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BRIEF COMMUNICATION

Somatic embryogenesis and plant regeneration in diploid and triploid *Arachis pintoi*

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Abstract

Plants of two cytotypes (2n=2x=20, and 2n=3x=30) of pinto peanut (*Arachis pintoi* Krapov. & W.C. Gregory) were regenerated through somatic embryogenesis. Embryogenic calli were induced from shoot tips or immature leaves dissected from *in vitro* growing plants. In the case of the diploid peanut the best somatic embryogenesis was achieved when shoot tips were cultured on Murashige and Skoog (MS) medium supplemented with 10 mg dm^{-3} *Picloram* (PIC) and 0.1 mg dm^{-3} 6-benzylaminopurine (BAP) or when explants from immature leaves were cultured on MS + 10 mg dm^{-3} PIC + 0.01 mg dm^{-3} BAP. In the case of triploid peanut the highest number of somatic embryos was obtained when shoot tips were cultured on MS + 10 mg dm^{-3} PIC + 0.01 mg dm^{-3} BAP or when immature leaves were cultured on MS + 20 mg dm^{-3} PIC + 0.01 mg dm^{-3} BAP. Somatic embryos were converted into plants by culture on MS + 0.01 mg dm^{-3} naphthaleneacetic acid + 0.01 mg dm^{-3} BAP. Plants were successfully transferred to pots in greenhouse.

Additional key words: forage legume, growth regulators, *in vitro* cultivation, leaf culture, *Leguminosae*, shoot tip.

One of the essential requirements for the successful application of plant biotechnology to agriculture is to have suitable *in vitro* protocols to regenerate whole plants through organogenesis or somatic embryogenesis. Somatic embryogenesis is more efficient than plant regeneration *via* organogenesis (Ammirato 1983), because *1*) somatic embryos are bipolar structures, both root and shoot are present simultaneously; *2*) somatic embryos can be encapsulated generating the synthetic or artificial seeds; *3*) somatic embryos can be used for long-term storage in genebanks; *4*) somatic embryos are of single cell origin and chimeric plant are less likely to develop and therefore more useful for genetic transformation.

Plant regeneration *via* somatic embryogenesis occurs in a wide number of plant species including some species of *Arachis,* such as *A. hypogaea* (Sellars *et al.* 1990, Baker and Wetzstein 1992, Durham and Parrott 1992, Eapen *et al.* 1993, Chengalrayan *et al.* 1994, Joshi *et al.* 2003), *A. paraguariensis* (Sellars *et al.* 1990), *A. pintoi* (Rey *et al.* 2000) and *A. glabrata* (Vidoz *et al*. 2004).

Pinto peanut (*Arachis pintoi*), a perennial herb, is an important forage legume in tropical and subtropical areas of the world (Pizarro and Rincón 1994). Cytogenetic studies have permitted find two cytotypes. There is a diploid cytotype, 2n=2x=20 (Fernández and Krapovickas 1994) and a triploid cytotype, 2n= 3x=30 (Peñaloza *et al*. 1996).

The aim of the present research was to set up a plant regeneration protocol for diploid and triploid Pinto peanut through somatic embryogenesis.

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Abbreviations: BAP - 6-benzylaminopurine, MS medium - Murashige and Skoog (1962) medium + 3 % sucrose + 0.65 % agar, NAA - 1-naphthaleneacetic acid, PIC - picloram (4-amino-3,5,6-trichloropicolinic acid), REYI - relative embryogenic yield index = (% of calli with SE) × (number of SE per callus), SE - somatic embryos.

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Seven-month-old field grown plants of *Arachis pintoi* Krapov. & W.C. Gregory were used as primary source of explants. The plants of the triploid cytotype were provided by Francisco M. Valls (Embrapa/Cenargen, Brasilia, Brazil). A herbarium specimen of this plant material is deposited in CTES as Lavia 90. Seedlings of the diploid cytotype were obtained from seeds collected by A. Krapovickas and W. Gregory in Cruz das Almas, Bahia, Brazil and a herbarium specimen is deposited in CTES, as Gregory and Krapovickas 12787.

Two source of explants were used: *1*) field grown plants and *2*) *in vitro* growing plantlets obtained by culture of shoot tips (2 - 3 mm in length) dissected from field grown plants. Shoot tips were previously surface sterilized by immersion in 70 % ethanol (30 s) followed by immersion in 0.9 % sodium hypochlorite (12 min) and then rinsed three times with autoclaved distilled water. Finally, the shoot tips were cultured on Murashige and Skoog (1962; MS) medium, 0.65 % agar (*Sigma Chemical Company*, St. Louis, MO, USA), supplemented with 0.01 mg dm^{-3} of 1-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP). After a incubation period of 60 d in a growth room (temperature of 27 ± 2 °C, 14-h photoperiod, irradiance of 116 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps) the plantlets were ready for to be used as source of explants.

In the first experiment, mature leaves (approximately 50 - 60 % of the final size) from field grown plants were cultured on MS + 10 mg dm^{-3} picloram (PIC) + 1 mg dm^{-3} BAP. This medium was described as one of the best in order to induce somatic embryos in a diploid cytotype of *Arachis pintoi* (Rey *et al*. 2000). In another experiment three kinds of explants from *in vitro* plants, obtained as was mentioned above, were cultured: *1*) shoot tips (2 - 3 mm in length); *2*) immature leaves (2 - 4 mm in length), and *3*) mature leaves consisted of the first expanded leaflets.

Explants from field grown plants were surface sterilized as it is mentioned above, meanwhile, explants from *in vitro* plants were dissected directly (without sterilization) from 60-d old plants. Then the explants were placed on 3 cm^{-3} culture medium in a 11-cm^{-3} glass tube previously autoclaved at 1.46 kg cm^{-2} for 20 min.

The tubes containing one explant each were covered with *Resinite AF-50*® film (*Casco S.A.C.*, Buenos Aires, Argentina) and incubated in a growth room at 14-h photoperiod with an irradiance of 116 μmol m^{-2} s^{-1} and temperature of 27 ± 2 °C.

The culture medium employed consisted of MS salts and vitamins with 3 % sucrose, supplemented with various concentrations and combinations of PIC and BAP. Ten explants (shoot tips or explants from leaves) were cultured per treatment. After 90 d the numbers of calli with somatic embryos (SE) as well as the numbers of SE per callus were recorded. With these data the relative embryo yield index (REYI) was calculated as: REYI = (% calli with SE) (number of SE per callus). Data were subjected to analysis of variance (*ANOVA*) and with the least significant difference test at the 5 % level of probability.

The medium for maturation and conversion of the embryos was composed by MS + 0.01 mg dm^{-3} NAA + 0.01 mg dm^{-3} BAP.

In the first experiment using explants from field grown triploid and diploid plants, callus growth occurred after 2 weeks of culture. In the diploid cytotype 17.3 % of the explants dissected from mature leaves produced somatic embryo. This fact confirmed the results previously reported (Rey *et al.* 2000).

When using explant dissected from *in vitro* plants, the earliest visible sign of expansion of the explants cultured was noticeable within one week of culture. After that, approximately 30 - 50 d of culture in both, diploid and triploid cytotypes, on either shoot tips or explants dissected from immature leaves, in most of the media tested, it was possible to observe somatic embryos together with a small mass of callus (Fig. 1*A*). After 90 d of culture, in various media, the numbers of somatic embryos were considerably higher (Fig 1*B*).

Table 1 Effect of eight culture media on somatic embryogenesis on shoot tips or immature leaves of a diploid and a triploid cytotype of *Arachis pintoi.*

PIC + BAP	Diploid						Triploid					
	shoot tips			leaves			shoot tips			leaves		
	calli with SE [%]	number of SE [$callus^{-1}$]	REYI	calli with SE [%]	number of SE [$callus^{-1}$]	REYI	calli with SE [%]	number of SE [$callus^{-1}$]	REYI	calli with SE [%]	number of SE [$callus^{-1}$]	REYI
10+0.00	30.0^{ab}	5.8^{abc}	174	66.6^{cd}	6.1^{b}	406	46.6^{c}	3.6^{abc}	167	56.0^{de}	6.0^{bc}	336
10+0.01	53.3^{c}	5.3^{abc}	282	83.3^{d}	6.0^{b}	499	46.6^{c}	7.1^{cd}	330	33.3^{bc}	4.2^{ab}	139
10+0.10	46.6^{bc}	6.7^{bc}	312	43.3^{b}	2.4^{a}	103	26.6^{b}	5.1^{bcd}	135	20.0^{ab}	2.8^{a}	56
10+1.00	16.6^{a}	3.5^{ab}	58	10.0^{a}	2.1^{a}	21	3.3^{a}	1.0^{a}	3	16.6^{a}	2.8^{a}	46
20+0.00	46.6^{bc}	4.5^{abc}	209	73.3^{d}	4.6^{ab}	337	36.6^{bc}	5.3^{bcd}	193	43.3^{cd}	2.2^{a}	95
20+0.01	26.6^{a}	7.7^{c}	204	53.3^{bc}	3.3^{ab}	175	36.6^{bc}	7.8^{d}	285	60.0^{e}	6.9^{c}	414
20+0.10	20.0^{a}	3.8^{ab}	76	46.6^{b}	5.5^{b}	256	26.6^{b}	3.1^{abc}	82	6.6^{a}	3.0^{a}	19
20+1.00	16.6^{a}	2.8^{a}	46	16.6^{a}	2.1^{a}	34	6.6^{a}	2.6^{ab}	17	6.6^{a}	1.6^{a}	10

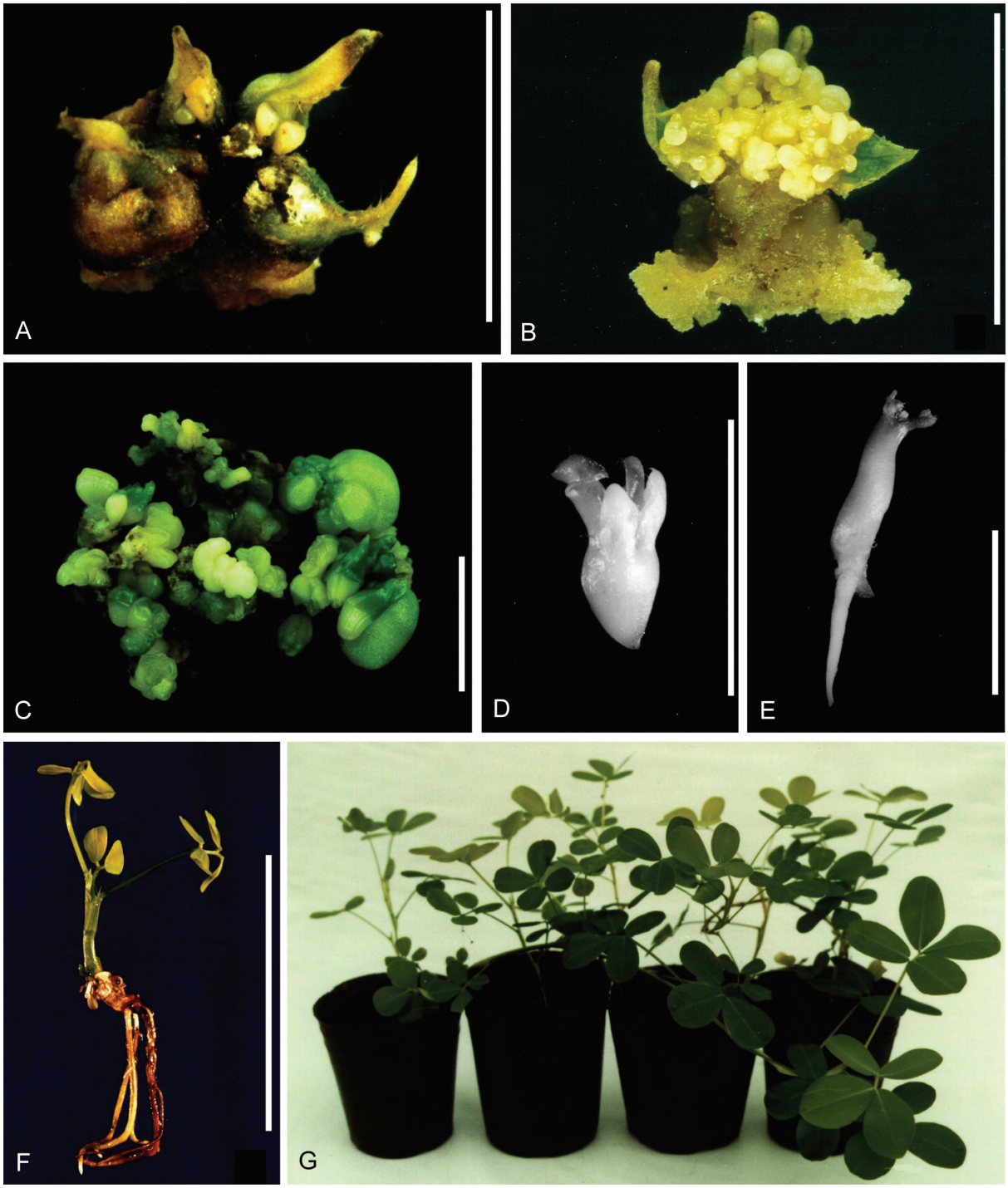

Fig. 1. Developmental stages in the plant regeneration in *Arachis pintoi* (2n=3x=30). *Bars* represent 5 mm. *A* - immature leaves after 35 d of culture; *B* - immature leaves after 90 d of culture; *C* - somatic embryos in different stages of development; *D* - an isolated somatic embryos; *E* - germination of a somatic embryo; *F* - plant derived from a somatic embryo; *G* - plants regenerated through somatic embryogenesis growing in pots.

The induction of somatic embryogenesis was highly affected by the kind of explants cultured and by the medium of culture. In both cytotypes, while either explants from shoot tips or immature leaves produced somatic embryos in all the media tested (Table 1), explants from mature leaves did not produce any embryo. In the diploid cytotype the highest values of REYI were obtained when shoot tips were cultured on a medium

composed by MS + 10 mg dm^{-3} PIC + 0.1 mg dm^{-3} BAP or when explants from immature leaves were cultured on MS + 10 mg dm^{-3} PIC + 0.01 mg dm^{-3} BAP. However, in the triploid cytotype the highest values of REYI were obtained when shoot tips were cultured on 10 mg dm^{-3} PIC + 0.01 mg dm^{-3} BAP or when immature leaves were cultured on 20 mg dm^{-3} PIC + 0.01 mg dm^{-3} BAP (Table 1). The values obtained in this work show a considerable improvement in comparison with those reported previously (Rey *et al*. 2000). This results clearly show that the source of the explants and its the physiological stage were essential for inducing somatic embryogenesis as was pointed out in various plant species (*e.g*. Halámková *et al.* 2004, Martin 2004), including members of the *Arachis* genus (Baker and Wetztein 1998).

When somatic embryos (Fig. 1*C*,*D*) were cultured on conversion medium, as much as 25 - 30 % produced plants (Fig. 1*E*,*F*), which have been successfully transplanted to soil (Fig. 1*G*).

To conclude, the present report describes and efficient and reliable protocol for regeneration of both diploid and triploid cytotype of *Arachis pintoi* through somatic embryogenesis.

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