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In vitro plant regeneration of *Arachis correntina* (Leguminosae) through somatic embryogenesis and organogenesis

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Abstract

In vitro protocols for plant regeneration of *Arachis correntina* through both somatic embryogenesis and organogenesis were developed using immature leaves as explants. Morphologically normal somatic embryos were obtained on culture media composed of 20.70 or 41.41 μM picloram (PIC) with the addition of 0.044 μM 6-benzylaminopurine (BA), resulting in a 33 and 24% of conversion into plants, respectively. The source of explants and the developmental stage of the leaves had a marked effect on somatic embryogenesis. The second folded immature leaves from *in vitro* growing plants were the most responsive producing up to 30% embryogenesis in MS + 41.41 μM PIC. Embryos converted into plants after transfer to MS medium devoid of growth regulators and these plants were successfully acclimatised. Adventitious shoots were obtained on culture media supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthaleneacetic acid (NAA) with or without 0.044 μM BA, achieving plant regeneration in the induction media. The highest percentage of bud formation was obtained on culture medium composed of MS + 10.74 μM NAA + 0.044 μM BA (12.5%). Roots were formed on all culture media tested. Regenerated plants were transferred to pots and grew well under greenhouse conditions.

Abbreviations: BA – 6-benzylaminopurine; 2,4-D – 2,4-dichlorophenoxyacetic acid; NAA – naphthaleneacetic acid; PIC – picloram-4-amino-3,5,6-trichloropicolinic acid

The groundnut, *Arachis hypogaea*, is the most widely spread of the 69 species of the genus *Arachis*, all of which are native to South America (Krapovickas and Gregory, 1994). However, several other species, such as *A. pintoi* Krapov. and W.C. Gregory, *A. glabrata* Bentham and *A. repens* Handro, are also useful as forage and ornamental or ground cover species (Dos Santos et al., 2003). The growing concern over the collection, rescue, conservation, multiplication and characterisation of wild species of *Arachis* germplasm lays in the fact that they contain useful genes for the genetic improvement of peanut (Gagliardi et al., 2000).

Cultivated and many wild peanuts are stored as seeds and, even with optimum storage practices, seed germinability and germplasm losses are inevitable (Dunbar et al., 1993). Consequently, *in vitro* germplasm conservation constitutes a viable option for their preservation providing that efficient *in vitro* protocols for plant regeneration are developed (Gagliardi et al., 2000; Vijaya Laxmi and Giri, 2003). Additionally, these protocols are required for the utilisation of genetic engineering in the improvement of *Arachis* species (Ozias-Akins and Gill, 2001).

Diploid wild peanuts of the genus *Arachis* section *Arachis* are close relatives of the cultivated peanut and present potential usefulness as tropical forages. Among these, *Arachis correntina*

($2n = 2x = 20$ chromosomes) occupies a prominent position for its resistance to rust, peanut mottle virus, tomato spotted wilt virus, aphids, mites, thrips and jassids (Kameswara Rao et al., 2003). *A. correntina* (Burkart) Krapov. and W.C. Gregory, is a perennial species that grows in the North West of the province of Corrientes (Argentina) as a component of natural pastures (Krapovickas and Gregory, 1994). *In vitro* plant regeneration from leaf explants *via* organogenesis has been recently achieved in *A. correntina* using thidiazuron (Mroginski et al., 2004) whereas somatic embryogenesis has never been reported in this species previously.

In this paper, we report two simple *in vitro* regeneration systems for *Arachis correntina* through either somatic embryogenesis or organogenesis from immature leaf explants.

Seeds of *Arachis correntina*, a predominantly autogamous species, were collected from different plants growing in the same area in Pirayui, Corrientes, Argentina by Luis Mroginski (herbarium specimen Krapovickas and others 11905 deposited in CTES) and cultivated in the garden of the Instituto de Botánica del Nordeste (IBONE). Explants were obtained from one-year-old plants growing: (a) in the garden of the IBONE; (b) in an acclimatised room with 27 ± 2 °C, a photoperiod of 14 h and 336 μmol m^{-2} s^{-1}; and (c) *in vitro* with 27 ± 2 °C, a photoperiod of 14 h and 116 μmol m^{-2} s^{-1}, obtained by *in vitro* culture of shoot tips from the plants growing in the garden according to Rey and Mroginski (2003). Explant tissues, randomly collected within the three groups of plants mentioned before, consisted of: (I) shoot tips (1–2 mm long), (II and III) first and second immature folded leaves beyond the shoot tip respectively, (IV) portions of the third emerging folded leaflets, and (V) portions of unfolded leaflets of the fourth leaf.

Murashige and Skoog (1962) (MS) basal medium with 3% sucrose was used. For culture establishment, PIC (20.70, 41.41, 62.11 and 82.82 μM), 2,4-D (9.05, 22.62, 45.25 and 67.87 μM) and NAA (10.74, 26.85, 53.71 and 80.56 μM) were tested alone or in combination with 0.044 μM BA. To achieve somatic embryo maturation and conversion, MS devoid of plant growth regulators was utilised. Media pH was adjusted to 5.8 with KOH or HCl before the addition of 0.7% agar (Sigma A-1296). Media were sterilised by autoclaving for 20 min (0.101 MPa). Explants were placed with the abaxial side down on 3 ml of culture medium in 11 ml glass tubes. Tubes were sealed with Resinite AF 50® (Casco SACIF Buenos Aires, Argentina) and incubated in a growth room at 27 ± 2 °C with 14 h photoperiod (116 μmol s^{-1} m^{-2} provided by cool-white fluorescent tubes – Philips TLD 84).

Rooted shoots and germinated somatic embryos were washed under running tap water and transferred to pots containing a mixture of soil, sand and perlite (1:1:1). Plantlets were acclimatised in a growth chamber at 27 ± 2 °C with 14 h photoperiod (336 μmol s^{-1} m^{-2} provided by cool-white fluorescent tubes – Philips TLD 84).

Ten explants were cultured per treatment, and experiments were repeated three times. Results are presented as the means of the replications with the standard error (mean ± SEM). Analysis of variance (ANOVA) was performed followed by Duncan's multiple comparison test ($p < 0.05$).

In order to study the influence of PIC on somatic embryogenesis, 2–4 mm long immature leaves (explant types II and III) collected from *in vitro* plants were cultured using the PIC concentrations mentioned above, with and without the addition of 0.044 μM BA. Within 7 days of culture, nodular calli were observed in most cultures, which gave rise to somatic embryos after 20 days. Direct somatic embryogenesis was also observed (Figure 1a).

The highest percentages of somatic embryogenesis were obtained in the media composed of MS + 41.41 or 62.11 μM PIC (45 and 56% respectively), the last one being significantly better than the rest. These media also produced more embryos per explant, especially the first one (43 embryos per explant). Globular, heart, torpedo and cotyledonary shaped normal embryos were observed, with numerous variations of abnormal embryos (two or multiple fused, horn-shaped embryos) similar to those described by Wetzstein and Baker (1993). Embryo quality was better in media composed of MS + 20.70 or 41.41 μM PIC + 0.044 μM BA, with up to 35% of normal embryos in the latter, conducing to a higher rate of *ex vitro*-established plants (33 and 24%, respectively).

To evaluate the types of explant, the best media in the previous experiment (MS + 41.41 μM PIC and MS + 41.41 μM PIC + 0.044 μM BA) were used. The stage of development, the size of the

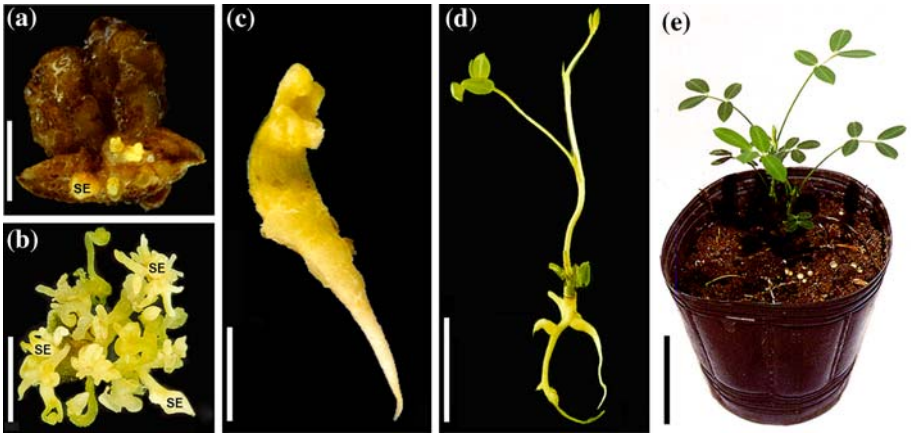

Figure 1. Somatic embryogenesis from immature leaves of *A. correntina*. (a) Somatic embryos emerging from the basal part of an immature leaf after 30 days of culture, bar: 5 mm. (b) Somatic embryos before being transferred to MS medium devoid of PIC, after 60 days of culture, bar: 5 mm. (c) Somatic embryo germinating on MS medium 10 days after transfer, bar: 10 mm. (d) Well-developed plantlet 30 days after being transferred to MS medium, bar: 20 mm. (e) Acclimatised plant growing under greenhouse conditions, after 60 days of transfer to soil, bar: 40 mm.

leaves and the source of explants considerably affected somatic embryo induction of *A. correntina*. Overall, the multiple comparisons test showed that the *in vitro* origin is superior in both percentage of somatic embryogenesis and number of embryos per explant. Regarding the type of explants, type III (second immature folded leaf) was superior (Table 1).

The percentage of somatic embryogenesis obtained in the culture medium experiments was higher than that of the explant type experiments (56% versus 30%). Such results could be ascribed to the fact that the first experiment was performed with plants that were established *in vitro* for 40 days while for the second experiment, the plants had been growing *in vitro* for 120 days.

Somatic embryos were isolated and transferred to MS without growth regulators (Figure 1b), where embryo maturation and conversion into plants was achieved 90 days after immature leaves were cultured. These plants were transferred to pots (Figure 1c–e), with an *ex vitro* survival rate of 80%, where they grew normally and produced seeds.

Table 1. Effect of explant type on the percentage of somatic embryogenesis (SE) and mean number of embryos per explant (N°) of *A. correntina*, after 40 days of culture

Type of explants	MS + 41.41 μM PIC						MS + 41.41 μM PIC + 0.044 μM BA					
	Explant origin						Explant origin					
	Garden		Acclimatised room		*In vitro*		Garden		Acclimatised room		*In vitro*	
	% SE	N°	% SE	N°	% SE	N°	% SE	N°	% SE	N°	% SE	N°
I	0	0	10	2	6.7	4	6.7	3.7	3.3	3.3	16.7	7.3
II	13.3	4.3	10	8	10	4.7	3.3	4	3.3	4	16.7	7.7
III	6.7	4.7	13.3	8.7	30*	19*	10	7	10	2.7	13.3	14.7
IV	0	0	6.7	2.7	10	6.3	10	5	0	0	13.3	5
V	0	0	0	0	0	0	0	0	0	0	3.3	2.3

(I) Shoot tips (1–2 mm long), (II and III) first and second immature folded leaves beyond the shoot tip respectively, (IV) portions of the third emerging folded leaflets, and (V) portions of unfolded leaflets of the fourth leaf.
*Represents significant difference according to Duncan's multiple comparison test ($p \leq 0.05$).

Our results coincide with those that reported the effectiveness of PIC on the induction of somatic embryos of *A. hypogaea* (Sellars et al., 1990; Eapen and George, 1993; Little et al., 2000), *A. pintoi* (Rey et al., 2000), *A. glabrata* (Vidoz et al., 2004) and *A. paraguariensis* (Sellars et al., 1990).

In *A. hypogaea*, the addition of cytokinins decreased the number of somatic embryos per explant (Eapen and George, 1993) or was ineffective in enhancing somatic embryogenesis (Chengalrayan et al., 1994). In our studies, although the highest percentage of somatic embryogenesis was obtained in a culture medium devoid of cytokinins, a better morphology and greater number of established plants were obtained when embryos developed on media containing 0.044 μM BA. This is in agreement with a previous report of the positive influence of BA addition on somatic embryogenesis of *A. pintoi* (Rey et al., 2000). The positive influence of BA on *A. correntina* somatic embryo conversion is probably due to its effect on embryo maturation, since it was successfully used in *A. hypogaea* for this purpose (Venkatachalam et al., 1999).

After 50 days of culturing folded leaves (explant types II and III) in media containing 2,4-D or NAA, several responses were obtained: expanded leaves, friable calli, roots with or without callus, and shoot buds. Only the lowest concentration of 2,4-D (9.05 μM) originated buds, regardless the addition of 0.044 μM BA (Figure 2). However, bud formation was achieved employing several concentrations of NAA with or without the addition of 0.044 μM BA. The highest percentage (12.5%) was obtained when the culture medium was composed of MS + 10.74 μM NAA + 0.044 μM BA. Shoot elongation and shoot rooting occurred in the induction media, and plant regeneration was achieved in only one step in 90% of the cases.

In our studies, 2,4-D induced direct and indirect adventitious shoot and root formation. Similar results were reported for *A. pintoi*, (Rey et al., 2000), but not for *A. hypogaea*, in which somatic embryogenesis occurred in media containing 2,4-D (Lakshmanan and Taji, 2000; Little et al., 2000).

Bud and shoot formation was observed in almost all culture media containing NAA, regardless BA addition to the media. Similarly, shoot organogenesis was obtained in a number of *Arachis* species using different combinations of NAA and BA (Bajaj et al., 1981; McKently et al., 1991; Cheng et al., 1992; Mansur et al., 1993; Rey et al., 2000). Additionally, in our experiments roots were produced with all NAA concentrations tested. Our results differ from a previous report in *A. correntina* (Mroginski et al., 2004) in which explants produced only callus when PIC, 2,4-D or NAA were added to the induction medium. This difference in responses could be attributed to the type of explant used: fully expanded leaves in the former while immature leaves were used in the present study.

Our findings provide a fast, simple procedure for regenerating plants via somatic embryogenesis or organogenesis, with little or no callus formation. The minimization of the callus stage is a desirable

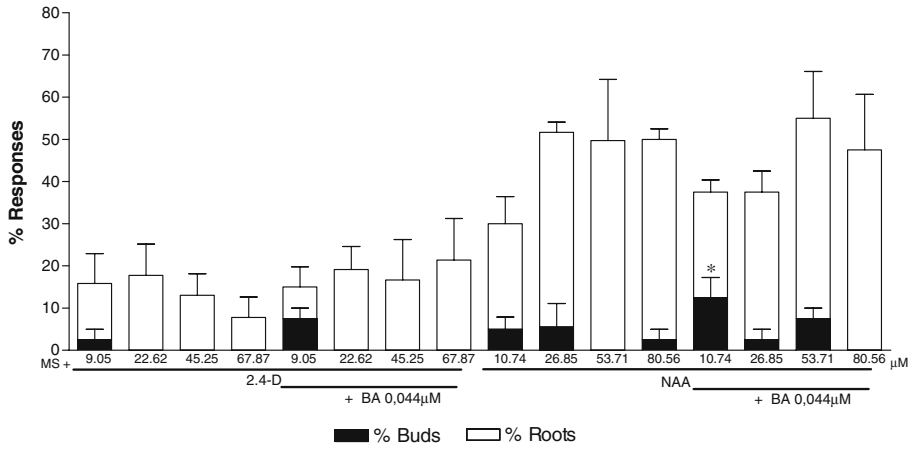

Figure 2. Effect of 2,4-D, NAA and BA supplemented to MS on adventitious bud and root formation from immature leaves of *A. correntina*, after 50 days of culture. *Represents significant difference according to Duncan's multiple comparison test ($p \leq 0.05$).

feature for *in vitro* protocols because callus cells are more genetically unstable than differentiated ones (Pittman et al., 1983). Moreover, an abbreviated tissue culture phase is preferred in order to reduce somaclonal variation and detrimental effects on the regeneration of transformed plants (Ozias-Akins and Gill, 2001). Consequently, *in vitro* culture of immature leaves can be used for an effective system of plant regeneration through either somatic embryogenesis or organogenesis. To induce somatic embryogenesis, two steps and the use of PIC were necessary whereas organogenesis was achieved in only one step employing NAA or 2,4-D. Plantlets obtained were normal and could be acclimatised successfully.

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