Solasodine accumulation in regenerated plants of *Solanum torvum* Sw.

MOREIRA, C.B.[1]; LIMA, S.S.[1]; ESQUIBEL, M.A.[1]; SATO, A.[1,2]*

[1] *Laboratório de Fisiologia Vegetal, Programa de Pós-Graduação em Biotecnologia Vegetal, Instituto de Biofísica Carlos Chagas Filho, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, CEP: 21941-590, Rio de Janeiro-Brasil* [2] *Laboratório de Cultura de Tecidos Vegetais, Departamento de Botânica, Escola de Ciências Biológicas, Universidade Federal do Rio de Janeiro, CEP: 22290-240, Rio de Janeiro-Brasil* * *alicesato@unirio.br.*

RESUMO: **Acúmulo de solasodina em plantas micropropagadas de *Solanum torvum* Sw.** A regeneração de *Solanum torvum* e a avaliação do conteúdo de solasodina foram os objetivos de cultura de segmentos nodais. A influência de auxinas (ácido 3-indolacético, ácido naftalenoacético) e de 6-benzilaminopurina no crescimento de *S. torvum* na micropropagação foi investigado. Cultura de segmentos nodais foi iniciada por sementes germinadas em meio básico MS acrescido de GA_3 e cultivadas em diferentes concentrações de AIA, AIA + BAP e ANA + BAP. Plantas da cultura *in vitro* com 60 dias foram coletadas, congeladas e liofilizadas e o método de alaranjado de metila foi utilizado para quantificação de solasodina para o ensaio espectrofotométrico. Os melhores resultados para regeneração vegetal e acúmulo de solasodina foram alcançados no meio MS sem adição de reguladores de crescimento havendo, porém grande produção de calos de base friáveis. Esses resultados mostram que a partir desses calos, cultura de células pode ser iniciada com aplicação de outras auxinas e citocininas para o aumento da produção de solasodina além de diferentes eliciadores, intensidades luminosas e concentrações de sacarose.

Palavras-chave: Solanaceae, jurubeba, auxina, citocinina, alcalóide esteroidal

ABSTRACT: A nodal segment culture was developed in order to assess *Solanum torvum* Sw. regeneration and solasodine levels. The influence of auxins (indoleacetic acid, 1-Naphthaleneacetic acid) and benzyl adenine on *S. torvum* growth in micropropagation was investigated. A nodal segment culture was initiated with seeds germinated in MS basal medium added of GA_3 and grown in different concentrations of IAA, IAA + BAP and NAA + BAP. Sixty-day-old plants from the *in vitro* culture were collected, frozen and lyophilized; then, the methyl orange method was used to quantify solasodine for the spectrophotometric assay. The best results regarding plant regeneration and solasodine accumulation were obtained by using the MS basal medium without addition of plant growth regulators; however, there was great production of calluses presenting friable bases. Based on these results, cell cultures can be initiated from such calluses with application of other auxins and cytokinins to enhance solasodine production, besides different elicitors, light intensities and sucrose concentrations.

Key words: Solanaceae, “Jurubeba”, auxin, cytokinin, steroidal alkaloid

INTRODUCTION

Alkaloids are a class of nitrogen compounds structurally diverse, found in all plant groups, occurring most of them in angiosperms (Henriques et al., 2002; Hughes & Shanks, 2002). Solanaceae family presents several alkaloid groups and many plants accumulate steroidal alkaloids based on a C_{27} cholestane skeleton, an important source of raw material to partial synthesis of steroidal hormones (Vieira & Carvalho, 1993; Esteves-Souza et al., 2002). The steroidal alkaloid solasodine has been reported to be accumulated in relatively high concentrations in a number of *Solanum* species. It can be converted to starter compound 16-dehydropregnenolone and has been used for the semi-synthetic production of pharmaceutical and contraceptive steroids in many parts of the world. In spite of the steroidal saponin diosgenin is the most important steroid from raw material (Figure 1), the high demand of this compound

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and problem with the supply of it have led researchers to look for alternative compounds (Galanes et al., 1984; Eltayeb et al., 1997).

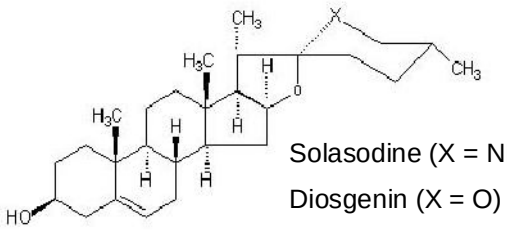

FIGURE 1. Solasodine and its closely related diosgenin.

The production of alkaloids in plant tissue culture by some solanaceous species have been presented in many reports as shown in Table 1 (Yamada & Hashimoto, 1982; Conner, 1987; Savary & Dougall, 1990; El-Ashaal et al., 1999; Khanam et al., 2000; Khanam et al., 2001; Okršlar et al., 2002; Kang et al., 2004; Jacob & Malpathak, 2005a; Jacob & Malpathak, 2005b; Bhat et al., 2008).

Solanum species which are found solasodine are easy to be cultivated because their high occurrence as herbs and ruderal shrubs, many times harmful in plant breeding (Vieira, 1998). *Solanum torvum* Sw. (Solanaceae), a medicinal plant known in Brazil as Jurubeba is indicated as sedative, diuretic and digestive (Mahmood et al., 1983; Corrêa, 1984; Ajaiyeoba, 1999; Chah et al., 2000). It is also considered a weed because its rapid spread in disturbed lands (Francis, 2007) but very common ingredient in Thai cuisine (Arthan et al., 2002).

The aim of this work was to study the *in vitro* development and solasodine production of the *S. torvum* plants from nodal segments culture.

MATERIAL AND METHOD

Fruits of *S. torvum* were collected in the Valonguinho campus of the Fluminense Federal University, Niterói, Rio de Janeiro and were identified by Drª Lúcia D'Ávila F. de Carvalho of Rio de Janeiro Botanical Garden Research Institute. A voucher specimen of this plant material is deposited in the Herbarium (RB 377.641) at Rio de Janeiro Botanical Garden Research Institute.

For the establishment of the tissue culture, seeds were surface sterilized twice with commercial liquid detergent for 15 minutes each time. In sterile chamber, they were washed with 70% (v/v) alcohol and then with 20% (v/v) commercial hypoclorite (2% active chlorine). Seeds were rinsed with sterile distilled water between steps. Seeds were germinated on Murashige and Skoog (MS) basal medium (Murashige & Skoog, 1962) added to 2.0 mg L^{-1} gibberellic acid (GA_3).

Nodal segments were grown in sterile flasks with MS basal medium hormone free and added with 0.2 mg L^{-1} indole acetic acid (IAA), 0.2 mg L^{-1} IAA + 0.7 mg L^{-1} benzyl adenine (BA), 0.2 mg L^{-1} IAA + 1.6 mg L^{-1} BA, 0.2 mg L^{-1} naphthalene acetic acid (NAA) + 0.7 mg L^{-1} BA at 28ºC $\pm$ 2ºC with 16 hours photoperiod and light intensity of 23 µmoles m^{-2} s^{-1}. The development and elongation of new shoots and roots formation were verified after 60 days of culture.

The methyl orange method was used for solasodine quantification (Vieira, 1998). Each sample was divided in three aliquots and it was analyzed by spectrophotometric assay. Harvested plants from in vitro culture with 60 days old were frozen and lyophilized. The dried plants (3 g) were extracted in commercial ethanol-glacial acetic acid system (95:5), in reflux, for two hours. Afterwards, the extract was evaporated to dryness and reconstituted with 50 mL glacial acetic acid 10% (v/v) and boiled for thirty minutes. Then the extract was chilled, it was separated by filtration with paper filter. The material was warmed up about 70ºC and basified with NH_4OH to pH 9-10. The extract was frozen for twenty-four hours and then centrifuged for thirty minutes to 3500 rpm.

The waste obtained was dissolved in warm methanol and then added chloridric acid for 5 hours of reflux. Afterwards, the extract was evaporated to dryness and solubilised with methanol-glacial acetic acid (95:5) until 100 mL. From the methanol-glacial acetic acid solution 1 mL was solubilised with distilled water in a volumetric balloon (50 mL). From the aqueous solution 2 mL was passed to a volumetric balloon and added 5 mL of buffer solution of phosphates (pH 5.0) and 1 mL of methylorange solution 0.05%. The mixture was extracted with 25 mL of chloroform (5 X 5 mL) and to the chloroformic solution was added 2 mL of ethanol-sulphuric acid solution (98:2). The absorbance was read in 520 nm. The values found were transformed in percentage of solasodine/dry weight for the equation of the calibration curve obtained. The calibration curve was obtained according to Vieira (1998) utilizing external standard of solasodine.

In all of the experiments the layout was totally randomized. For growth regulators, tests were done in triplicate, n = 40 each time. Data was analyzed by variance test (ANOVA) and mean data were compared by Tukey test (significance of 5%). Rooting percentage was analyzed by difference of percentage test (p_1 and p_2), significance of 5%.

RESULT AND DISCUSSION

After 60 days of culture, plants grown in MS showed the best results for bud neoformation, shoot elongation and rooting. In this study, growth regulators supplementation reduced the height; bud neoformation number and rooting in regard to MS control (Table 2).

TABLE 1. Alkaloids produced in tissue culture by some solanacean species.

Species	Initial Explant	Basal Medium	Used Hormones (μM)	Alkaloids Produced	Analyzed Structure	Quantification of *in vitro* alkaloids (DW)	Reference
Datura innoxia	Leaf	B5	4.52 2,4-D + 0.46 Kin	Scopolamine (Sc) Hyoscyamine (Hy)	Callus	0.019-0.24 μg g^{-1} (Sc + Hy)	Savary & Dougall, 1980
D. kymatocarpa	Leaf	B5	4.52 2,4-D + 0.46 Kin	Scopolamine (Sc) Hyoscyamine (Hy)	Callus	0.052-0.22 μg g^{-1} (Sc + Hy)	
D. lanosa	Leaf	B5	4.52 2,4-D + 0.46 Kin	Scopolamine (Sc) Hyoscyamine (Hy)	Callus	0.084-0.22 μg g^{-1} (Sc + Hy)	
D. pruinosa	Leaf	B5	4.52 2,4-D + 0.46 Kin	Scopolamine (Sc) Hyoscyamine (Hy)	Callus	0.030-0.16 μg g^{-1} (Sc + Hy)	
D. stramonium	Leaf	B5	4.52 2,4-D + 0.46 Kin	Scopolamine (Sc) Hyoscyamine (Hy)	Callus	0.73-3.7 μg g^{-1} (Sc + Hy)	
Duboisia myoporoides	Leaf	MS	0.1 2,4-D 10.0 BA + 1.0 NAA 10.0 BA + 0.1 2,4-D 10.0 Kin + 0.1 2,4-D 10.0 BA + 0.1 IBA 10.0 BA + 1.0 NAA 1.0 BA + 10.0 IBA	 Nicotine (Ni) Hyoscyamine (Hy) Scopolamine (Sc)	Stem Root 	5.8-1018.0 μg g^{-1} (Ni) 7.8-38.5 μg g^{-1} (Hy) 3.6-6.5 μg g^{-1} (Sc) 86.0-91.0 μg g^{-1} (Ni) 69.0-82.0 μg g^{-1} (Hy) 37.3-48.2 μg g^{-1} (Sc)	Khanam et al., 2000
D. myoporoides	Leaf	MS	0.1-10.0 BA + 0.5-27.0 NAA 1.0-10.0 BA + 0.1-10.0 IBA 22.0 BA 25.0 IBA	Hyoscyamine (Hy) Scopolamine (Sc)	Callus Stem Root 	0.41-6.9 μg g^{-1} (Hy) 0.23 μg g^{-1} (Sc) 7.8-38.5 μg g^{-1} (Hy) 3.6-6.5 μg g^{-1} (Sc) 69.0-82.0 μg g^{-1} (Hy) 37.3-48.2 μg g^{-1} (Sc)	Khanam et al., 2001
Hyocyamus niger	Leaf	LS	5 NAA + 5 BA	Hyoscyamine (Hy) Scopolamine (Sc)	Callus	Dark - 0.015% (Hy) Light - 0.02% (Hy) Dark - 0.001% (Sc) Light - 0% (Sc)	Yamada & Hashimoto, 1982
H. niger	Leaf	LS	10 NAA + 5 BA	Hyocyamine (Hy) Scopolamine (Sc)	Cell suspension	Dark - 0.01% (Hy) Light - 0.007% (Hy) Dark - 0.002% (Sc) Light - 0% (Sc)	
Scopolia parviflcra	Rhizome	---	---	Hyocyamine (Hy) Scopolamine (Sc)	Root Stem Leaf 	1.68 mg g^{-1} (Hy) 0.52 mg g^{-1} (Sc) 1.12 mg g^{-1} (Hy) 1.25 mg g^{-1} (Sc) 1.06 mg g^{-1} (Hy) 1.56 mg g^{-1} (Sc)	Kang et al., 2004
Solanum khasianum	Root	MS/B5	---	Solasodine	Transformed Hairy Root	0.6-6.9 mg g^{-1} (2 wk) * 0.9-6.3 mg g^{-1} (4 wk) * 1.3-8.2 mg g^{-1} (6 wk) *	Jacob & Malpathak, 2005a

Continua

TABLE 1. Alkaloids produced in tissue culture by some solanacean species.

Continuação

S. khasianum	Leaf	MS	---	Solasodine (in the hairy roots)	NSR 30-45% > 45%	4.4 mg g^{-1} 4.1 mg g^{-1} 3.8 mg g^{-1}	Jacob & Malpathak, 2005b
S. khasianum	Leaf	MS	---	Solasodine (released into the medium)	NSR 30-45% > 45%	0.72 mg g^{-1} 0.31 mg g^{-1} 0.22 mg g^{-1}	
S. laciniatum	Leaf	MS	1.0 BA + 1.0 NAA	Solasodine	Callus Stem	0.09 mg g^{-1} 0.04 - 2.23 mg g^{-1}	Conner, 1987
		½ MS	---		Hairy Roots	0.3-1.0%	
S. laciniatum	Leaf	MS	10 BA + 1.0 NAA	Solasodine	Shoots	0.5 %	
S. nigrum	Leaf	MS	2.22 BA 3.33 BA + 150 mM NaCl 0.90 2,4-D + 100 mM NaCl	Solasodine	Leaf Reg. callus Non reg. callus	2.02 mg g^{-1} # 2.40 mg g^{-1} # 1.0 mg g^{-1} #	Bhat et al., 2008
Solanum nigrum	Leaf	MS	2.22 BA + 2.69 NAA	Total Glyccalkaloids ▪	Leaf Stem	2.33-4.29% ▪ 0.96-4.56% ▪	El-Ashaal et al., 1999
	In vitro leaf	MS	2.22 BA + 2.69 NAA		Root	5.33-5.94% ▪	
S. nigrum var. *judaicum*	Leaf	MS	2.22 BA + 2.69 NAA	Total Glyccalkaloids ▪	Leaf Stem	1.60-2.25% ▪ 2.37-3.10% ▪	
	In vitro leaf	MS	2.22 BA + 2.69 NAA		Root	1.78-7.57% ▪	

#Major values in each culture; ▪Total glycoalkaloids (Solamargine + Solasonine + Solanine); DW – Dry weight; B5 – Gamborg et al. (1968); LS – Linsmaier and Skoog (1965); MS – Murashige and Skoog (1962); *Manipulation of media (MS and B5) components, production in weeks (wk); NSR – root culture with no shoot regeneration; 30-45% - root culture with 30-45% of shoot regeneration; > 45% - root culture with > 45% of shoot regeneration; Reg. callus – regenerative callus; Non reg. callus – non regenerative callus.

TABLE 2. *In vitro* development of nodal segment of *S. torvum* and solasodine accumulation after 60 days culture. Values are means ± standard error (SE) for n = 40 with triplicate.

Culture medium	Neoformation of buds/plant (±SE)[1]	Shoot elongation (cm) (±SE)[1]	Rooting rate (%)	% Solasodine content/DW (±SE)[1]
MS hormone free (control)	$5.5^{a} \pm 0.18$	$11.3^{a} \pm 0.34$	100.0^{a}	$1.34^{a} \pm 0.10$
MS + 0.2 mg L^{-1} IAA	$5.0^{a} \pm 0.19$	$9.9^{b} \pm 0.34$	98.3^{a}	$0.20^{bc} \pm 0.01$
MS + 0.2 mg L^{-1} IAA + 0.7 mg L^{-1} BA	$1.9^{b} \pm 0.09$	$6.8^{c} \pm 0.36$	98.5^{a}	$0.40^{b} \pm 0.02$
MS + 0.2 mg L^{-1} IAA + 1.6 mg L^{-1} BA	$1.5^{b} \pm 0.09$	$3.6^{d} \pm 0.14$	75.2^{b}	$0.41^{b} \pm 0.01$
MS + 0.2 mg L^{-1} ANA + 0.7 mg L^{-1} BA	$1.1^{b} \pm 0.06$	$2.6^{d} \pm 0.21$	90.8^{a}	$0.09^{c} \pm 0.02$

[1] Values in the same column followed by the same letter do not differ statistically at $p \leq 0.05$

The formation of white to white-brown basal calli was observed on nodal segments under effect of growth regulators tested and concomitantly reduction of shoots production and elongation.

The induction of small organogenic callus in leaf explants of *Duboisia myoporoides* (Solanaceae) grown in MS medium added with 1 µM IAA and 10 µM BA was reported after four weeks of culture (Khanam et al., 2000). In the present work, *S. torvum* nodal segments showed the formation of basal calli but there was no organogenesis. Hypocotyl explants of *S. torvum* under effect of NAA (0.1-1.0 mg L^{-1}) and BA (0.2-2.0 mg L^{-1}) on MS medium had developed shoots and organogenic calli (Jaiswal & Narayan, 1985). On leaf explants of *Solanum nigrum* and *S. nigrum* var. *judaicum*, calli were formed in NAA (0.5-4.0 mg L^{-1}) with BA (0.5 mg L^{-1}) on MS medium. In this case, the combination of 0.5 mg L^{-1} for both regulators resulted on the highest index of regeneration (El-Ashaal et al., 1999). From these studies it can be concluded that the combination of NAA + BA was very efficient to induction of indirect organogenesis, although in *S. torvum* nodal segments, the formation of basal calli were observed only without organogenesis.

Nodal segments of *S. torvum* presented roots in over 98% of the explants in MS medium added to 0.2 mg L^{-1} IAA and when it was added 1.6 mg L^{-1} BA to the medium, rooting rate have dropped. In MS medium added to NAA and BA concentrations nodal segments produced over 90% of plants rooted in 60 days of culture.

The production of solasodine by *in vitro* culture was affected to a significant extent by plant growth regulators added to medium culture. Plants grown in MS media produced more solasodine than those grown in MS with auxins and cytokinin added (Table 2).

Auxins have been shown to inhibit alkaloid biosynthesis and accumulation. In *Catharanthus roseus* cell cultures, auxins exerted a dramatic reduction on alkaloid terpenoid precursors availability (Gantet et al., 1997). Auxins have also been shown to down-regulate gene expression in alkaloid biosynthesis. In *C. roseus* cell cultures the *sss* gene (for strictosidine synthase) and *tdc* gene (for tryptophan decarboxylase) was rapidly down regulated by auxin (Pasquali et al., 1992). However, it was showed that NAA applications did not affect leaf camptothecin concentration (Li & Liu, 2003).

Cytokinins have also been shown to be involved in plant secondary metabolism. For example, cytokinins stimulate alkaloid biosynthesis in cell lines of *C. roseus* and *Fagara zanthoxyloides* (Decendit et al., 1992; Coillerot et al., 1996; Carpin et al., 1997). On the other hand, it was reported that BA applications do not improve total leaf camptothecin (Li & Liu, 2003).

Regenerated plants that had been developed high basal calli, presented lower elongation consequently less photosensitizing tissues. In *S. laciniatum* this fact alters solasodine biosynthesis on leaves since the synthesis is dependent or promoted in active chloroplast (Conner, 1987). It was also observed on green root culture of *Datura stramonium* the increment of alkaloid production and subsequent decrease of the production when this culture was transferred to dark conditions (Flores et al., 1993). This type of culture might be the key for high production of the solasodine since this compound was firstly detected and this accumulation occurs on green parts of the plants, e.g. leaves and immature fruits (Kittipongpatana et al., 1998).

It was reported a great production of glycoalkaloids (solasonine, solamargine and solanine) on leaf culture of *Solanum nigrum* and *Solanum nigrum* var. *judaicum* on MS + 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} BA (El-Ashaal et al., 1999). The results of this work are controversy since lower solasodine accumulation on regenerated plants were obtained

when cultivated on the same combination of plant growth regulators.

The evaluation of solasodine content on *in vitro* plants of *S. torvum* showed that on MS medium with no plant growth regulators, the content was 13.4 mg solasodine g^{-1} DW (1.34%) as higher as the most competitive species that produced on leaves, from field plants, from 1.6 to 3.8% (Vieira & Carvalho, 1993).

It was mentioned that a number of factors might influence the in vitro accumulation of solasodine on *S. aviculare* as basic medium of culture, sucrose concentration, type and concentration of plant growth regulators and the combinations of overall factors (Kittipongpatana et al., 1998). Also, the salinity stress in *S. nigrum* offers possibility of enhanced production of solasodine, using NaCl as an efficient and economical elicitor source (Bhat et al., 2008).

From the biotechnological point of view, *S. torvum* is a species that must be investigated in future work since the in vitro production of solasodine have been a desired target and it has been potential alternative to diosgenin for commercial steroid drug synthesis (Galanes et al., 1984). Also the price of diosgenin increased dramatically in the 90's, therefore the search for suitable alternative remains a viable area of research. The success in obtaining a method for high production of solasodine, might very well be one of the solutions for this problem in the future.

It was investigated the relation between growth and solasodine production manipulating the components in MS and Gamborg's B5 media in hairy root cultures of *Solanum khasianum* (Jacob & Malpathak, 2005a). Thus it was concluded that optimization of appropriate root line with a high growth rate and secondary metabolism shall help in a scale up of solasodine production in suitable bioreactors. Meanwhile, in hairy roots culture of *S. khasianum*, the presence of shoots as an alternate sink for the steroidal alkaloid extended the exponential growth phase of the culture and it can be used to achieve optimum hairy root culture performance for increased solasodine production and low permeabilization into the medium (Jacob & Malpathak, 2005b)

In vitro plants of *Solanum torvum* showed the capacity to produce alkaloids. MS basal medium was the best culture conditions for solasodine accumulation (1.34% DW). IAA (0.2 mg L^{-1}) and BA (0.7 and 1.6 mg L^{-1}) and also NAA (0.2 mg L^{-1}) and BA (0.7 mg L^{-1}) applications in growth media decreased the bud neoformation/explant, shoot elongation and rooting percentage in nodal segment culture and also inhibited the solasodine accumulation in regenerated plants, however growth regulators combinations induced high production of friable basal calli. These results show that, for further studies, *in vitro* cell culture can be initiated with others auxin and cytokinin for enhancing *in vitro* production of solasodine besides different elicitors, light intensities and sucrose concentrations.

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