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In vitro regeneration of cocona (*Solanum sessiliflorum*, Solanaceae) cultivars for commercial production

A.R. Schuelter[1], A.K. Grunvald[1], A.T. Amaral Júnior[2], C.L. da Luz[1], C.L. Luz[1], L.M. Gonçalves[1], S. Stefanello[1] and C.A. Scapim[3]

[1]Laboratório de Biotecnologia, Universidade Paranaense, Toledo, PR, Brasil
[2]Laboratório de Melhoramento Genético Vegetal, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, RJ, Brasil
[3]Departamento de Agronomia, Universidade Estadual de Maringá, Maringá, PR, Brasil

Corresponding author: A.R. Schuelter
E-mail: adilson_schuelter@yahoo.com.br

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ABSTRACT. Cocona (*Solanum sessiliflorum* Dunal) is a solanaceous shrub native to the Amazon region that produces an edible fruit. This species has numerous advantages, particularly a high nutritional value and productivity. However, due to irregular germination and rapid loss of seed viability, there are few plantations for production on a large scale. Development of alternative propagation strategies is essential for the production of homogeneous seedlings of genotypes with superior agronomic performance. We developed techniques for *in vitro* regeneration of the cocona varieties Santa Luzia and Thaís for large-scale production of healthy plantlets. Twenty days after seeding, seedling segments germinated *in vitro* were used as explant sources. Three successive experiments were performed: one to test the effect of the explant source and combinations of two growth regulators, auxin (indole acetic acid, IAA) and kinetin (KIN), on the morphogenetic response; another to investigate the effect of the combination of growth regulators on the morphogenetic response of hypocotyl segments, and another to evaluate

how sucrose concentration affects the development of adventitious shoots. The best shoot induction was obtained using hypocotyl segments and stem apices, while rhizogenesis was greatest in leaves with a petiole. The number of adventitious shoots per explant on hypocotyl segments increased with 10 and 20 mg/L KIN, combined with 0.02 mg/L IAA in the variety Santa Luzia. Sucrose combined with these growth regulator levels increased the average number of calli; these were optimally produced when 45 g/L sucrose and 0.01 mg/L IAA + 20 mg/L KIN were applied. Only sucrose concentration influenced shoot proliferation in the two *S. sessiliflorum* varieties, with a maximum at 17.5 g/L.

Key words: Organogenesis; Tissue culture; Regeneration; *In vitro* culture; Explants; *Solanum sessiliflorum*

INTRODUCTION

Cubiu (*Solanum sessiliflorum* Dunal) is native to the Amazon and widely distributed across the humid equatorial regions of Brazil, Peru and Colombia. The fruits are characteristically rich in iron, niacin, citric acid, and pectin. For this reason, they are used as food, consumed fresh or as juice, jam, compote, jelly, or seasoning, as well as in the production of cosmetics (Silva Filho, 2002; Silva Filho et al., 2003; Souza et al., 2006). Native people left a legacy that contributed to the discovery of the value of the species for medicinal purposes; it is currently used, although at incipient levels, to fight skin diseases and reduce cholesterol, glucose and uric acid levels in the blood (Silva Filho, 1998; Pardo, 2004).

The form of sexual reproduction of the species is still debatable among scientists. Pahlen (1977) and Paiva (1999) consider cubiu as being autogamous with frequent allogamy. Studies on the floral biology of *Solanum sessiliflorum* var. *sessiliflorum*, however, led Storti (1988) to consider cubiu an allogamous plant. In recent studies, Luz et al. (2008) and Pizzinato et al. (2008) concluded that cubiu is an allogamous plant, since plants grown in a greenhouse and covered with organza, preventing the action of pollinators, produced no fruits with seeds. However, there is evidence that stigmata are receptive and pollen grains germinate when the flowers are closed, indicating the possibility of selfing (Pizzinato et al., 2008).

Since the evolutionary process is still wild, the fruits tend to be irregular and fruit ripening not synchronous. Besides, seed germination is irregular and depends on specific conditions such as temperature and humidity (Lopes and Pereira, 2005), storage (Stefanello et al., 2008) and fruit harvesting (Souza et al., 2008). Moreover, the production of seedlings of sexual origin is rather time-consuming (Boufleuher et al., 2008).

For the use of cubiu in agrobusiness, a viable alternative is to use tissue culture for the large-scale production of genetically uniform seedlings that are free of phytopathogenic agents (Torres et al., 1998; Boufleuher et al., 2008). Among the rare studies on micropropagation in *S. sessiliflorum* one can cite Hendrix et al. (1987), Cordeiro and Mattos (1991) and Boufleuher et al. (2008).

Driven by increased market demand, the industry has slowly grown in the cultivation and marketing of the Santa Luzia and Thaís varieties, especially in the southern and southeastern regions of Brazil. In this respect, the lack of synchronous flowering of these varieties and the occurrence of panmixia with a low production of fertile seeds (Pizzinato et al., 2008)

indicate the need for research to investigate the possibility of seedling production for *in vitro* cultivation and to determine the most appropriate means of cultivation of the species.

As a result, this study was conducted to establish a protocol for large-scale *in vitro* regeneration of the Santa Luzia and Thaís cubiu varieties with the aim of producing healthy seedlings.

MATERIAL AND METHODS

Material and cultivation conditions

Cubiu (*S. sessiliflorum* Dunal) seeds of the Santa Luzia (round fruit) and Thaís (long fruit) varieties from the Santa Luzia Experimental Station (Guareí, SP) were disinfected with 70% ethanol for 2 min and 40% sodium hypochlorite for 20 min, and then washed with distilled water three times. After sterilization, the seeds were placed in vials containing 50 mL MS culture medium (Murashige and Skoog, 1962) with half the mineral concentration, supplemented with 30 g/L sucrose, 200 mg/L myo-inositol, vitamins (0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine and 0.1 mg/L thiamine), 2 mg/L glycine and 6. 5 g/L agar. The pH was adjusted to 5.8 before autoclaving.

The cultures were maintained in a growth chamber with a photoperiod of 16 h, under 30 $\mu moL \cdot m^{-2} \cdot s^{-1}$ light radiation from two fluorescent lamps (20 W, Osram, Brazil). The temperature in the growth chamber was maintained at 24 ± 2°C. The seedlings germinated *in vitro* were used as explant sources 20 days after seeding.

Three successive experiments were performed to verify the effect of the explant source and plant growth regulators on morphogenetic response in the first, the combination of growth regulators on morphogenetic response in the second, and sucrose concentrations to obtain adventitious shoots in the third.

Effect of explant source and growth regulators on morphogenetic response

The explant sources from leaf segments, leaf with petiole, petiole, hypocotyl segments, and stem apices of the Thaís and Santa Luzia varieties, from seedlings germinated *in vitro*, were grown on Petri dishes with 10 mL MS basal culture medium supplemented with MS vitamins, 30 g/L sucrose, 200 mg/L myo-inositol, vitamins (0.5 mg/L nicotinic acid, 0.1 mg/L pyridoxine and 0.1 mg/L thiamine), 2.0 mg/L glycine, 6.5 g/L agar, and kinetin - KIN (20 mg/L) - combined with or without indole acetic acid - IAA (0.01 and 0.1 mg/L); the pH was adjusted to 5.8 before autoclaving.

The experimental design was completely randomized with the (2 x 5 x 2), factorial and a total of 20 treatments. Three Petri dishes were inoculated with each one of the culture media with these combinations. Every experimental unit consisted of ten explants, except the stem apices, which consisted of five explants per dish. The Petri dishes were kept in a growth chamber with a photoperiod of 16 h at 24 ± 2°C, and were evaluated 60 days after inoculation for shoot, callus and root induction.

Effect of kinetin and indole acetic acid on adventitious bud induction on hypocotyl segments

Hypocotyl segments of seedlings of the Thaís and Santa Luzia cubiu varieties with a length of about 5 mm, germinated *in vitro*, were placed on Petri dishes containing 10 mL

MS culture medium supplemented with 30 g/L sucrose, 200 mg/L myo-inositol, vitamins (0.5 mg/L nicotinic acid, 0.1 mg/L pyridoxine and 0.1 mg/L thiamine), 2.0 mg/L glycine, and 6.5 g/L agar, at different KIN (10, 20 and 30 mg/L) and IAA (0.01 and 0.02 mg/L) concentrations. The pH was adjusted to 5.8 before autoclaving.

The experimental design was completely randomized with the (3 x 3), factorial and a total of nine treatments and four replications. Each experimental unit consisted of one Petri dish with ten explants. Cultures were placed in a growth chamber, with a photoperiod of 16 h, at 24 ± 2°C. The mean shoot number and callus and root frequency were evaluated after 30 and 60 days.

Influence of different sucrose concentrations on shoot induction in hypocotyl segments

Hypocotyl segments of 5 mm were removed from seedlings germinated *in vitro* and placed on Petri dishes with MS basal culture, supplemented with 30 g/L sucrose, 200 mg/L myo-inositol, vitamins (0.5 mg/L nicotinic acid, 0.1 mg/L pyridoxine and 0.1 mg/L thiamine), 2.0 mg/L glycine, and 6.5 g/L agar, with combinations of different sucrose concentrations (0, 15, 30, 45, and 60 g/L) and KIN (20 mg/L), IAA (0.01 and 0.02 mg/L). The pH was adjusted to 5.8 before autoclaving.

The experimental design was completely randomized with 2 varieties x 2 phytohormone concentrations x 5 sucrose concentrations, factorial, resulting in 20 treatments. The experimental unit consisted of a Petri dish with ten explants and three replications. Cultures were kept in a growth chamber with a photoperiod of 16 h at 24 ± 2°C, and were evaluated 60 days after inoculation for the mean number of shoots, roots and calli.

After 60 days, the hypocotyl segments in the experiment involving the determination of the effect of sucrose on the proliferation of adventitious buds were transferred to vials containing MS culture medium supplemented with MS basal vitamins, 15 g/L sucrose, 200 mg/L myo-inositol, vitamins (0.5 mg/L nicotinic acid, 0.1 mg/L pyridoxine and 0.1 mg/L thiamine), 2.0 mg/L glycine and 6.5% agar. The pH of the culture medium was adjusted to 5.8. The cultures were maintained for 30 days in a growth chamber with a photoperiod of 16 h at 24 ± 2°C. After the incubation period, shoot size and root formation were evaluated.

Statistical analysis

In the three experiments, the basic assumptions for ANOVA were implemented by Levene and Shapiro-Wilk tests at 1% probability (SAS, 2001). The analysis of variance and mean grouping by the Scott-Knott test, applied in the first two experiments, as well as analysis of variance and regression implemented in the third experiment, were performed using the statistical program SISVAR (Ferreira, 1999).

RESULTS AND DISCUSSION

Effect of explant type, culture medium and variety on adventitious bud induction

The size of induced shoots in the different treatments was very small, mostly less than 1 cm. Therefore, the analysis was performed with data of the total number of shoots (TNS) and number of shoots smaller than 1 cm (NSS).

Significant differences ($P < 0.05$) were observed for TNS, NSS, number of roots (NR), number of calli (NC), and for interaction between the explant type, hormone combination and variety.

The analysis of partitioning of the five explants for two varieties and two combinations of IAA and KIN for variable TNS (Table 1) showed that for the Santa Luzia variety shoot formation on the petiole and hypocotyl segment was highest in culture medium supplemented with 0.01 mg/L IAA and 20 mg/L KIN. For Thaís, in the same combination of regulators, the number of shoots on hypocotyl segments was higher than on other explants. The *in vitro* culture of petioles, hypocotyl segments and stem apices of Santa Luzia and Thaís in culture medium supplemented with 0.1 mg/L IAA and 20 mg/L KIN resulted in a higher formation rate of shoots as well as calli per explant. Similar results were observed for NSS (Table 1). The different responses of the five explant types are probably due to the endogenous hormonal balance in the plant tissue (Grattapaglia and Machado, 1998), while the variety effect can be attributed to the genetic constitution (Faria et al., 2007).

Table 1. Analysis of partitioning of five cubiu explants for the two varieties Santa Luzia and Thaís and two culture medium types.

Culture medium	0.01 mg/L IAA + 20 mg/L KIN		0.1 mg/L IAA + 20 mg/L KIN	
	Santa Luzia	Thaís	Santa Luzia	Thaís
		Shoots/explant (TNS)		
Leaf segment	0.75 bA	0.75 cA	0.79 bA	0.75 bA
Leaf with petiole	0.95 bA	0.91 cA	0.77 bA	0.85 bA
Petiole	1.50 aA	1.24 bA	1.24 aA	1.33 aA
Hypocotyl segment	1.34 aB	1.76 aA	1.36 aA	1.31 aA
Stem apex	1.49 bA	1.26 bA	1.93 aA	1.23 aA
		Shoots/explant (smaller than 1 cm) (NSS)		
Leaf segment	0.73 bA	0.75 cA	0.79 bA	0.71 bA
Leaf with petiole	0.79 bA	0.91 cA	0.91 bA	0.85 bA
Petiole	1.50 aA	1.24 bA	1.09 aA	1.33 aA
Hypocotyl segment	1.34 aB	1.76 aA	1.36 aA	1.32 aA
Stem apex	1.36 aA	1.21 bA	1.18 aA	1.22 aA
		Calli/explant (NC)		
Leaf segment	0.16 bA	0.06 bA	0.20 aA	0.20 bA
Leaf with petiole	0.10 bA	0.16 bA	0.23 aA	0.13 bA
Petiole	0.36 aA	0.20 bA	0.16 aA	0.30 bA
Hypocotyl segment	0.63 aA	0.63 aA	0.46 aB	0.80 aA
Stem apex	0.00 bA	0.20 bA	0.23 aA	0.13 bA
		Roots/explant (NR)		
Leaf segment	0.20 cA	0.26 aA	0.56 bA	0.13 aB
Leaf with petiole	1.00 aA	0.46 aB	0.90 aA	0.40 aB
Petiole	0.16 cA	0.36 aA	0.16 cA	0.16 aA
Hypocotyl segment	0.56 bA	0.30 aA	0.40 cA	0.10 aB
Stem apex	0.13 cA	0.13 aA	0.20 cA	0.16 aA

KIN = kinetin, IAA = indole acetic acid; TNS = total number os shoots; NSS = number of shoots smaller than 1 cm; NC = number of calli; NR = number of roots. Means followed by the same lower case letter in a column do not differ from each other, at 5% probability by the Scott-Knott test, and means followed by the same capital letter in a row do not differ from each other, at 5% probability by the F-test.

Despite the low genetic variability detected by Luz et al. (2008) of Thaís compared with Santa Luzia, Scherer (2004) observed differential germination *in vitro*, which may be linked to a variety-specific totipotential effect resulting in differences in shoot induction. Moreover, the results of this study differ from those obtained by Hendrix et al. (1987) for cubiu, who detected the greatest number of adventitious shoots and roots in leaf segments.

The analysis of partitioning of the two medium types for the two varieties and five explants showed an increased shoot production from stem apices of Santa Luzia in the culture medium containing 0.1 mg/L IAA and 20 mg/L KIN. Hypocotyl segments of Thaís grown in culture medium supplemented with 0.01 mg/L IAA and 20 mg/L KIN resulted in higher shoot formation. For the latter variety, the same result was found when shoots smaller than 1 cm were evaluated (Table 2).

Table 2. Analysis of partitioning of two types of culture medium for five explant types for the Santa Luzia and Thaís varieties.

Culture medium	Santa Luzia		Thaís	
	0.01 mg/L IAA + 20 mg/L KIN	0.1 mg/L IAA + 20 mg/L KIN	0.01 mg/L IAA + 20 mg/L KIN	0.1 mg/L IAA + 20 mg/L KIN
	Shoots/explant (TNS)			
Leaf segment	0.75 A	0.79 A	0.75 A	0.75 A
Leaf with petiole	0.95 A	0.77 A	0.91 A	0.85 A
Petiole	1.50 A	1.24 A	1.24 A	1.33 A
Hypocotyl segment	1.34 A	1.36 A	1.76 A	1.31 B
Stem apex	1.49 B	1.93 A	1.26 A	1.23 A
	Shoots/explant (smaller than 1 cm) (NSS)			
Leaf segment	0.73 A	0.79 A	0.75 A	0.71 A
Leaf with petiole	0.79 A	0.91 A	0.91 A	0.85 A
Petiole	1.50 A	1.09 A	1.24 A	1.33 A
Hypocotyl segment	1.34 A	1.36 A	1.76 A	1.32 B
Stem apex	1.36 A	1.18 A	1.21 A	1.22 A
	Roots/explant (NR)			
Leaf segment	0.20 B	0.56 A	0.26 A	0.13 A
Leaf with petiole	1.00 A	0.90 A	0.46 A	0.40 A
Petiole	0.16 A	0.16 A	0.36 A	0.16 A
Hypocotyl segment	0.56 A	0.40 A	0.30 A	0.10 A
Stem apex	0.13 A	0.20 A	0.13 A	0.16 A

Means followed by the same capital letter, in a row, do not differ from each other by the F-test at 5% probability. For abbreviations, see legend to Table 1.

Gubiš et al. (2004) obtained a high rate of shoot regeneration per explant from hypocotyl segments and cotyledon leaves of *Lycopersicum esculentum* Mill. grown in MS culture medium supplemented with 1 mg/L zeatin + 0.1 mg/L IAA.

For the analysis of partitioning for variable NR, the mean grouping test of Scott and Knott (1974) showed that root formation was greatest on leaves with petiole of Santa Luzia, in the two

culture medium types (Table 2). According to Barceló Coll et al. (1988), shoots are sites of intense auxin production, which when translocated to the stem base stimulates rhizogenesis. Nevertheless, the quality of shoots of the propagation stage generally determines the success of rooting (Grattapaglia and Machado, 1998). However, there were no significant differences between the different explant sources of the Thaís variety (Table 1). Leaf explants with petiole of Santa Luzia performed better than of Thaís in shoot formation in the medium supplemented with 0.01 mg/L IAA and 20 mg/L KIN. The superiority of this variety was also observed when leaf segment, leaf with petiole and hypocotyl segments were grown in medium containing 0.1 mg/L IAA and 20 mg/L KIN.

When we evaluated the relationship between the culture media within the varieties and five explant types, significant differences were detected only in leaf segments of Santa Luzia, where a supplement of 0.1 mg/L IAA and 20 mg/L KIN resulted in a better root development (Table 2).

The different treatments induced the formation of calli with a spongy and whitish appearance, similar to those obtained by Cordeiro and Mattos (1991), which did not induce adventitious shoots or embryogenic structures. Petioles and hypocotyl segments of the variety Santa Luzia were more responsive in callus formation when grown in medium supplemented with 0.01 mg/L IAA and 20 mg/L KIN. For Thaís, differences were only significant for hypocotyl segments. For the same explant at a concentration of 0.1 mg/L IAA and 20 mg/L KIN, the number of regions with calli in Thaís exceeded that in Santa Luzia (Table 1).

Cytokinins participate in the regulation of many plant processes that induce callus cell division in the presence of auxin, leading to bud or root formation directly on the explant or from calli, among others (Taiz and Zeiger, 2004). The cytokinin type and concentration are key factors for the performance of *in vitro* multiplication. According to Grattapaglia and Machado (1998), the cytokinin 6-benzylaminopurine and KIN are very effective in promoting proliferation.

A preliminary analysis showed that the use of hypocotyl segments and stem apices may be an alternative to establish a regeneration protocol for the Santa Luzia and Thaís varieties. However, it is worth mentioning that the induced adventitious shoots were small-sized (Figure 1A and B), usually less than 5 mm, and not prone to differentiation. Thus, it is extremely important to enhance the regeneration protocol by increasing the induction and formation rate of larger and well-defined shoots.

Effect of indole acetic acid kinetin and variety on adventitious bud induction in stem segments

For the variables shoot, root and callus formation from hypocotyl segments, the differences for the main effect of phytohormones and the interaction of phytohormones x varieties were significant ($P < 0.05$).

Comparing the types of culture media in the two varieties for shoot formation (TNS) showed that the culture media M3 and M6 were superior in shoot production in relation to other treatments for the variety Santa Luzia (Table 3). However, the best treatments for shoot production of the Thaís variety were M4, M5, and M6 (Table 3).

The analysis of partitioning for the two varieties comparing the nine types of culture media (Table 3) showed that the media M3 and M5 induced the highest shoot formation in Santa Luzia, and M5, M4, and M7 for Thaís. This difference between the two varieties can be explained by changes in levels of endogenous hormones and the expression of genes encoding hormone receptors, as proposed by Close and Gallagher-Ludeman

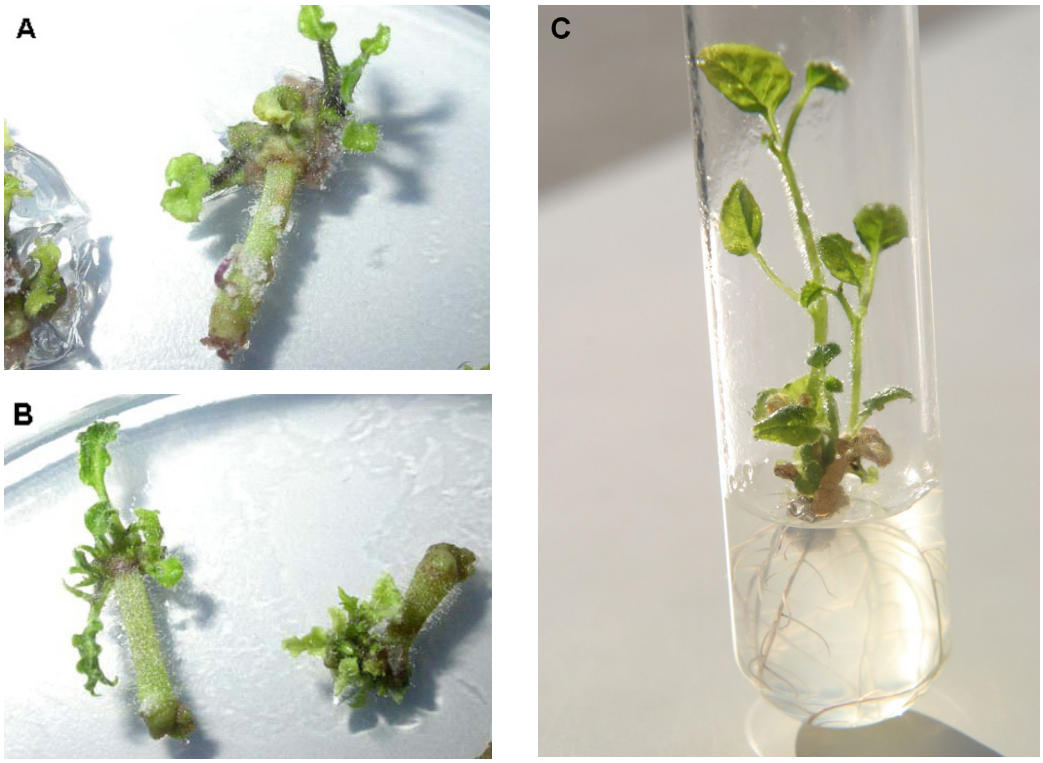

Figure 1. Morphogenetic response. **A.** Shoot formation of the Santa Luzia variety in the culture medium M3. **B.** Shoot formation of the Thaís variety in the culture medium M7. **C.** Elongation and rooting of adventitious shoots of Santa Luzia in culture medium without growth regulators.

Table 3. Analysis of the partitioning of the Santa Luzia and Thaís varieties within the two types of media (rows), and the breakdown of the types of means of varieties in Santa Luzia and Thaís (columns).

Culture medium	Shoots/explant (TNS)		Calli/explant (NC)		Roots/explant (NR)	
	Santa Luzia	Thaís	Santa Luzia	Thaís	Santa Luzia	Thaís
M1 - 10 mg/L KIN	1.65 bA	1.57 bA	0.37 aA	0.35 bA	0.67 aA	0.30 aB
M2 - 10 mg/L KIN + 0.01 mg/L IAA	1.60 bA	1.72 bA	0.80 aA	1.15 aA	0.70 a A	0.12 aB
M3 - 10 mg/L KIN + 0.02 mg/L IAA	2.57 aA	1.47 bB	0.42 aA	0.72 aA	0.57 a A	0.15 aB
M4 - 20 mg/L KIN	1.92 bA	2.60 aA	0.30 aA	0.15 bA	0.25 bA	0.10 aA
M5 - 20 mg/L KIN + 0.01 mg/L IAA	2.77 aA	2.67 aA	0.37 aA	0.37 bA	0.60 aA	0.02 aB
M6 - 20 mg/L KIN + 0.02 mg/L IAA	2.17 aA	1.92 bA	0.12 aA	0.30 bA	0.42 aA	0.17 aA
M7 - 30 mg/L KIN	1.00 bB	2.07 aA	0.22 aA	0.27 bA	0.22 bA	0.02 aA
M8 - 30 mg/L KIN + 0.01 mg/L IAA	1.72 bA	1.07 cA	0.40 aA	0.22 bA	0.47 aA	0.15 aB
M9 - 30 mg/L KIN + 0.02 mg/L IAA	1.52 bA	0.60 cB	0.20 aA	0.02 bA	0.22 bA	0.07 aA

Means followed by the same lower case letter in a column do not differ from each other at 5% probability by the Scott-Knott test, and the same capital letters in a row do not indicate a difference at 5% probability by the F-test. For abbreviations, see legend to Table 1.

(1989). According to Galiba et al. (1986), a polygenic system may be involved in *in vitro* regeneration. It is worth mentioning that an analysis of the composition of these three methods indicated that the presence of cytokinin in the culture medium may have been essential for shoot development in Santa Luzia.

In fact, according to Preece (1995), cytokinins play a primary role in cell division and also break the apical dominance and influence shoot induction and growth. Grattapaglia and Machado (1998) argue that the cytokinin type and concentration are the factors that most influence the success of *in vitro* multiplication. It is, however, of utmost importance that an appropriate protocol be established, since an excess of growth regulators in cultures causes a phytotoxic effect. In this respect, Narayanaswamy (1977) reports that the toxicity caused by an excess of growth regulators in the culture medium, or the extended period of time in which the culture is exposed to them, may cause genetic, physiological and morphological changes, resulting in a reduction of the proliferation rate *in vitro*.

Regarding the variable callus formation (NC), no significant difference between the nine different culture media was found when hypocotyl segments of the Santa Luzia variety were grown. However, there were differences in the media M2 and M3 for Thaís (Table 3). The addition of growth regulators to the culture is extremely important, since the auxins are able to start cell division and control the growth processes and cell elongation. According to Mendoza and Kaeppler (2002), the use of auxin extends the influence on the process of seedling regeneration and callus formation and, in combination with cytokinin, leads to rapid cell division, forming a large number of relatively small and undifferentiated cells (Raven et al., 2001; Taiz and Zeiger, 2004).

In the partitioning for root formation (NR) of the different media separately for the two varieties, the Scott-Knott cluster analysis grouped the media M1, M2, M3, M5, M6, and M8 in a single group for Santa Luzia, indicating superior performance of this treatment (Table 3). For the Thaís variety, the nine medium types did not differ. When the two varieties were analyzed separately for the different media, it was found that Santa Luzia performed better than Thaís in M1, M2, M3, M5, and M8 for this NR. Apart from M1, the media of this group consisted of different auxin and cytokinin combinations.

A comparison of the two varieties showed a higher overall mean for the average number of shoots in Santa Luzia than in Thaís. Moreover, it was shown that regardless of the variety and composition of the culture medium the adventitious shoots were characteristically small-sized and not prone to differentiation, as in the previous experiment. Along this line, research results with different species have shown the importance of osmotic agents such as sucrose for the growth and development of adventitious shoots (Cao et al., 2003; Faria et al., 2006; Maldaner et al., 2006). The process of improving the regeneration protocol of *S. sessiliflorum* was therefore continued.

Effect of sucrose concentration, medium type and variety on adventitious bud induction

An analysis of the five sucrose concentrations by quadratic polynomial regression showed that in the medium containing 0.01 mg/L IAA + 20 mg/L KIN, callus formation was most intense (1.4 calli per explant) at a concentration of 33.22 g/L sucrose (Figure 2A). When evaluated with the medium supplemented with 0.02 mg/L IAA + 20 mg/L KIN by cubic re-

gression, the maximum NC was 1.84 at 19.46 g/L sucrose (Figure 2B). Sucrose proved to be important for callus development in both growth regulator combinations, since production was reduced in sucrose-free culture medium.

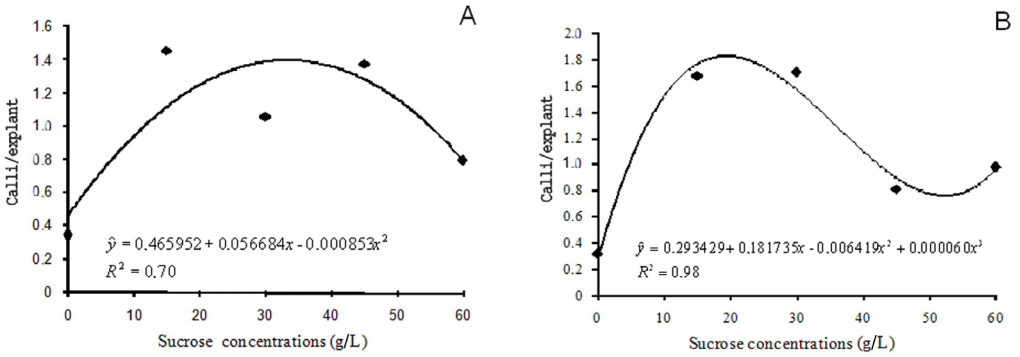

Figure 2. Variation in the mean number of calli per explant for five sucrose concentrations (0, 15, 30, 45, and 60 g/L) in the following combinations: **A.** 0.01 mg/L IAA + 20 mg/L KIN; **B.** 0.02 mg/L IAA + 20 mg/L KIN.

The addition of carbohydrate sources to the medium is indispensable, since *in vitro* cultures are unable to perform photosynthesis to sustain organ growth, induction and differentiation (George, 1993). Carbohydrates provide the metabolic energy and carbon skeletons of all organic compounds required for cell growth and development (Caldas et al., 1988). On the other hand, high concentrations are not beneficial, as shown here. The addition of sucrose after the point of maximum regression reduced the average NC. This shows that the high osmotic potential of the culture medium had a major influence on the results, since a high osmotic pressure reduces growth and affects cellular metabolism, as claimed by Caldas et al. (1988).

The highest average NC per explant was obtained when the stem segments were grown in culture medium with 0.01 mg/L IAA + 20 mg/L KIN plus 45 g/L sucrose. However, when grown in medium with 0.02 mg/L IAA + 20 mg/L KIN, an increased production was observed at a sucrose concentration of 30 g/L (Table 4). This relationship of the factors can be explained by the inactivation of molecules of plant growth regulators in the combination with sugars derived from sucrose, glucose and fructose hydrolysis (Coruzzi and Zhou, 2001). In this process, the increase in sucrose level alters the balance of the active auxin and cytokinin molecules and in the end, the regulator concentration is not high enough to induce the same physiological response (Souza et al., 2007).

Table 4. Analysis of partitioning of the average number of calli per explant at five sucrose concentrations, supplemented with different indole acetic acid (IAA) and kinetin (KIN) concentrations.

Phytohormone concentration	Sucrose concentration (g/L)				
	0	15	30	45	60
0.01 mg/L IAA + 20 mg/L KIN	0.35a	1.46a	1.06b	1.38a	0.80a
0.02 mg/L IAA + 20 mg/L KIN	0.31a	1.68a	1.71a	0.81b	0.98a

Means followed by the same lower case letter in a column do not differ by the F-test at 5% probability.

For the average number of shoots, only the main effect of sucrose was significant ($P < 0.05$). Regardless of the regulator concentrations and the varieties tested, regression analysis indicated a maximum number of 3.18 shoots per explant at a sucrose concentration of 17.52 g/L (Figure 3). Rodrigues et al. (2006) examined *in vitro* vegetative propagation of apple trees and found that sucrose-free culture media led to death or atrophy of the explant; the highest number of shoots was obtained at sucrose concentrations of 30 and 45 g/L, similar to results of Flores et al. (1999) with the same species.

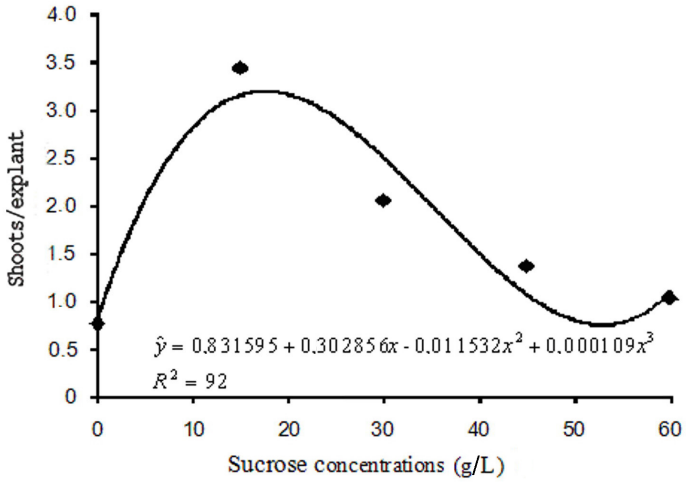

Figure 3. Variation in the mean number of shoots per explant at five sucrose concentrations (0, 15, 30, 45, and 60 g/L).

The differences in the cubiu varieties regarding the optimal sucrose concentration may be a result of differences in the genetic constitution of the species, leading to different responses in micropropagation. Nagao et al. (1994) and Santos (2003) reported that the nutritional requirements for tissue growth in *in vitro* conditions vary among species, among varieties and even within the plant itself, and thus the culture medium needs to be optimized.

Just as for calli, a moderate sucrose concentration in the culture medium is essential for shoot production. It was observed here that the sucrose concentration required for an increased production of shoots is lower than for calli. In this regard, it is of utmost importance to recall that in most cases the osmotic potential of a medium is controlled by the carbon source, as emphasized by Paiva Neto and Otoni (2003).

Studies with different sucrose concentrations have also been conducted to optimize *in vitro* rooting. Calvete et al. (2002), for example, cultivated strawberry plantlets in MS medium plus 0.005 mg/L 6-benzylaminopurine and observed that supplementation with 45 g/L sucrose increased rooting. For cubiu, however, the addition of IAA and KIN combined with different sucrose concentrations did not enhance root formation.

After 60 days of culture, the adventitious shoots were transferred to vials containing MS culture medium free of plant growth regulators. After 30 days (Figure 1C), the shoots had a length of 4.2 ± 0.8 cm and a well-formed root system, i.e., the plantlets were ready for acclimatization.

CONCLUSIONS

The Santa Luzia variety was more totipotent in shoot formation; however, on average, Thaís produced shoots with greater mean sizes. Sucrose levels combined with growth regulators (IAA and KIN) influenced the average number of calli, whereas shoot proliferation was influenced only by sucrose for both varieties of *S. sessiliflorum*.

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