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# CRYOPRESERVATION OF *IN VITRO* GROWN SHOOT TIPS AND APICAL MERISTEMS OF THE FORAGE LEGUME *ARACHIS PINTOI*

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## Abstract

A cryopreservation protocol using the encapsulation-dehydration procedure was established for shoot tips (2-3 mm in length) and meristems (0.3-0.5 mm) sampled from *in vitro* plantlets of diploid and triploid cytotypes of *Arachis pintoi*. The optimal protocol was the following: after dissection, explants were precultured for 24 h on establishment medium (EM), encapsulated in calcium alginate beads and pretreated in liquid EM medium with daily increasing sucrose concentration (0.5, 0.75, 1.0 M) and desiccated to 22-23% moisture content (fresh weight basis). Explants were frozen using slow cooling (1ºC $min^{-1}$ from 25ºC to -30ºC followed by direct immersion in liquid nitrogen), thawed rapidly and post-cultured in liquid EM medium enriched with daily decreasing sucrose concentrations (0.75, 0.50, 0.1 M). Explants were then transferred to solid EM medium in order to achieve shoot regeneration, then on Murashige and Skoog medium supplemented with 0.05 µM naphthalene acetic acid to induce rooting of shoots. With this procedure, 53% and 56% of cryopreserved shoot tips of the diploid and triploid cytotypes, respectively, survived and formed plants. However, only 16% of cryopreserved meristems of both cytotypes regenerated plants. Using ten isozyme systems and seven RAPD profiles, no modification induced by cryopreservation could be detected in plantlets regenerated from cryopreserved material.

**Keywords:** apical meristems, cryopreservation, encapsulation-dehydration, genetic stability, shoot tips.

**Abbreviations:** BAP: benzylaminopurine; EM, establishment medium: MS supplemented with 0.05 µM BAP and 0.05 µM NAA; LN: liquid nitrogen; NAA: naphthaleneacetic acid; MS: Murashige & Skoog (1962) salts and vitamins with 3% sucrose; MC: moisture content (fresh weight basis).

## INTRODUCTION

The genus *Arachis* belongs to the Leguminosae family. Eighty species are recognized, which are found in tropical and temperate regions of the world, and originate from South America. The cultivated peanut (*A. hypogaea*) is economically the most important species because of the value of its seeds (19, 20, 35). The wild peanut species, *A. pintoi* (common name: perennial peanut or Pinto peanut), belongs to the Section Caulorrhizae. It is a perennial herb which is an important forage legume in tropical and subtropical areas of the world (1, 25).

Originally, *A. pintoi* was described as a diploid plant (2n = 2 x = 20; 13), but, more recently, Peñaloza *et al.* (24) found a triploid cytotype (2n = 3x =30), characterized by its vigorous growth rate. The diploid types produce relatively few seeds and, like other *Arachis* species, these seeds show high viability loss after a few months of storage (7, 15). The triploid cytotype does not produce seeds and it is an obligate vegetatively propagated plant. In consequence, for the conservation of its germplasm, it is necessary to maintain collections of plants in the field with all the disadvantages of this procedure, such as loss of plant material due to diseases, pests or natural calamities. Additionally, a great deal of labour and time are required for maintaining these field collections. For short- and medium-term storage of *A. pintoi* germplasm, Rey & Mroginski (29) have recently reported a technique based on slow growth storage of *in vitro* shoot tips which permits conservation for one year. However, for an efficient and economic long-term conservation of these cytotypes, cryopreservation is the alternative strategy, as demonstrated for other plant species (8, 23) including *A. hypogaea* (3, 16) and some members of the Extranervosae section (16, 17).

Cryopreservation is considered an ideal procedure of germplasm maintenance and conservation because it requires limited space, the plant material can be stored without genetic modifications and protected from contamination and it requires limited space and maintenance (10). Various techniques are available for cryopreservation of plant germplasm. Among them is the encapsulation/dehydration technique, which has been introduced by Dereuddre *et al.* (6). It is based on the technology developed for the production of synthetic seeds (27). This technique has been applied to numerous plant species (18). In this technique, freezing tolerance is induced by preculturing encapsulated samples in liquid medium enriched with sucrose (generally about 0.7- 1 M) and by dehydrating them to moisture contents around 20-25% (fresh weight basis).

The present work aimed at investigating the efficiency of the encapsulation-dehydration technique for cryopreservation of *A. pintoi in vitro*-grown shoot tips and apical meristems.

## MATERIALS AND METHODS

*Plant material*

Nine-month old field grown plants of *A. pintoi* Krapov. & W.C. Gregory were used as the primary source of explants. Seedlings of the diploid cytotype (2n = 2x =20) were obtained from seeds collected by A. Krapovickas and W. Gregory in Cruz das Almas, Bahia, Brazil, whose herbarium specimen is deposited in CTES, as Gregory and Krapovickas 12787. Plants of the triploid cytotype (2n = 3x = 30) were kindly supplied by Francisco Valls (Embrapa/Cenargen, Brasilia, Brazil). A herbarium specimen of this cytotype is deposited in CTES as Lavia 90. Shoot tips of these materials (Fig. 1) 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 plus one drop of Tween 20®) for 12 min, then rinsed three times with autoclaved distilled water. The shoot tips were placed in individual test tubes on EM medium solidified with 0.65% Sigma agar (A-1296). The tubes

were then covered with Resinite AF-50® and after an incubation period of 60 days in a growth room (temperature of 27 ± 2ºC, 14 h light/10 h dark photoperiod, irradiance of 116 µmol $m^{-2}$ $s^{-1}$ provided by cool white fluorescent lamps), plantlets were ready to be used as source of explants. Subculturing was performed every 4 weeks. Two types of explants were excised from these stock cultures for experiments: shoot tips (2 to 3 mm in length) and apical meristems (0.3 to 0.5 mm in length, consisting of the dome and a pair of leaf primordia).

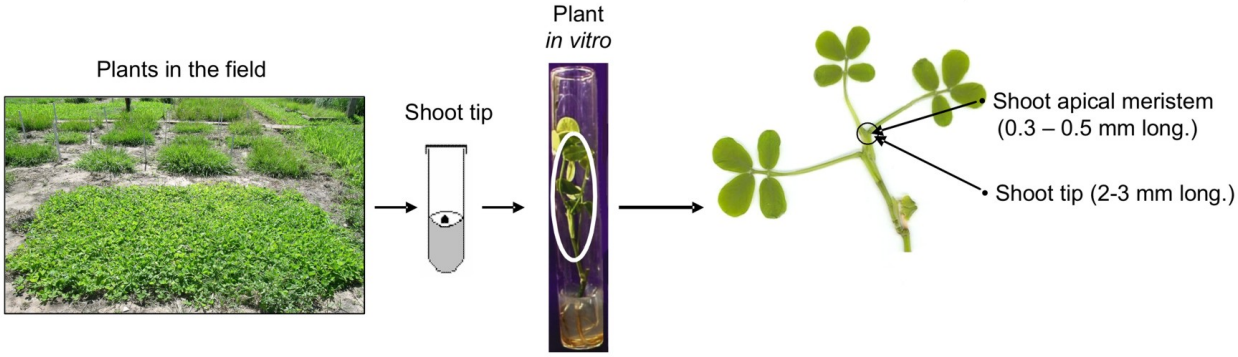


**Figure 1.** Source and types of explants used for cryopreservation experiments.

*Encapsulation-dehydration and cryopreservation*

Table 1 presents the 20 treatments tested for cryopreservation of *A. pintoi*. Explants were cultured for 24 h on EM solidified with 0.65% agar (Sigma A-1296). They were then encapsulated individually in 3% calcium alginate beads (about 4-5 mm in diameter). The beads were then precultured on a rotary shaker (80 rpm) in EM enriched with progressively increasing sucrose concentration, using the following sequence: 24 h in 0.5 M sucrose, 24 h in 0.75 M sucrose, and 24 h in 1.0 M sucrose.

Desiccation was performed by placing 10 beads on an aluminum net 15 mm above 30 g silica gel in an hermetically closed sterile plastic container (50 ml capacity). Beads containing meristems or shoot tips were desiccated for 0 to 6 h, thereby reaching moisture contents (MC, fresh weight basis) between 66 (no desiccation) and 7% (6 h desiccation) (Fig. 2). The MC of beads was measured gravimetrically after each desiccation period by drying the beads in an oven at 100°C until two successive weighings gave the same value. MC was calculated using the equation [fresh weight (FW)-dry weight (DW) / FW] x 100.

After desiccation, beads were placed in 5.0 ml sterile polypropylene Nalgene cryotubes (10 beads/tube), which were either directly immersed in LN (rapid freezing) or submitted to a two-step freezing protocol (slow freezing), consisting of slow prefreezing at 1°C $min^{-1}$ from 25°C to -30°C using a Controlled Rate Freezing System Model 9000 (Gordinier Electronics Inc., USA) followed by direct immersion of the cryotubes in LN. Cryotubes were kept in LN for a minimum of 1 h.

*Rewarming, post-culture, and reculture*

Cryotubes which had been immersed in LN were rewarmed rapidly in a water-bath at 30ºC for 2 min. In cases where beads had been precultured with sucrose, they were post-cultured on a rotary shaker (80 rpm) in liquid EM medium containing progressively decreasing sucrose concentrations using the following sequence: 24 h in 1.0 M sucrose, 24 h in 0.75 M sucrose, and 24 h in 0.75 M sucrose. Explants were then transferred to solidified EM medium under standard culture conditions in order to achieve shoot regeneration. Regenerated shoots were cultured on MS medium (22) supplemented with 0.05 µM NAA in order to induce rooting (28). In cases where no preculture with sucrose was performed, explants were transferred directly to solidified EM medium. The plants obtained after 3 months in culture were transferred to the greenhouse, planted in a mixture of soil and sand

(1:1) and initially covered with plastic bags for 2 weeks to prevent desiccation and to allow acclimatization.

*Genetic stability tests*

For genetic stability testing, isozyme and RAPD analyses were carried out on ten plantlets selected at random from control plants (T 1) and cryopreserved plants by rapid freezing (T 18) or slow freezing (T 20) (See Table 1).

Enzymes and genomic DNA were extracted from leaf tissue of *in vitro* plants of diploid and triploid *A. pintoi* cytotypes. DNA samples were extracted according to Doyle & Doyle (5). The standard protocol for Random Amplified Polymorphic DNA (36) 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 and 08). The reaction mixture for the PCR contained 50 ng genomic DNA, 0.2 µM primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs Promega and 2 U GoTaq® DNA polymerase Promega, in 25 µL of the reaction buffer. The reaction was carried out in a DNA Thermal Cycler from Biometra, 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.

Ten isozyme systems were analysed: 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), on discontinuous native polyacrylamide gels (PAGE) 3% - 7.5% mini slab according to Laemmli (1970).

Homogenates were obtained by mechanically grinding 0.1 g leaf tissues with 1 mL of Tris - HCl 0.1 M buffer (pH = 6.8) + glycerol 2% + β-mercaptoethanol 0.1% + bromophenol blue 0.01%. Protein extracts were centrifuged at 14,000 rpm for 10 min. The supernatants were used immediately for isozyme analyses or stored frozen at -70ºC.

Depending on the isozyme system, samples of 10 - 20 µl were loaded. Electrophoresis was performed at 4ºC with a constant current of 1.2 mA $cm^{-1}$. The staining of the 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 & Parfitt (2), Cardy et al. (4) and Soltis et al. (32) were followed. The relative electrophoretic mobility ($R_f$) was calculated for each isozyme band.

Cluster analysis was used for testing genetic stability between control plants and plants cryopreserved by rapid or slow cooling of the diploid and triploid cytotypes. RAPD fragments were scored for each of 30 OTUs (Operational Taxonomy Unit). 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 these matrices, the Dice similarity coefficient was applied to construct a similarity matrix. The distortion of phenogram was measured by computing the cophenetic correlation coefficient (r). The computational work was done using NTSYS-pc (Numerical Taxonomy of Multivariate Analysis System) software package version 2.11W Rohlf, (30).

*Statistical analysis*

All treatments consisted of 10 explants and all experiments were repeated at least three times. In experiments aiming at constructing the dehydration curves, explant survival was determined after 7 days of culture as the percentage of explants remaining green, whereas in all other experiments, explant survival was determined after 30 days of culture as the percentage of explants forming plants. 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

*Cryopreservation*

Typical curves of evolution of MC of precultured beads during dehydration are presented in Fig. 2A and B. In the experiment with beads containing shoot tips (Fig. 2A), MC decreased from an initial 79% to 22% after 5 h desiccation. Survival of non-cryopreserved (-LN) shoot tips remained high (above 81%) up to 5 h desiccation (22 % MC), then decreased rapidly, reaching 7% survival after 6 h. Survival of cryopreserved (+LN) shoot tips increased progressively from 0% after 2 h or 3 h desiccation for slow and rapid cooling, respectively to an optimum of 75% survival for slow cooling and of 53% for rapid cooling after 5 h desiccation, and decreased rapidly afterwards.

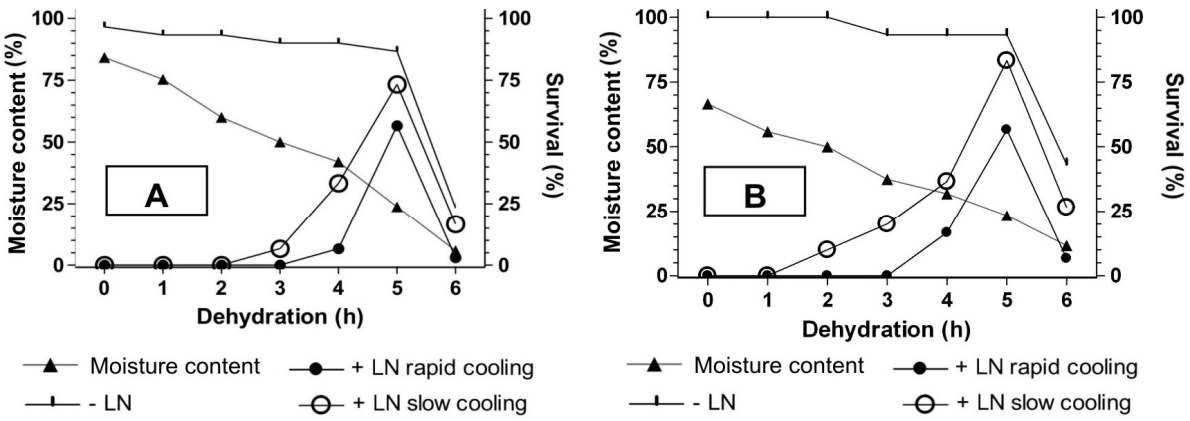


**Figure 2.** Moisture content (fresh weight basis) and survival of shoot tips (A) and meristems (B) of the diploid cytotype of *Arachis pintoi* after desiccation (-LN) and after desiccation followed by slow or rapid cooling in liquid nitrogen (+LN)


**Figure 3** Regrowth of cryopreserved *Arachis pintoi* meristems,
**a.** meristem in alginate bead,
**b.** 20 days;
**c.** 30 days;
**d.** 40 days;
**e.** 60 days;
**f.** 90 days after rewarming;
**g.** regenerated plantlets. (Vertical bars indicate 5 mm in a, b, c, d, e, f; and 4 cm in g).

In the experiment with beads containing meristems (Fig. 2B), the initial MC was 66%. It decreased progressively to about 23% within 5 h and reached 10% after 6 h. Survival of non cryopreserved (-LN) meristems was high (about 90%) up to 5 h and dropped to around 40% after 6 h. Survival of meristems cryopreserved by slow or rapid cooling displayed a similar pattern as that observed with shoot tips. The highest survival was achieved after 5 h desiccation, with 79 and 55% survival after slow or rapid cooling, respectively. Surviving cryopreserved explants, when recultured on EM, rapidly gave rise to *in vitro* plantlets which grew into phenotypically normal plants after transfer into pots (Fig. 3). Survival of control (-LN) shoot tips and meristems of the triploid cytotype of *A.* pintoi cryopreserved (+LN) by slow or rapid cooling displayed a pattern similar to that observed with the diploid genotype, with the highest survival (48-77%) of cryopreserved samples also noted after 5 h desiccation (data not shown).

Without freezing (T 1-4), the percentage of shoot regeneration from shoot tips and meristems of the diploid and triploid cytotype was generally higher than after freezing following the corresponding treatments (T 17-20) (Table 2). Freezing was lethal for non-encapsulated explants (data not shown) as well as for encapsulated explants which were not adequately dehydrated (T 5-8 and T 13-16) or which were not submitted to sucrose preculture (T 9-12). After cryopreservation, positive results were obtained only when encapsulated explants were both precultured with sucrose and desiccated to 22-23% MC (T 17-20). After freezing, the best regeneration percentage was achieved using slow cooling with both types of explants (T20). Sucrose post-treatment significantly improved survival after cryopreservation.

**Table 1.** Treatment matrix for the cryopreservation of meristems and shoot tips of *Arachis pintoi*.

| Treatment | Encapsulation | Preculture with Sucrose | Dehydration | -30ºC | -196ºC | Thawing | 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 | + | + | + | + | + | + | + |

Without encapsulation (--); with encapsulation (+)

**Table 2.** Effect of treatment on shoot regeneration from meristems and shoot tips of the diploid and triploid cytotype of *Arachis pintoi* cryopreserved using the encapsulation-dehydration technique. See Table 1 for details of treatments T1-T20.

| | Shoot regeneration (% ± SE) | | | |
|---|---|---|---|---|
| | Diploid cytotype | | Triploid cytotype | |
| Treatment | meristems | shoot-tips | meristems | shoot-tips |
| T 1 | 56.67 ± 3.33[d] | 86.67 ± 8.81[c] | 53.33 ± 6.66[c] | 86.67 ± 13.33[c] |
| T 2 | 13.33 ± 6.66[ab] | 33.33 ± 8.81[ab] | 16.67 ± 6.66[ab] | 33.33 ± 3.33[ab] |
| T 3 | 33.33 ± 3.33[bc] | 53.33 ± 6.66[b] | 30 ± 5.77[b] | 56.67 ± 6.66[bc] |
| T 4 | 50 ± 5.77[cd] | 93.33 ± 6.66[c] | 63.33 ± 3.33[c] | 90 ± 10[c] |
| T 5-16 | 0 | 0 | 0 | 0 |
| T 17 | 3.33 ± 3.33[a] | 10 ± 5.77[a] | 3.33 ± 3.33[a] | 6.66 ± 3.33[a] |
| T 18 | 13.33 ± 3.33[ab] | 36.67 ± 8.81[ab] | 16.67 ± 3.33[ab] | 40 ± 5.77[ab] |
| T 19 | 13.33 ± 3.33[ab] | 10 ± 5.77[a] | 6.66 ± 6.66[a] | 6.66 ± 3.33[a] |
| T 20 | 16.67± 3.33[ab] | 53 ± 6.66[b] | 16.67 ± 3.33[ab] | 56.67 ± 3.33[bc] |

*Genetic stability*

Isozyme and RAPD analysis were employed for assessing genetic stability of control plants and plants cryopreserved by rapid or slow freezing.

1) Isozyme analysis

Ten isozyme systems were analyzed and good quality zymograms were provided using young leaves of *in vitro* plantlets.

The isozyme patterns presented bands at $R_f$ 0.15 to 0.35 and 0.80 to 0.95 for ACP; 0.35 to 0.60 for DIA and 0.60 to 1.0 for EST. The zymograms LAP, PER and ME showed bands with $R_f$ ranging 0.48 to 0.80, $R_f$ 0.20 to 0.70 and $R_f$ 0.25, respectively. Band patterns for PGD, PGM and SKDH appeared at $R_f$ 0.45 to 0.55 and for PGI at $R_f$ 0.36 to 0.45

Esterase and peroxidase isozyme systems revealed polymorphic profiles for diploid and triploid cytotypes, but LAP and PGD were polymorphic systems only for the triploid cytotype.

All polymorphic bands appeared in controls and in plants cryopreserved by rapid or slow freezing and could be related with the genotype of the control plants. Figure 4 shows the EST isozyme patterns of control and cryopreserved plants from diploid and triploid cytotypes. Comparison of the zymograms of 10 isozyme systems indicated no signs of genetic changes as a result of cryopreservation treatment.

2) RAPD analysis

RAPD analysis was carried out with seven of the nine primers assayed, from which amplified products were clear and reproducible. The primers generated 53 and 59 fragments for diploid and triploid cytotypes, respectively, between 380 and 3300 base pairs, with an average of 42.9% of polymorphic fragments. Figure 5 shows the RAPD pattern produced using primer OPG-02 for the diploid and triploid cytotypes.

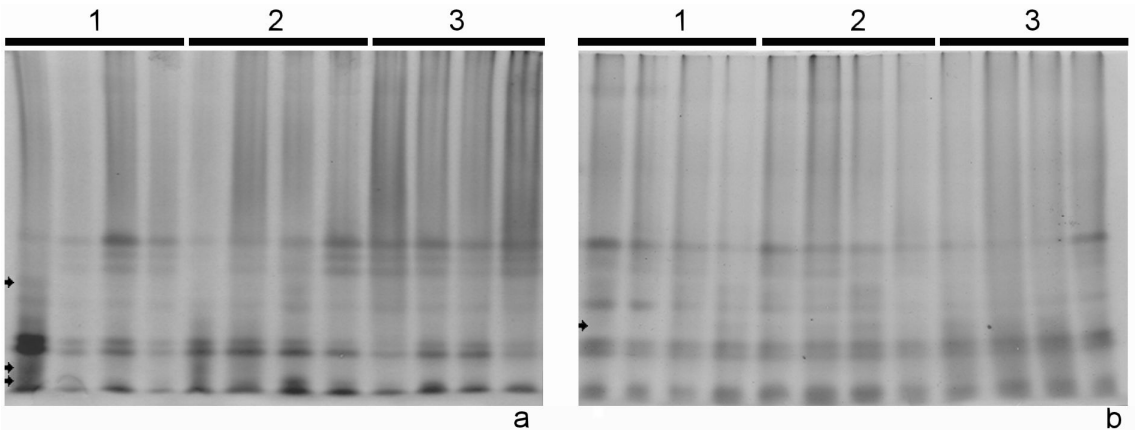


**Figure 4.** Esterase zymogram of *in vitro* plantlets originating from meristems of *Arachis pintoi*. (**a**) diploid cytotype and (**b**) triploid cytotype. Control plants (**1**) and meristems cryopreserved by rapid (**2**) and slow freezing (**3**). Arrows indicate polymorphic bands.

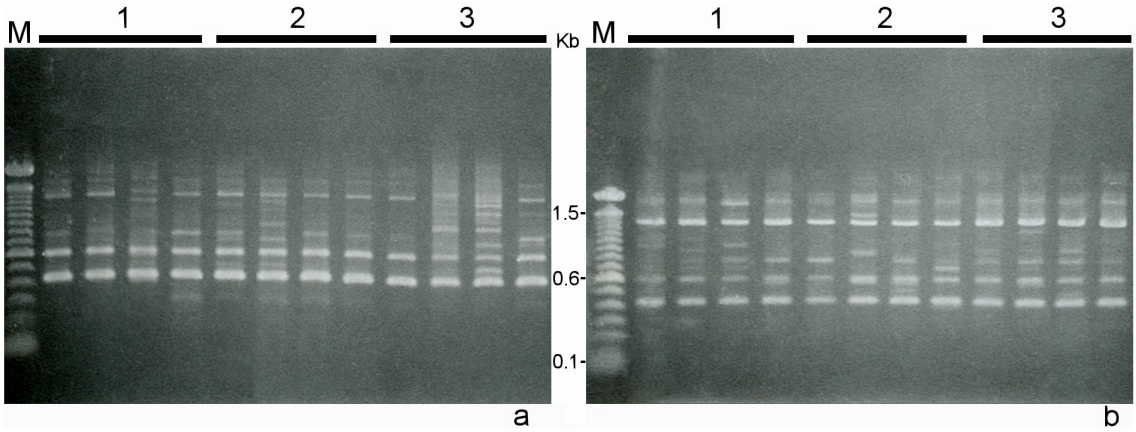


**Figure 5**. RAPD banding profiles using primer OPG-02 of DNA samples extracted from *in vitro* plantlets originating from meristems of *Arachis pintoi.* Amplification products were generated from the diploid (**a**) and triploid (**b**) cytotype. Control plants (**1**) and meristems cryopreserved by rapid (**2**) or slow freezing (**3**). **M**: 100 bp DNA ladder.

The UPGMA phenograms based on Dice similarity matrix clustered all the individuals assayed with similarity coefficients higher than 0.87 (Figures 6 and 7). The similarity range from 1 to 0.87 indicated that there was a high similarity level. The cophenetic correlation coefficients were 0.72 and 0.89 for diploid and triploid cytotypes, respectively, reflecting a small amount of distortion introduced during the clustering.

Four main groups were observed, at a cut off line of 0.90 for the diploid cytotype and five groups for the triploid cytotype. Such clustering was a consequence of the genotype differences among the control plants, and the same level of variability was also identified in the plants recovered from freezing.

Cryopreserved individuals were not separated from control plants and were intermixed through all groups.

The UPGMA analysis based on RAPD band polymorphisms suggests that cryopreservation did not generate more variability than that already existing. In addition, no differences between cryopreservation treatments were found.

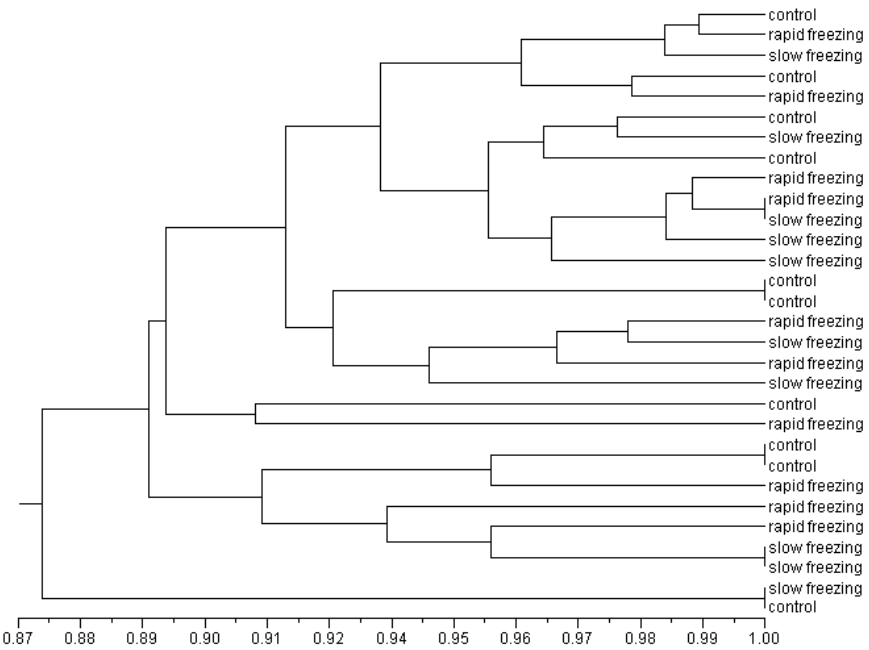


**Figure 6.** Phenogram of 30 OTU's of *in vitro* plantlets originating from the diploid cytotype of *Arachis pintoi* resulting from the UPGMA cluster analysis of the OTUxOTU similarity matrix. Cophenetic correlation coefficient (r) = 0.78.

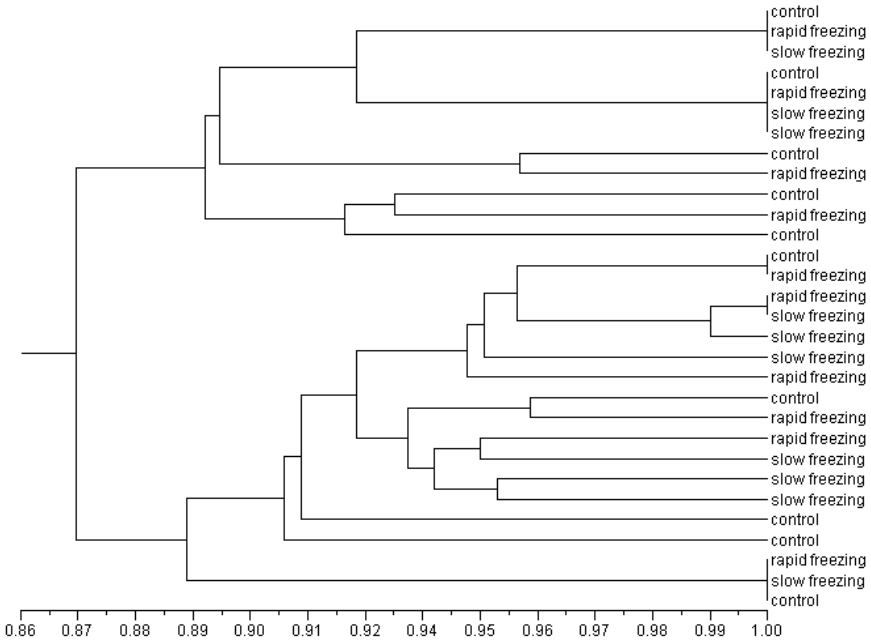


**Figure 7.** Phenogram of 30 OTU's of *in vitro* plantlets originating from the triploid cytotype of *Arachis pintoi* resulting from the UPGMA cluster analysis of the OTUxOTU similarity matrix. Cophenetic correlation coefficient (r) = 0.80.

## DISCUSSION

In the present work, cryopreservation of *in vitro* grown shoot tips and meristems of *A. pintoi* was successfully achieved using the encapsulation-dehydration procedure. With this procedure, 53% and 56% of shoot tips of the diploid and triploid cytotypes, respectively withstood cryopreservation and formed plants. Likewise, 16% of cryopreserved meristems of both cytotypes survived and formed plants. This difference in survival between shoot tips and meristems is related to the higher difficulty in regenerating plants from meristems compared with shoot tips (10).

In this study, encapsulation of explants, preculture with stepwise increasing sucrose concentration and adequate desiccation were necessary to achieve plant regeneration after cryopreservation. Similar results have been found with most plant materials cryopreserved using this technique (10, 34). Encapsulating meristems and shoot tips in alginate beads facilitated manipulations, particularly when small explants were employed (2-3 mm in the case of shoot tips and 0.3-0.5 mm in the case of meristems). It is interesting to note that encapsulation did not induce any detrimental effect compared with non-encapsulated controls. This result has been frequently observed with other plant species (18) and moreover it was found that in some cases encapsulation enhanced plant regeneration as reported during cryopreservation of meristems of *Melia azedarach* (31) and with the orchid *Oncidium bifolium*, for which encapsulation improved the *in vitro* response of seeds and protocorms in comparison with non-encapsulated samples (14). Although the mechanism behind this positive effect requires further clarification, a possible explanation could be the greater availability of nutrients to encapsulated samples (12).

Preculture with high sugar concentrations enhanced the tolerance level to dehydration and subsequent freezing by partial dehydration and intracellular sugar accumulation (20). A progressive increase in sucrose concentration was employed with *A. pintoi* in order to reduce the toxic effect of high sucrose concentrations, as has been also noted with grape (26).

Desiccation was necessary to ensure survival of cryopreserved explants of both cytotypes tested. The optimal MC for both types of explants was 22-23%, which is within the range of optimal MCs (about 20-25%) required for cryopreservation of plant tissues (10).

Although both freezing procedures tested ensured survival, the highest percentage of plant regeneration was achieved using the slow freezing procedure. A similar result has been observed notably during cryopreservation of grape shoot tips (26). An hypothesis to explain this result could be that higher survival was due to additional (freeze-induced) dehydration, which took place in the beads and samples during slow prefreezing.

After rewarming, postculture of encapsulated shoot tips in media with decreasing sucrose concentration increased survival. This procedure, which is recommended for cell suspensions (9), was successfully employed first for oil palm somatic embryos (11) and applied to other differentiated materials such as *Oncidium bifolium* protocorms (14) and *Bactris gasipaes* shoot tips (33). The improvement in survival noted is related to the reduction of the osmotic shock imposed on cells due to the progressive decrease in sucrose level during postculture.

Finally, using ten isozyme systems and seven RAPD profiles, we could not detect any modification induced by cryopreservation in plantlets regenerated from cryopreserved material. This is yet another confirmation of the efficiency and safety of

cryopreservation as regards the preservation of genetic stability of cryopreserved material (10).

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