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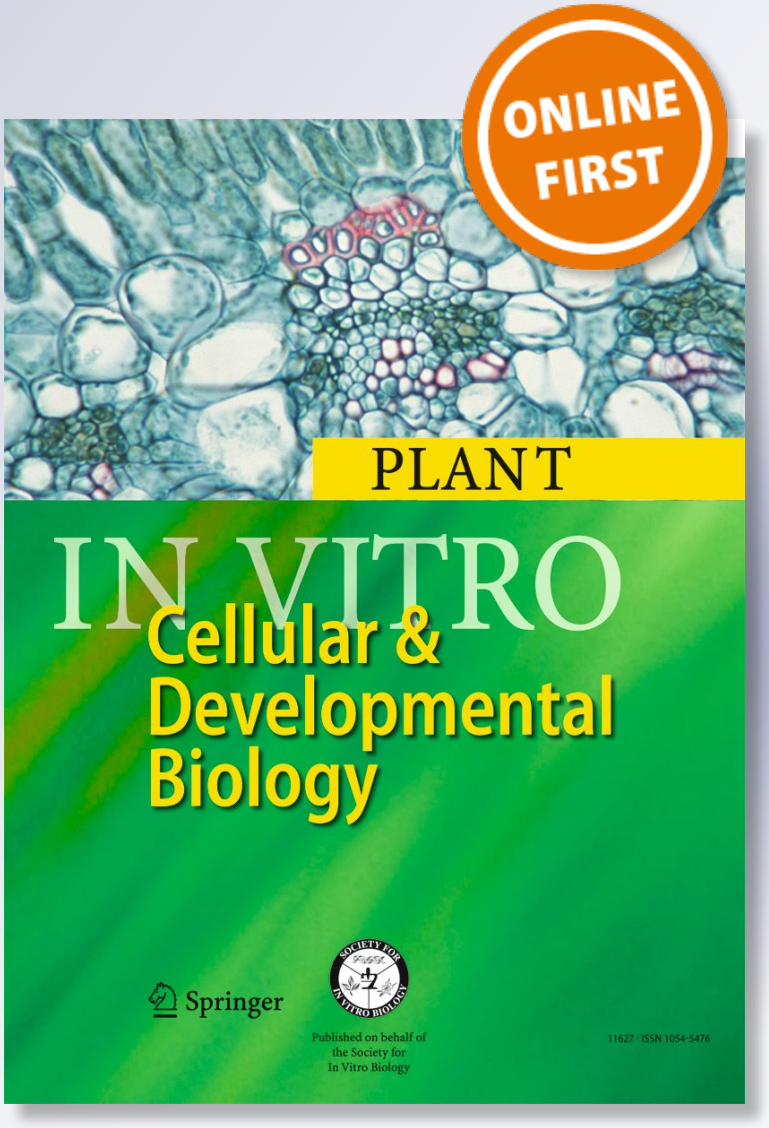
In Vitro Cellular & Developmental Biology - Plant

ISSN 1054-5476

In Vitro Cell.Dev.Biol.-Plant
DOI 10.1007/s11627-017-9865-y



Springer

In Vitro Cell.Dev.Biol.—Plant
https://doi.org/10.1007/s11627-017-9865-y

MICROPROPAGATION

In vitro plant regeneration and cryopreservation of *Arachis glabrata* (Fabaceae) using leaflet explants

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Received: 14 March 2017 / Accepted: 9 October 2017 / Editor: Ewen Mullins

Abstract *Arachis glabrata* Benth (perennial peanut) is a rhizomatous legume with high forage value and great potential for soil conservation as well as it displays valuable plant genetic resources for the cultivated edible peanut improvement. In this study, we developed for the first time successful protocols for micropropagation and cryopreservation of *A. glabrata*. First fully expanded leaflets from greenhouse-growing plants were efficiently established *in vitro* (93%) and displayed high frequency of bud induction (58%) on MS medium with 6 mg L^{-1} 1-fenil-3-(1,2,3-tiadiazol-5-il)urea [TDZ]. Whole plant regeneration was achieved *via* direct organogenesis by transferring the induced buds to MS media. Immature unexpanded leaves from micropropagated plants were effectively cryopreserved by using the droplet-vitrification technique. Maximum survival (~ 70%) and further regeneration (60–67%) were obtained by preconditioning immature leaves on semisolid MS with 0.3 M sucrose (1 d), exposing to loading solution consisting of 0.4 M sucrose plus 2 M glycerol (30 min) followed by glycerol-sucrose plant vitrification solution PVS3 (150 min in ice), and direct plunging into liquid nitrogen in droplets of PVS3 deposited on cryoplates. Tissues were rewarmed by plunging the aluminum foils directly in liquid MS enriched with 1.2 M sucrose (15 min) at room temperature. Growth recovery and plant regeneration were efficiently achieved *via* shoot organogenesis, and somatic embryogenesis by culturing cryostored explants on MS added with 6 mg L^{-1} TDZ. Genetic stability of plants derived from cryopreserved leaves was confirmed by random amplified polymorphic DNA markers. The protocols established in this study have great potential for rapid multiplication and conservation of selected *A. glabrata* genotypes.

Keywords Shoot organogenesis · Somatic embryogenesis · Thidiazuron · Droplet-vitrification · Genetic stability · Perennial peanut

Introduction

Rhizomal perennial peanut, *Arachis glabrata* Benth., is a relative of the cultivated edible peanut (*A. hypogaea* L.) that naturally grows in northeastern Argentina, eastern Paraguay and the state of Mato Grosso do Sul in Brazil between 13° S and 28° S (Krapovickas and Gregory 1994), but with a wide area of adaptation ranging from 31° N to 35° S latitude (Prine *et al.* 1981). *A. glabrata* is a legume with high forage value for intensively grazed pastures on infertile, acid soils. Furthermore, it has great potential for soil conservation and as an ornamental since it develops excellent ground cover even on poor soils (French *et al.* 1993; Krapovickas and Gregory 1994). Once established, perennial peanut competes well with native grasses and does not require additional fertilizer, allowing growers to reduce fertilizer applications by up to 40% (Rouse and Mullahey 1997).

Because rhizomal perennial peanut is a legume, its culture increases the soil nitrogen availability by fixation of

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Published online: 01 December 2017

atmospheric nitrogen and decomposition of mowed peanut clippings. Moreover, studies of perennial peanut have indicated that ruminant animals can assimilate nitrogen more efficiently from its hay and haylage than from other legume forages, which results in positive economic and environmental effects (Foster *et al.* 2009, 2011; Sullivan and Foster 2013).

The high forage value of *A. glabrata* is evidenced by the release of several cultivars in Florida (USA), such as "Florigraze" (is cold tolerant, having survived − 14°C in the winter after establishment) (Prine *et al.* 1981) and "Arbrook" (is better adapted to excessively drained soils than Florigraze, but is less cold tolerant) (Prine *et al.* 1986). Additionally, rhizomal perennial peanut has proved to be resistant to multiple pest and diseases (Angelici *et al.* 2008), and some of these resistances were transferred to *A. hypogaea* (Mallikarjuna 2002).

Besides *A. glabrata*, also other wild species of *Arachis*, *e.g.*, *A. pintoi*, *A. repens*, and *A. correntina*, display great potential as new agricultural options and valuable plant genetic resources for the cultivated edible peanut improvement. Unfortunately, according to recent biogeographic data, the natural distribution areas of most *Arachis* species are being severely reduced due to the anthropogenic impact. As a result, *ex situ* preservation actions are necessary to safeguard the threatened diversity of this genus (Pacheco *et al.* 2009).

Ex situ preservation of *Arachis* germplasm is usually carried out as living plants or seed banks. However, living plant collections are at the risk of disease, pest, fire, drought, and human damage, besides the genetic erosion (Zhang *et al.* 2014). Moreover, since *Arachis* seeds display a sub-orthodox behavior (Vásquez-Yanes and Aréchiga 1996), loss of seed viability occurs even under adequate storage conditions (Dunbar *et al.* 1993). In addition, despite often dense flowering of *A. glabrata*, scarce seed output is frequent in this species (Smartt and Stalker 1982), so that it is usually planted vegetatively. Consequently, *in vitro* propagation and cryoconservation methodologies represent important complementary tools for *ex situ* conservation of *Arachis* germplasm and are particularly useful for problem species as *A. glabrata*.

In this work, we developed for the first time efficient *in vitro* systems for whole plant regeneration (*via* direct organogenesis) and cryopreservation (by using the droplet-vitrification technique) of *A. glabrata*. Although indirect shoot organogenesis was previously reported from leaflet explants of this species, such procedure has demonstrated not to be effective because the low rate of shoot development (10%) (McKently *et al.* 1991) besides shoot bud formation cannot be reproduced consistently (Dunbar and Pittman 1992). Moreover, somatic embryogenesis was reported in two *A. glabrata* cultivars, but conversion of embryos into plants was very low (1–6%) (Vidoz *et al.* 2004). On the other hand, cryopreservation had not been earlier reported for this species. Thus, this is the first documented cryopreservation procedure of rhizomal perennial peanut tissues. We also tested the genetic stability of plants derived from cryopreserved explants by random amplified polymorphic DNA (RAPD) markers.

Materials and methods

Plant material, *in vitro* establishment and multiplication conditions Adult plants of *A. glabrata* (herbarium specimen Lavia 6 deposited in CTES), growing under greenhouse conditions (belonging to the *in vivo* germplasm bank of the IBONE) provided the primary explants in this study. Apical shoot tips (~2 mm long), nodal segments (10–15 mm long segment of stem with an axillary bud), and the first expanded leaflets (~ 80% of the final size) were excised and surface sterilized by immersing them in 70% (*v/v*) ethanol (30 s), followed by an aqueous solution at different concentrations of sodium hypochlorite containing 0.5, 1.0, 1.5, or 2.0% hypochlorite (*v/v*) plus 0.1% (*v/v*) Triton® X-100 (Merk, Darmstadt, Germany) (12 min) and finally rinsed three times with sterile distilled water. Leaf explants consisted of ~4 mm^2 squares from the middle section of the leaflet blade. Explants were individually cultured on 3 mL of solidified (6.5 g L^{-1} agar A-1292, Sigma-Aldrich Ltd. Co., St. Louis, MO) establishment medium contained into 11-mL tubes. The establishment media consisted of MS medium (Murashige and Skoog 1962), with 30 g L^{-1} sucrose and 3, 6, or 9 mg L^{-1} 1-fenil-3-(1,2,3-tiadiazol-5-il)urea (TDZ) or free of plant growth regulators (hereinafter PGR-free MS). The pH of all media was adjusted to 5.8. Tubes with culture media were autoclaved at 1.46 kg·cm^{-2} and 120°C for 20 min.

Once the explants were aseptically cultured, the tubes were sealed with Resinite AF 50® (Casco S.A.I.C. Company, Bs. As., Argentina) and incubated at standard conditions (*i.e.*, 27 ± 2°C and 14 h photoperiod with a photosynthetic flux of 116 μmol·m^{-2}·s^{-1}). After 10 d of *in vitro* establishment, explants without contamination were transferred to PGR-free MS medium and incubated under the same conditions for another 35 d for buds and shoots development.

The *in vitro* shoots obtained in above experiment were used for achieving further multiplication through culture of nodal segments about 15–20 mm in length on 15-mL glass tubes containing 4 mL of MS with 30 g L^{-1} sucrose, 0.2 g L^{-1} activated charcoal (AC), and 6.5 g L^{-1} agar. Incubation was carried out at standard conditions and subcultures of nodal segments were performed every 4–6 wk.

Cryopreservation: droplet-vitrification procedure Immature unexpanded leaves (3–4 mm long) were excised from micropropagated plants and used as explants for cryopreservation studies. Leaves were preconditioned on semisolid MS medium added with 0.3 M sucrose (1 d) and then exposed to loading solution consisting of 0.4 M sucrose combined with

2 M glycerol (30 min). After loading, leaves were exposed to PVS3 vitrification solution [50% sucrose (*w/v*) + 50% glycerol (*w/v*); Nishizawa *et al.* 1993] (30–180 min in ice). Samples were then cryostored (1 d) by immersing them directly into liquid nitrogen (LN) in droplets of PVS3 solution deposited on cryoplates (6 × 25 mm aluminum foil strips). After cryostorage, samples were rapidly warmed by immersing the cryoplates in 35 mL of liquid MS supplemented with 1.2 M sucrose at room temperature (15 min). Leaves were then placed on filter papers (~2 min) to remove excess liquid and cultured on semisolid induction medium for growth recovery.

Growth recovery Immature leaves from both non-cryostored (−LN) and cryostored (+LN) treatments were recovered on induction media consisting on solidified MS added with 6 mg L^{-1} TDZ or 10 mg L^{-1} picloram-4-amino-3,5,6-trichloropicolinic acid (PICL). Cultures were first kept in the dark (1 wk) and then moved to standard incubation conditions. After 10 or 30 d of culture on media with TDZ or PICL, respectively, the explants were transferred to PGR-free MS and incubated under standard conditions for plant regeneration. Further multiplication was carried out as mentioned above.

After the different steps of the cryopreservation procedure, both from non-cryostored (−LN) and cryostored (+LN) treatments, the survival was evaluated (percentage of explants that remained green 15 d after recovery) as well as the regeneration capacity of explants (percentage of leaves that displayed any morphogenetic developmental pathway after 60 d of culture). Both measurements were expressed relative to the total number of leaves treated.

Rooting and hardening Shoots (~2 cm) derived from non-cryostored (−LN) and cryostored (+LN) leaves were cultured on solidified MS added with 2 g L^{-1} AC for rooting. Cultures were incubated at standard conditions. The resulting plantlets were gently rinsed with tap water and placed into plastic pots with a mixture of soil, peat moss, and sand (1:1:1 *v/v/v*). Pots were individually covered with a transparent polyethylene bag and kept at standard conditions in a growth room for 3–4 wk. After that, the acclimatized potted plants were transferred to the greenhouse.

Histological studies Fresh tissue samples were fixed in FAA (5% formaldehyde, 5% acetic acid, and 63% ethanol) and then dehydrated with "Deshidratante Biológico Biopur". Dehydrated samples were embedded in paraffin wax (Gonzalez and Cristóbal 1997), serially sectioned with a rotative microtome (10 μm thick), stained with Safranin-Astra blue (Luque *et al.* 1996), and examined with a light microscope.

Genetic fidelity assessment For testing genetic stability, RAPD analyses were carried out on 15 plantlets selected at random from both non-cryostored (−LN) and cryostored (+LN) plants. Genomic DNA was purified from leaf tissue of *in vitro* plants of *A. glabrata* according to Doyle and Doyle (1990) method. To generate RAPD patterns, the standard protocol for RAPD analyses (Williams *et al.* 1990) was followed, using ten arbitrary 10-mers from Operon Technologies, Alameda, California (OPG-08, -11, -14, -16, and -19; OPP-02, -04, -08, -17, and -18). The PCR mixture consisted of 40 ng genomic DNA, 0.2 μM primer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs Promega, and 1.6 U GoTaq® DNA polymerase Promega, in 20 μL as final volume. Biometra DNA Thermal Cycler programmed for 45 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min, was employed to carry out PCR reaction. Agarose gels 1.5% (*w/v*) were using to separate the resultant products. The gels were stained with ethidium bromide and visualized with UV light.

Genetic stability between non-cryostored (−LN) and cryostored (+LN) plants were tested using cluster analysis. RAPD profiles were evaluated for each of 30 OTUs (operational taxonomy unit) scoring each character as [1] for presence or [0] absence. The Dice similarity coefficient was useful to construct a similarity matrix. The cophenetic correlation coefficient (r) was calculated for testing the phenogram distortion. NTSYS-pc (Numerical Taxonomy of Multivariate Analysis System) software package version 2.11W Rohlf (Rohlf 1994) was employed to calculate the resultant phenograms.

Statistical analyses A completely randomized design was used for *in vitro* establishment, multiplication, and cryopreservation experiments. Three replicates of 10–20 explants were carried out for each treatment. Data were subjected to analysis of variance (ANOVA) and, previous verification of the normal distribution of data, the significance of mean differences was determined by using the Tukey's multiple comparison test ($P < 0.05$).

Results and discussion

***In vitro* establishment** For apical shoot tips and nodal segments, the sodium hypochlorite concentrations and the exposure time assayed in this study were not effective for *in vitro* establishment of these explants, showing 100% contamination with microorganisms (bacteria and/or fungi) or oxidative injury due to the high levels of chlorine in the disinfectant solution, according to the treatment. For expanded leaflets, the treatment of 1% sodium hypochlorite for 12 min was the most effective, with 93.3% of successful *in vitro* establishment (Table 1).

Table 1 Effect of explant type and sodium hypochlorite (NaOCl) concentration on the success of *in vitro* establishment of *A. glabrata*

Explants	[NaOCl] (%)	*In vitro* establishment (%)[a]
Apical shoot tips	0.5	0.0 c
	1.0	0.0 c
	1.5	0.0 c
	2.0	0.0 c
Nodal segments	0.5	0.0 c
	1.0	0.0 c
	1.5	0.0 c
	2.0	0.0 c
Leaflet segments	0.5	40.0 ± 7.6 b
	1.0	93.3 ± 4.4 a
	1.5	58.3 ± 6.0 b
	2.0	0.0 c

Data are presented as means ± SE. Different *letters* within *column* indicate significant differences by Tukey's multiple comparison test ($P < 0.05$).

[a] Measured as the percentage of explants without contamination after 10 d of culturing ($n = 60$)

High *in vitro* establishment rates by using leaflet explants from field or greenhouse-grown plants have been previously reported for *Arachis* species (Mroginski *et al.* 2004; Vidoz *et al.* 2004). On the other hand, inefficient establishment have been informed when *A. pintoi* apical shoot tips were used as primary explants to initiate a micropropagation system (Rey and Mroginski 2003). *In vitro* culture initiation from explants dissected from field or greenhouse-grown plants is a critical step. The contamination could be significantly reduced by using more sanitized plant stock, treated with appropriate combination of chemicals to reduce infection with pathogens (Cassells 2001). Furthermore, it would be interesting to evaluate the effectiveness of other disinfecting agents such as hydrogen peroxide and calcium hypochlorite when it is necessary the establishment of problem explants (*e.g.* apical shoot tips and nodal segments).

***In vitro* shoot induction** Between 30 and 58% of leaflet segments displayed shoot organogenesis when cultured on media supplemented with TDZ. Numerous bud initiation occurred from all the adaxial side of the explants without previous formation of callus within 15–20 d of induction initiation. Maximum bud induction (58.3%) was reached on MS added with 6 mg L^{-1} TDZ with an average of 13.2 buds per explant after 45 d of culture, while bud differentiation was not observed when explants were established on PGR-free MS (Table 2). A high percentage (80–90%) of the TDZ-induced buds were capable of continued growth and shoot elongation after transfer to PGR-free MS and then efficiently rooted (~ 90%) when transferred to MS supplemented with AC.

The balance between PGRs, particularly cytokinins and auxins, in the induction medium is a determinant for plant regeneration *via* organogenesis (Evans *et al.* 1981). Successful shoot organogenesis has been reported in several *Arachis* species by combining α-naphthaleneacetic acid (NAA) or 2,4-dichlorophenoxyacetic acid (2,4-D) with 6-benzylaminopurine (BAP) or kinetin (KIN) (Mroginski *et al.* 1981; Pittman *et al.* 1983; Mroginski and Kartha 1984; McKently *et al.* 1991; Rey *et al.* 2000; Vidoz *et al.* 2006). Moreover, the use of TDZ (a substitute of phenylurea which displayed a potent activity as a cytokinin in several plant species; Murthy *et al.* 1998; Mithila *et al.* 2001) has been reported in *A. hypogaea* (Kanyand *et al.* 1997; Gill and Ozias-Akins 1999; Akasaka *et al.* 2000), *A. correntina* (Mroginski *et al.* 2004), and *A. villosa* (Fontana *et al.* 2009).

Indirect shoot organogenesis was previously observed in *A. glabrata* from leaflet explants cultured on media supplemented with BAP. However, such procedure has demonstrated not to be very effective because the low rate (10%) of bud meristems that continued through growth and differentiation to shoot development (McKently *et al.* 1991). Also, adventitious shoot formation cannot be reproduced consistently (Dunbar and Pittman 1992). Moreover, somatic embryogenesis was reported in two *A. glabrata* cultivars from leaflet explants cultured on media supplemented with PICL, with 17–20% of explants giving rise to embryos and only 1–6% conversion into plants (Vidoz *et al.* 2004). Additionally, the competence of TDZ in comparison with other cytokinins [BAP, KIN, isopentenyladenine (2iP)] to induce shoot organogenesis in *A. glabrata* was previously evaluated in our laboratory. Data from that study demonstrated that only TDZ exhibited an ability for bud formation and plant regeneration from leaflet explants (unpublished data). In the present work, the potent

Table 2 Effect of TDZ concentration added to MS basal medium on bud induction from leaflet explants of *A. glabrata*

TDZ (mg L^{-1})	Percentage of explants with buds	Mean number of buds per explant
0	0.0 c	0.0 d
3	43.3 ± 4.4 ab	10.4 ± 0.5 b
6	58.3 ± 7.3 a	13.2 ± 0.5 a
9	30.0 ± 5.0 b	7.3 ± 0.4 c

Results were recorded after 45 d of *in vitro* establishment. Data are presented as means ± SE. Different *letters* within the same *column* indicate significant differences by Tukey's multiple comparison test ($P < 0.05$).

Table 3 Effect of preconditioning, loading treatment and different exposure duration to vitrification solution (PVS3) on survival and regrowth from both non-cryostored (−*LN*) and cryostored (+*LN*) *A. glabrata* immature leaves, cultured on induction media with *TDZ* (6 mg L^{-1}) or *PICL* (10 mg L^{-1})

Treatments	MS + TDZ				MS + PICL			
	-LN		+LN		-LN		+LN	
	Survival[a]	Regrowth[b]	Survival[a]	Regrowth[b]	Survival[a]	Regrowth[b]	Survival[a]	Regrowth[b]
Control	100 a	97 ab	0 m	0 m	100 a	83 abcde	0 m	0 m
Preconditioning	100 a	93 abc	0 m	0 m	100 a	77 abcde	0 m	0 m
Loading	100 a	97 ab	0 m	0 m	97 ab	80 abcde	0 m	0 m
PVS3 30 min	100 a	90 abcd	13 jklm	0 m	100 a	77 abcde	7 klm	0 m
PVS3 60 min	100 a	93 abc	23 ijklm	7 klm	100 a	73 abcdef	17 jklm	3 lm
PVS3 90 min	100 a	90 abcd	37 ghijk	23 ijklm	97 ab	77 abcde	33 hijkl	17 jklm
PVS3 120 min	93 abc	87 abcd	63 cdefgh	53 efghi	97 ab	70 abcdef	63 cdefgh	43 fghij
PVS3 150 min	97 ab	87 abcd	73 abcdef	67 bcdefg	93 abc	73 abcdef	67 bcdefg	60 defgh
PVS3 180 min	90 abcd	83 abcde	70 abcdef	60 defgh	93 abc	70 abcdef	67 bcdefg	53 efghi

Data are presented as means of three replicates. Different *letters* within the same *column* indicate significant differences by Tukey's multiple comparison test ($P < 0.05$).

[a] Measured as the percentage of explants that remained green after 15 d of rewarming ($n = 30$)

[b] Measured as the percentage of explants that displayed any morphogenetic developmental pathway after 60 d of culture ($n = 30$)

activity of TDZ was confirmed on the regulation of *A. glabrata* shoot organogenesis from leaf explants. In addition, the results obtained here allowed the development of an efficient and reproducible methodology for whole plant regeneration of this species *via* direct organogenesis. This method may be useful with many other *Arachis* species.

Cryopreservation and growth recovery Control explants (immature leaves cultured on induction media immediately after excision) displayed high frequency of survival (100%) and morphogenetic regeneration (83–97%). The effect of the preconditioning and loading treatment as well as the exposure duration to vitrification solution on survival and regrowth was also considered for both non-cryostored (−LN) and cryostored (+LN) leaves (Table 3). The preconditioning and loading treatment did not affect the survival (100%) and regrowth (77–97%) of non-cryostored leaves. Moreover, none of the PVS3 exposure duration used to ensure vitrification of cell content proved to be cytotoxic. Control explants exhibited high survival (≥ 90%) and regeneration (≥ 70%) rates even after the longest periods of exposure. On the other hand, recovery after cryostorage was strongly affected by the PVS3 exposure duration in addition to the culture medium. The recovery was improved as the exposure to vitrification solution was prolonged, reaching the highest survival (~ 70%) and regeneration (60–67%) rates from leaves treated with PVS3 for 150 min followed for LN storage.

As it is known, successful cryopreservation of live tissues is only possible if the development of intracellular ice crystals is avoided. This can be achieved by vitrification, *i.e.*, the non-crystalline solidification of water (Sakai 2000). Two requirements are necessary for a solution to vitrify: (i) a concentrated cellular solution and (ii) rapid cooling rates (Panis *et al.* 2005). Data from this work confirmed that pretreatments of explants before exposure at ultralow temperatures was an essential prerequisite for the survival of *A. glabrata* immature leaves following cryopreservation. Particularly the vitrification solutions (*VS*) play an important role for tissues dehydration and cryoprotection. The beneficial result of sugars in cryopreservation can be a consequence of an osmotic dehydration (Tanaka *et al.* 2004). Moreover, it is known that sucrose stabilizes membranes and proteins during dehydration (Crowe *et al.* 1990). However, regardless of their positive effect for cryopreservation, *VS* can exhibit high osmotic and chemical toxicity. Consequently, the osmotic dehydration process needs to be optimized for each plant tissue by using the proper *VS* and adjusting the exposure duration.

On the other hand, results from this study prove that high recovery frequencies can be achieved by using the droplet-vitrification technique. The main benefits of using this procedure are the very high cooling/warming rates achieved, due to the slight quantity of *VS* surrounding each explant and the use of aluminum cryoplates (Leunufna and Keller 2003; Panis *et al.* 2005). Effective cryopreservation protocols by droplet-vitrification approaches have been previously reported for apical shoot tips and meristems of several species, *e.g.*, potato (Schäfer-Menuhr *et al.* 1997; Panta *et al.* 2014), sweet potato (Pennycooke and Towill 2000), mint (Towill and Bonnart 2003), *Musa* spp. (Panis *et al.* 2005), *Malus* (Halmagyi *et al.* 2010), and *Limonium serotinum* (Barraco *et al.* 2011). However, there are no reports on leaf explants cryopreservation by using any of the available techniques. In this work, we

describe for the first time, the use of immature unexpanded leaf as explant to be successfully cryopreserved through the droplet-vitrification technique.

Once recovery, all treated leaves (preconditioned, loaded, PVS3 exposed), both non-cryostored and cryostored, showed similar morphogenetic developmental pathway to that of the control ones after culture on induction media. Immature unexpanded leaves cultured on medium with TDZ displayed high percentages of shoot organogenesis (Figs. 1 and 2). Numerous bud initiation occurred from all the adaxial side of the explants within 15–20 d of culture, without previous formation of callus. The shoot apex exhibited a usual structure for the dicotyledons: the caulinar apical meristem with the tunica-corpus zonation, meristematic cells small and isodiametric, with dense cytoplasm (which stained darkly) and small vacuoles. The apical meristem is surrounded by several leaf primordia, helically arranged. The cells derived from the meristem are organized in protoderm, ground meristem, and procambial strands (Fig. 2). After 30 d of culture, new buds were developed in the axil of primary buds. High percentage of the TDZ-induced buds were able to continuing growth and elongation after transferred to PGR-free MS (Table 4). Elongated shoots efficiently rooted (~ 90%) after 15–20 d of transferred to MS supplemented with AC (Fig. 1).

On the other hand, immature leaves cultured on medium supplemented with PICL followed the regeneration pathway *via* direct somatic embryogenesis (Figs. 1 and 2). Leaves took

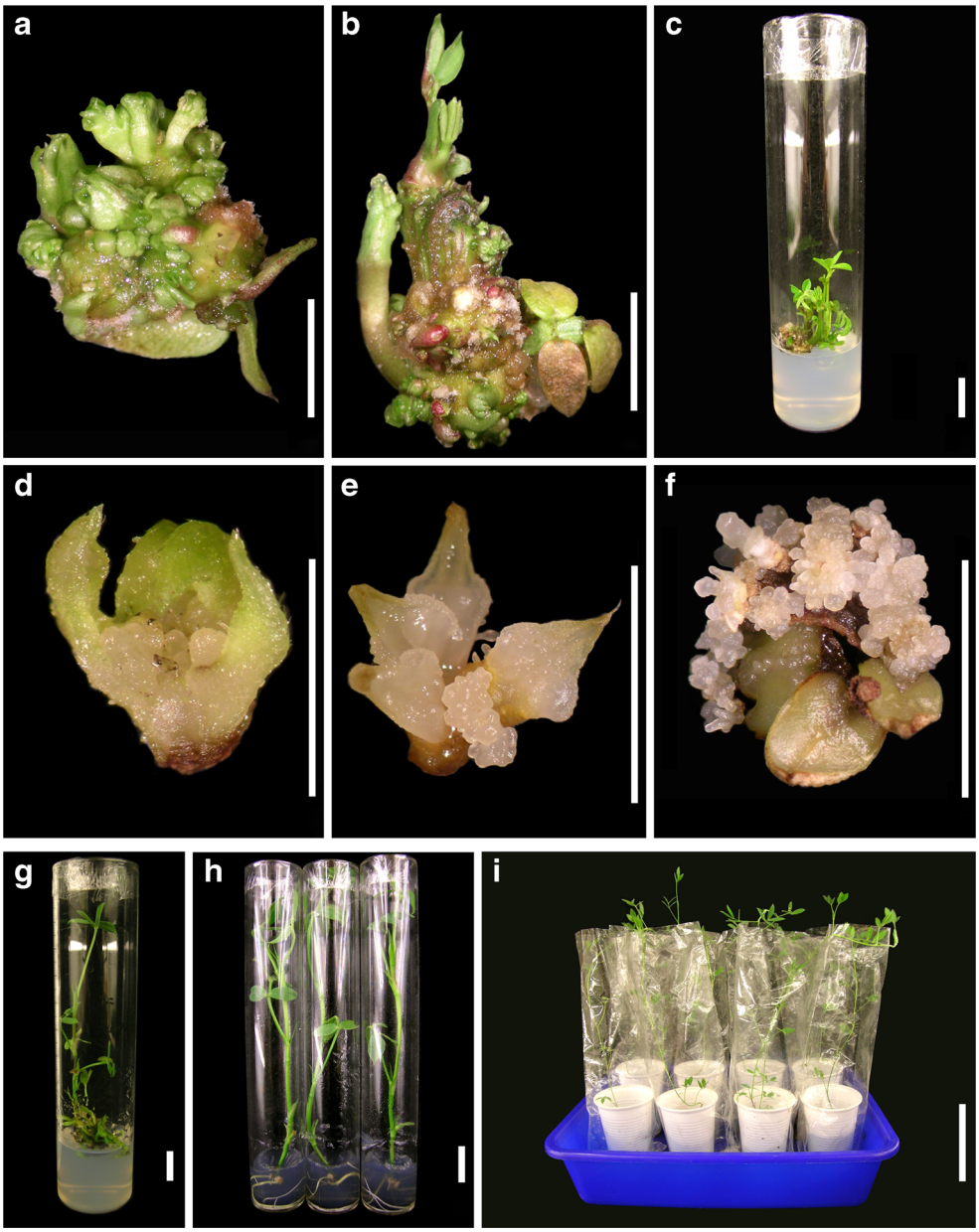

Figure 1. *In vitro* plant regeneration from immature unexpanded leaves of *A. glabrata* cultured on MS with 6 mg L^{-1} TDZ or 10 mg L^{-1} PICL. (*a–c*) Shoot organogenesis (TDZ), (*d–f*) Somatic embryogenesis (PICL), (*g–i*) Plant regeneration and hardening. (*a–f*) Details of leaf explants showing buds and shoots/somatic embryos development after 30 (*a*, *d*), 45 (*b*, *e*), and 75 d (*c*, *f*) of initiated the culture on induction media. (*g–h*) TDZ-derived elongated (*g*) and rooted (*h*) shoots after 30 d transferred to PGR-free MS and 20 d culture of nodal segments on PGR-free MS supplemented with 0.2 g L^{-1} AC, respectively. (*i*) *In vitro* regenerated plants successfully transferred to soil and acclimatized to *ex vitro* conditions. *Bars*, 5 mm in *a–b*, *e–f*; 1 cm in *b*, *g–h*; 2 mm in *d*; 10 cm in *i*.

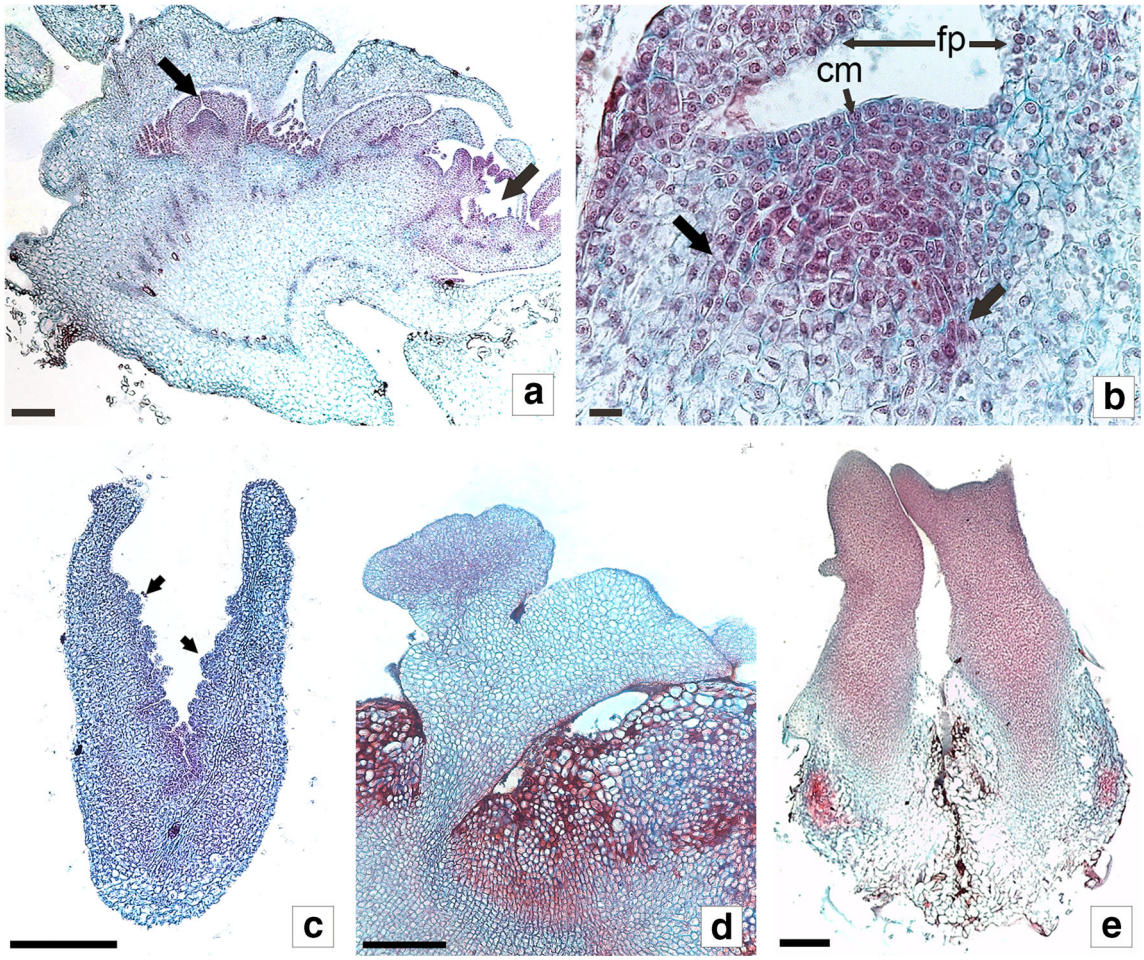

Figure 2. Histological sections of *A. glabrata* showing regeneration of shoots and embryos. (*a–b*) Shoot organogenesis (TDZ). (*c–e*) Somatic embryogenesis (PICL). (*a*) Longitudinal section showing pair of shoot with multiple leaf primordia (*arrows*). (*b*) Detail of meristematic zone showing apical meristem (*cm*), procambium strands (*arrows*) and leaf primordia (*lp*). (*c*) Transversal section of explant showing embryo initial development (*arrows*) from adaxial side of the leaf. (*d–e*) Bipolar embryos, note the lack of apical meristems. *Bars*, 200 μm in *a–b*, *c–e*; 20 μm in *b*.

a little longer to start growing and somatic embryos began differentiating ~30 d after initiation of culture as globular structures proliferating from the adaxial side of the leaflets. After 45–60 d of culture, some of the globular embryos progressively enlarged and organized as bipolar structures. However, resulting embryos rarely germinated and converted

Table 4 The effect of cryopreservation and induction medium on the morphogenetic response and plant regeneration from immature leaves of *A. glabrata*

Treatments	Morphogenetic responses (%)[a]		Mean number of buds/somatic embryos per explant[b]	Mean number of plants per explant[c]
	Shoot organogenesis	Somatic embryogenesis		
−LN +TDZ	86.7 ± 8.8	0.0	25.4 ± 0.6	18.5 ± 0.5
+LN +TDZ	66.7 ± 8.8	0.0	23.8 ± 0.4	16.7 ± 0.5
−LN +PICL	0.0	76.7 ± 3.3	18.2 ± 0.8	0.42 ± 0.1
+LN +PICL	0.0	56.7 ± 6.7	17.6 ± 0.6	0.28 ± 0.1

Non-cryostored (−LN) and cryostored (+LN) leaves were cultured on induction medium composed of MS supplemented with TDZ (6 mg L^{-1}) or PICL (10 mg L^{-1}). Data were presented as means of three replicates ± SE.

[a] Measured as the percentage of explants which displayed buds/somatic embryos after 60 d of culturing on induction medium

[b] Measured after 60 d of culturing on induction medium

[c] Measured after 120 d of culturing on induction medium

Figure 3. Flowchart for *in vitro* propagation and cryopreservation of *A. glabrata*. (*a*) Greenhouse-growing plants (initial source of explants). (*b*) First fully expanded leaflets (surface-sterilized, sliced into squares and *in vitro* established for bud induction). (*c*) Primary bud and shoot regeneration (MS + 6 mg L^{-1} TDZ). (*d*) *In vitro* regenerated plant (second source of explants). (*e*) Immature unexpanded leaves (used as explants for the cryopreservation studies). (*f*) Droplet-vitrification procedure. (*g*) Cryostorage. (*h*) Recovery and bud and shoot regeneration (MS + 6 mg L^{-1} TDZ). (*i*) *In vitro* regenerated plants. (*j*) Hardened plant.

into plants (Table 4). Histological studies demonstrated the development of nodular structures directly from adaxial side of the leaflets, which displayed small cells with dense cytoplasm and darkly stained nucleus. These structures gave rise gradually to globular and bipolar somatic embryos, which had no vascular connections with the primary explant. Anatomical analysis also revealed that most embryos lacked apical meristems (Fig. 2), which explains the low rate of conversion into plants. Similar results were previously observed in *A. hypogaea* (Wetzstein and Baker 1993) and two accessions of *A. glabrata* (Vidoz *et al.* 2004). Thus, it would be interesting to evaluate different culture media to promote further maturation of resulting somatic embryos. There are several reports highlighting the importance of a two-step procedure to achieve successful plant regeneration *via* somatic embryogenesis, *i.e.*, by culturing explants on induction medium (generally added with auxins) and subculturing embryos on germination medium (manipulating different concentrations of sucrose and cytokinins or devoid PGR) (Rey *et al.* 2000; Vila *et al.* 2003; Vidoz *et al.* 2004, 2006; Bhattacharya *et al.* 2016).

It is important to note here that even though the recovery rate was similarly high in both morphogenetic developmental pathways, a high percentage of the TDZ-induced buds were able to develop whole plants, and most PICL-derived somatic embryos failed on conversion (Table 4). These results emphasize the importance of adjusting culture conditions by providing the proper PGR during recovery and regeneration induction.

Rooting and hardening Elongated shoots derived from non-cryostored and cryostored leaves efficiently formed roots (90–95%) within 10–15 d after subculture on PGR-free MS added with 2 g L^{-1} AC. Rooted plants were successfully (~ 90%) transferred to soil and acclimatized to *ex vitro* conditions. Hardened plants were healthy and showed normal morphology.

Although rooting was previously induced in *A. pintoi* and *A. correntina* by using NAA (Rey *et al.* 2000, 2009; Mroginski *et al.* 2004), in this study we achieved both further shoot growth and rooting by culturing nodal segments of *A. glabrata* on MS with AC but lacking PGR. Moreover, we obtained high rates of plant acclimatization to *ex vitro* conditions, which makes possible the successful

use of this methodology for mass propagation and *in vitro* conservation of this species.

Figure 3 shows a flowchart for the *in vitro* culture establishment, plant regeneration and cryopreservation system developed in this study, by using *A. glabrata* leaf explants. It involves: (a) Greenhouse-growing plants as source of primary explants. (b) Excision and surface-sterilization of first fully expanded leaflets, dissection of leaflet blade into squares, and *in vitro* establishment on bud induction medium (MS + 6 mg L^{-1} TDZ). (c) Transfer of induced explants to PGR-free MS medium for continued growth and differentiation to shoot development. (d) Further multiplication and plant regeneration through culture of elongated shoots and nodal segments on MS + 0.2 g L^{-1} AC. (e) Excision of immature unexpanded leaves from *in vitro* regenerated plants. (f) Cryopreservation by droplet-vitrification procedure. (g) Cryostorage in LN. (h) Rewarming and recovery (by culturing immature leaves on MS + 6 mg L^{-1} TDZ for bud induction). (i) Further multiplication and plant regeneration (as in d). (j) Plant acclimatization to *ex vitro* condition.

Genetic fidelity assessment With the aim to confirm whether this cryopreservation procedure allows maintaining genetic stability of *A. glabrata* germplasm, a comparison of RAPD patterns generated by ten primers of arbitrary nucleotide sequences randomly chosen was carried out. Nine of the ten RAPD primers resulted in clear and reproducible amplification products. The number of scorable loci for each RAPD primer varied from 4 (OPG-11) to 11 (OPG-19). They generated 68 total loci with average of 7.5 loci per primer. The amplified fragments were between 740 and 3400 base pairs in length. Four RAPD primers produced monomorphic fragments and the other five were slightly polymorphic.

The UPGMA phenogram based on Dice similarity matrix clusters all the individuals assayed with similarity coefficients higher than 0.966 (Fig. 4). At a cutoff line of 0.966, we observed two groups, a large group containing 80% of the assayed individuals and a small group including the 20% remaining. The similarity range from 1 to 0.966 indicated a high similarity level within the individuals tested. The cophenetic correlation coefficient was 0.875, reflecting a very small amount of distortion introduced during the clustering.

RAPD profile homogeneity analysis has been successfully used to evaluate genetic fidelity of plant material submitted to different cryogenic techniques in *Arachis* species (Gagliardi *et al.* 2003; Rey *et al.* 2009, 2013). In those studies, the amplified RAPD fragments revealed a large number of monomorphic bands with a low percentage of polymorphic bands in all tested individuals. Moreover, the Dice similarity coefficient values displayed a high genetic similarity between cryostored

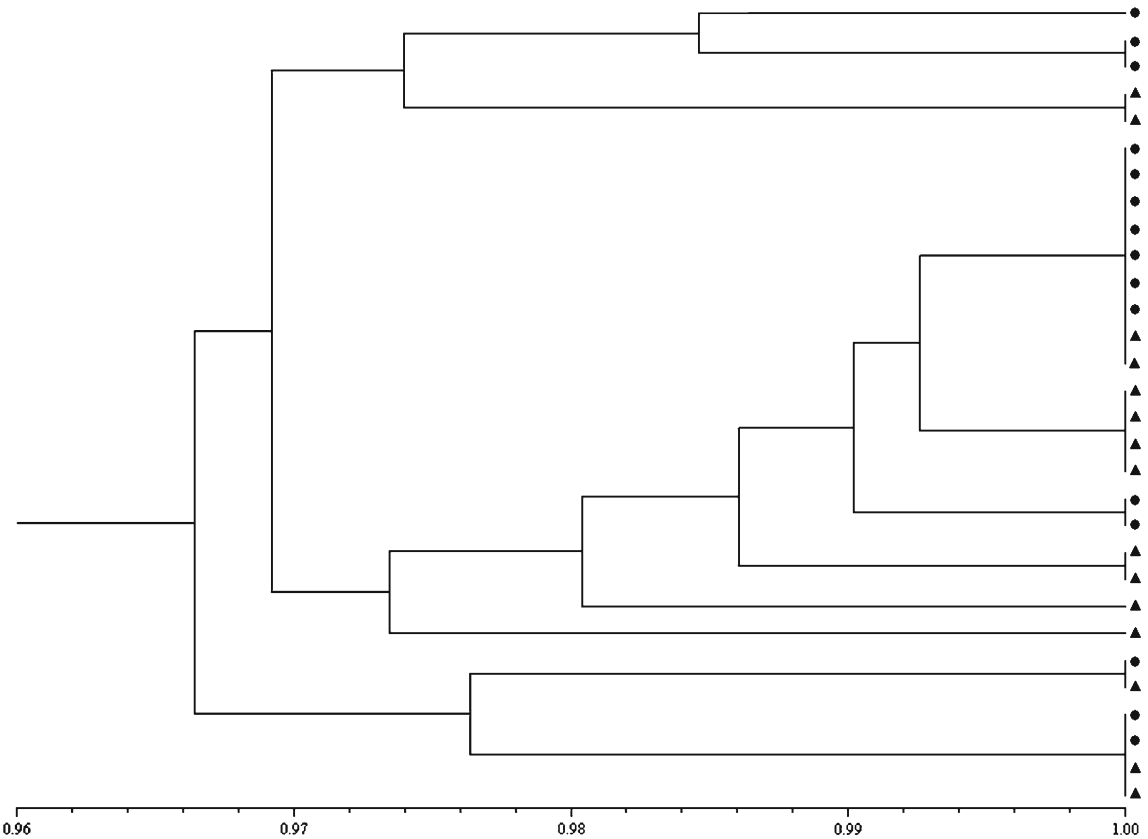

Figure 4. Phenogram of 30 OTU's of *in vitro* non-cryostored (*black dots*) and cryostored (black triangles) plants of *A. glabrata* resulting from the UPGMA cluster analysis of the OTUxOTU similarity matrix. Cophenetic correlation coefficient (r) = 0.875.

and non-cryostored plants. The UPGMA analysis based on RAPD band polymorphisms revealed that the slightly variability existing in cryostored plants is not higher than the original variability observed in the mother plants.

In this work, using nine RAPD profiles, we could not identify any modification induced by the cryopreservation procedure when plantlets derived from non-cryostored and cryostored material were comparatively analyzed. This is another confirmation of efficiency and safety of cryopreservation with regards to genetic stability of the cryopreserved material.

Perspective and concluding remarks

The landscape of biotechnology applied to micropropagation and cryoconservation of *Arachis* germplasm is encouraging given the increased number of species and cultivars that have been studied in the last 35 yr. Micropropagation of *Arachis* species was first reported by Mroginski *et al.* (1981), who described the plant regeneration of six *A. hypogaea* cultivars *via* organogenesis from immature leaf culture. This methodology was extended for plant propagation of other *A. hypogaea* cultivars and some wild *Arachis* species (see review of Pacheco *et al.* 2009). However, no effective protocol was available so far for plant regeneration of *A. glabrata* either *via* organogenic or embryogenic pathway. On the other hand, cryopreservation of *Arachis* germplasm was first applied to *A. hypogaea* apical meristems (showed 20–30% survival rates) by involving a cryoprotectant solution treatment previous to freeze preservation (Bajaj 1979). In the last decades, cryopreservation protocols for embryonic axes, whole seeds, *in vitro* apical shoot tips or somatic embryos have been reported for eight *Arachis* species by using desiccation combined with a two-step freezing technique (Runthala *et al.* 1993), vitrification (Gagliardi *et al.* 2002, 2003; Rey and Mroginski 2009), or encapsulation-dehydration (Rey *et al.* 2009, 2013). However, *A. glabrata* cryopreservation has not been reported yet.

Since a suitable protocol for plant regeneration is a critical requirement for the development of cryopreservation systems, effort must be devoted to the development of methodologies that ensure effectiveness and reproducibility. In this study, we have developed a successful procedure for micropropagation of *A. glabrata via* direct shoot organogenesis both starting with leaflet segments from greenhouse-growing plants and with unexpanded leaves from *in vitro* regenerated plants. Subsequently, successful cryopreservation of rhizomal perennial peanut tissues was achieved by using the droplet-vitrification procedure. It is interesting to note here that this is the first report of using immature leaves as explants for germplasm cryopreservation. Moreover, plants derived from cryopreserved leaves showed genetic stability when analyzed by RAPD markers.

The cryopreservation methodology described here is easy to execute and allowed high survival (73%) and recovery (67%) rates after cryopreservation when leaves were properly treated with the vitrification solution. Additional research will determine the applicability of this procedure to a wider range of rhizomal perennial peanut accessions as well other *Arachis* species, so that this approach could be used for the establishment of cryogenic collection for *Arachis* clonal germplasm.

Acknowledgements The authors would like to thank CONICET (PIP 11220150100398 CO) and SGCyT-UNNE (PI 12A007 and 16A010) for the financial support. We are also grateful to Dr. H. Rey for helpful collaboration and Dr. G. Lavia for providing the plant material.

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