

Somatic embryogenesis and plant regeneration through leaf culture in *Arachis glabrata* (Leguminosae)

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Abstract

Plants of two accessions of *Arachis glabrata* were regenerated via somatic embryogenesis. Embryogenic calli were initiated from leaflet explants on Murashige and Skoog medium supplemented with picloram alone or picloram in combination with 6-benzylaminopurine. Leaflets of accession A6138 induced the highest percentage of somatic embryos in media composed of 10 mg·dm⁻³ and 15 mg·dm⁻³ picloram. In contrast, 5 mg·dm⁻³ picloram with 0.1 mg·dm⁻³ 6-benzylaminopurine was one of the most effective combinations in accession AF385. MS medium supplemented with 2 g·dm⁻³ activated charcoal (AC) used for 30 days was the most effective for embryo maturation. After 20 days of culture on MS medium devoid of growth regulators, 6 % of embryos converted into plantlets in accession A6138.

List of abbreviations: AC, activated charcoal; BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; MS, Murashige and Skoog (1962) medium; NAA, α -naphthaleneacetic acid; PIC, picloram- (4-amino-3,5,6-trichloropicolinic acid).

Introduction

The genus *Arachis* consists of 69 species originated in South America. *Arachis glabrata* (2n=4x=40 chromosomes) is a perennial and rhizomatous legume with high forage value (Krapovickas and Gregory 1994), and has proved to be persistent under defoliation or continuous grazing. However, seed set is less frequent probably due to an irregular meiosis (Smartt and Stalker 1982). This species is resistant to multiple diseases, and this resistance was transferred to *A. hypogaea* L. through gene introgression (Mallikarjuna 2002).

Gene transformation enables the improvement of plants in which traditional breeding methods are limited due to sexual incompatibility and sterility barriers (Baker and Wetsztein 1992). Nevertheless, gene transfer systems require a reliable protocol for transformed plant regeneration (Little *et al.* 2000). Consequently, the development of these protocols is the first step for utilizing this new technology (Ventakachalam *et al.* 1999). Recently, somatic embryogenesis has become a practical tool not only for incorporating useful genes but also for

micropropagation (Griga 1999). Although somatic embryogenesis has been achieved in many legumes, an efficient protocol for plant regeneration of *A. glabrata* through somatic embryogenesis is still lacking. The previous reports of this species describe plant regeneration via organogenesis in culture media supplemented with NAA and BA (McKently *et al.* 1991), and pollen-embryos and pollen-callus induction (Bajaj *et al.* 1980).

In this communication, we report the development of a somatic embryogenic system for plant regeneration from *A. glabrata* leaflets.

Materials and methods

Plants of five *Arachis glabrata* Benth. (Table 1) accessions were used in this study. The source of explants were immature but fully expanded leaflets (approximately 80 % of the final size) from adult plants. Leaflets from 1 year old plants growing in greenhouse conditions were surface sterilized with 70 % ethanol for 30 s followed by 0.9 % NaOCl plus one drop of Tween 20[®] for 12 min and then rinsed three times in sterile distilled water. Explants consisted of squares of approximately 4 mm² of the median portion of the laminae, including the midrib. Each explant was placed with the abaxial side in contact with 3 cm³ of the culture medium, in an 11 cm³ glass tube. The medium was composed of Murashige and Skoog (1962) salts and vitamins, with 3 % sucrose, 0.7 % agar and growth regulators in several combinations (2 - 15 mg·dm⁻³ PIC; 2 - 15 mg·dm⁻³ 2,4-D; 0.01 - 0.1 mg·dm⁻³ BAP). The medium pH was adjusted to 5.8 with KOH or HCl prior to adding the agar. The tubes were covered with aluminium foil and autoclaved at 1.46 kg·cm⁻² for 20 min.

Ten to thirteen explants were used for each treatment with three to five replicates. Means are given with standard error (\pm SE). Data were statistically analysed using Duncan's test and significant difference was accepted when $P \leq 0.05$.

Cultures were incubated at 27 ± 2 °C, under a photoperiod regime of 14 h (116 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ provided by cool white fluorescent tubes). Observations on the development of somatic embryos were recorded after 90 days of culture.

Table 1. List of plant material of *Arachis glabrata* employed in this study.

Accession number*	Collector	Place of collection	Botanical variety
A6138	M.M. Arbo	Santa Rosa (Mnes), Paraguay	glabrata
A6146	M.M. Arbo	Santa Rosa (Mnes), Paraguay	glabrata
AF385	A. Fernández	Caacupé, Paraguay	Hagenbeckii
L5	G. Lavia	Ituzaingó (Ctes), Argentina	Hagenbeckii
L6	G. Lavia	Posadas (Mnes), Argentina	glabrata

*All the herbarium specimenes are deposited in CTES

Somatic embryos were transferred to conversion media composed of 25, 50 or 100 % strength of MS medium without growth regulators, with or without AC (2 g·dm⁻³) for 30 days and then transferred to hormone free MS medium.

Well-developed plantlets were rinsed under running tapwater and planted individually in pots containing a mixture of soil, sand and peatmoss (1:1:1). These plants were acclimatized at 27 ± 2 °C under a light intensity of 336 $\mu\text{m}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and then moved to the greenhouse.

Histology was performed according to Gonzalez and Cristóbal (1997). Samples of callus were fixed in FAA (formaline, acetic acid, 70 % ethanol 5:5:90), dehydrated with "Deshidratante histológico Biopur[®]", followed by paraffin embedding as described by Johansen (1940). Embedded tissue was cut with a rotatory microtome into 10- μm thick sections, stained with Safranin-Astra blue (Luque *et al.* 1996) and observed under a light microscope.

Results and discussion

The *in vitro* establishment of cultures was successful. Leaflet explants began to enlarge within the first week of culture with less than 2 % contamination. On longer incubation, calli induction was observed in high frequencies (93 % of the cultured

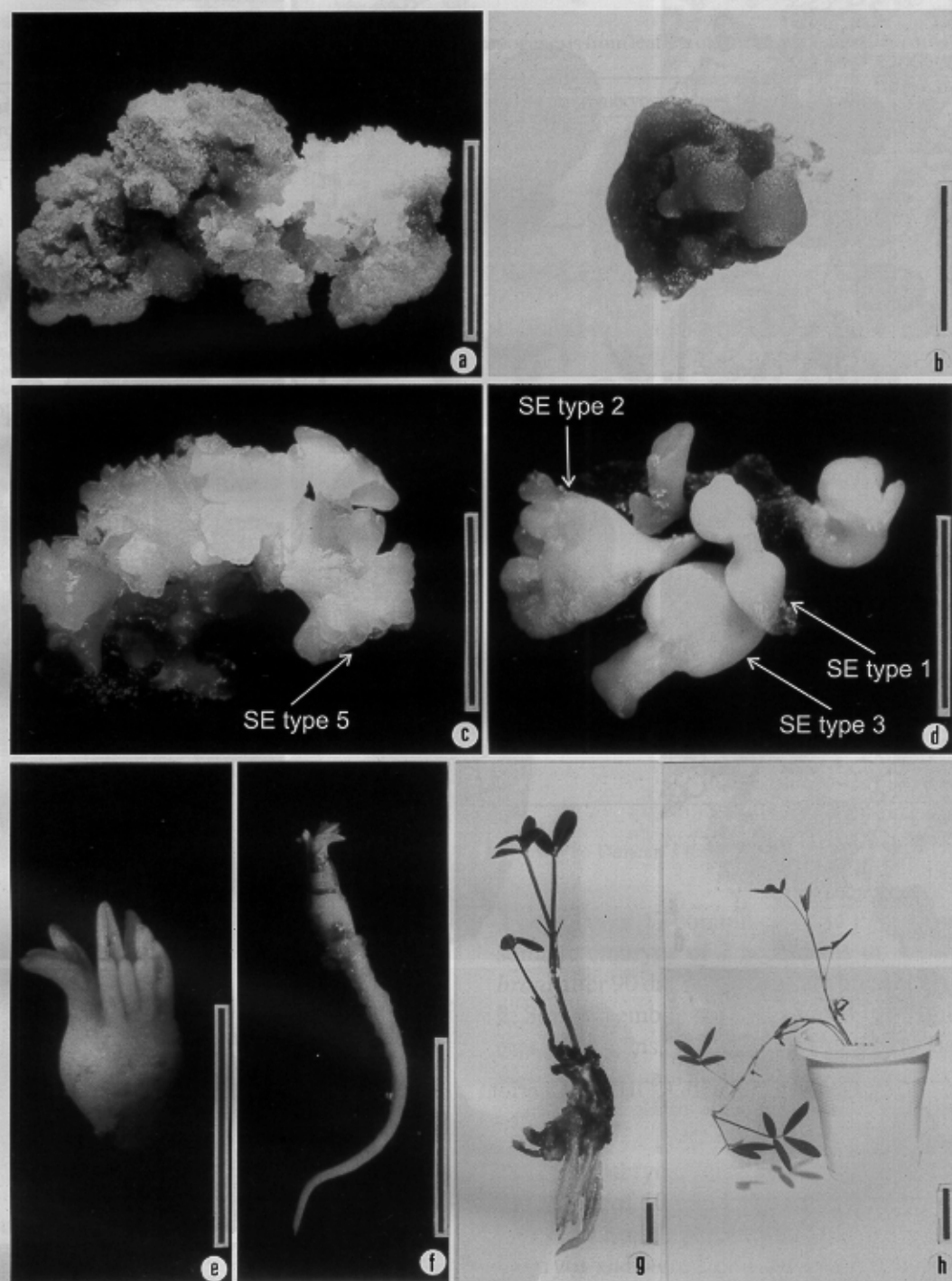


Fig. 1a-d. Somatic embryogenesis in *A. glabrata*. Bar: 0.5 cm. a: Friable callus. b: Mucilaginous callus with globular regions. c: Callus with somatic embryos (SE). d: Morphological variation of regenerated somatic embryos. e: Somatic embryo type 1. Bar: 3mm. f: Somatic embryo germinated. Bar: 0.5 cm. g: Plant regenerated from a somatic embryo. Bar: 0.5 cm. h: Plant growing in soil. Bar: 2 cm.

Fig. 2. (next page) Histological sections of somatic embryos derived from leaflets of *Arachis glabrata*. Bar: 0.1 mm. a-d: Ontogenic development. a-c: Globular somatic embryos. d: Transversal section showing a callus with somatic embryos. e: Bipolar embryo type 1 exhibiting shoot and root apical meristems. f: Longitudinal section of differentiated shoot apical meristem, with leaf primordia. g-h: Embryo like structure without shoot apical meristem (type 3). i: Longitudinal section of non functional shoot apical meristem. j: Transversal section of an embryo similar to h showing the absence of shoot apical meristem.

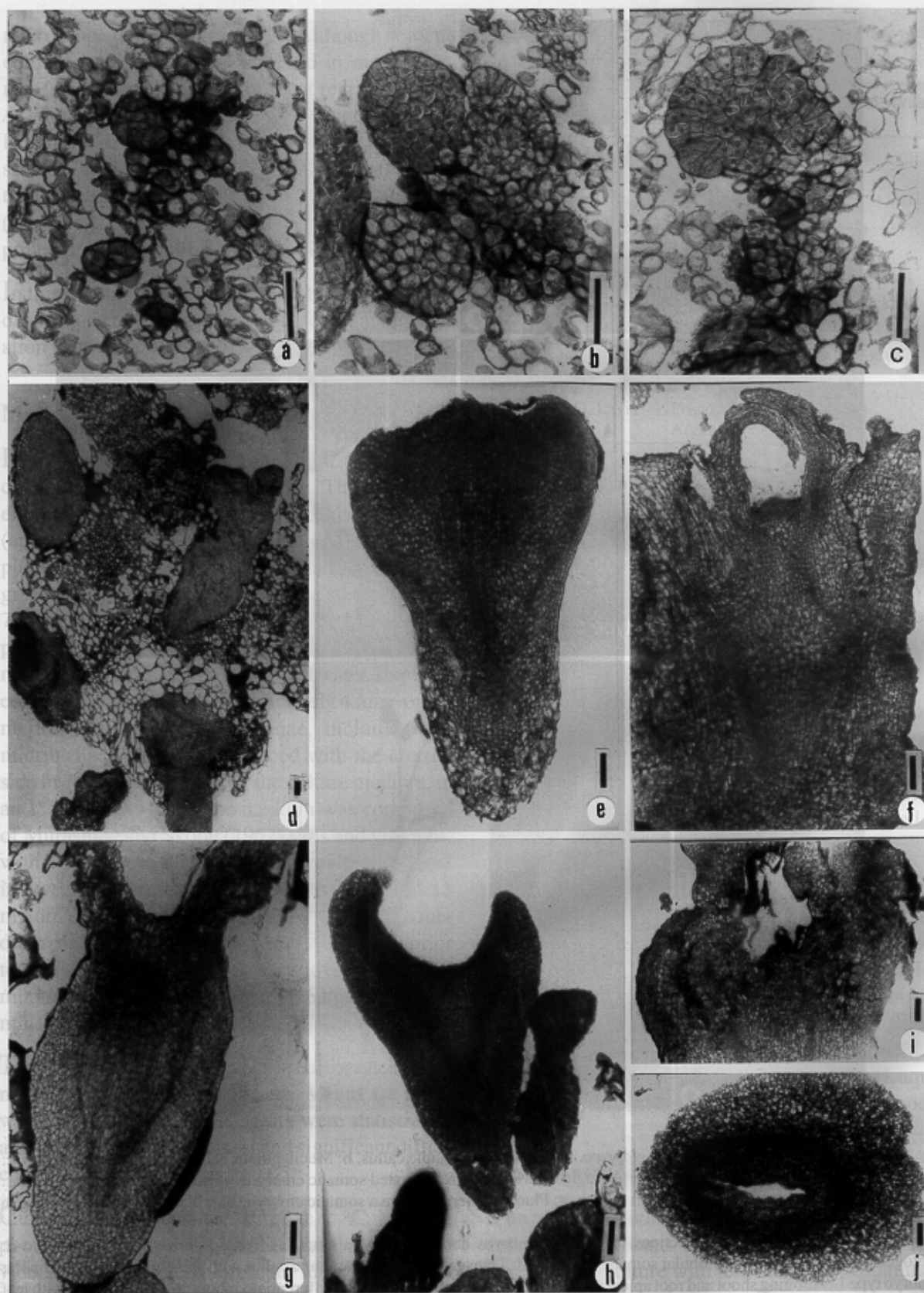


Table 2. Effect of induction media on the percentage of somatic embryogenesis from leaflets of two accessions of *Arachis glabrata*.

Induction media Growth regulators added to MS medium	Percentage of calli with somatic embryos ^a		Mean number of embryos per explant ^a	
	A6138	AF385	A6138	AF385
2 mg·dm ⁻³ PIC	4±4 ^{bcd}	-	2±0 ^b	-
5 mg·dm ⁻³ PIC	15±5 ^{bcd}	-	12±3 ^b	-
8 mg·dm ⁻³ PIC	16±4 ^{cde}	-	12±5 ^b	-
10 mg·dm ⁻³ PIC	20±6 ^e	-	17±5 ^{bc}	-
15 mg·dm ⁻³ PIC	19±1 ^{de}	-	34±24 ^c	-
2 mg·dm ⁻³ PIC + 0.01 mg·dm ⁻³ BAP	5±2 ^{bcd}	1±0 ^b	2±0 ^b	1±0 ^b
5 mg·dm ⁻³ PIC + 0.01 mg·dm ⁻³ BAP	9±5 ^{bcd}	-	16±6 ^{bc}	-
8 mg·dm ⁻³ PIC + 0.01 mg·dm ⁻³ BAP	15±6 ^{bcd}	-	7±1 ^b	-
10 mg·dm ⁻³ PIC + 0.01 mg·dm ⁻³ BAP	19±8 ^{de}	-	8±2 ^b	-
15 mg·dm ⁻³ PIC + 0.01 mg·dm ⁻³ BAP	9±1 ^{bcd}	-	11±6 ^b	-
2 mg·dm ⁻³ PIC + 0.1 mg·dm ⁻³ BAP	8±5 ^{bcd}	1±0 ^b	4±1 ^b	7±1 ^b
5 mg·dm ⁻³ PIC + 0.1 mg·dm ⁻³ BAP	3±2 ^{bc}	1±1 ^b	7±2 ^b	12±0 ^b
8 mg·dm ⁻³ PIC + 0.1 mg·dm ⁻³ BAP	9±3 ^{bcd}	-	17±9 ^{bc}	-
10 mg·dm ⁻³ PIC + 0.1 mg·dm ⁻³ BAP	7±4 ^{bcd}	1±0 ^b	7±3 ^b	18±4 ^b
15 mg·dm ⁻³ PIC + 0.1 mg·dm ⁻³ BAP	5±5 ^{bcd}	-	8±0 ^b	-
0.01 mg·dm ⁻³ BAP	- _b	-	-	-
0.1 mg·dm ⁻³ BAP	- _b	-	-	-

^a Mean±SE; ^{b,c,d,e} different letters indicate significant difference according to Duncan's test ($P \leq 0.05$)

explants) in all the accessions and in all the media tested.

After 50 days of culture in the same media, three types of calli could be distinguished: type A: friable nonembryogenic calli which were generally pale yellow in colour (Fig. 1a); type B: mucilaginous nonembryogenic calli with a smooth surface (Fig. 1b), most of which were brown coloured and had transparent to white round growing areas similar to those structures reported in *A. hypogaea* by Baker and Wetsztein (1992) and Gill and Ozias-Akins (1999); and type C: mucilaginous embryogenic calli with embryos (Fig. 1c,d). Similarly to *A. hypogaea* type C calli, these were smaller than non-embryogenic ones (Baker and Wetsztein 1992). After 90 days of culture, the number of embryogenic calli and embryos per explant increased (in average 46 %, data not shown).

The effect of 17 combinations of PIC and BAP on somatic embryos of 2 accessions of *Arachis glabrata* after 90 days of culture are presented in Table 2. Somatic embryos were induced from only these two accessions, in different culture media. Even though there were no significant differences among most of the culture media employed in this work concerning the percentage of explants giving rise to somatic embryos, for accession A6138 the media consisting of 10 mg·dm⁻³ PIC or 15 mg·dm⁻³ PIC produced a high percentage of calli with somatic embryos and were significantly better than the rest regarding the number of embryos per explant.

On the other hand, the best media for accession A6138 were not the same as in accession AF385, where the percentage of response was considerably lower. Although explants giving embryos were obtained in culture media containing 2, 5 or 10 mg·dm⁻³ PIC with 0.1 mg·dm⁻³ BAP, and 2 mg·dm⁻³ PIC with 0.01 mg·dm⁻³ BAP, these media

were not significantly different from the rest. The highest mean number of embryos per explant was obtained in culture medium consisting of MS media supplemented with 10 mg·dm⁻³ PIC and 0.1 mg·dm⁻³ BAP (Table 2). It was observed in both accessions that secondary somatic embryogenesis occurred at least during two subcultures.

The protocol optimized in this work, based on maintaining cultured explants in an induction medium and then transferring embryogenic calli with somatic embryos onto fresh maturation medium is similar to the procedure developed for *A. hypogaea* (Chengalrayan *et al.* 1994, Baker and Wetsztein 1995, Ventakachalam *et al.* 1999). Our results are in concurrence with *A. paraguariensis* (Sellars *et al.* 1990) and *A. pinto*, in which somatic embryos were originated from cultures developed on media containing PIC and BAP (Rey *et al.* 2000). However, the highest percentage of embryo induction in *A. pinto* was achieved on higher concentrations of PIC and BAP. Moreover, these results were not in agreement with those reported for *A. hypogaea*, where the addition of a cytokinin to a culture media containing an auxin resulted in either lowered number of embryos per explant (Eapen and George 1993) or was simply ineffective (Chengalrayan *et al.* 1994).

Ontogenic studies showed that the regeneration process is *via* somatic embryogenesis. A wide morphological variety of somatic embryos was seen, comprising globular- (Fig. 2a-c), torpedo- (Fig. 2d-e) and cotyledonary-shaped embryos (Fig. 2f). Histological studies also showed many tubular or horn shaped embryos without shoot meristem.

Regardless of the hormone concentration, most of the embryos obtained could be classified according to Wetsztein and Baker (1993) as type 1 embryos (single embryos, torpedo-shaped, well defined axis) (Fig. 1d, Fig. 2e), type 2 (single embryos, torpedo-shaped, well defined axis with distinct hypocotyl and epicotyl, epicotyl with multiple foliar structures and clearly differentiated apex) (Fig. 1d, Fig. 2f), type 3 (single embryos, tubular or horn shaped, with no visible apex) (Fig. 1d, Fig. 2g-j) and type 5 embryos (multiple fused embryos, with individual embryo axis defined, fasciated with broadened and distorted hypocotyl bases) (Fig. 1c).

Type 1 and 2 embryos regenerated plants when transferred to maturation media (Fig. 1e-h), whereas type 3 and 5 embryos did not convert into plants. The type of embryos obtained was not influenced by the composition of the culture medium.

The percentage of explants giving rise to embryos was only 20 % compared to peanut, where the percentage of somatic embryogenesis was higher than 80 % (Baker *et al.* 1995). However, the embryogenic response was similar to that reported for *A. pinto* (Rey *et al.* 2000).

All efforts to obtain somatic embryos in accessions L5, L6 and A6146 have so far remained unsuccessful. In some legumes, the inability to induce somatic embryogenesis in many important cultivars constitutes a limitation to the application of embryogenic culture for commercial propagation (Lakshmanan and Taji 2000). In peanut, although the frequency of somatic embryo induction as well as the mean number of embryos per culture are highly dependent on genotype, all the genotypes tested induced somatic embryos in various degrees (George and Eapen 1993, Baker *et al.* 1995, Chengalrayan *et al.* 1998).

Explants cultured on medium containing BAP alone failed to induce somatic embryos in all the accessions tested, and most explants did not respond or induced friable nonembryogenic calli. Nevertheless, some buds (11 %) were differentiated in leaflet-explants of accession AF385 cultured on MS + 0.01 mg·dm⁻³ BAP. However, these buds did not show further elongation.

In order to achieve maturation and conversion, somatic embryos were transferred to culture media with 100, 50 or 25 % strength of MS medium with or without the addition of AC. Some of these media were employed for *A. hypogaea* by Chengalrayan *et al.* (1994). In accession A6138, 6 % of the embryos converted into plants (54 plants) upon transferring to medium containing 2 g·dm⁻³ AC (for 30 days) and followed by transfer onto MS medium devoid of AC for 20 days (Fig. 1e-h). These plantlets were transplanted to pots containing soil mixture and maintained in a controlled environment. The establishment rate was 76 % (41 plants) in that accession. On the other hand, in accession AF385, the conversion rate was lower than 1 %.

Unfortunately, many embryos failed to convert into plants. Histological studies have shown that this was due to a poorly-developed apical meristem. Similar results were also observed in peanut (Wetsztein and Baker 1993), resulting in a lack of further germination/development (Griga 1999). Chengalrayan *et al.* (1998) indicated that failure of somatic embryos to undergo conversion into plantlets could be genotype-dependent, even though it has often been attributed to malformed plumules or to immature embryos.

In all the accessions tested neither somatic embryogenesis nor organogenesis were observed when culture media contained 2,4-D instead of PIC (data not shown). These results partially agree with those reported for *A. pintoi*, where the addition of 2,4-D to the culture media produced only shoots but not somatic embryos. In contrast, somatic embryos were obtained in peanut when 2,4-D was added to the medium (Baker and Wetsztein 1995, Chengalrayan *et al.* 1998, Griga 1999, Little *et al.* 2000, Lakshmanan and Taji 2000).

In conclusion, our results indicate that it is possible to regenerate plants of certain accessions of *Arachis glabrata* through leaf culture.

References

- Bajaj Y.P.S., Labana K.S., Dhanju M.S. 1980.** Induction of pollen-embryos and pollen-callus in anther cultures of *Arachis hypogaea* and *A. glabrata*. *Protoplasma* 103: 397-399.
- Baker C.M., Wetzstein H.Y. 1992.** Somatic embryogenesis and plant regeneration from leaflets of peanut, *Arachis hypogaea*. *Plant Cell Rep.* 11: 71-75.
- Baker C.M., Durham R.E., Burns J.A., Parrott W.A., Wetzstein H.Y. 1995.** High frequency somatic embryogenesis in peanut (*Arachis hypogaea* L.) using mature, dry seed. *Plant Cell Rep.* 15: 38-42.
- Baker C.M., Wetzstein H.Y. 1995.** Repetitive somatic embryogenesis in peanut cotyledon cultures by continual exposure to 2,4-D. *Plant Cell Tissue Organ Cult.* 40: 149-254.
- Chengalrayan K., Sathaye S.S., Hazra S. 1994.** Somatic embryogenesis from mature embryo-derived leaflets of peanut (*Arachis hypogaea* L.). *Plant Cell Rep.* 13: 578-581.
- Chengalrayan K., Mhaske V.B., Hazra S. 1998.** Genotypic control of peanut somatic embryogenesis. *Plant Cell Rep.* 17: 522-525.
- Eapen S., George L. 1993.** Somatic embryogenesis in peanut: Influence of growth regulators and sugars. *Plant Cell Tissue Organ Cult.* 35: 151-156.
- George L., Eapen S. 1993.** Influence of genotype and explant source on somatic embryogenesis in peanut. *Oléagineux* 48: 361-364.
- Gill R., Ozias-Akins P. 1999.** Thidiazuron-induced highly morphogenic callus and high frequency regeneration of fertile peanut (*Arachis hypogaea* L.) plants. *In Vitro Cell Dev. Biol-Plant.* 35: 445-450.
- Gonzalez A.M., Cristóbal C.L. 1997.** Anatomía y ontogenia de semillas de *Helicteres lhotzkyana* (*Sterculiaceae*). *Bonplandia* 9: 287-294.
- Griga M. 1999.** Somatic embryogenesis in grain legumes. In: *Advances in Regulation of Plant Growth and Development*, ed. by M. Strnad, P. Peč, E. Beck, Peres Publ., Prague: 223-250.
- Johansen D.A. 1940.** *Plant microtechnique*. McGraw Hill Book Co, New York. 551 p
- Krapovickas A., Gregory W. 1994.** Taxonomía del género *Arachis* (*Leguminosae*). *Bonplandia* 8: 1-186.
- Lakshmanan P., Taji A. 2000.** Somatic embryogenesis in Leguminous plants. *Plant Biol.* 2: 136-148.
- Little E.L., Magbanua Z.V., Parrot W.A. 2000.** A protocol for repetitive somatic embryogenesis from mature peanut epicotyls. *Plant Cell Rep.* 19: 351-357.
- Luque R., Sousa H.C., Kraus J.E. 1996.** Métodos de coloracao de Roeser (1972) e Kropp (1972) visando a substituição do azul do astra por azul de alcaio 8GS ou 8GX. *Acta Bot. Bras.* 10: 199-212.
- Mallikarjuna N. 2002.** Gene introgression from *Arachis glabrata* into *A. hypogaea*, *A. duranensis* and *A. diogeni*. *Euphytica* 124: 99-105.
- McKently A.H., Moore G.A., Gardner F.P. 1991.** Regeneration of peanut and perennial peanut from cultured leaf tissue. *Crop Sci.* 31: 833-837.
- Murashige T., Skoog F. 1962.** A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15: 473-497.
- Rey H.Y., Scocchi A.M., Gonzalez A.M., Mroginski L.A. 2000.** Plant regeneration in *Arachis pintoi* (*Leguminosae*) through leaf culture. *Plant Cell Rep.* 19: 856-862.
- Sellers R.M., Southward G.M., Phillips G.C. 1990.** Adventitious somatic embryogenesis from cultured immature zygotic embryos of peanut and soybean. *Crop Sci.* 30: 408-414.

Smartt J., Stalker H.T. 1982. Speciation and cytogenetics in *Arachis*. In: Peanut Science and Technology, ed. by H.E. Patee, C.T. Young, Yoakum, Texas: 21-49.

Venkatachalam P., Geetha N., Khandelwal A., Shaila M.S., Sita G.L. 1999. Induction of direct somatic

embryogenesis and plant regeneration from mature cotyledon explants of *Arachis hypogaea* L. Current Science 77: 269-273.

Wetzstein H.Y., Baker C.M. 1993. The relationship between somatic embryo morphology and conversion in peanut (*Arachis hypogaea* L.). Plant Sci. 92: 81-89.

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