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Thidiazuron Promotes *In Vitro* Plant Regeneration of *Arachis correntina* (*Leguminosae*) via Organogenesis

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ABSTRACT

Arachis correntina (Burkart) Krapov. & W.C. Gregory is a herbaceous perennial leguminous plant growing in the Northeast of the Province Corrientes, Argentina. It is important as forage. The development of new *A. correntina* cultivars with improved traits could be facilitated through the application of biotechnological strategies. The purpose of this study was to investigate the plant regeneration potential of mature leaves of *A. correntina* in tissue culture. Buds were induced from both petiole and laminae on 0.7% agar-solidified medium containing 3% sucrose, salts and vitamins from Murashige and Skoog (MS) supplemented with 0.5–25 μM thidiazuron (TDZ). Shoot induction was achieved by transference of calli with buds to MS supplemented with 5 μM TDZ. Fifty-four percent of the regenerated shoot rooted on MS + 5 μM naphthaleneacetic acid. Histological studies revealed that shoots regenerated via organogenesis.

Key words: *Arachis correntina*; *In vitro* plant regeneration; *Leguminosae*; Organogenesis; Thidiazuron (TDZ)

INTRODUCTION

Modern biotechnological methods of direct gene transfer have facilitated plant improvement programs. New pest- and disease-resistant varieties of crops have been created and the nutritional value has been enhanced in other varieties (Jauhar 2001; Vega and others 1999). However, for plant genetic engineering to be successful, a suitable protocol for plant regeneration is an absolute requirement (Jähne and others 1995).

Plant regeneration *in vitro* via organogenesis is a complex and little understood process. The effects of genotype, explant and growth regulators on regeneration *in vitro* have been reported for many plant species. With *Arachis*, a leguminous genus, encompassing more than 69 species (Krapovickas and Gregory 1994). Mroginski and others (1981) were able to regenerate plants of six cultivars of *A. hypogaea* through organogenesis by immature leaf culture. Various authors have extended this ability to other cultivars of cultivated peanut (Cheng and

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others 1992; McKently and others 1991; Pittman and others 1983; Seitz and others 1987; Utomo and others 1996) and to some wild species of *Arachis* (Mansur and others 1993; McKently and others 1991; Pittman and others 1983). Plant regeneration in *Arachis* spp. via organogenesis was also reported by *in vitro* culture of mature leaves (Burtnik and Mroginski 1985; Rey and others 2000). Although several species in the genus *Arachis* have been regenerated, *in vitro* regeneration of *A. correntina*, a herbaceous perennial grown in the sandy soil in the Northeast of the Province Corrientes, Argentina (Krapovickas and Gregory 1994) has not been reported. Several explants of *A. correntina* formed callus when grown *in vitro* on media with 2,4-dichlorophenoxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and/or kinetin (KIN) or N6-benzyladenine (BA) but exhibited no ability for plant regeneration (Rouvier, personal communication).

Plant regeneration *in vitro* via organogenesis is controlled primarily by the interaction of plant hormones, specifically cytokinins and auxins, in the culture medium (Evans and others 1981). The combination of NAA or 2,4-D and BA or KIN have been successfully used for plant regeneration in *Arachis* spp (Mroginski and Kartha 1984). However, few authors have used thidiazuron (TDZ) with *A. hypogaea* (Akasaka and others 2000; Gill and Ozias-Akins 1999; Kanyand and others 1994). TDZ is a substituted phenylurea compound, shown to possess potent activity as a cytokinin in the regulation of shoot organogenesis in several plant species (Huetteman and Preece 1993; Mithila and others 2001; Murthy and others 1998). Our study sought to identify the role and interaction of explant source and growth regulators in callus-mediated organogenesis of *A. correntina*. This work also investigated the histological changes in the leaf-derived calli of *A. correntina* and documented the sequence of events leading to plant regeneration via organogenesis.

Material and Methods

Plant Materials and Surface Sterilization

Seeds of *Arachis correntina* (Burkart) Krapov. & W.C. Gregory were germinated in a potting mixture of soil and sand (1:1) in the garden of the Instituto de Botńnica del Nordeste, Corrientes, Argentina. The seeds were collected by Luis Mroginski in Pirayui (herbarium specimen Krapovickas and others 11905 deposited in CTES).

The first fully expanded leaflets from adult plants were used as a source of two kinds of explants: A-petiole: (3 mm long); B-laminae: a square of approximately 4 mm^2 of the median portion of the laminae containing the midrib. The explants were surface-sterilized with 70% ethanol for 30 sec and soaked in 1% commercial NaOCl containing one drop of Tween 20® for 12 min. Thereafter, they were rinsed thoroughly with sterile distilled water.

Media and Culture Conditions

The nutrient medium (MS) consisted of major and minor salts as well as vitamins according to Murashige and Skoog (1962), 3% sucrose, 0.7% agar (Sigma Chemical Co A-1296) and supplemented with or without cytokinins, BA or KIN (0.05 μM, 0.5 μM, 5 μM, 15 μM, or 25 μM), TDZ (0.05 μM, 0.5 μM, 5 μM, 15 μM, 25 μM, 40 μM, 50 μM, or 65 μM), and auxins, 2,4-D, picloram-4-amino-3,5,6-trichloropicolinic acid (PIC) or NAA (0.1 μM, 1 μM, 5 μM, 25 μM, 50 μM, or 100 μM). The pH of each medium was adjusted to 5.8 with KOH or HCl before the addition of agar. Glass tubes 11 ml containing 3 ml of culture medium were covered with aluminum foil and autoclaved at 1.46 kg/cm^2 for 20 min.

Explants (1 per tube) were aseptically cultured. The tubes were sealed with Resinite AF 50® (Casco S.A.I.C. Company, Buenos Aires), and incubated in a growth room under light-dark (14–10 h) conditions at 27 ± 2°C. The light source was provided by cool fluorescent tubes (116 μm ($m^{-2}.s^{-1}$). After one month of culture, the calli were subcultured on fresh medium containing MS + 5 μM TDZ.

Rooting and Hardening

Elongated shoots (1–2 cm) were excised and transferred to root induction medium consisting of MS + (0, 0.05 μM, 0.5 μM, or 5 μM) NAA. The environmental conditions were as described above for callus and shoot induction. Plantlets resulting from rooted shoots were rinsed gently under running tapwater to remove adhering cultured media and subsequently transferred to plastic pots containing a mixture of soil, sand and peat moss (1:1:1). Each pot was covered with a polyethylene bag. The potted plants were acclimatized and after 2–3 weeks moved to the greenhouse.

Histological Analysis

Histological studies were performed according to Gonzalez and Cristóbal (1997). Tissue samples were

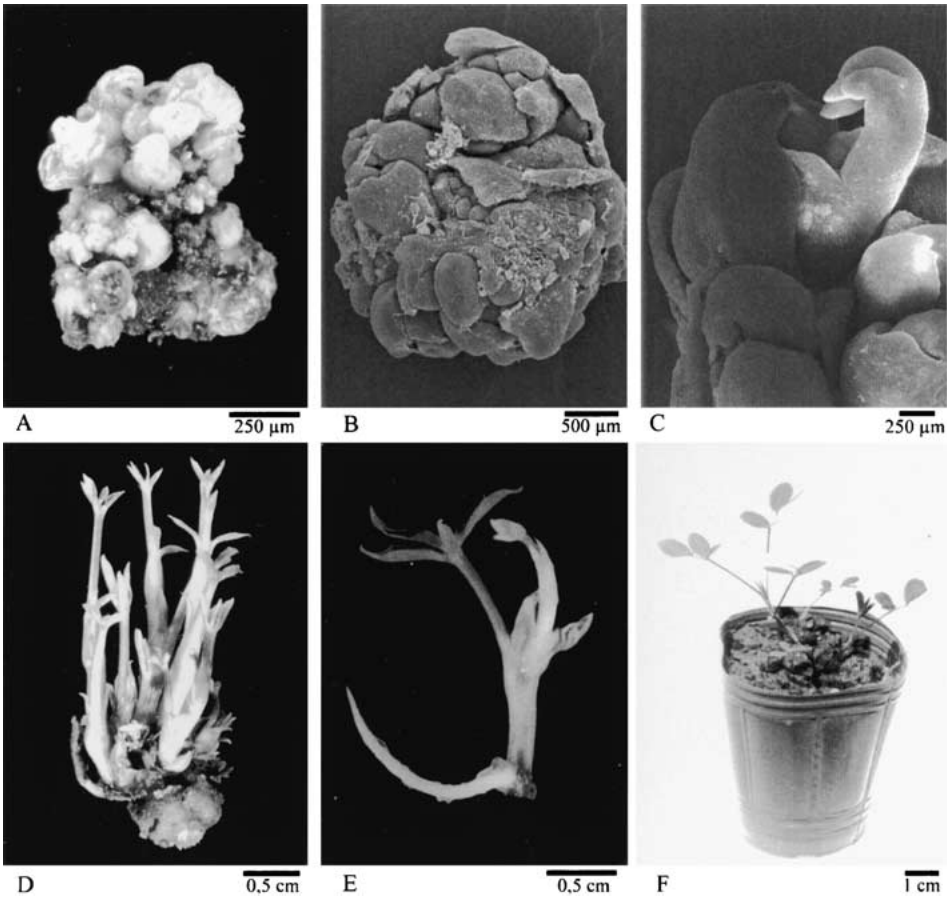

Figure 1. Plant regeneration in calli derived from leaflet explants of *Arachis correntina*. (**A**) Callus with buds obtained by culture of explants from laminae on MS + 15 μM TDZ. (**B**) Same callus with buds observed in a scanning electron microscope. (**C**) Bud formation. (**D**) Multiple shoot differentiation when callus with buds produced by culture of laminae on MS + 15 μM TDZ were subcultured on MS + 5 μM TDZ. (**E**) Rooted regenerated shoot. (**F**) Plantlet growing in soil.

fixed in FAA (5% formaldehyde, 5% acetic acid and 63% ethanol). They were dehydrated with ''Deshidratante Biológico Biopur®'' and embedded in paraffin wax. Serial sections (10 μm thick) were stained with Safranin-Astra blue-(Luque and others 1996) and examined under a light microscope.

Tissue samples for Scanning Electronic Microscopy (SEM) were fixed in FAA. After ethanol dehydration the samples were critical point dried with CO_2 and coated with gold-palladium.

Experimental Design and Statistical Analysis

The experiments were performed with 10–15 explants for each replication and three or four replications per treatment. Treatments were arranged randomly in the shelves of the growth room. All data were subjected to analysis of variance (ANOVA) and comparisons of means were made by Duncan's multiple range test at $p < 0.05$.

Results

Approximately 80–95% of explants from both laminae and petiole started to grow shortly after incubation. Callus initiation occurred from the cut surface of the explant within one week of culture in practically all the media with a growth regulator. Contamination with microorganisms (bacteria and/or fungi) was low (less than 5% of the explants). The calli showed a great variability in color, texture and size, depending on the culture medium. When BA or KIN in combination with 2,4-D, PIC, or NAA were used, the calli were yellowish and friable and reached 1.5 cm in diameter. However, calli generated in the presence of TDZ were compact and green with a diameter of no more than 1 cm. Although callus induction readily occurred in all the combinations of growth regulators tested, bud regeneration only occurred when MS was supplemented with 15 μM TDZ. On this medium, after 10–15 days of culture, the explants produced calli with green compact nodules, indicating the initiation of adventitious bud regeneration. This phenomenon began simultaneously on the entire surface of the calli (Figure 1A–C). The frequencies of calli with buds were relatively low, 15 and 22% in cultured laminae and petiole, respectively.

Because MS + 15 μM TDZ was the only growth regulator that induced bud differentiation, a second experiment was done to determine the opti-

Table 1. Effects of eight Concentrations of TDZ Added to MS Medium on the Induction of Callus with Buds from Leaflet Explants of *A. correntina* after 30 days of Culture

TDZ (in μM)	Laminae		Petiole	
	% calli with buds	Number of bud per explant	% calli with buds	Number of buds per explant
0.05	0[a]	0	0[a]	0
0.5	0[a]	0	18[1)ab]	4
5	19[abc]	6	31[bc]	6
15	25[bc]	4	25[bc]	8
25	34[d]	9	27[bc]	15
40	10[ab]	6	40[d]	8
50	16[abc]	4	27[bc]	7
65	22[abc]	5	29[bc]	5

[1]*Different letters within columns indicate significant differences at $p < 0.05$, using Duncan's multiple range test*

Table 2. Effect of the Initial Medium for Callus + Bud Induction on the Production of Buds and Shoots from Leaflet Explants of *A. correntina* when Subcultured for 30 days on MS + 5 μM TDZ

Initial medium TDZ (μM)	Laminae				Petiole			
	% Calli with only buds	% Calli with buds + shoots[1]	Number of buds/ explants	Number of shoots/ explants	% Calli with only buds	% Calli with buds + shoots	Number of buds/ explants	Number of shoots/ explants
0.5	0	0	0	0	52[2a]	48	24	2
5	95[b]	5	16	2	81[b]	19	23	3
15	100[b]	0	13	0	96[b]	4	22	1
25	71[a]	29	10	1	95[b]	0	17	1

[1)]*Only shoots of more than 0.5 cm in length were scored*
[2)]*Different letters, within columns, indicate significant differences at $p < 0.05$, using Duncan's multiple range test*

mal concentration of TDZ for promotion of this morphogenetic response. Table 1 summarizes the results obtained when explants from laminae and petiole were cultured on MS supplemented with 8 concentrations of TDZ. MS + 25 μM TDZ was optimal for regeneration (34% of the calli differentiated buds) when laminae of leaves were cultured. However, for petioles, MS + 40 μM TDZ generated the highest percentage of calli with buds (40%), whereas MS + 25 μM TDZ produced the highest number of buds/explants (15 buds/explant). Intermediate concentrations of TDZ resulted in optimal bud differentiation indicating that a suitable concentration range was tested. The percentage of calli with buds and the number of buds/explants were greatly promoted when calli that originated on 0.5–25 μM TDZ were then subcultured on MS + 5 μM TDZ (Table 2). As many as 95–100% of the calli, independent of the origin of the calli, showed bud differentiation and from 29–48% of the calli resulted in multiple shoot development (Figure 1D).

Regenerated shoots formed roots (Figure 1E) within 5–10 days after subculture on the rooting medium containing MS + 0.5 or 5 μM NAA with 45 and 54%, respectively, of the shoots rooted. Media without a growth regulator or with 0.05 μM NAA were not effective. Rooted plants were successfully transferred to soil (Figure 1F) where they grew well and appeared morphologically normal.

Histological examination to determine the origin of regenerating buds revealed that within one week of culture the meristematic zones developed from both the epidermic cells and from 4–7 layers of subepidermic cells. These meristematic cells were smaller, isodiametric, thin-walled and exhibited a dense cytoplasm, which stained darkly, with small vacuoles (Figure 2A). In the epidermis cells, mainly anticlinal division occurred, whereas both anticlinal and periclinal divisions were observed in the subepidermal layer generating a protuberance with a shoot apical meristem in the center (Figure 2 B,C). The origin of the leaf primordia began with divisions of cells surrounding the shoot apical meristem

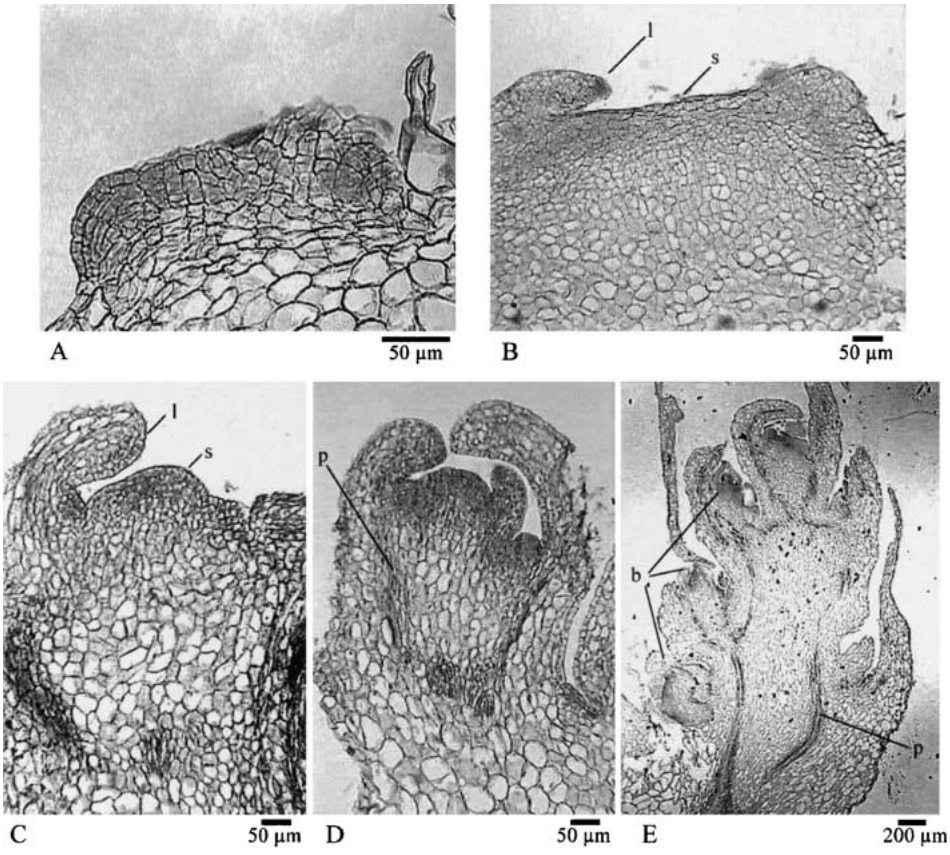

Figure 2. Histological evidence of regeneration of plantlets of *Arachis correntina* through organogenesis (**A**) Group of initial cells. (**B, C**) Leaf primordium-(l) and shoot primordium (s) differentiation. (**D**) Procambium (p). (**E**) New bud (b) differentiation and procambium (p).

(Figure 2 B,C). After a few days more of culture, shoot buds surrounded by a pair of leaf primordia, differentiated from these meristematic structures. Vascular tissue connecting shoot buds to the explant were observed (Figure 2D). Within 3 weeks of culture, new meristematic zones in the axil of each shoot bud were observed which originated new shoots (Figure 2E).

Discussion

With the exception of *Arachis pintoi* (Burtnik and Mroginski 1985; Rey and others 2000) other members of the genus *Arachis* have been recalcitrant to regeneration via organogenesis when explants dissected from mature leaves were cultured. Our results provide the first successful report of organogenesis induction and reproducible plant regeneration from both petiole and laminae of mature leaves of *A. correntina*. This finding raises the possibility of achieving plant regeneration from mature leaf-tissues from other species of *Arachis*.

The most significant finding of our research was that TDZ supplementation of the medium was essential for plant regeneration. Media containing combinations of 2,4-D or PIC, or NAA with KIN or BA failed completely and only produced calli.

The effect of TDZ in inducing plant regeneration in immature tissues of *Arachis hypogaea* was reported previously, however, in these reports juvenile explants were used. Seedlings grown in the presence of TDZ formed somatic embryos (Murch and Saxena 1997; Murch and others 1999; Murthy and others 1995; Saxena and others 1992) as well as multiple shoots when explants derived from young plantlets (8 days old) were used (Kanyand and others 1994, 1997). Media with TDZ also promoted organogenesis and plant regeneration by culturing immature cotyledons and embryo axes of *Arachis hypogaea* (Akasaka and others 2000; Gill and Ozias-Akins 1999). The optimal TDZ concentration for peanut shoot organogenesis was reported to be in a range of 0.5–150 µM TDZ (Akasaka and others 2000; Gill and Ozias-Akins 1999; Kanyand and others 1994). Our results are in agreement with these works and extend findings to mature tissues.

The histological observations suggest that shoots developed following a pattern of organogenesis were similar to that previously described (Thorpe 1980). This study revealed that the development of buds and shoots was normal. Abnormal shoot

morphologies associated with the use of TDZ in peanut (Akasaka and others 2000) and in other plant species (Huettemann and Preece 1993) were absent in our experiments.

In conclusion, the regeneration protocol developed in the present study consisted of three stages: (1) bud induction, by *in vitro* culture of explants dissected from mature leaves on MS + 25 μM TDZ or by *in vitro* culture of petiole on MS + 40 μM TDZ; (2) shoot elongation by subculture of calli and buds on MS + 5 μM TDZ and (3) rooting of shoots on MS + 0.5 or 5 μM NAA.

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REFERENCES

Akasaka Y, Daimon H, Mii M. 2000. Improved plant regeneration from cultured leaf segments in peanut (*Arachis hypogaea* L.) by limited exposure to thidiazuron. Plant Science 156:169–175.

Burtnik OJ, Mroginski LA. 1985. Regeneración de plantas de *Arachis pintoi* (*Leguminosae*) por cultivo in vitro de tejidos foliares. Oléagineux 40:609–611.

Cheng M, Hsi DCH, Phillips GC. 1992. *In vitro* regeneration of Valencia-type peanut (*Arachis hypogaea* L) from cultured petiolules and other seedling explant. Peanut Science 19:82–87.

Evans DA, Sharp WR, Flick CE. 1981. Growth and behavior of cell cultures: embryogenesis and organogenesis In: Thorpe TA Plant tissue culture. Methods and applications in agriculture New York: Academic Press. pp 45–113.

Gill R, Ozias-Akins P. 1999. Thidiazuron-induced highly morphogenic callus and high frequency regeneration of fertile (*Arachis hypogaea* L.) plants. In Vitro Cell Dev Biol Plant 35:445–450.

Gonzalez AM, Cristóbal CL. 1997. Anatomía y ontogenia de semillas de *Helicteres lhotzkyana* (*Sterculiaceae*). Bonplandia 9:287–294.

Huetteman CA, Preece JE. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. Plant Cell Tissue Organ Culture 33:105–119.

Jähne A, Becker D, Lörz H. 1995. Genetic engineering of cereal crop plants: a review In: Cassells AC, Jones PW The methodology of plant genetic manipulation: criteria for decision making Dordrecht: Kluwer Academic Publ. pp 85: 1–3reprinted for Euphytica.

Jauhar PP. 2001. Genetic engineering and accelerated plant improvement: opportunities and challenges. Plant Cell Tissue Organ Cult 64:87–91.

Kanyand M, Dessai AP, Prakash CS. 1994. Thidiazuron promotes high frequency regeneration of peanut (*Arachis hypogaea*) plants *in vitro*. Plant Cell Reports 14:1–5.

Kanyand M, Peterson CM, Prakash CS. 1997. The differentiation of emergences into adventitious shoots in peanut *Arachis hypogaea* (L). Plant Science 126:87–95.

Krapovickas A, Gregory WC. 1994. Taxonomía del género *Arachis* (*Leguminosae*). Bonplandia 8:1–186.

Luque R, Sousa HC, Kraus JE. 1996. Métodos de coloracao de Roeser (1972) e Krapp visando a subtituicao do azul do astra de alcico 8 GS ou 8 GX. ActaBotBras 10:199–212.

Mansur E, Lacorte C, Rabello ACG, Cordeiro AR. 1993. *In vitro* regeneration of *Arachis villosulicarpa* Hehne from cotyledon segments, leaves and cell suspension. Pesq Agropec Bras 28:1143–1146.

McKently AH, Moore GA, Gardner FP. 1991. Regeneration of peanut and perennial peanut from cultured leaf tissue. Crop Sci 31:833–837.

Mithila J, Murch SJ, Krishnaraj S, Saxena PK. 2001. Recent advances in *Pelargonium in vitro* regeneration systems. Plant Cell Tissue Organ Culture 67:1–9.

Mroginski LA, Katha KK, Shyluk JP. 1981. Regeneration of peanut (*Arachis hypogaea*) by *in vitro* culture of immature leaves. Can J Bot 59:826–830.

Mroginski LA, Kartha KK. 1984. Tissue culture of legumes for crop improvement. Plant Breeding Reviews 2:215–264.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Phys Plant 15:473–497.

Murech SJ, Saxena PK. 1997. Modulation of mineral and free fatty acid profiles during thidiazuron mediated somatic embryogenesis in peanut (*Arachis hypogaea* L.). J Plant Physiol 151:358–361.

Murch SJ, Victor JMR, Krishnaraj S, Saxena PK. 1999. The role of proline in thidiazuron-induced somatic embryogenesis of peanut. In Vitro Cell Dev Plant 35:102–105.

Murthy BNS, Murch SJ, Saxena PK. 1995. Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea*): endogenous growth regulator levels and significance of cotyledons. Phys Plant 94:268–276.

Murthy BNS, Murch SJ, Saxena PK. 1998. Thidiazuron: a potent regulator of *in vitro* plant morphogenesis. In Vitro Cell Dev Biol Plant 34:267–275.

Pittman RN, Banks DJ, Kirby JS, Mitchell ED, Richardson PE. 1983. *In vitro* culture of immature peanut (*Arachis* spp.) leaves: morphogenesis and plantlet regeneration. Peanut Sci 10:21–25.

Rey HY, Scocchi AM, Gonzalez AM, Mroginski LA. 2000. Plant regeneration in *Arachis pintoi* (*Leguminosae*) through leaf culture. Plant Cell Reports 19:856–862.

Saxena PK, Malik KA, Gill R. 1992. Induction by thidiazuron of somatic embryogenesis in intact seedlings of peanut. Planta 187:421–424.

Seitz MH, Stalker HT, Green CC. 1987. Genetic variation for regenerative response in immature leaflet cultures of the cultivated peanut, *Arachis hypogaea*. Plant Breeding 98:104–110.

Thorpe TA. 1980. Organogenesis *in vitro*, structural, physiological and biochemical aspects. Rev Cytol 11A:71–111.

Utomo SD, Weissinger AK, Isleib TG. 1996. High efficiency peanut regeneration using a nonimbibed immature leaflet culture method. Peanut Science 23:71–75.

Vega M, Bontoux L, Llobell A. 1999. Biotechnology for environmentally safe agriculture. In Vitro Cell Dev Biol Plant 35:432–435.