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Regeneration of Plants from Apical Meristem Tips and Nodal Segments of *Arachis pintoi*

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ABSTRACT

The *in vitro* regeneration potential of shoot apical tips (2 to 3 mm in length), meristems (0.3 to 0.5 mm in length), and nodal segments (4 to 7 mm long with an axillary bud) of diploid ($2n = 2x = 20$) and triploid ($2n = 3x = 30$) cytotypes of *Arachis pintoi* was evaluated. Explants were cultured on MS medium supplemented with different concentrations and combinations of naphthaleneacetic acid (NAA) and benzyladenine (BA). In one experiment the effect of gibberellic acid was tested. The cultures were done in liquid and solid media. Plant regeneration can be readily achieved from all explants in one step of 30 d culture on MS + 0.01 mg/L each of NAA and BA or two steps consisting of 1) shoots regeneration through culture of explants on MS + 0.01 mg/L each of NAA and BA, and 2) induction of rooting in regenerated shoots by reculture on MS + 0.01 mg/L NAA. The plantlets were successfully transferred to pots in a greenhouse.

Key Words: Meristem culture, plant regeneration, tissue culture, *Arachis* species.

Plant tissue culture, especially apical meristem tips and axillary bud culture, has been widely used as a useful tool in rapid clonal propagation of selected genotypes of a broad range of crop species. The use of meristem culture for virus elimination was extensively applied for a number of species, including *Arachis hypogaea* L. (Chen and Sherwood, 1990) and interspecific hybrids of the genus *Arachis* (Dunbar *et al.*, 1993; Morris *et al.*, 1997; Radhakrishnan *et al.*, 1999). Plant meristems, as well as shoot tips and axillary buds, have been considered as excellent material for *in vitro* germplasm preservation programs (Styer and Chin, 1983; Nehra and Kartha, 1994; Takagi, 2000; Gagliardi *et al.*, 2002).

The wild peanut species *Arachis pintoi* (Krapov. and W.C. Gregory), belonging to the section *Caulorrhizae*, grows as a perennial herb (Krapovickas and Gregory, 1994). There are diploid ($2n = 2x = 20$) (Fernández and Krapovickas, 1994) and triploid ($2n = 3x = 30$) cytotypes (Peñaloza *et al.*, 1996). *Arachis pintoi* (common name: perennial peanut or pinto peanut) is a novel and important forage legume in tropical and subtropical areas of the word (Pizarro and Rincón, 1994; Argel and Ramírez, 1996).

Although plant regeneration from leaf culture of *A. pintoi*, either through organogenesis or somatic embryogenesis (Burtnik and Mroginski, 1985; Rey *et al.*, 2000), have been reported, there is no report on successful *in vitro* plant regeneration from both shoot apical meristem and nodal segments. The present paper describes the cultural conditions employed to induce plant regeneration from *in vitro* cultured meristem as well as nodal segments from both diploid and triploid cytotypes of *A. pintoi*.

Materials and Methods

Plant Material. Seeds of the diploid accession of *A. pintoi* were originally collected by A. Krapovickas and W.C. Gregory in Cruz das Almas, Bahía, Brazil (herbarium specimen Gregory and Krapovickas 12787, deposited in CTES). Plant materials for this study were obtained from seeds germinated in a mixture of soil and sand (1:1). The plants of the triploid cytotype were provided by J. F. M. Valls from Embrapa/Cenargen, Brasilia, Brazil (herbarium specimen Lavia 90, deposited in CTES) and plant materials used in this study were obtained from

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adult field-grown plants.

Source of Explants. The explants were excised from 60-d-old *in vitro* grown plants obtained by culture of shoot tips dissected from field grown plants previously surface sterilized by immersion in 70% ethanol for 30 s followed by immersion in a solution of commercial bleach [0.9% sodium hypochlorite (final concentration)] plus one drop of Tween 20® for 12 min and then thoroughly washed three times with autoclaved distilled water. The medium employed consisted of Murashige and Skoog (1962) salts and vitamins with 3% sucrose (MS), supplemented with 0.01 mg/L benzyladenine (BA) and 0.01 mg/L naphthaleneacetic acid (NAA). The medium was solidified by using 0.65% Sigma agar (A-1296). The pH of the medium was adjusted to 5.7 with KOH or HCl prior to adding the agar. The tubes were covered with aluminum foil and autoclaved at 1.46 kg cm^{-2} for 20 min. One shoot tip was cultured in a 11 cc glass tube containing 3 cc of culture medium. The tubes were then covered with Resinite AF-50® film (Casco S.A.C., Buenos Aires) and incubated in a growth room at 27 ± 2 C under a 14/10 h daylight cycle with an irradiance of 4.5 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps.

Protocols for Explants Culture. Three kinds of explants were cultured: shoot apical meristem (0.3 to 0.5 mm in length, consisting of the dome and a pair of leaf primordia), shoot tips (2 to 3 mm in length), and nodal segments (4 to 7 mm long segments of stem bearing a node and a portion of underlying internode). Explants were dissected directly (with no surface sterilization) from 60-d-old *in vitro* grown plants and cultured in the same conditions as were described above. The medium used was MS supplemented with BA or NAA (0, 0.01, 0.05, 0.1, or 1 mg/L) with 0, 0.1, 0.5, or 1 mg/L gibberellic acid (GA_3) incorporated to the media after sterilization through bacteriological filters (0.22 µm Millipore filter).

In one experiment the effect of liquid media (with a bridge consisting of a filter paper as support of the explants) on the morphogenic responses of cultured meristems was tested. In the other experiment the effect of different gelling agents from Sigma Chemical Co. (agar A-1296, agar A-9799, agar A-7921, agargel A-3301, agarose A-0169 and Phytagel, P-8169), liquid media agitated at 100 rpm, and membrane rafts (Life Raft®, Membrane Raft M 1917, and M 7288) on the morphogenic responses of meristems were studied.

Experimental Design and Results Analysis. Ten explants (shoot tip, meristem, or nodal segment) were cultured per treatment. Treatments were arranged randomly on the shelves in the growth room. Each experiment was repeated at least three times. After 30 d culture the morphogenic responses of the cultured explants were recorded. Means are presented with the standard error (SE).

Rooting of Regenerated Shoots. Shoots of more than 5 mm in length with the primary explants were induced to form roots after 30 to 40 d of subculture on MS supplemented with 0.01 mg/L NAA. The plants obtained directly in the establishment medium or from the shoots rooted in the rooting medium were transferred in pots containing a mix of soil and sand (1:1) and covered with plastic bags for 2 wk to prevent desiccation and to allow acclimatization.

Results and Discussion

In vitro Establishment of Shoot Tips From the Field. For both diploid and triploid cytotypes, culture initiation from explants dissected from plants grown in the field was a critical step because of the high rate (on average 60%) of contamination of the cultures with microorganisms (bacteria and/or fungus). Since shoot tips were dissected from plants grown outside of greenhouse, these results were expected and have also been reported for many plant species. The contamination could probably be reduced significantly by using more hygienic stock of plants grown in greenhouses and treated with an appropriate combination of chemicals to reduce infection with pathogens (Cassells, 2001). On the other hand, the browning of the explants cultured was another source of losses during establishment (on average 20%).

However, approximately 20% of the cultured explants remained green and showed the earliest sign of growth within 15 d of culture. The shoot tips enlarged considerably and by 60 d culture, regenerated shoots or complete plants were 5 to 6 cm in length with at least 5 to 6 expanded leaves. It was never possible to observe new shoot buds developed from the axils. These results are not in agreement with those obtained in peanut by Eapen *et al.* (1998), but in this case it is interesting to note that they used other plant species and shoot tips from immature seeds. In addition, they supplemented MS medium with a high concentration (5 mg/L) of BA. This medium could induce the rupture of apical dominance, and consequently promote the development of shoots from axillary buds.

Shoot Tips and Nodal Segment Cultures from Diploid Cytotype. Unlike the *in vitro* establishment of the field-grown plants, when shoot tips and nodal segments were dissected from *in vitro* shoots, the rates of contamination with microorganisms and the browning of the cultured explants were insignificant. Contamination and browning levels were not greater than 5%. Figure 1 summarizes the effect of the supplementation to MS medium with 10 combinations of NAA and BA on plant regeneration from shoot tips and nodal segments. Although both explants permitted plant regeneration independently of the culture medium, nodal segments appeared to be slightly better than the shoot tips. On the other hand, the best culture medium consisted of MS supplemented with 0.01 mg/L each of NAA and BA. Concentrations of NAA higher than 0.05 mg/L in combination with 0.01 or 0.1 BA were not effective for inducing plant regeneration. Similar results were obtained by using 0.01 mg/L NAA in conjunction with 1 mg/L BA. As many as 90% of the nodal segments cultured during 30 d on MS + 0.01 mg/L NAA + 0.01 mg/L BA regenerated whole plants directly from the buds without callus formation. Gagliardi *et al.* (2002) worked with *A. retusa* (Krapov., W.C. Gregory, and Valls), *A. macedoi* (Krapov. and W.C. Gregory), and *A. burchellii* (Krapov. and W.C. Gregory) tissues and recommended the culture of nodal segments on MS in the presence of 2.7 µM (± 0.5mg/L) NAA as the sole growth regulator.

Meristem Cultures from Diploid Cytotype. The contamination with microorganisms and the browning of

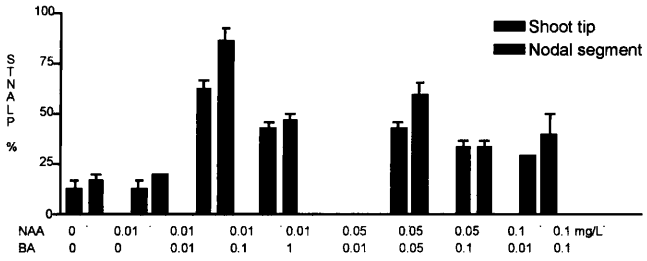

Fig. 1. Effect of 10 combinations of NAA and BA supplemented to MS medium on the plant regeneration by *in vitro* culture of shoot tips or nodal segments of *A. pintoi*. (Vertical bars represents ± SE).

the cultures meristems were relatively low (≤ 5% of the explants). After 30 d of culture, the remaining meristems expressed four typical growth responses: 1) regeneration of whole plants (Fig. 2d), 2) regeneration of shoots (with expanded leaves) of more than 5 mm in length (Fig. 2c), 3) only expansion of leaves (Fig. 2b), or 4) regeneration of shoots (without expanded leaves) of less than 3 mm in length (Fig. 2a). The relative proportion of these responses was greatly affected by the culture medium employed. An appropriate combination of NAA and BA seems to be

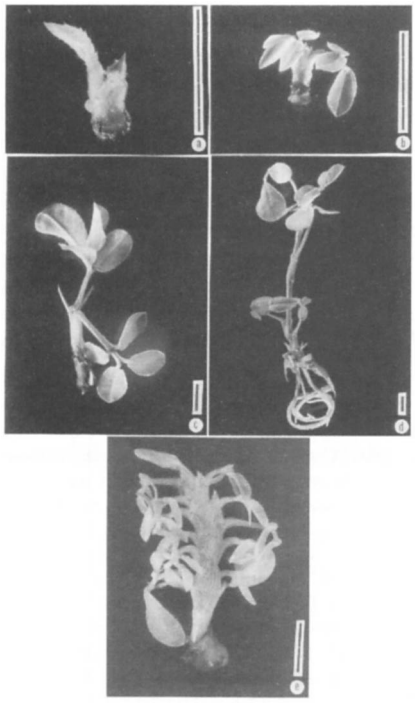

Fig. 2. Morphogenic responses of meristems of *A. pintoi* cultured *in vitro* (bars 5 mm). a) Regenerated shoot (without expansion of leaves) less than 3 mm. b) Regenerated shoot (with expanded leaves) of more than 3 mm. c) Regenerated shoot (with expanded leaves) of more than 5 mm. d) Regenerated plant. e) Regenerated shoot in a medium with high level of BA (3 mg/L).

a very important factor to consider for obtaining plant regeneration (Fig. 3). Here again, although plant regeneration and/or shoots greater than 5 mm in length were obtained in various media of culture, the best results were obtained by using MS supplemented with 0.01 mg/L each of NAA and BA. This medium permitted 17% of the cultured meristems to regenerate into plants (Fig. 3). However, by reculture of the remaining explants on the same medium for 60 d, as many as 55% of the explants differentiated whole plants and 45% produced shoots (with expanded leaves) of greater than 3 mm in length. These

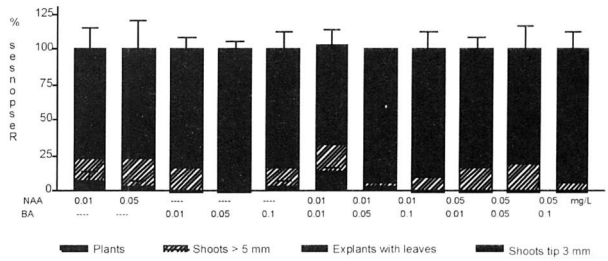

Fig. 3. Effect of various combinations between NAA and BA supplemented to MS medium on the morphogenic responses of meristems of *A. pintoi* cultured *in vitro*. (Vertical bars represent ± SE).

shoots were successfully rooted when they were cultured on rooting medium.

It is interesting to note that when meristems were cultured on MS supplemented with a relatively high concentration of BA (i.e., 3 mg/L), shoots with short internodes and several expanded leaves were obtained (Fig. 2e). The regeneration of new shoots from axillary buds occurred in a few cases. The frequencies of plant regeneration are not significantly modified if the medium composed by MS + 0.01 mg/L NAA + 0.01 mg/L BA is supplemented with gibberellic acid using either semisolid or liquid media (Fig. 4). On the other hand, although plant and shoot regeneration from meristems of *A. pintoi* can be readily achieved (with similar percentages) by using various systems of culture involving different types of gelling agents and membranes (Table 1), most of them produced hyperhydric plants or shoots which causes a serious prob-

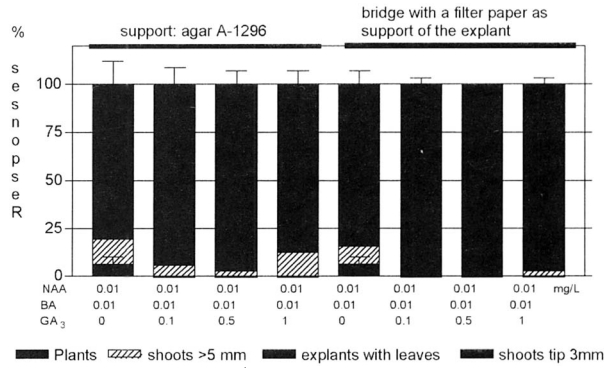

Fig. 4. Effect of various combinations of NAA, BA and GA3 supplemented to MS medium on the morphogenic responses of meristems of *A. pintoi* cultured *in vitro* in solid and liquid media. (Vertical bars represent ± SE).

lem in tissue culture and limits the micropropagation.

The consistency of the medium and the type and concentration of the gelling agent have been related to the development of hyperhydricity (Gaspar, 1991; Debergh *et al.*, 1992). The use of Membrane Life-Raft (M-1917) was the only culture system tested which was effective in eliminating hyperhydricity, whereas one of the agars (A-1296) usually employed in tissue culture produced a few hyper-

Table 1. Effect of six gelling agents and membrane rafts on the regeneration of plants or shoots from meristems of *A. pintoi* cultured on MS + 0.01 mg/L NAA + 0.01 mg/L BA (Results after 60 d culture).

System of culture	Support of explants	Meristems forming plants or shoots[a]	Plants or shoots hyperhydric
		%	%
Solid	Agar (A-1296)[b] 0.65%	64 (± 10)[c]	3 (± 1)
	Agar (A-9799) 0.50%	64 (± 5)	9 (± 1)
	Agar (A-7921) 0.70%	68 (±7)	23 (± 4)
	Agarose (A-0169) 0.89%	54 (±12)	31 (± 6)
	Agargel (A-3301) 0.40%	59 (±6)	18 (± 3)
	Phytagel (P-8169) 0.18%	52 (±6)	40 (± 2)
Liquid	With agitation 120 rpm	71 (±8)	42 (± 4)
	Membrane Life – Raft (M-1917)	69 (± 5)	0
	Membrane Life – Raft (M-7288)	45 (± 8)	20 (± 4)

[a]Only shoots of more than 5 mm in length were scored.
[b]Information within parenthesis is the code of the Sigma product.
[c]Numbers in parenthesis are standard errors.

hydric plants (Table 1).

Based on the morphogenic responses of the meristems of *A. pintoi* included in the present study, it is possible to conclude that the concentration and combination of growth regulators govern plant regeneration. The beneficial effect of the presence of NAA and BA in the culture medium for caulogenesis or plant regeneration from meristems of *A. hypogaea* was demonstrated previously (Kartha *et al.*, 1981; Morris *et al.*, 1997; Radhakrishnan *et al.*, 1999). However, the concentrations of NAA and BA recommended as optimum by these authors were slightly different than the ones obtained in this study. Kartha *et al.* (1981) reported that whole plant regeneration, with a frequency of 75%, occurred when 0.1 µM (0.02 mg/L) BA was applied in combination with 10 µM (1.86 mg/L) NAA. However, Morris *et al.* (1997) and Radhakrishnan *et al.* (1999) recommended using 1 mg/L each of NAA and BA. On the other hand, Bajaj (1983) used 2 mg/L indolacetic acid + 0.2 mg/L BA to obtain shoots in 70 to 80% of the excised meristems within 4 to 6 wk.

Shoot Tips, Meristem and Nodal Segment Cultures from Triploid Cytotype. Like the diploid cytotype, three types of explants from a triploid cytotype were cultured *in vitro* on MS + 0.01 mg/L each of NAA and BA and permitted plant or shoot regeneration (Table 2). Shoots were induced to form roots by culturing them on a rooting medium. The frequency of rooted shoots was high (ca. 80% in all cases) and the plantlets were successfully transplanted to soil.

Conclusions

The results suggest that plant regeneration from both diploid and triploid cytotypes of *A. pintoi* can readily be achieved through shoot tip, meristem, or nodal segment culture on MS + 0.01 mg/L each NAA and BA. Whole plants can be produced in only one step of 30 d culture or in two steps involving regeneration of shoots and then rooting the regenerated shoots in MS + 0.01 mg/L NAA. Rapid progress in the application of these protocols to micropropagation, elimination of viruses, and germplasm preservation of *A. pintoi* is expected.

Table 2. Plant and shoot regeneration from shoot tips, meristems and nodal segments of triploid ($2n = 3x = 30$) *A. pintoi* cultured during 30 d on MS + 0.01 mg/L NAA + 0.01 mg/L BA.

Explants	Explants forming Shoots[a,b]	Plants
	%	%
Shoot tips	29 (± 5)	12 (± 5)
Meristems	26 (± 5)	6 (± 5)
Nodal segments	37 (± 7)	60 (± 8)

[a]Only shoots more than 5 mm in length were scored.
[b]Numbers in parenthesis are standard error.

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