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Organogenesis and plant regeneration of *Arachis villosa* Benth. (Leguminosae) through leaf culture

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Key words: *in vitro* cultivation, leaflet explants, micropropagation, thidiazuron, wild groundnut

ABSTRACT: With the aim of developing an efficient plant regeneration protocol, leaflet explants of three accessions of *Arachis villosa Benth.* (S2866, S2867 and L97) were cultured on basic Murashige and Skoog medium supplemented with different combinations of plant growth regulators: α-naphthalenacetic acid, indole-3-butyric acid, 6-benzylaminopurine, kinetin and thidiazuron. The accession L97 was the only one able to differentiate buds through indirect organogenesis. The most suitable combination for bud regeneration was the basic medium added with 13.62 μM thidiazuron and 4.44 μM 6-benzylaminopurine. These results show the important role of the genotype in morphogenetic responses and the organogenetic effect of thidiazuron in *Arachis villosa* accession L97. A thidiazuron lacking media (only 0.54 μM α-naphthalenacetic acid, 13.95 μM kinetin and 13.32 μM 6-benzylaminopurine were added) promoted the elongation of the regenerated buds. Adventitious rooting was achieved 90 days after the isolated shoots were transferred to a rooting medium containing 0.54 μM α-naphthalenacetic acid.

Introduction

Arachis villosa Benth. (2n=2x=20) is a perennial legume of forage interest (Fernández *et al*., 1998). This species inhabits dunes and sandy grounds of Argentina and Uruguay, along the Uruguay River and in the Uruguayan coast of the La Plata River (Krapovickas and Gregory, 1994).

The greatest interest of the search, rescue, multiplication and characterization of *Arachis* wild species resides in the fact that they contain useful genes for the improvement of the cultivated peanut (*Arachis hypogaea*) (Stalker and Moss, 1987). *A. villosa*, in particular, has high oil content, is drought tolerant and shows resistance to tikka disease (*Cercosporidium personatum*) and insect pests (Cherry, 1977; Subramanyam *et al*., 1985). At the same time, *Arachis* seeds are considered as sub-orthodox and, even under optimal store conditions in seed banks, they may lose viability, impair renewal and be led to the loss of valuable germplasm (Dunbar *et al*., 1993). Therefore, *in vitro* preservation techniques, which are based on micropropagation procedures, are considered important for the recovery, multiplication, and distribution of wild species of this genus (Pacheco *et al*., 2008). In this way, the development of suitable protocols for plant regeneration is one of the main prerequisites for the genetic improvement of crop plants and germplasm conservation using biotechnological methods (Mroginski and Kartha, 1984; Roca *et al*., 1993).

At the moment only two reports exist on *in vitro* regeneration of *A. villosa*; one from anthers and pollen grains culture (Bajaj *et al*., 1981), and the other from shoot base-derived callus cultures (Vijaya Laxmi and Giri, 2003), both with a limited plant regeneration effi-

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ciency. The objective of the present work was to develop an efficient protocol for the regeneration of *A. villosa* plants through leaflet culture.

Materials and Methods

Plant Material

Plants of three *Arachis villosa* accessions (Table 1) were used in this study. The first fully expanded leaflets of plants growing in greenhouse conditions were employed as explant source. The leaflets were surface-sterilized by immersion in 70% ethanol (30 s) followed by immersion in a solution of commercial bleach (0.9% NaOCl, final concentration) plus one drop of Tween 20® (12 min) and then rinsed three times with autoclaved distilled water.

Experimental media and culture conditions

The basic medium used was a salts and vitamins solution (according to Murashige and Skoog, 1962) to which 3% sucrose and 0.65% agar (Sigma Chemical Co) were added. Either no plant growth regulators, or various concentrations and combinations of α-naphthalenacetic acid (0.054, 0.54, 5.4 μM), indole-3-butyric acid (0.049; 0.49; 4.9 μM), 6-benzylaminopurine (4.44; 13.32; 22.20; 26.64; 44.40 μM), kinetin (4.65; 13.95; 27.90 μM) and thidiazuron (4.54; 13.62; 27.24 μM) were added to the basic medium, pH was adjusted to 5.8 with KOH or HCl prior to adding the agar. The tubes were covered with aluminium foil and autoclaved 20 min at 0.101 MPa. One square of approximately 3 mm^2 of the laminae was placed with the abaxial side down in an 11 cm^3 glass tube with 3 cm^3 of medium. The tubes were covered with Resinite AF-50® (Casco S.R.L. Company, Buenos Aires). Cultures were incubated at 27 ± 2ºC during a 14 h photoperiod (116 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes). Shoots were obtained when the regenerated buds were transferred to an elongation media to which 0.54 μM α-naphthalenacetic acid, 13.95 μM kinetin and 13.32 μM 6-benzylaminopurine were added.

In each treatment ten replicates were taken and each experiment was repeated three times. Data were transformed ($y = 0.5 + x^{0.5}$) and analyzed by one-way ANOVA followed by Duncan post-test (0.05% significance level) using the *Infostat* software (InfoStat, 2008).

Rooting, transfer to soil and hardening

For root induction, the regenerated shoots obtained through organogenesis were transferred to a medium to which 0.54 μM α-naphthalenacetic acid was added. The regenerated plantlets were rinsed gently under running tap water to remove the culture medium and immediately planted in pots containing a mixture of soil and sand (1:1) (v/v). The potted plants were acclimatized and subsequently moved to the greenhouse.

Results

After 90 days of culture in the media containing either α-naphthalenacetic acid alone or in combination with 6-benzylaminopurine, oxidation (blackening of explants due to oxidation of phenolic compounds) was the only response in accessions S2867 and L97. In accession S2866, besides oxidized explants (7-26%), there were friable-green colour calluses with (8-47%) and without (53-100%) roots (Table 2). The use of indole-3-butyric acid and 6-benzylaminopurine, either alone or combined, induced callus production in accessions

TABLE 1.

List of plant material of *Arachis villosa* employed in this study.

Accession[a]	Collector	Place of collection
S2866	G. Seijo and V. Solís Neffa	Laguna Mansa, Paso de los Libres, Corrientes (Argentina)
S2867	G. Seijo and V. Solís Neffa	Parque Perón, Paso de los Libres, Corrientes (Argentina)
L97	P. Millot	Cultivated in greenhouse of IBONE Origin: Uruguay

[a]All the herbarium specimens are deposited in CTES

TABLE 2.

Morphogenetic responses of leaflet explants of three accessions of *Arachis villosa* (S2866, S2867 and L97) after 90 days of culture.

Plant Growth Regulators (µM)			Morphogenetic responses (%)											
			S2867				S2866				L97			
NAA	IBA	BAP	Oxidation	Callus only	Callus + roots	Callus + buds	Oxidation	Callus only	Callus + roots	Callus + buds	Oxidation	Callus only	Callus + roots	Callus + buds
0.054	-	-	100	0	0	0	26±2.6	66±1.7	8±2	0	100	0	0	0
0.54	-	-	100	0	0	0	0	100	0	0	100	0	0	0
5.4	-	-	100	0	0	0	19±9.6	59±4	22±6.1	0	100	0	0	0
-	0.049	-	71±8.1	29±8.1	0	0	89±8.1	11±8.1	0	0	100	0	0	0
-	0.49	-	100	0	0	0	69±5	31±5	0	0	100	0	0	0
-	4.9	-	100	0	0	0	76±5.3	24±5.3	0	0	100	0	0	0
-	-	4.44	100	0	0	0	45±6	55±5	0	0	100	0	0	0
-	-	13.32	100	0	0	0	16±3.6	84±3.6	0	0	100	0	0	0
-	-	22.20	100	0	0	0	77±2.9	23±2.9	0	0	100	0	0	0
-	-	44.40	100	0	0	0	72±2.9	28±2.9	0	0	100	0	0	0
0.054	-	4.44	100	0	0	0	0	100	0	0	100	0	0	0
0.054	-	13.32	100	0	0	0	0	100	0	0	100	0	0	0
0.054	-	22.20	100	0	0	0	0	100	0	0	100	0	0	0
0.054	-	44.40	100	0	0	0	0	100	0	0	100	0	0	0
0.54	-	4.44	100	0	0	0	0	100	0	0	100	0	0	0
0.54	-	13.32	100	0	0	0	0	100	0	0	100	0	0	0
0.54	-	22.20	100	0	0	0	0	100	0	0	100	0	0	0
0.54	-	44.40	100	0	0	0	0	100	0	0	100	0	0	0
5.4	-	4.44	100	0	0	0	0	73±2.6	27±2.6	0	100	0	0	0
5.4	-	13.32	100	0	0	0	0	53±8.1	47±8.1	0	100	0	0	0
5.4	-	22.20	100	0	0	0	0	80±8.7	20±8.7	0	100	0	0	0
5.4	-	44.40	100	0	0	0	7±2.5	81±1.5	11±1.2	0	100	0	0	0
-	0.049	4.44	3±1	97±1	0	0	12±1.5	88±1.5	0	0	100	0	0	0
-	0.049	13.32	6±1.8	95±1.8	0	0	36±7.9	64±7.9	0	0	100	0	0	0
-	0.049	22.20	29±7.4	72±7.4	0	0	22±4.9	78±4.9	0	0	100	0	0	0
-	0.049	44.40	38±7.6	62±7.6	0	0	35±13.2	65±13.2	0	0	100	0	0	0
-	0.49	4.44	8±2.5	93±2.5	0	0	34±3.6	66±3.6	0	0	100	0	0	0
-	0.49	13.32	14±1.7	86±1.7	0	0	21±3.6	79±3.6	0	0	100	0	0	0
-	0.49	22.20	12	88	0	0	11±1.2	89±1.2	0	0	100	0	0	0
-	0.49	44.40	19±3.2	81±3.2	0	0	25±5	75±5	0	0	100	0	0	0
-	4.9	4.44	20±4.8	81±4.8	0	0	22±2	78±2	0	0	100	0	0	0
-	4.9	13.32	6±1.5	94±1.5	0	0	27±5	73±5	0	0	100	0	0	0
-	4.9	22.20	6±2.6	94±2.6	0	0	24±4.7	76±4.7	0	0	100	0	0	0
-	4.9	44.40	87±6.6	13±6.6	0	0	64±6.4	36±6.4	0	0	100	0	0	0

Each value represents mean ± SD
NAA= α-naphthalenacetic acid
IBA=indole-3-butyric acid
BAP=6-benzylaminopurine

S2866 and S2867 whereas in L97 accession 100% of the explants were oxidized and no bud or shoot differentiation occurred (Table 2).

The effect of the addition of another cytokinin (kinetin) to the combination of α-naphthalenacetic acid and 6-benzylaminopurine was evaluated, and results showed that the employed plant growth regulators did not induce bud regeneration. The tested media offered three types of response in the accession S2866: explant oxidation between 47 and 86%; callus in 14% and callus + roots between 18 and 53%. In L97 accession all the explants became oxidized (Table 3).

In the media thidiazuron and 6-benzylaminopurine were added, a high percentage of explants from accessions S2866 and S2867 became oxidized (80-90%), and the regenerated callus never differentiated buds, even after three subcultures. With several combinations of these growth regulators, accession L97 showed organogenetic capacity, being this genotype the only one able to produce callus + buds (Fig. 1A). Initially, the explants showed two types of responses: (1) callus formation without buds (3-33%) and (2) oxidized explants (67-100%). After 30 days of subculture in the same media, the calluses of five treatments (4.54 μM thidiazuron + 13.32 μM 6-benzylaminopurine, 4.54 μM thidiazuron + 26.64 μM 6-benzylaminopurine, 13.62 μM thidiazuron + 4.44 μM 6-benzylaminopurine, 13.62 μM thidiazuron + 13.32 μM 6-benzylaminopurine and 27.24 μM thidiazuron + 4.44 μM 6-benzylaminopurine) regenerated shoot buds. The other plant growth regulator combinations only induced callus formation (Fig. 2).

The maintenance of the organogenetic calluses in the original media did not make possible the bud elongation. Only when the calluses were transferred to a thidiazuron lacking media composed by the basic medium supplemented with 0.54 μM α-naphthalenacetic acid, 13.32 μ M 6-benzylaminopurine and 13.95 μM kinetin, bud elongation occurred and shoots were obtained (Fig. 1B).

Adventitious rooting of the isolated shoots occurred 90 days after transference to a media composed by 0.54 μM α-naphthalenacetic acid (Fig. 1C). Four-week-old rooted shoots were transplanted to soil contained in pots (Fig. 1D, E).

Discussion

The responses achieved by culturing leaf explants in media with α-naphthalenacetic acid and α-naphthalenacetic acid + 6-benzylaminopurine differ from those reported for other legumes. In *Arachis pintoi*, the combination of these plant growth regulators on mature leaves made possible plant regeneration through orga-

TABLE 3.

Morphogenetic responses of leaflet explants of two accessions of *Arachis villosa* (S2866 and L97) after 90 days of culture.

Plant Growth Regulators (µM)			Morphogenetic responses (%)							
			S2866				L97			
NAA	BAP	KIN	Oxidation	Callus only	Callus + roots	Callus + buds	Oxidation	Callus only	Callus + roots	Callus + buds
0.54	4.44	4.65	82±7.6	0	18±7.6	0	100	0	0	0
0.54	13.32	13.95	100	0	0	0	100	0	0	0
0.54	26.64	27.9	86±6.5	14±6.5	0	0	100	0	0	0
5.4	4.44	4.65	47±5.8	0	53±5.8	0	100	0	0	0
5.4	13.32	13.95	57±2.9	0	43±2.9	0	100	0	0	0
5.4	26.64	27.9	67±2.5	0	33±2.5	0	100	0	0	0

Each value represents mean ±SD
NAA= α-naphthalenacetic acid
BAP=6-benzylaminopurine
KIN=kinetin

nogenesis (Burtnik and Mroginski, 1985). In the same manner, in *Desmodium uncinatum* and *Desmodium affine,* buds were differentiated when a dose of 26.64 μM of 6-benzylaminopurine was used in combination with α-naphthalenacetic acid (0.054; 0.54; 5.4 μM) (Rey and Mroginski, 1997). However, the results obtained with accession S2866 (Table 2) resembled the data reported by Rey and Mroginski (1996) for *Aeschynomene sensitiva*, in which the culture of leaf portions in media containing basic medium + 5.4 μM α-naphthalenacetic acid or in combination with 0.44 or 0.044 μM 6-benzylaminopurine produced only calluses and roots.

Coincidences were not found between the results obtained with *Arachis hypogaea* (Venkatachalam *et al*., 1996) and those achieved with *Arachis villosa* in the present study. Media with several doses of α-naphthalenacetic acid, 6-benzylaminopurine and kinetin induce shoot bud regeneration in peanut (*A. hypogaea*), a response which was not reached in *A. villosa* cultures in the current study.

The incidence of genotype in the morphogenetic responses and the organogenic effect of thidiazuron were demonstrated with the results obtained with this plant growth regulator in *A. villosa*, where only the accession L97 was able to regenerate buds. Besides culture conditions, genotype plays a major role in culture response (Mroginski *et al*., 1981; McKently *et al*., 1991) and it can explain the differential plant growth regulator requirements by varieties of one plant species (Banerjee *et al*., 2007). Morphogenesis using thidiazuron was reported in cultures of *A. hypogaea*, in which explants derived of 8 days-old plantlets produced multiple shoot

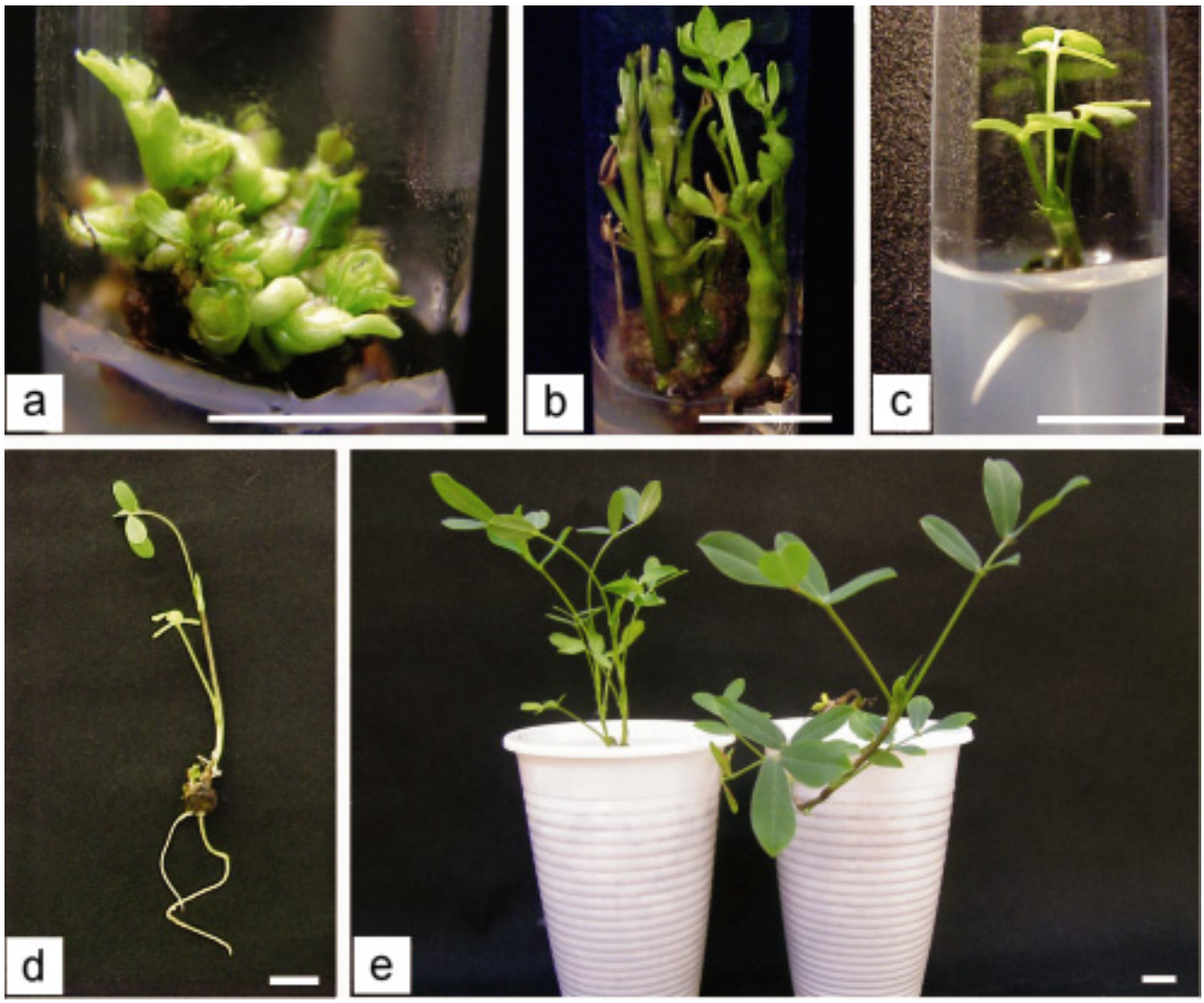

FIGURE 1. *In vitro* plant regeneration of *Arachis villosa* accession L97 from leaflet culture. (A) Bud regeneration. (B) Bud elongation. (C) Rooted shoot. (D) Plantlet obtained by rooting a regenerated shoot. (E) Plants growing in soil. Bar = 1 cm.

primordia (Kanyand *et al*., 1994, 1997), and in *Arachis correntina*, in which the culture of leaves regenerate organogenic callus (Mroginski *et al*., 2004).

Several authors have indicated that optimal concentrations of thidiazuron for caulinary organogenesis of peanut range from 0.45 to 136.2 μM (Kanyand *et al*., 1994; Gill and Ozias-Akins, 1999; Akasaka *et al*., 2000), within which are those that made possible the formation of buds in *A. villosa*. The dose of thidiazuron is critical in regeneration studies. In *Cajanus cajan*, lower concentrations of thidiazuron induces multiple shoots while a higher concentration completely switches the regeneration pathway by inducing a cluster of well formed somatic embryos instead of shoot formation at the cotyledonary nodal region (Singh *et al*., 2003). In *Echinacea purpurea*, the same concentration of thidiazuron induces two kinds of regeneration responses: somatic embryos and shoots (Jones *et al*., 2007).

Most works on *Arachis* genus have reported organogenesis obtained from explants cultivated on media containing auxins and cytokinins (Bajaj *et al*., 1981; Burtnik and Mroginski, 1985; McKently *et al*., 1991; Cheng *et al*., 1992; Chengalrayan *et al*., 2001); other reports have informed plant regeneration of some *Arachis* species and other legumes using only cytokinins, but the majority of these studies used highly meristematic tissues as explant source: seeds, embryonic axes, cotyledons and hypocotyls (Gagliardi *et al*., 2000; Gill and Ozias-Akins, 1999; Radhakrishnan *et al*., 2000; Singh *et al*., 2003).

The results obtained in this investigation are similar to those obtained by Vijaya Laxmi and Giri (2003) where the combination of thidiazuron and 6-benzylaminopurine managed to regenerate plants of *Arachis stenosperma* and *A. villosa* from shoot-base derived callus. Also, the use of two cytokinins (6-benzylaminopurine and kinetin) induced caulinary organogenesis in leaf explants of *A. hypogaea* (Sarker and Islam, 2000).

Regenerated buds elongation was possible on Murashige and Skoog media supplemented with 0.54 μM α-naphthalenacetic acid, 13.32 μM 6-benzylaminopurine and 13.95 μM kinetin devoid of thidiazuron (Fig. 1B). Shoot buds induced on thidiazuron containing media were clustered with only slight or no elongation. This effect was observed in several species when organogenesis was achieved with thidiazuron (Ahmad *et al*., 2006; Gill and Ozias-Akins, 1999; Pelah *et al*., 2002). Cytokinins usually stimulate shoot proliferation and inhibit their elongation. Thidiazuron, unlike the rest of the cytokinins containing adenine in their chemical structure, is a substance derived from the fenilurea that in addition to its cytokinin activity has a wide spectrum of responses to the *in vitro* culture (Mok *et al*., 1987), inducing organogenic responses in lower concentrations than the adenine-type cytokinins. Therefore, inhibition of shoot elongation by thidiazuron may be consistent with its high

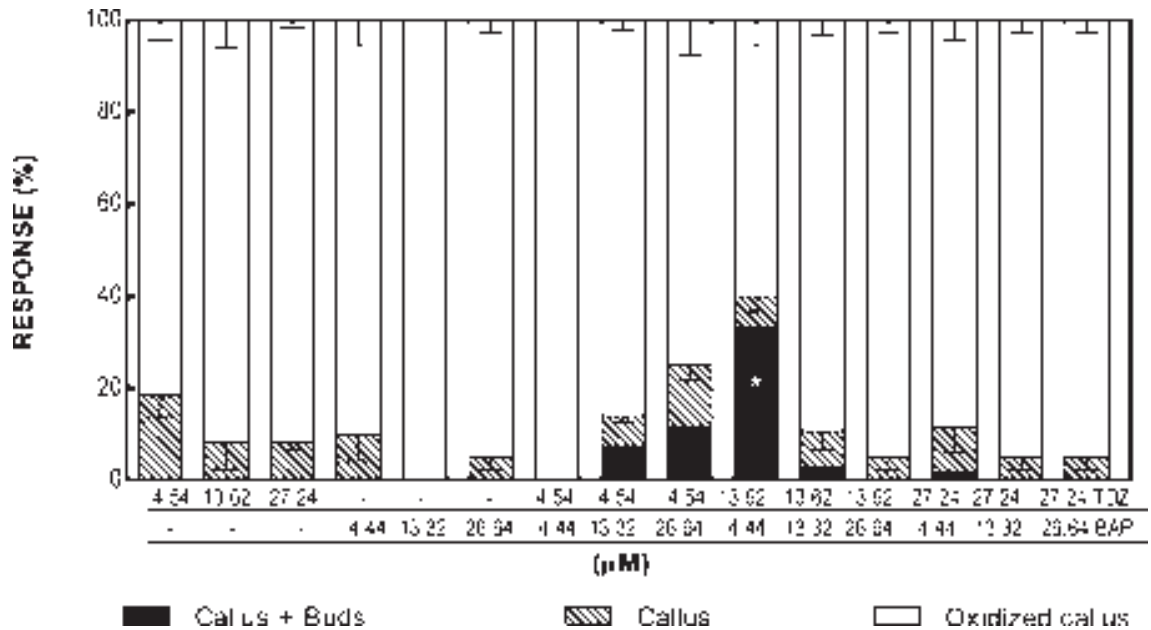

FIGURE 2. Effect of subculture in the differentiation of buds of *Arachis villosa* L97. Responses after 30 days (120 days from the initial culture). Bar represents mean ± SE.
* indicates significant difference (P=0.05) among treatments respect to callus + buds morphogenetic response. TDZ=thidiurazon, BAP=6-benzylaminopurine.

cytokinin activity and should not be considered a toxic effect (Huetteman and Preece, 1993). These authors mention that the problem of shoot elongation can be overcome by transfer of shoot cultures to a secondary medium often lacking thidiazuron or with a different balance of plant growth regulators. In this case, an auxin was incorporated to the medium and thidiazuron was replaced by two cytokinins with less activity.

In conclusion, our results indicate that is possible to regenerate plants of accession L97 of *Arachis villosa* through leaf culture by culturing portions of the first fully expanded leaf in a medium with 13.62 μM thidiazuron and 4.44 μM 6-benzylaminopurine. Then the differentiated buds must be transferred for their elongation to a medium to which 0.54 μM α-naphthalenacetic acid, 13.95 μM kinetin and 13.32 μM 6-benzylaminopurine were added. Finally, for root induction, isolated shoots have to be cultured in a rooting media.

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