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In vitro regeneration and conservation of wild species of *Arachis*

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SUMMARY
Wild species of *Arachis* are restricted to South America and generally occur in regions under intensive environmental disturbance. Both *in situ* and *ex situ* conservation strategies are required in order to maintain the availability of these genotypes. This work developed *in vitro* regeneration systems from seed explants of 17 wild species of *Arachis* from six Sections (*Heteranthae*, *Caulorrhizae*, *Triseminatae*, *Erectoides*, *Procumbentes* and *Arachis*). After seed disinfection, embryonic axes, leaflets and cotyledons were excised aseptically and cultured on Murashige and Skoog (MS) medium supplemented with 8.8 μM, 22 μM or 110 μM 6-benzylaminopurine (BAP). Cultures were maintained in a growth chamber at 28° ± 2°C with a 16 h photoperiod. Regeneration patterns from seed explants were similar among species from all Sections. Embryonic axes produced plants through meristematic amplification or multiple shoot formation, while cotyledons and embryonic leaflets produced shoots at significantly lower frequencies through direct and indirect organogenesis, respectively. Shoots obtained from all explants were transferred to MS medium without growth regulators to induce root formation.

The genus *Arachis* is native to South America and consists of 80 described species, divided into nine Sections (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). Cultivated groundnut (*Arachis hypogaea* L., Section *Arachis*) is the fifth most important oilseed worldwide, and is considered a key crop in subsistence agriculture in several countries due to the high oil and protein content of its seeds. Wild relatives are considered potential sources of novel genes for groundnut breeding because of their high genetic variability, which includes resistance to pests and diseases, and tolerance to adverse climatic conditions.

Wild species of *Arachis* are restricted to five South American countries, including Argentina, Bolivia, Brazil, Paraguay and Uruguay. The highest genetic diversity occurs in Brazil, where four Sections (*Caulorrhizae*, *Extranervosae*, *Heteranthae* and *Triseminatae*) are endemic (Stalker and Simpson, 1995). However, these species generally occur in regions under intensive environmental disturbance, which leads to habitat destruction and genetic erosion. Urgent conservation measures are required to maintain the availability of these genotypes.

Arachis germplasm conservation is usually achieved in seed banks or as live plants. Storage under seed bank conditions requires constant germplasm renewal and multiplication, since *Arachis* seeds display sub-orthodox behaviour due to their high lipid content and thin seed coat (Vásquez-Yanes and Aréchiga 1996). However, frequent loss of viability can occur even under optimal storage conditions. Moreover, plant multiplication under field conditions can be limited by specific soil and environmental requirements, and by the low number of seeds produced. Therefore, complementary *ex situ* conservation strategies that allow storage and multiplication should be considered to guarantee the conservation of *Arachis* germplasm.

In vitro conservation methods are considered an important tool for *ex situ* conservation of *Arachis* species. Different *in vitro* regeneration systems have already been described for wild species of *Arachis*, based on both organogenesis and somatic embryogenesis. Many organogenic systems have been reported using embryonic tissues and mature explants such as cotyledons (Mansur *et al*., 1993; Rani and Reedy, 1996, Gagliardi *et al*., 2000), embryonic axes (Dunbar *et al*., 1993; Gagliardi *et al*., 2000), nodal segments (Gagliardi *et al*., 2002a; Rey and Mroginski, 2003), leaves (Pittman *et al*., 1983; 1984; Burtnik and Mroginski, 1985; McKently *et al*., 1991; Mansur *et al*., 1993; Mroginski *et al*., 2004), protoplasts (Li *et al*., 1993) and anthers (Mroginski and Fernández, 1980; Bajaj *et al*., 1981). On the other hand, regeneration through somatic embryogenesis was achieved mainly from leaves, and has been reported comparatively less frequently (Sellars *et al*., 1990; Rey *et al*., 2000; 2006; Vidoz *et al*., 2004).

We have already described an efficient protocol for recovering *in vitro* plants from non-viable seeds of species from the Section *Extranervosae* (Gagliardi *et al*., 2000). These techniques were also used to establish cryopreservation systems using seed explants (Gagliardi *et al*., 2002b; 2003). However, there is still a need to

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extend this study to other *Arachis* species from different Sections, mainly those that have low seed production or have had their distribution areas drastically reduced by human actions. In addition, it is desirable to have a general regeneration protocol that can be applied to *Arachis* species, since the optimisation process for each species is compromised, in some cases, by lack of sufficient material. Therefore, this work was directed towards the development of *in vitro* regeneration systems from seed explants of wild species of *Arachis* from six different Sections.

MATERIALS AND METHODS

Plant material and culture conditions

Seeds from 17 wild species of *Arachis* from six Sections (Table I), stored at 10ºC and 25% relative humidity for 6 – 12 months, were provided by the Embrapa Genetic Resources and Biotechnology Seed Bank, Brasília.

The basal medium consisted of MS salts and vitamins (Murashige and Skoog, 1962), supplemented with 3% (w/v) sucrose and solidified with 7% (w/v) granulated and purified agar (Merck, Germany). The pH of all media was adjusted to 5.8 before autoclaving for 15 min at 121ºC. Cultures were maintained in a growth chamber at 28° ± 2°C with a 16 h photoperiod at a total irradiance of 46 µmoles m^{-2} s^{-1} provided by cool-white fluorescent lamps.

Plant regeneration from seed explants

Seeds were surface sterilised with 0.2% (w/v) $HgCl_2$ for 45 min with gentle agitation and washed three-times with sterile de-ionised water. Disinfected seeds were then peeled and used as a source of explants for the establishment of *in vitro* cultures. Embryonic axes, embryonic leaflets and cotyledons were excised aseptically and transferred to culture vessels (250 ml) containing MS medium supplemented with 8.8 µM, 22 µM or 110 µM 6-benzylaminopurine (BAP) (Gagliardi *et al*., 2000). Responsive cotyledons of all species were transferred to fresh medium supplemented with 5 µM $AgNO_3$ after excision of the first shoots (Pestana *et al*., 1999).

For root induction, shoots obtained from seed explants were transferred to MS medium without growth regulators.

Statistical analysis

Regeneration rates and mean numbers of shoots produced per explant were recorded during 2 months of culture, at 2-week intervals. Data were subjected to analysis of variance (ANOVA), and comparisons of means were carried out using the Tukey-Kramer comparisons test (at the 0.05% significance level) using GraphPad Instat version 3.0 (GraphPad Software Inc., San Diego, CA, USA).

RESULTS AND DISCUSSION

Plant regeneration from embryonic axes

Embryonic axes excised from seeds of all *Arachis* species showed shoot formation through meristematic amplification in response to 8.8 µM BAP (Figure 1A, B). Explants of *A. archeri* and *A. porphyrocalix* (Section *Erectoides*) and *A. appressipila* (Section *Procumbentes*) also showed the formation of friable calli from the hypocotyl region of the embryo, with subsequent somatic embryo production, at frequencies between 40% and 80%, after 2 months of culture (Figure 1C).

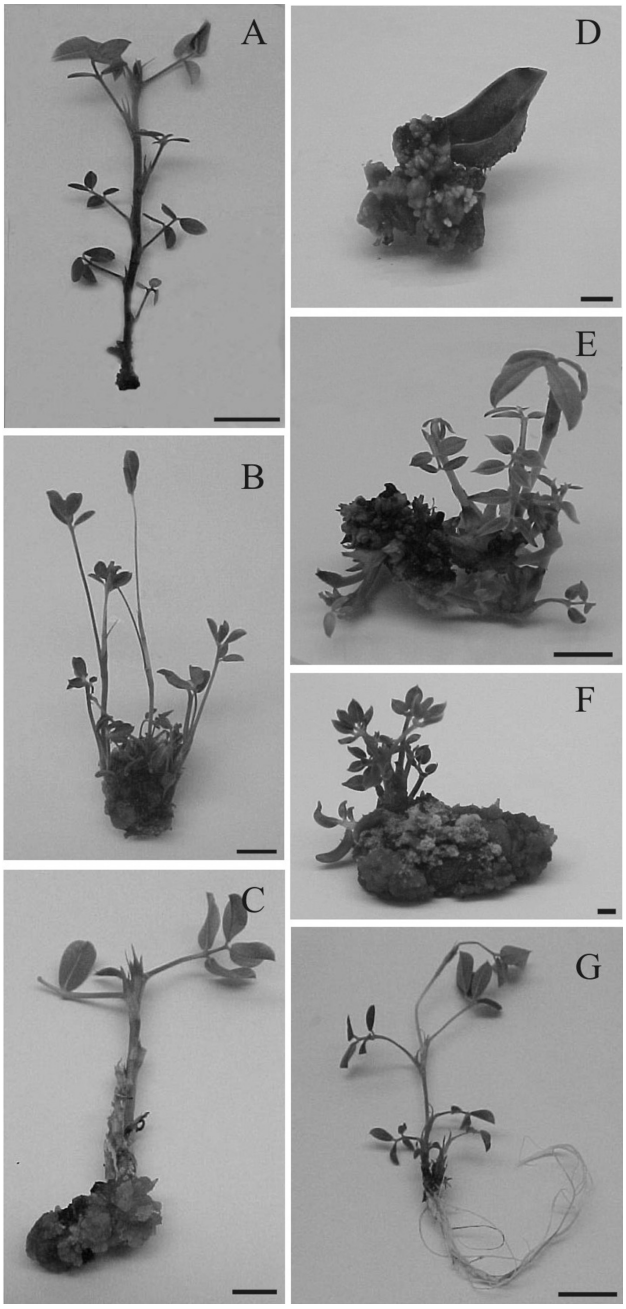

FIG. 1
In vitro regeneration of wild species of *Arachis*. Panel A, shoot of *A. sylvestris* V 6001 (Section *Heteranthae*) originated from an embryonic axis cultured on MS medium supplemented with 8.8 µM BAP. Panel B, multiple shoot formation from an embryonic axis of *A. pusilla* V 10833 (Section *Heteranthae*) on MS medium supplemented with 8.8 µM BAP. Panel C, shoot of *A. appressipila* GKP 10002 (Section *Procumbentes*), with a friable callus, originated from the hypocotyl region of the embryo on MS medium supplemented with 8.8 µM BAP. Panel D, compact callus formation from an embryonic leaflet of *A. paraguariensis* V 14204 (Section Erectoides) after 15 d of culture in the presence of 22 µM BAP. Panel E, same explant as in Panel E after 2 months. Panel F, direct organogenesis from embryo-free cotyledon of *A. magna* V 14750 (Section *Arachis*) in response to 110 µM BAP. Panel G, rooted shoot of *A. dardanii* V 13383 (Section *Heteranthae*) cultured on MS medium without growth regulators. Bars in each Panel = 1 cm.

TABLE I
Wild species of Arachis *used for* in vitro *regeneration from seed explants*

Section	Species	Accession	Storage period (months)
Heteranthae	*A. dardanii*	V 13383	12
	A. pusilla	V 10833	12
	A. seridöensis	V 10969	12
	A. sylvestris	V 6001	06
Caulorrhizae	*A. pintoi*	V 6791-wf	06
		W 647	06
Triseminatae	*A. triseminata*	W 820	ND†
Erectoides	*A. archeri*	V 13494	ND
	A. paraguariensis	V 13556	06
		V 14204	06
	A. porphyrocalix	V 7303	12
Procumbentes	*A. appressipila*	GKP 10002	06
Arachis	*A. decora*	Sv 4533	06
	A. glandulifera	V 14730	06
	A. gregoryi	V 6389	12
	A. magna	V 14750	06
	A. palustris	V 13023	06
	A. praecox	V 14682	06
	A. schininii	V 9923	06

†ND, not determined.

The regenerative response of embryonic axes was higher in species from the Sections *Arachis*, *Triseminatae* and *Erectoides*, and ranged from 78 – 100 %, except for *A. archeri*, which displayed a plant regeneration frequency of 43%. Explants of species from the Sections *Heteranthae*, *Caulorrhizae* and *Procumbentes* showed regeneration frequencies of approx. 70%, except for *A. seridöensis* (Section *Heteranthae*), which had the lowest regeneration rate (29%; Table II). Shoot formation was not positively correlated with the regeneration frequencies displayed by the embryonic axes, since some species from the Section *Erectoides* that showed high regeneration rates produced the lowest numbers (1.0 – 1.7) of shoots per explant. The highest shoot formation rates (5.8 shoots per explant) were observed in embryonic axes of *A. pintoi* V 6791, from the Section *Caulorrhizae* (Table III). The regeneration potentials observed here contrast with a previous study on *in vitro* plant regeneration from embryonic axes of *Arachis* species from the Section *Extranervosae*, in which higher multiple shoot formation rates (3.7 – 43.3 shoots per explant) were obtained in response to 8.8 µM BAP (Gagliardi *et al*., 2000). This difference may be due to genetic factors that determine the regeneration potential or to physiological alterations occurring during seed storage.

Plant regeneration from embryonic leaflets

Leaflets of *A. archeri*, *A. porphyrocalix* (Section *Erectoides*) and *A. appressipila* (Section *Procumbentes*) produced friable embryogenic calli in response to BAP. Induction of somatic embryogenesis from leaf tissues of wild *Arachis* is commonly associated with the presence of auxins in the culture media. For example, Rey *et al*. (2000; 2006) and Vidoz *et al*. (2004) described *in vitro* regeneration systems for *A. pintoi* and *A. glabrata* from mature leaves through indirect somatic embryogenesis, using high concentrations of picloran (20.7 µM and 41.4 µM) and low concentrations of BAP (0.04 µM – 4.4 µM). Somatic embryogenesis from seed explants of *A. archeri*, *A. porphyrocalix* and *A. appressipila*, in response to BAP at concentrations that induced meristematic amplification and indirect organogenesis in species of other Sections, suggests a strong influence of genotype, since these are closely-related species.

Embryonic leaflets of all other species studied gave rise to shoots through indirect organogenesis when cultured on MS medium supplemented with 22 µM BAP. Leaflets enlarged in size during the first week, and the morphogenic process started with the formation of green, compact, organogenic callus from the petioles, after a further 2 weeks in culture (Figure 1D). These results agree with previous reports on *in vitro* regeneration of wild species of *Arachis* from embryonic leaflets. Pittman *et al*. (1984) and Mansur *et al*. (1993) described indirect shoot formation from immature leaflets of *A. villosulicarpa* in response to combinations of BAP and α-naphthaleneacetic acid (NAA), while McKently *et al*. (1991) reported indirect organogenesis from leaflets of *A. glabrata* in response to BAP alone. In this work, regeneration frequencies and shoot production from embryonic leaflets varied considerably among species from the same Section. In Section *Arachis*, *A. magna* V 14750 showed a regeneration frequency of 90%, whereas explants of *A. praecox* failed to regenerate. The highest regeneration frequency

TABLE II
Regeneration frequencies of seed explants of wild species of Arachis *after 2 months of culture*

			Regeneration frequency (%)		
Section	Species	Accession	Embryonic axes*	Embryonic leaflets**	Cotyledons***
Heteranthae	*A. dardanii*	V 13383	60	71	0
	A. pusilla	V 10833	80	55	0
	A. seridöensis	V 10969	29	16	0
	A. sylvestris	V 6001	70	67.5	30
Caulorrhizae	*A. pintoi*	V 6791	70	50	10
		W 647	70	0	20
Triseminatae	*A. triseminata*	W820	100	ND†	0
Erectoides	*A. archeri*	V 13494	43	100	0
	A. porphyrocalix	V 7303	80	95	10
	A. paraguariensis	V 13556	78	54	0
		V 14204	100	87.5	25
Procumbentes	*A. appressipila*	GKP 10002	71	95	0
Arachis	*A. decora*	Sv 4533	80	43	50
	A. glandulifera	V 14730	80	59	70
	A. gregoryi	V 6389	86	8.9	7
	A. magna	V 14750	100	90	75
	A. palustris	V 13023	90	16	10
	A. praecox	V 14682	80	0	45
	A. schininii	V 9923	90	72.5	20

*MS medium supplemented with 8.8 µM BAP; **MS medium supplemented with 22 µM BAP; ***MS medium supplemented with 110 µM BAP.
†ND, not determined.

TABLE III
Shoot production from seed explants of wild species of Arachis *after 2 months of culture*

Section	Species	Accession	Shoots per explant		
			Embryonic axes*	Embryonic leaflets**	Cotyledons***
Heteranthae	*A. dardanii*	V13383	2.7 ± 0.8ab	1.3 ± 0.5a	0
	A. pusilla	V10833	2.4 ± 0.6ab	1.8 ± 0.5a	0
	A. seridöensis	V10969	2.7 ± 1.2ab	2.5 ± 0.5a	0
	A. sylvestris	V6001	4.2 ± 0.8a	1.9 ± 0.3a	2.7 ± 1.3a
Caulorrhizae	*A. pintoi*	V6791	5.8 ± 0.8ac	1.3 ± 0.3a	0
		W647	2.7 ± 0.6ab	1.2 ± 0.2a	0
Triseminatae	*A. triseminata*	W820	1.2 ± 0.9ab	ND†	0
Erectoides	*A. archeri*	V13494	1.0 ± 0.1b	0	0
	A. porphyrocalix	V7303	1.0 ± 0.4b	0	0
	A. paraguariensis	V13556	1.7 ± 0.4ab	1.5 ± 0.5a	0
		V14204	1.4 ± 0.3ab	9.6 ± 2.3b	1.6 ± 0.6a
Procumbentes	*A. appressipila*	GKP10002	1.7 ± 0.5ab	0	0
Arachis	*A. decora*	Sv4533	3.4 ± 0.6a	4.0 ± 0.1a	3.5 ± 2.0a
	A. glandulifera	V14730	1.7 ± 0.7ab	1.0 ± 0.1a	3.2 ± 0.6a
	A. gregoryi	V6389	2.6 ± 0.5ab	0	0
	A. magna	V14750	2.8 ± 0.2ab	1.5 ± 0.5a	1.8 ± 1.3a
	A. palustris	V13023	4.3 ± 0.8a	0	0
	A. praecox	V14682	3.1 ± 0.5a	0	0
	A. schininii	V9923	2.6 ± 0.4ab	0	0

Values are means ± standard error. Means followed by the same lower-case letters in a column were not statistically different by Tukey-Kramer multiple comparisons test ($P < 0.05$).
*MS medium supplemented with 8.8 µM BAP; **MS medium supplemented with 22 µM BAP; ***MS medium supplemented with 110 µM BAP.
†ND, not determined.

(100%) was observed in *A. archeri* V 13494 (Table II), while *A. paraguariensis* V 14204 (Section *Erectoides*) showed the highest rate of shoot production (9.6 shoots per explant; Table III; Figure 1E).

Despite the high regeneration potential displayed by embryonic leaflets, both indirect organogenesis and somatic embryogenesis are not considered suitable regeneration systems for the establishment of *in vitro* conservation protocols, since these processes involve a callus phase, with the attendant risk of somaclonal variation.

Plant regeneration from cotyledons

Cotyledons cultured on MS medium supplemented with 110 µM BAP showed shoot formation through direct organogenesis (Figure 1F). Four species from the Section *Arachis* (*A. decora*, *A. glandulifera*, *A. magna* and *A. praecox*) showed higher regeneration rates from cotyledons when compared to species from other Sections. *A. magna* V 14750 showed the highest regeneration frequency (75%), whereas *A. decora* Sv 4533 showed the highest rate of shoot production (3.5 shoots per explant; Table II; Table III). The low regeneration potential displayed by cotyledons in this work is in contrast to other reports. Rani and Reedy (1996) described higher rates of shoot formation (14 – 16 shoot per explant) from cotyledons of cultivated and wild species of *Arachis* in response to MS medium supplemented with 30.8 µM BAP in combination with 2.85 µM indole-3-acetic acid (IAA). Cotyledons of species from the Section *Extranervosae* also showed higher multiple shoot production rates (37.5 shoot per explant) in response to 110 µM BAP (Gagliardi *et al.*, 2000). In addition, this reduced regeneration potential from cotyledons was significantly lower than the potential displayed by embryonic axes and leaflets excised from the same seed. Differences in regenerative potential observed in different explants from the same donor seed may be attributed to oxidative injuries during seed collection and/or storage, which may result in metabolic abnormalities (Benson, 1990; Morris *et al.*, 1995).

After excision of the first shoots, all responsive cotyledons became necrotic. Cotyledons that were transferred to fresh medium containing 5 µM $AgNO_3$, after excision of the first shoots, showed a significant reduction in necrosis and could be maintained in culture for an additional period of 2 months without losing their proliferative potential. The oxidative response observed was probably related to the liberation of ethylene promoted by the stress of excision. In tissue culture, one of the main physiological consequences of ethylene liberation is inhibition of morphogenesis. $AgNO_3$ is considered an important ethylene inhibitor, as it reduces interactions between the hormone and its receptors by competition with Ag^{2+} ions (Ma *et al.*, 1998).

Shoots obtained from embryonic axes, embryonic leaflets and cotyledons were transferred to MS medium without growth regulators to induce root formation (Figure 1G). Rooting was achieved at frequencies approaching 100%. Nodal segments obtained from these primary plants were sub-cultured periodically onto MS medium without growth regulators to induce *in vitro* multiplication, according to Gagliardi *et al.* (2000).

In conclusion, an *in vitro* regeneration protocol for seed explants of 17 species from six different Sections of *Arachis* was developed, using different media compositions and explant types. Meristematic amplification and direct organogenesis were obtained from embryonic axes and cotyledons, respectively. Although not all species showed both regeneration pathways, these methods can be considered suitable to establish *in vitro* conservation protocols, taking into account that they do not involve a callus phase and therefore reduce the risk of somaclonal variation.

Two important aspects of the present study should be emphasised. First, the use of seed explants provides for a wide representation of available genetic diversity. Second, although distinct regeneration pathways were observed according to explant type and genotype, we have demonstrated that the same regeneration system could be applied for the rescue, conservation and multiplication of *Arachis* germplasm from different

Sections. The similarity in behaviour suggests that *Arachis* species have conserved physiological characteristics related to their response to growth regulators, despite their morphological, cytological and molecular variability. This is especially important for species that produce low numbers of seeds, which impairs testing different media compositions.

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