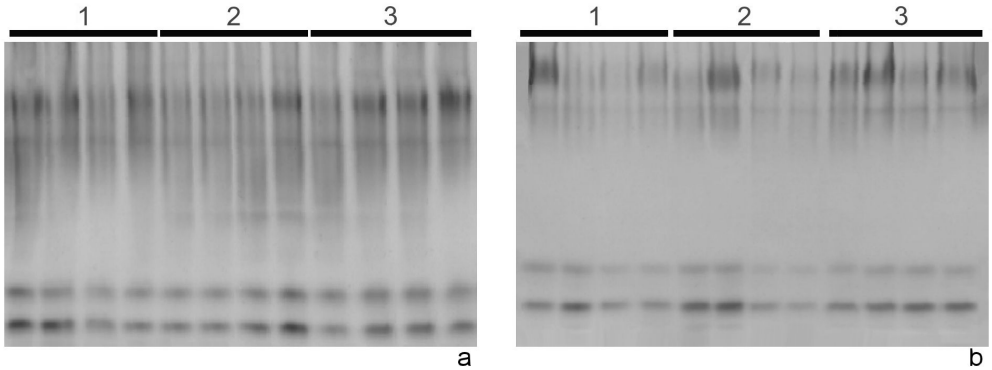

Figure 3. Acid phosphatase zymogram of *in vitro* plants derived from *A. pintoi* somatic embryos. Diploid (a) and triploid cytotypes (b). Control plants (1) and plants derived from somatic embryos cryopreserved using rapid (2) or slow cooling (3).

2) *RAPD analysis*

The nine primers tested generated 68 and 51 fragments for diploid and triploid cytotypes, respectively, which scored between 350 and 3200 base pairs in length, with an average of six DNA fragments/primer. All primers produced clear and reproducible patterns. No qualitative differences were detected with five of the nine assayed primers for diploid and triploid cytotypes. The other four primers were polymorphic. Figure 4 shows the RAPD patterns generated using primer OPG-02 for the diploid cytotype and primer OPG-10 for the triploid cytotype.

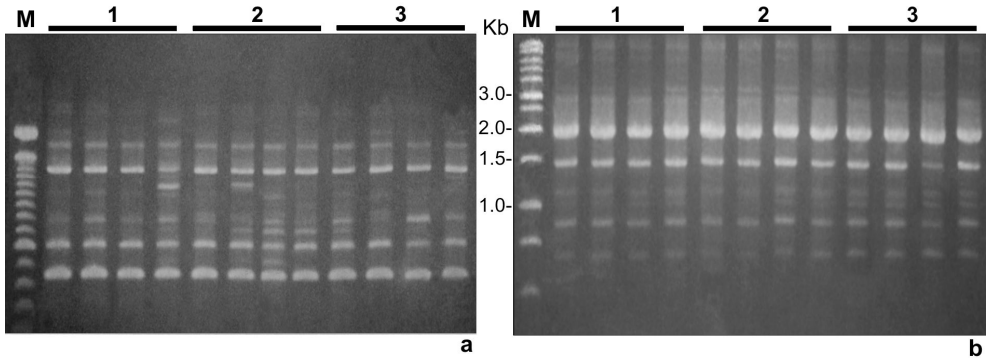

Figure 4. RAPD banding profiles of DNA samples from *in vitro* plants derived from *A. pintoi* somatic embryos. a. Amplification products generated using primer OPG-02 from diploid cytotype; b. Amplification products generated using primer OPP-08 from triploid cytotype. Control plants (1) and plant derived from somatic embryos cryopreserved using rapid (2) or slow cooling (3). M: 100 bp DNA Ladder.

The UPGMA phenogram based on similarity matrix generated from RAPD profile showed a range of variation, from 1 to 0.95 for the diploid (Fig. 5) and from 1 to 0.96 for the triploid cytotype (Fig. 6). The cophenetic correlation coefficient was 0.72 and 0.89 for diploid

and triploid cytotypes, respectively, reflecting the small amount of distortion introduced during clustering. The phenogram of the diploid cytotype (Fig. 5) showed two main groups, which were clustered at 0.95. Both groups included plants derived from non-cryopreserved and cryopreserved somatic embryos. No cluster for only cryopreserved individuals was observed.

The phenogram based on UPGMA clustering analysis of the triploid cytotype (Fig. 6) showed a small group consisting of two separated non-cryopreserved plants, clustered at 0.965. The other non-cryopreserved and cryopreserved plants were intermixed in the main group. The UPGMA phenograms based on RAPD band polymorphisms suggested that cryopreservation did not generate any variability. In addition, no differences between cryopreservation treatments were found.

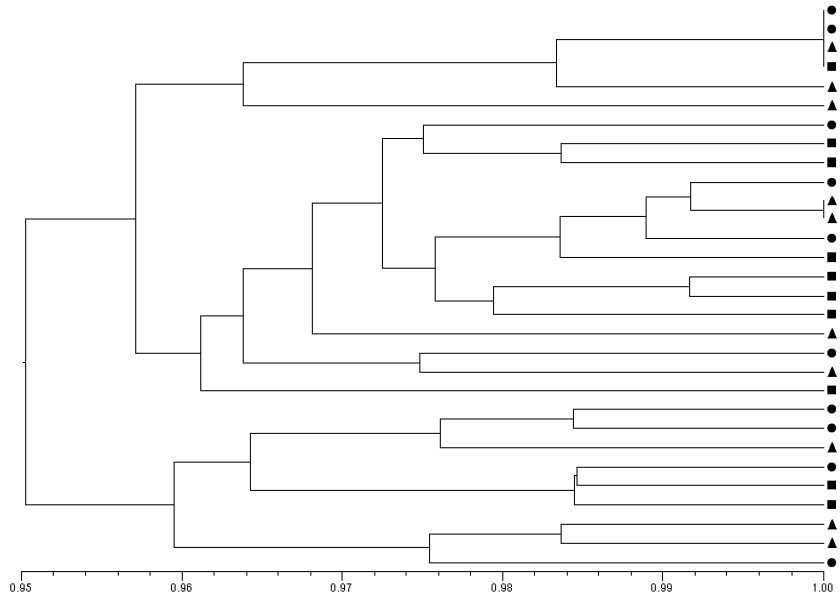

Figure 5. UPGMA phenogram based on RAPD banding patterns of *in vitro* plants of *A. pintoi* diploid cytotype. Control plants (●) and plants derived from somatic embryos cryopreserved by rapid (▲) or slow cooling (■). Cophenetic correlation coefficient (r) = 0.72.

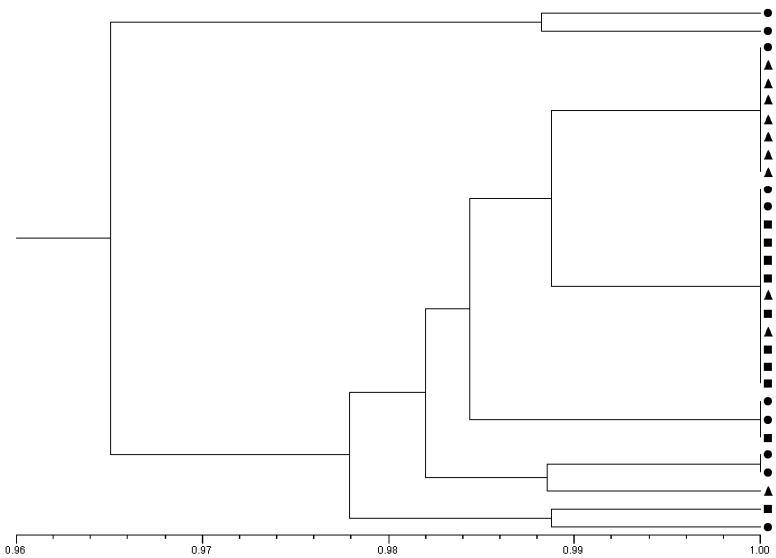

Figure 6. UPGMA phenogram based on RAPD banding patterns of *in vitro* plants of *A. pintoi* triploid cytotype. Control plants (●) and plants derived from somatic embryos cryopreserved by rapid (▲) or slow cooling (■). Cophenetic correlation coefficient (r) = 0.89.

DISCUSSION

In the present work, we report for the first time the successful cryopreservation of *A. pintoi* somatic embryos using the encapsulation-dehydration technique, which was originally developed for shoot-tips and apical meristems (40). With this procedure, a maximum of 27% and 30% somatic embryos of the diploid and triploid cytotypes, respectively, showed regrowth after LN exposure and formed plants, which were transferred into pots to the greenhouse for further growth. The cryopreservation protocol comprises the following steps:

1. Production of somatic embryos - from immature leaves using the procedure recommended by Rey and Mroginski (38).
2. Encapsulation of somatic embryos in 3% calcium alginate beads using the procedure described for production of synthetic seeds (35). Since somatic embryos are small (2-3 mm in diameter) encapsulation in alginate beads facilitated manipulations. Encapsulation of somatic embryos did not induce any detrimental effect compared with non-encapsulated controls. This result is in agreement with those obtained in other plant species (21), as well as for cryopreservation of shoot tips and apical meristems of *A. pintoi* (40).
3. Preculture of encapsulated somatic embryos - in medium with progressively increased sucrose concentration. This step was necessary to achieve plant regeneration after cryopreservation. Similar results have been obtained with most plant materials cryopreserved using this technique (47, 11), including for cryopreservation of shoot-tips and apical meristems of *A. pintoi* (40). This treatment with high sugar concentrations probably enhanced the level of tolerance to dehydration and subsequent LN exposure by partial dehydration and intracellular sugar accumulation (51, 43).
4. Dehydration of encapsulated somatic embryos - with silica gel for 5 h (to 20% MC). This step was critical to achieve survival of cryopreserved explants of both cytotypes tested. Like with other plant species, successful cryopreservation required a precise control of MC of target tissues (33, 53). The somatic embryos used in this study showed a steady MC decrease in line with increasing dehydration durations. The MC ensuring optimal survival of embryos of both cytotypes was 20%, which was similar to the optimal MC for cryopreservation of *A. pintoi* shoot tips and apical meristems (40). This value is within the range of optimal MCs (20-25%) for cryopreservation of plant tissues (11).
5. Cryopreservation of encapsulated somatic embryos - by using slow or rapid cooling. Although the results obtained with rapid cooling did not differ significantly from those obtained with slow cooling, we recommend the use of rapid cooling because it is simpler than slow cooling.
6. Rewarming, postculture, and reculture of somatic embryos - cryopreserved somatic embryos were rapidly rewarmed in a water-bath at 30ºC for 2 min and cultured in liquid media with progressively decreased sucrose concentrations. This procedure, which has been recommended for explants of various plant species (10, 12, 16, 46), improved survival probably via the reduction of the osmotic shock imposed on cells due to the progressive decrease in sucrose level during postculture. Culture in liquid medium could also aid an easier rehydration and nutrition of cryopreserved somatic embryos. Finally, somatic embryos were transferred to solidified EM where they grew rapidly. They emerged from the alginate beads and gave rise to *in vitro* plantlets, which were transferred into pots to the greenhouse for further growth.

No morphological abnormality was observed on any of the plants obtained after cryopreservation and their genetic stability was confirmed based on 10 isozyme systems and nine RAPD profiles. Rey *et al.* (40) also reported no genetic modifications induced by

cryopreservation of encapsulated-dehydrated *A. pintoi* shoot tips and meristems using isozyme and RAPD analyses.

In conclusion, the present study showed that it was possible to cryopreserve *A. pintoi* somatic embryos using encapsulation-dehydration. The protocol established ensured not only high survival of somatic embryos but also their development in whole plants, which were successfully grown in greenhouse conditions. Recovery percentages could be improved by refining pretreatment conditions, e.g. by increasing the duration of treatment with the successive sucrose media used, or by increasing the final sucrose concentration. In addition, the genetic stability of plants regenerated from cryopreserved *A. pintoi* somatic embryos was confirmed based on isozyme systems and RAPD profiles. This study provides a strong basis for the establishment of cryopreservation protocols for other *Arachis* species.

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