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In vitro Propagation of *Solanum sessiliflorum* as Affected by Auxin and Cytokinin Combinations and Concentrations

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Abstract: This study aimed to establish a protocol for the micropropagation of the cubiu (*Solanum sessiliflorum*) varieties Santa Luzia (SL) and Thaís (TH). Firstly, nodal segments were cultured onto MS-based medium supplemented with vitamins, mio-inositol (200 mg L^{-1}), glycine (400 mg L^{-1}), sucrose (30 g L^{-1}) and agar (6.5 g L^{-1}), plus NAA (0, 0.25 and 0.5 mg L^{-1}) and BAP (0, 1.0, 2.0 and 3.0 mg L^{-1}). In the second experiment, nodal segments were placed on MS medium, plus vitamins, sucrose (15 g L^{-1}), agar (6.5 g L^{-1}) and IAA (0 and 0.01 mg L^{-1}) in combination with KIN, TDZ or ZEA at 0, 0.64, 1.25, 2.50, 5.0, 10.0 and 20.0 mg L^{-1}. The combinations of NAA and BAP inhibited shoot-bud growth and maintenance of the apical dominance. High TDZ concentrations induced prolific shoot formation with the greatest number of axillary shoots, although with indefinite morphology in many cases. The combination of 0.01 mg L^{-1} IAA and 5.0 mg L^{-1} TDZ induced the most intense axillary shoot proliferation in SL, whereas 2.5 mg L^{-1} TDZ and 0.01 mg L^{-1} IAA, combined with 1.25 mL^{-1} TDZ, were most effective for adventitious shoot-bud differentiation. For TH, the proliferation of axillary and adventitious buds was improved in the treatment with 5.0 mg L^{-1} TDZ and 2.5 mg L^{-1} TDZ, respectively. After 60 days of cultivation, the adventitious and axillary shoot-buds on MS-based medium lacking growth regulators were 4.2±0.32 cm long and the plants presented a well-developed root system.

Key words: Organogenesis, Amazonian species, axillary buds, adventitious buds, growth regulators

INTRODUCTION

Cubiu (*Solanum sessiliflorum* Dunal) is a native Amazonian species, common in the Brazilian, Peruvian, Colombian and Venezuelan Amazon (Rascio *et al*., 2002; Volpato *et al*., 2004). Recently, the market price of cubiu has been risen (Silva Filho *et al*., 2005) and cubiu has been introduced in other regions of Brazil (Brancher and Tagliari, 2004; Pires *et al*., 2006).

For modern agro-industry, on the search for multifunctional raw material, cubiu appears as a promising crop with its fruits of pleasant taste and flavor, displaying a versatile use for food and cosmetics, besides phytotherapic properties with high vitamin and mineral salt concentrations (Silva Filho *et al*., 2003; Yuyama *et al*., 2007). Moreover, it is a high-yielding, easy-to-cultivate annual plant and depending on the genotype produces 4 to 89 fruit number per plant, e fruit weight from 18.5 to 301 g (Silva Filho *et al*., 2005).

Due to the rise of the economic value of the fruits (Silva Filho *et al*., 2005) and the adaptation of particular genotypes to conditions of milder temperature and relative humidity, the species has also become interesting for farmers in the South (Brancher and Tagliari, 2004) and Southern (Pires *et al*., 2006) of Brazil as an excellent new agricultural option. However, the species is allogamy (Storti, 1988; Luz *et al*., 2008) and germination of seeds is dependent on temperature conditions and specific substrates used for planting (Lopes and Pereira, 2005). In this context, a reliable methodology must be established to supply homogenous and healthy propagative material in sufficient amounts to meet the demand of agro-industrial production and to allow the conservation of cubiu germplasm. In this context, tissue culture is an alternative to the *in vitro* propagation technique and is being applied in various species of agrosilvicultural interest (Ledo *et al*., 2001; Alves *et al*., 2004; Wendt *et al*., 2008).

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The studies focusing on *in vitro* propagation of cubiu (Hendrix *et al*., 1987; Cordeiro and Mattos, 1991) provide no information on the number and average sizes of the shoot-buds and elongated shoots. Besides, the varieties Santa Luzia (SL) and Thaís (TH) developed and traded at the Experimental Station Santa Luzia/Guareí (SP), Brazil and currently under evaluation for the cultivation potential in the states of the South and Southeast of Brazil, have not yet been evaluated for their *in vitro* morphogenic potential.

Present study focused on the establishment of a protocol for *in vitro* regeneration of cubiu by the evaluation of the morphogenetic response of nodal segments of the varieties SL and TH to different auxin and cytokinin concentrations.

MATERIALS AND METHODS

Single nodal segments derived from elongated adventitious shoot-buds of the two cubiu varieties, namely Santa Luzia (SL) and Thaís (TH), were used as explants; they were grown for 30 days in culture medium without growth regulators, induced by direct organogenesis following (Hendrix *et al*., 1987) methodology in MS (Murashige and Skoog, 1962) culture medium.

Two successive experiments were undertaken in 2005 at the Laboratory of Biotechnology of the University Paranaense, Toledo (PR). Experiment I was aimed to study the effect of naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) on the morphogenetic response in nodal segments. Thus, nodal segments (average 0.5 cm) were placed in 500 mL glass flasks containing 50 mL of MS culture medium, enriched with vitamins (0.5 mg L^{-1} pyridoxine HCl; 0.5 mg L^{-1} nicotinic acid and 0.1 mg L^{-1} thiamine HCl), glycine (2 mg L^{-1}), mio-inositol (200 mg L^{-1}), sucrose (15 g L^{-1}) and 6.5 g L^{-1} granulated bacteriological agar and different NAA (0, 0.25 and 0.5 mg L^{-1}) and BAP concentrations (0, 1.0, 2.0 and 3.0 mg L^{-1}). The pH was adjusted to 5.7, previously to autoclaving.

The experimental design was completely randomized, with three replications per treatment. The experimental units consisted of six explants per flask that were sealed with transparent PVC film (Goodyear, Brazil). Unless otherwise stated, cultures were maintained at 24±2°C under a 16/8 h light/dark regime with 12.6 μmol/m^2/sec light radiation provided by two fluorescent lamps (Osram®). The formation of shoot-buds, roots and calli and the axillary shoot-bud length were evaluated after 30 days of cultivation. The data of shoot-bud length were subjected to analysis of variance and the method of Scott-Knott (5%) using the software package GENES (Cruz, 2001).

Table 1: Culture medium and concentrations of indolacetic-3-acid (IAA), kinetin (KIN), thidiazuron (TDZ) and zeatin (ZEA) used to evaluate the morphogenetic responses in nodal explants of the cubiu varieties Santa Luzia and Thaís

Culture medium	Growth regulators (mg L^{-1})
M1	-*
M2	IAA 0.01
M3	KIN 0.64
M4	KIN 1.25
M5	KIN 2.50
M6	KIN 5.0
M7	KIN 10.0
M8	KIN 20.0
M9	IAA 0.01 + KIN 0.64
M10	IAA 0.01 + KIN 1.25
M11	IAA 0.01 + KIN 2.50
M12	IAA 0.01 + KIN 5.0
M13	IAA 0.01 + KIN 10.0
M14	IAA 0.01 + KIN 20.0
M15	TDZ 0.64
M16	TDZ 1.25
M17	TDZ 2.50
M18	TDZ 5.0
M19	TDZ 10.0
M20	TDZ 20.0
M21	IAA 0.01 + TDZ 0.64
M22	IAA 0.01 + TDZ 1.25
M23	IAA 0.01 + TDZ 2.50
M24	IAA 0.01 + TDZ 5.0
M25	IAA 0.01 + TDZ 10.0
M26	IAA 0.01 + TDZ 20.0
M27	ZEA 0.64
M28	ZEA 1.25
M29	ZEA 2.50
M30	ZEA 5.0
M31	ZEA 10.0
M32	ZEA 20.0
M33	IAA 0.01 + ZEA 0.64
M34	IAA 0.01 + ZEA 1.25
M35	IAA 0.01 + ZEA 2.50
M36	IAA 0.01 + ZEA 5.0
M37	IAA 0.01 + ZEA 10.0
M38	IAA 0.01 + ZEA 20.0

*Control

In experiment II, nodal segments (average 0.5 cm) were transferred to test tubes (190×30 mm) containing 10 mL complete MS medium plus vitamins (0.5 mg L^{-1} pyridoxine HCl; 0.5 mg L^{-1} nicotinic acid and 0.1 mg L^{-1} thiamine HCl), glycine (2 mg L^{-1}), mio-inositol (200 mg L^{-1}), sucrose (15 g L^{-1}) and agar (6.5 g L^{-1}) and different concentrations of IAA, KIN, thidiazuron (TDZ) and zeatin (ZEA) (Table 1). The pH of the culture media was adjusted to 5.7 before autoclaving and the test tubes sealed with PVC film. All tubes were incubated at 24±2°C under Osram® 75W cool white fluorescent lamps providing a 16 h photoperiod and a light intensity of 12.6 μmol/m^2/sec at culture level.

The experimental design was completely randomized in a factorial arrangement (2 varieties and 38 treatments), with three replications per treatment. Each experimental unit consisted of five test tubes. The callogenic and rhizogenic responses and shoot-bud length were assessed after 30 and 60 days of growth. The intensity of the morphogenetic response (absence, low, moderate and

high) in callogenesis and rhizogenesis was evaluated. The data of shoot-bud length were subjected to analysis of variance with the method of Scott-Knott (5% of significance), using the software GENES (Cruz, 2001).

After 60 days of cultivation, responsive explants that displayed shoot-bud formation were transferred to MS medium devoid of growth regulators. After 30 days, the mean number of adventitious and axillary shoot-buds was evaluated. The mean shoot-bud size was measured 30 days after the second subculture.

RESULTS AND DISCUSSION

Effect of NAA and BAP on the morphogenetic response of nodal segments: After 30 days of cultivation, the growth habit in the explants of the varieties SL and TH presented apical dominance. There was no proliferation of axillary buds in any of the evaluated NAA/BAP combinations. For shoot-bud length however, a statistical difference of 5% probability was detected between the treatments tested in both varieties by the analysis of variance.

In the analysis of means (Table 2) the culture medium lacking growth regulators (MW) presented the most intense shoot-bud growth (6.29 and 3.33 cm for SL and TH, respectively). These results demonstrate that NAA and BAP played an inhibitory role in shoot-bud growth and differentiation in cubiu, as confirmed by earlier study carried out at the laboratory, in similar experiments using IAA and KIN (data not shown).

The callus proliferation was null in the growth regulators-free treatments, but prolific in those supplemented with NAA, in accordance with results by Hendrix *et al.* (1987). Cicatricial callogenic responses initiated between 10 and 15 days in the cut surfaces of the explants, regardless the NAA and BAP concentration used. These callus further spread out over the explant area and had a pale yellow color and a semi-friable texture. After some time, there was a marked swelling of the cell masses, thought without adventitious regenerative capacity.

For SL, an intense rhizogenesis occurred in the treatments MW and MV (0.25 mg L^{-1} NAA), whereas for TH this response was observed only in hormone-free nutrient medium (MW) (Table 2). However, morphogenetic responses in cubiu accessions were reported with NAA/BAP combinations (Cordeiro and Mattos, 1991), evidencing that explant source and the NAA and BAP concentration led to callus and root formation. Likewise, nodal segments of *Cissus sicyoides* presented a proliferation which could have been caused by a hormonal imbalance between the endogenous content of the explant and the growth regulator concentration in the culture medium (Abreu *et al.*, 2003).

Since the cytokinin BAP separately or in combination with NAA failed to induce proliferation of axillary buds, other cytokinins were evaluated in experiment II, separately or in combination with IAA, as suggested for cubiu in earlier studies (Hendrix *et al.*, 1987; Cordeiro and Mattos, 1991).

Effect of different cytokinins in combination with auxin on the morphogenetic response of nodal segments: In the analysis of variance for the mean shoot-bud length after 30 and 60 days of cultivation, significant differences were detected for the effects of culture medium, variety and interaction. The grouping of means of the shoot-bud length (Table 3) showed that the varieties differed in the treatments M1 (MS) and M4 (1.25 mg L^{-1} KIN) after 30 and 60 days and in the treatment M9 (0.01 mg L^{-1} IAA + 0.64 mg L^{-1} KIN) after 30 days and M10 (0.01 mg L^{-1} IAA+ 1.25 mg L^{-1} KIN) after 60 days; in these treatments SL presented the best results.

Table 2: Morphogenetic response and mean length of axillary shoot-buds in nodal explants of the varieties Santa Luzia (SL) and Thaís (TH) cultivated on culture media supplemented with different concentrations 6-benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA)

	Callogenesis		Rhizogenesis		Shoot length (cm)	
Culture medium	SL	TH	SL	TH	SL	TH
MW (MS)	-	-	+++	+++	6.29a	3.33a
MW I (MS + 1. 0 BAP)	+	++	-	++	0.70b	0.38b
MW II (MS + 2. 0 BAP)	+	+	-	+	0.51b	0.69b
MW V(MS + 3. 0 BAP)	+	+	-	-	0.70b	0.49b
MV (MS + 0. 25 NAA)	++	+++	+++	+	0.96b	0.32b
MVI (MS + 0. 25 NAA + 1. 0 BAP)	+++	+++	++	-	0.24b	*
MVII (MS + 0. 25 NAA + 2. 0 BAP)	+++	+++	-	-	0.14b	0.19b
MVIII (MS + 0. 25 NAA + 3. 0 BAP)	+++	+++	-	-	0.16b	0.20b
MW V (MS + 0. 5 NAA)	++	+++	+	+	0.36b	0.38b
MX (MS + 0. 5 NAA + 1. 0 BAP)	+++	+++	-	-	0.12b	*
MXI (MS + 0. 5 NAA + 2. 0 BAP)	+++	+++	-	-	*	0.12b
MXII (MS + 0. 5 NAA + 3. 0 BAP)	++	+++	+	+	0.21b	1.45b

Intensity of morphogenetic responses: Absence (-); Low (+); Moderate (++) and High (+++). Equal letter(s) in the columns correspond to equal means by the Scott-Knott test at 5% significance. *Lost observations

Table 3: Influence of culture media (CM) on the morphogenetic responses and means of the axillary shoot-bud length differentiated in nodal explants of the varieties Santa Luzia (SL) and Thaís (TH), after 30 and 60 days of *in vitro* cultivation

	Callogenesis				Rhizogenesis				Mean length (cm)			
	30 days		60 days		30 days		60 days		30 days		60 days	
CM	SL	TH	SL	TH	SL	TH	SL	TH	SL	TH	SL	TH
M1	+	+++	+	+++	+++	-	+++	-	2.87Aa	0.15Ba	6.60Aa	0.20Ba
M2	+	+++	++	+++	++	++	++	++	1.52Ab	1.15Aa	2.87Ab	1.71Aa
M3	+++	++	+++	++	++	+	++	++	1.43Ab	0.60Aa	3.90Ab	2.50Aa
M4	++	+++	++	+++	+	-	+++	-	1.70Ab	0.07Ba	4.27Ab	0.09Ba
M5	++	+++	++	+++	-	-	++	+	0.30Ac	0.17Aa	0.70Ac	0.33Aa
M6	+++	+++	+++	+++	+	-	++	-	0.07Ac	0.09Aa	0.26Ac	0.15Aa
M7	+	+	+	++	+	-	-	-	0.13Ac	0.45Aa	0.23Ac	0.40Aa
M8	-	+++	+	+++	-	-	-	-	0.22Ac	0.08Aa	0.20Ac	0.17Aa
M9	++	+++	+++	+++	+++	++	+++	+++	2.63Aa	0.60Ba	4.33Ab	3.63Aa
M10	+	+++	++	+++	+++	+	+++	+	1.67Ab	1.69Aa	5.80Aa	3.47Ba
M11	+	+++	+	+++	++	+	+++	++	0.17Ac	0.18Aa	2.07Ac	1.13Aa
M12	+++	+++	+++	+++	-	+	+++	+	0.31Ac	0.07Aa	0.27Ac	0.09Aa
M13	++	++	++	++	++	-	+	+	0.09Ac	0.60Aa	0.19Ac	1.15Aa
M14	+	++	+	+++	+	-	+	-	0.23Ac	0.44Aa	0.23Ac	0.44Aa
M15	+	+++	+	+++	-	-	+	-	0.33Ac	0.70Aa	0.40Ac	0.83Aa
M16	+++	+++	+++	+++	-	-	-	-	0.23Ac	0.23Aa	0.23Ac	0.50Aa
M17	+	+	+	+	-	-	-	-	0.20Ac	0.16Aa	0.40Ac	0.57Aa
M18	+	++	+	+++	-	-	-	-	0.29Ac	0.20Aa	0.40Ac	0.40Aa
M19	++	+++	++	+++	-	-	-	-	0.10Ac	0.12Aa	0.20Ac	0.40Aa
M20	+	-	+	+	+	-	+	-	0.16Ac	0.08Aa	0.16Ac	0.33Aa
M21	++	+++	++	+++	+	-	+	-	0.32Ac	0.15Aa	0.32Ac	0.35Aa
M22	+	+++	++	+++	-	-	+	-	0.17Ac	0.07Aa	0.30Ac	0.14Aa
M23	+++	+++	+++	+++	-	-	-	-	0.16Ac	0.32Aa	0.30Ac	0.60Aa
M24	+++	+++	+++	+++	-	-	-	-	0.17Ac	0.12Aa	0.23Ac	0.37Aa
M25	-	++	+	++	+	-	+	-	0.23Ac	0.56Aa	0.40Ac	0.87Aa
M26	+	-	+	+	-	-	-	-	0.32Ac	0.30Aa	0.40Ac	0.63Aa
M27	+	+++	+	+++	-	+	++	+	0.39Ac	0.37Aa	0.69Ac	0.77Aa
M28	++	+++	++	+++	+	+	-	++	0.57Ac	0.35Aa	0.70Ac	0.72Aa
M29	+	++	++	++	-	-	-	++	0.67Ac	0.47Aa	0.87Ac	1.17Aa
M30	+	++	+	++	-	-	-	-	0.67Ac	0.57Aa	1.03Ac	0.90Aa
M31	++	+	++	+	-	+	-	+	0.57Ac	0.73Aa	0.80Ac	1.40Aa
M32	+++	+++	+++	+++	-	-	-	-	0.60Ac	0.57Aa	1.20Ac	0.63Aa
M33	++	+++	++	+++	+	-	+	+	0.52Ac	0.76Aa	0.53Ac	0.70Aa
M34	+	+++	+	+++	-	-	-	-	0.70Ac	0.36Aa	1.23Ac	0.73Aa
M35	+++	+++	+++	+++	-	-	-	-	0.36Ac	0.26Aa	0.43Ac	0.40Aa
M36	+++	+++	+++	+++	+	-	-	-	0.90Ac	0.49Aa	1.53Ac	0.43Aa
M37	++	+++	++	+++	-	-	-	-	0.40Ac	0.40Aa	0.67Ac	0.33Aa
M38	++	+++	+++	+++	+	-	+	-	0.39Ac	0.40Aa	0.70Ac	0.73Aa

Morphogenetic responses: Absence (-); Low (+); Moderate (++) and High (+++). Equal capital letter(s) in a row for each evaluation date correspond to equal means at 5% significance by the F Fisher-Snedecor test and lower case letter(s), in the columns, represent equal means at 5% significance by the Scott-Knott test

For SL, the most intense shoot-bud growth after 30 days was observed in the culture media M1 and M9 (0.01 mg L^{-1} IAA + 0.64 mg L^{-1} KIN), with a mean length of 2.87 and 2.63 cm, respectively (Fig. 1A). After 60 days of cultivation, growth was most intense in the nutrient media M1 and M10 (0.01 mg L^{-1} IAA + 1.25 mg L^{-1} KIN), with means of 6.60 and 5.80 cm, respectively. The means for TH variety did not differ among the treatments in the two evaluated periods.

The morphogenetic response (Table 3) of the varieties in MS medium (M1) differed from the first experiment. For TH, the callus proliferation was intense with no root formation. Conversely, SL responded to this culture medium and rhizogenesis occurred in greater proportion in the nutrient media enriched with KIN or IAA or both.

These different *in vitro* morphogenetic responses in the varieties do exist, not only between species of the same genus, but also among genotypes of the same species, owing to their unique characteristics, determined by genetic factors. These differences call for the establishment of distinct protocols (Slater *et al*., 2003).

In general, intense callogenic responses were detected in practically all treatments, which may be the reason for the low mean shoot lengths verified. On the other hand, Schuelter *et al.* (2005) tested the effect of sucrose on the morphogenetic response in cubiu stem segments and verified greater callus formation at low sucrose concentrations (≤15 g L^{-1}) as compared to higher concentrations. This would explain the absence of callus formation in the MS medium and the weak formation in the medium supplemented with cytokinin only in the first

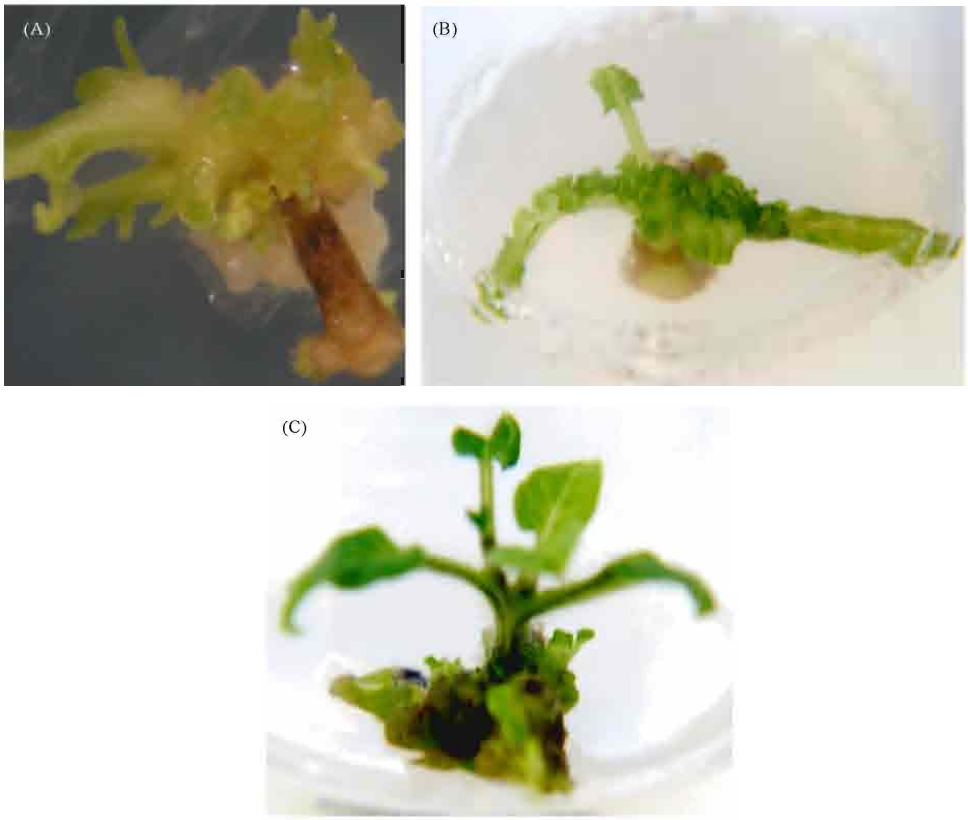

Fig. 1: Detail of cubiu shoot-buds derived from nodal segments after 60 days of culture initiation. (A) Adventitious buds of the variety Santa Luzia at 0.01 mg L^{-1} IAA + 0.64 mg L^{-1} KIN; (B) Abnormal axillary buds of the variety Thaís at 0.01 mg L^{-1} IAA + 10 mg L^{-1} TDZ and (C) Axillary and adventitious buds of the variety Santa Luzia at 0.01 mg L^{-1} IAA + 1.25 mg L^{-1} TDZ

experiment, in which 30 g L^{-1} sucrose was used instead of 15 g L^{-1}. Indeed, an over-formation of calli and roots would inhibit the development which is undesirable in the multiplication phase (Slater *et al.*, 2003).

From 15 days after the implantation of the experiment onwards, in some treatments the proliferation of axillary and adventitious buds began. After 60 days of cultivation, the TDZ nutrient media induced the richest proliferation of axillary and adventitious buds (Table 4).

High rates of axillary shoot-buds were observed in the treatments with concentrations of over 5 mg L^{-1} TDZ (M19, M20, M25 and M26) in both varieties (Table 4). However, the buds and internodes were poorly elongated and the former grouped in rosette-like shape (Fig. 1B). According to Kim *et al.* (1997), the number of shoot-buds differentiated in the presence of TDZ is considerably larger at higher concentrations (5.0 and 10 mg L^{-1}) although with rather weak elongation. A longer exposition to TDZ must therefore be avoided, since this growth regulator can cause abnormal morphology in the shoot-buds (Lu, 1993). On the other hand, the micropropagation of many species has become viable due to the efficiency of TDZ over other cytokinin types such as BAP and KIN, at extremely low concentrations (Nayak *et al.*, 1997), apart from being less costly than ZEA (Carvalho and Biasi, 2004). Thus, concentrations below 5 mg L^{-1} TDZ (M24 for SL and M18 for TH) induced a lower number of axillary buds per explant, which were normal (Fig. 1C). The main objective of the multiplication phase is the production of the largest number of plants in the shortest period of time. The most important aspect is to obtain a satisfactory mean shot-bud induction rate with a minimum of variation from one explant to another, since the quality and homogeneity of the aerial parts are essential for the rooting phase (Slater *et al.*, 2003).

The highest number of adventitious shoot-buds in both varieties was obtained under low TDZ concentrations ($\leq$5.0 mg L^{-1}) and no auxin (M17) (Table 4); the regenerated shoot-buds were normal in all culture media that contained TDZ.

Table 4: No. of axillary (A×B) and adventitious shoot-buds (AdB) in the cubiu varieties Santa Luzia (SL) and Thaís (TH), derived from culture media with different sources and concentrations of growth regulators and after 30 days of growth in culture medium (CM) devoid of growth regulators

	SL		TH			SL		TH	
CM	A×B	AdB	A×B	AdB	CM	A×B	AdB	AwB	AdB
M1	1.33	0	0	0	M20	6.0	0	12.33	1.0
M2	2.0	0	0	0	M21	0	5.66	0.5	0
M3	0	0	0	0	M22	0.66	11.66	0.5	0
M4	1.66	0	0	0	M23	1.0	0.66	0.66	3.0
M5	0	0	0	0	M24	9.66	2.0	7.66	3.67
M6	0	0	0	0	M25	3.66	1.33	14.0	1.33
M7	0	3.0	0	0	M26	13.66	0	14.66	0.66
M8	0	0	0	0	M27	0	2.33	0	1.0
M9	0	0	0	0	M28	0	1.66	0.66	0
M10	0	0	0	0	M29	1.33	4.0	0	1.66
M11	0	0	0	0	M30	0	1.0	1.33	2.33
M12	0	0	0	0	M31	1.33	6.0	3.33	2.33
M13	0	0.33	0.33	1.0	M32	4.0	2.33	2.33	0.66
M14	0	0	0	0	M33	0.66	1.0	0.66	0.66
M15	0.33	7.66	3.0	0.33	M34	1.33	1.66	0	0
M16	0	6.67	1.33	2.0	M35	0	1.66	0.33	0
M17	0	11.66	0.66	8.67	M36	0	1.66	0	0
M18	0.33	4.0	7.0	2.0	M37	0	4	0	2.66
M19	5.33	1.0	10.33	8.0	M38	0	5.33	1.38	1.0

Treatments that contained KIN and ZEA were not as efficient as those with TDZ in the proliferation of axillary and adventitious buds. ZEA, although rather costly, was especially effective for *in vitro* cultivation of persimmon (*Diospyros khaki*) root (Carvalho and Biasi, 2004), nodal segments of *Annona glabra* (Oliveira *et al.*, 2008) and hypocotyledonary segments of annatto (*Bixa orellana*) (Paiva Neto *et al.*, 2003).

The influence of cytokinin types and concentrations and the combinations with auxins on the morphogenetic responses *in vitro* has been widely discussed in the literature. Among the cytokinins, TDZ has been used in the regeneration of a wide range of species, from herbaceous to woody (Chen *et al.*, 2002; Paiva Neto *et al.*, 2003; Barik *et al.*, 2004; Thomas and Philip, 2005; Jones *et al.*, 2007; Corredoira *et al.*, 2008). A strong cytokinin activity together with a marked influence on the endogenous balance of cytokinins has been described to TDZ (Lu, 1993). Compared to purine cytokinins, those derived from phenylureas apparently present some peculiarities in the process of differentiation of buds from branches, resulting in multiple buds. Histological investigations demonstrated intense mitotic division in hypocotyledonary explants of annatto, resulting in significant meristemoid proliferation, though without elongation of individual buds (Paiva Neto *et al.*, 2003). Promising results regarding the formation of adventitious buds were related to leaf segments of cubiu cultivated in culture medium with 0.01 mg L^{-1} IAA and 20 mg L^{-1} KIN (Hendrix *et al.*, 1987). However, in stem segments-derived adventitious buds of variety SL were obtained at a concentration of 0.02 mg L^{-1} IAA + 20 mg L^{-1} KIN, which produced a mean of 2.18 buds per explant (Bonett *et al.*, 2005).

Fig. 2: Plants of the variety Santa Luzia (SL) cultivated during 60 days in MS lacking growth regulators

In the evaluation 30 days after the transference to the MS lacking growth regulators, the growth of the axillary and adventitious shoot-buds was weak. For the variety SL the mean size of the adventitious and axillary shoot-buds was 0.52±0.85 and 0.30±0.58 cm, respectively. For TH the mean rate for adventitious and axillary shoot-buds was 0.23±0.19 and 0.12±0.07 cm, respectively. However, the second subculture of these shoot-buds in similar medium resulted in the development of 4.0±0.32 cm long shoots with a well-developed root system (Fig. 2). This response may be linked to a residual effect of growth regulators

added to the earlier induction and, or proliferation phases, though lessened from one subculture to the other in medium devoid of growth regulators.

CONCLUSION

Present results allowed the conclusion that the chosen NAA and BAP concentrations were not adequate to induce axillary buds. However, the treatments with high TDZ concentration induced the most intense proliferation of axillary buds in both varieties, although the buds presented abnormalities. For the variety SL, the most efficient treatment for axillary shoot-bud formation was 0.01 mg L^{-1} IAA + 5.0 mg L^{-1} TDZ, whilst for the adventitious shoot-bud differentiation the combination of 2.5 mg L^{-1} TDZ and 0.01 mg L^{-1} IAA + 1.25 mL TDZ. For the variety TH, the treatment with 5.0 mg L^{-1} TDZ was most effective to obtain axillary shoot-buds, whereas adventitious shoot-buds were efficiently used with 2.50 mg L^{-1} TDZ. However, the presence of growth regulators inhibited bud growth, which was mitigated by two subcultures in freshly prepared medium lacking these substances.

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