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RESEARCH NOTE

The role of BAP in somatic embryogenesis induction from seed explants of *Arachis* species from Sections *Erectoides* and *Procumbentes*

G. Pacheco · R. F. Gagliardi · L. A. Carneiro · C. H. Callado · J. F. M. Valls · E. Mansur

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Abstract Somatic embryogenesis was induced from seed explants of *Arachis archeri*, *A. porphyrocalix* (Section *Erectoides*) and *A. appressipila* (Section *Procumbentes*) in response to 6-benzylaminopurine (BAP). Embryo axes first developed into single shoots in response to 4.4 μM BAP. Friable embryogenic calluses were produced from the hypocotyl region of these explants in response to different BAP concentrations. Embryonic leaflets also gave rise to friable calluses, but somatic embryos were only observed in explants of *A. archeri* and *A. appressipila*. Histological analyses revealed the presence of heart-shaped, torpedo and cotyledonary stages embryos, both as isolated and fused structures. A low frequency of embryo-to-plant conversion was achieved by inducing shoot development on medium solidified with 0.5% phytagel and supplemented with 1.5% or 3% sucrose. Rooting was induced on MS supplemented with indole-3-acetic acid (IAA).

Keywords *Arachis* · Groundnut · Histology · Micropropagation · Somatic embryogenesis

Abbreviations
BAP 6-Benzylaminopurine
IAA Indole-3-acetic acid

Wild relatives of the cultivated groundnut (*A. hypogaea* L.) are restricted to South America and show wide genetic variability, constituting valuable gene sources to breeding programs. *A. archeri, A. porphyrocalix* (Section *Erectoides*) and *A. appressipila* (Section *Procumbentes*, previously included in Section *Erectoides*) are closely related species, resistant to an insect complex (Stalker and Campbell 1983).

Extensive research on somatic embryogenesis of the cultivated groundnut (*A. hypogaea*) has been carried out by several authors (Baker and Wetzstein 1994; Chengalrayan et al. 1997; Victor et al. 1999). However, in wild species of *Arachis*, in vitro regeneration has been mainly reported through organogenesis or development of preexisting meristems (Gagliardi et al. 2000; Mroginski et al. 2004). Plant regeneration via somatic embryogenesis is comparatively less reported and has only been described for *A. paraguariensis* (Sellars et al.

G. Pacheco · R. F. Gagliardi · L. A. Carneiro · E. Mansur (✉)
Laboratório de Micropropagação e Transformação de Plantas, UERJ, Rio de Janeiro, Brazil
e-mail: mansur@uerj.br

C. H. Callado
Departamento de Botânica, UERJ, Rio de Janeiro, Brazil

J. F. M. Valls
Embrapa Recursos Genéticos e Biotecnologia, Brasilia, DF, Brazil

1990), *A. pintoi* (Rey et al. 2000; Rey and Mroginski 2006) and *A. glabrata* (Vidoz et al. 2004).

Somatic embryogenesis is generally promoted by auxins, either alone or in combination with cytokinins. In contrast, the use of cytokinins as the sole growth regulator is rarely adopted (Sagare et al. 2000; Kaparakis and Alderson 2002). Taking this into consideration, more studies are required in order to extend knowledge about the physiological mechanisms of cytokinin induced somatic embryogenesis. The present work was directed towards the induction of somatic embryogenesis in three closely related species of *Arachis*, exploring the embryogenic potential of seed explants in response to BAP.

Seeds of *A. archeri* V 13494, *A. porphyrocalix* V 7303 and *A. appressipila* GKP 10002 were provided by the Embrapa Genetic Resources and Biotechnology seed bank. After surface sterilization with 0.2% (w/v) $HgCl_2$ for 45 min under agitation, seeds were washed three times with sterile de-ionized water and peeled. Embryonic axes and leaflets were aseptically excised from disinfected seeds and cultured on basal medium supplemented with BAP at 4.4, 8.8 and 17.6 μM (embryo axes) or 4.4, 13.2, 22 and 30.8 μM (embryonic leaflets). The basal medium consisted of MS salts and vitamins (Murashige and Skoog 1962), plus 3% sucrose (w/v) and solidified with 0.7% agar (w/v). The pH was adjusted to 5.8 before autoclaving for 15 min at 121°C. Cultures were maintained in a growth chamber at 28°C ± 2°C with 16 h light/8 h darkness and a total irradiance of 46 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by cool-white fluorescent lamps.

Embryogenic calluses formed from embryonic axes and leaflets were transferred to fresh basal medium at 4 week-intervals. Isolated and fused somatic embryos at different developmental stages were detached from calluses using a stereomicroscope and then transferred to media containing 1.5 or 3% sucrose and solidified either with 0.7% agar or phytagel at different concentrations (0.2%, 0.5% and 1%). After 4 weeks, shoots were transferred to MS supplemented with different IAA concentrations (0, 0.57, 2.85 and 5.7 μM) for rooting.

Calluses were fixed in 2.5% glutaraldehyde and 4% paraformaldehyde in 0.05 M sodium phosphate buffer, pH 7.2 and stored at 4°C. Samples were dehydrated in a graded ethanol series, followed by paraffin embedding. Serial 12 μm thick sections were made with a rotary microtome and double-stained in astra blue-basic fuchsin. Photographs were obtained with the image capture system Image-Pro Plus for Windows using a video camera Optronics attached to an Olympus BX 40 microscope.

Regeneration rates, mean number of embryos per explant and conversion frequencies were recorded after 8 weeks of culture. Three to ten replicates were used in each treatment and each experiment was repeated three times. Data were subjected to analysis of variance (ANOVA) and comparisons of means were carried out with Tukey-Kramer comparisons test at 0.05% significance level using the software *GrapPad Instat*.

Embryo axes from the three species developed distinct regeneration patterns in response to BAP. First, single shoot formation from preexisting meristem was observed after 30 days of culture, with the highest regeneration frequencies occurring in response to 4.4 μM BAP (Table 1). This provided an efficient approach for recovery and in vitro conservation of germplasm of species from these sections, corroborating previous results obtained with Section *Extranervosae* (Gagliardi et al. 2000). Germplasm recovery is especially important for *A. archeri*, which was recently considered as threatened of extinction by habitat loss (Jarvis et al. 2003).

In addition to originating plants from preexisting meristems, embryo axes gave rise to friable embryogenic calluses (Fig. 1A and B) from the hypocotyl region, after approximately 15 days of culture. The highest frequency of callus formation was observed in explants of *A. porphyrocalix*. Embryogenic callus of *A. archeri* displayed significantly higher numbers of embryos/explant as compared to the other two species (Table 1).

Embryonic leaflets from the three species also developed friable calluses in response to all BAP concentrations, at different frequencies. Calluses of *A. archeri* and *A. porphyrocalix* were green, friable at the surface and more compact inside. In contrast, explants of *A. appressipila* formed pale yellow and highly friable calluses (Fig. 1C). In *A. archeri* and *A. appressipila*, these tissues

Table 1 In vitro regeneration from embryonic axes from wild *Arachis* species after 60 days of culture on MS medium supplemented with different BAP concentrations

Species	Culture medium	Development from seedling shoot meristem (%)	Embryogenic callus formation (%)	Embryos/explant
A. archeri	MS + 4.4 μM BAP	50	50	194.0 ± 4.8[a]
	MS + 8.8 μM BAP	29	43	214.0 ± 24.8[a]
	MS + 17.6 μM BAP	33	67	49.4 ± 4.0[b]
A. porphyrocalix	MS + 4.4 μM BAP	75	50	13.4 ± 0.9[a]
	MS + 8.8 μM BAP	40	80	26.4 ± 4.8[b]
	MS + 17.6 μM BAP	25	100	41.8 ± 0.7[c]
A. appressipila	MS + 4.4 μM BAP	75	50	1.8 ± 0.3[a]
	MS + 8.8 μM BAP	73	71	19.2 ± 2.7[b]
	MS + 17.6 μM BAP	40	60	2.4 ± 0.4[a]

Values are means ± standard error. Means followed by different letters indicate significant differences among treatments for each species by Tukey–Kramer multiple comparisons test ($P \leq 0.05$ level)

underwent differentiation and produced embryos (Fig. 1D), while calluses from *A. porphyrocalix* did not show embryogenic competence. Similarly to the number of embryos observed from callus from embryo axes explants, calluses derived from leaflets of *A. archeri* displayed the highest embryogenic potential (Table 2), showing a significantly higher embryo production as compared to *A. appressipila* and to previous reports on somatic embryogenesis in other wild species of *Arachis* (Sellars et al. 1990; Rey et al. 2000; Vidoz et al. 2004).

The histological analysis revealed the presence of torpedo and cotyledonary stages embryos in the calluses. The absence of vascular connections into the original explant and the presence of meristematic cells on both shoot and root poles confirmed somatic embryogenesis and excluded the possibility of shoot production through an organogenic pathway (Fig. 1E, F and G). Abnormalities such as fasciation of cotyledons and occurrence as fused structures were also detected (Fig. 1H). The occurrence of abnormal and fused somatic embryos has been previously reported for *Arachis* (Chengalrayan et al. 1997; Rey et al. 2000) and other species (Suhasini et al. 1996; Distabanjong and Geneve 1997).

Complete germination of somatic embryos with development of roots and shoots was observed at very low frequencies in the calluses, while in the induction media (Fig. 1D and I). Low conversion rates are an important limitation to the use of somatic embryogenesis for in vitro regeneration of many species, and different approaches have been adopted to overcome this problem (Suhasini et al. 1996; Lakshmanan and Taji 2000). In this work, embryos at distinct developmental stages showed an incomplete conversion process, developing only shoots in spite of the presence of meristematic cells in the root apical pole. Mature embryos of *A. archeri* and *A. porphyrocalix* showed the highest shoot pole developmental rates (69% and 60%) in media supplemented with 1.5% sucrose and solidified 0.5% or 1.0% phytagel, respectively (Table 3). The highest shoot pole development rates were observed in immature embryos of *A. appressipila* cultured on MS medium supplemented with 3% sucrose and solidified with 0.5% phytagel (Table 3).

The growth regulator used at the induction phase can play an important role in the embryo-to-plant conversion step (Sellars et al. 1990). According to some reports, somatic embryos induced on media supplemented with cytokinins alone show suppression of root development, despite the normal development of cotyledonary leaves (Sagare et al. 2000; Kaparakis and Alderson 2002). In view of the presence of both root and stem apical meristems in somatic embryos of *A. archeri*, it seems likely that the absence of root development described here may be related to the cytokinin-supplemented media used in the induction phase.

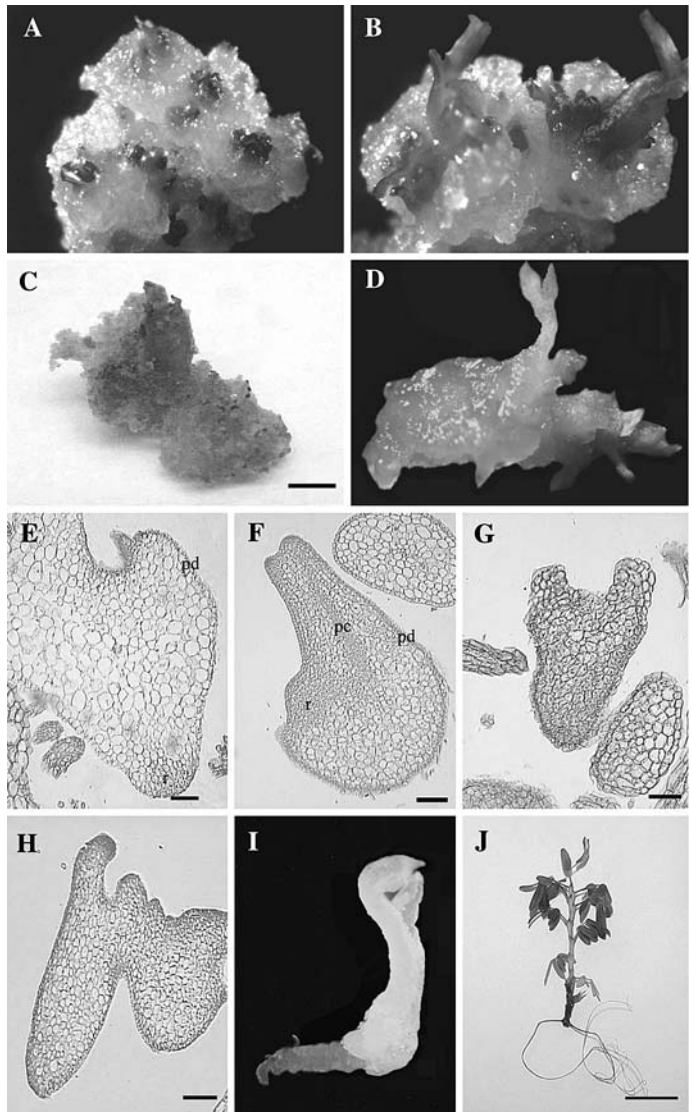

Fig. 1 Somatic embryogenesis in wild species of *Arachis*. (**A**) Friable embryogenic callus from embryo axes of *A. archeri* cultured on MS supplemented with 8.8 μM BAP (10×); (**B**) Embryos at early conversion stage (40×); (**C**) Friable embryogenic callus from *A. appressipila* cultured on MS supplemented with 22 μM BAP (bar = 1 cm); (**D**) Embryogenic callus of *A. archeri* obtained in response to 22 μM BAP showing bipolar somatic embryo after 120 days of culture (9×); (**E**) Transition from the heart-shaped to the torpedo-stage embryo of *A. archeri*, showing the establishment of the protoderm (pd) and the root apical meristem (r) (bar = 50 μm); (**F**) Torpedo-stage embryo of *A. archeri* showing protoderm (pd), procambium (pc), and lateral root apical meristem (r) (bar = 100 μm); (**G**) Early torpedo-stage embryo of *A. archeri* (bar = 50 μm); (**H**) Torpedo-stage embryo of *A. archeri* fused to a heart-shaped embryo (bar = 100 μm); (**I**) Normal, bipolar somatic embryo of *A. archeri* (9×); (**J**) Whole plant formed from a somatic embryo of *A. archeri* (bar = 1 cm)

Shoots of *A. porphyrocalix* and *A. appressipila* transferred to MS medium supplemented with different IAA concentrations did not produce roots irrespective of the IAA concentration. On the other hand, rooting frequencies of shoots of *A. archeri* were significantly influenced by the growth regulator concentration and by the developmental stage of the embryo when transferred to the conversion medium. The highest root formation frequency (50%) was achieved in response to 0.57 and 2.85 μM IAA, in shoots originated from mature embryos (Fig. 1J).

Table 2 In vitro regeneration from embryonic leaflets from wild *Arachis* species after 60 days of culture on MS medium supplemented with different BAP concentrations

Species	Accession	Culture medium	Callus formation (%)	Embryos/explant
A. archeri	V 13494	MS + 4.4 μM BAP	64	41.3 ± 7.1[a]
		MS + 13.2 μM BAP	34	29.0 ± 4.0[a]
		MS + 22 μM BAP	100	158.4 ± 38.0[b]
		MS + 30.8 μM BAP	17	0
A. porphyrocalix	V 7303	MS + 4.4 μM BAP	100	0
		MS + 13.2 μM BAP	100	0
		MS + 22 μM BAP	95	0
		MS + 30.8 μM BAP	59	0
A. appressipila	GKP 10002	MS + 4.4 μM BAP	100	3.8 ± 0.8 [a]
		MS + 13.2 μM BAP	90	4.0 ± 0.7 [a]
		MS + 22 μM BAP	95	7.0 ± 2.1 [a]
		MS + 30.8 μM BAP	85	5.0 ± 1.6 [a]

Values are means ± standard error. Means followed by different letters indicate significant differences among treatments for each species by Tukey–Kramer multiple comparisons test ($P \leq 0.05$ level)

Table 3 Shoot pole development from somatic embryos at different developmental stages, after 60 days of culture

Species	Conversion medium (MS)		Shoot pole development (%)	
	Sucrose concentration (%)	Solidifying agent	Immature embryos	Mature embryos
A. archeri	1.5	0.2% Phytagel	12	55
		0.5% Phytagel	0	69
		1.0% Phytagel	37	52
	3	0.7% Agar	0	11.1
		0.2% Phytagel	35	17
		0.5% Phytagel	40	40
		1.0% Phytagel	30	30
A. porphyrocalix	1.5	0.2% Phytagel	20	22
		0.5% Phytagel	16	35
		1.0% Phytagel	0	60
	3	0.7% Agar	100	20
		0.2% Phytagel	20	30
		0.5% Phytagel	6	43
		1.0% Phytagel	22	11
A. appressipila	1.5	0.2% Phytagel	0	0
		0.5% Phytagel	8	21
		1.0% Phytagel	12	14
	3	0.7% Agar	20	0
		0.2% Phytagel	7	0
		0.5% Phytagel	30	0
		1.0% Phytagel	13	0

To date, there have been no reports on the complete in vitro plant regeneration via somatic embryogenesis from species of Sections *Procumbentes* and *Erectoides*, except for *A. paraguariensis* (Sellars et al. 1990). In this study we have described in vitro regeneration of *A. archeri*, *A. porphyrocalix* and *A. appressipila* and showed, for the first time, that BAP as the sole growth regulator is able to induce embryogenesis in *Arachis*. Although somatic embryogenesis induced by cytokinins is not a common event, it has been reported for a number of species both from embryonic tissues or differentiated explants (Lakshmanan and Taji 2000; Sagare et al. 2000; Kaparakis and Alderson 2002). However, on previous reports of somatic embryogenesis in

other wild *Arachis*, cytokinins were only used associated to auxins, especially picloram (Sellars et al. 1990; Rey et al. 2000; Vidoz et al. 2004). The embryogenic response induced by BAP appears to be genotype dependent, as the three species studied in this work are closely related and the BAP concentrations used here are known to induce multiple shoot formation from embryo axes and indirect organogenesis from leaflets in other *Arachis* species (Gagliardi et al. 2000). This regeneration system could be used as a model to study cytokinin-induced cellular processes involved in the different stages of somatic embryogenesis.

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