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BRIEF COMMUNICATION

In vitro production of solasodine from *Solanum trilobatum*

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Abstract

Sucrose concentration in the culture medium affected chlorophyll content, trichome development and amount of solasodine in regenerated plantlets of *Solanum trilobatum*. High chlorophyll content and glandular trichomes were observed in the plants grown on Murashige and Skoog basal medium supplemented with 131.85 mM sucrose. The solasodine was quantified using reverse phase high performance liquid chromatography. The plantlets cultivated on this medium yielded 35.97 mg g^{-1} (d.m.) solasodine whereas the field plants used as control yielded only 2.32 mg g^{-1} (d.m.) of solasodine.

Additional key words: chlorophyll content, HPLC, *in vitro* regeneration, trichomes.

Solanum trilobatum Linn. is a straggling climber chiefly found in Deccan peninsula, India. The presence of solasodine, a glycoalkaloid, which can be used for steroidal drug biosynthesis, has been reported in this species (Krishnamurthy and Reddy 1996). The natural production of solasodine is restricted. Hence, the pharmaceutical industry is exploring alternative methods such as *in vitro* production of natural compounds. Manipulating the cultural conditions such as concentration and combinations of growth regulators, quality and quantity of radiation, carbon source, inoculum size, temperature and macronutrients alter the rate of secondary metabolite production (Nigra *et al*. 1989, Yu *et al*. 1996, Rao and Ravishankar 2002, Bhatnagar *et al*. 2004, Xu *et al*. 2008). *In vitro* regeneration potential was high in *S. trilobatum* (Rejitha *et al*. 2002, Alagumanian *et al*. 2004). In the present study we report the morphology and solasodine content of *in vitro* regenerated *Solanum trilobatum* plantlets with respect to the sucrose concentration in the medium.

Ripened fruits were collected from the Central Ayurvedic Research Institute, Poojappura, Thiruvanathapuram, Kerala, India. The fruits were washed in 1 % *Labolene*, a neutral liquid detergent, (*Qualigens*, Mumbai, India) for 5 min, surface sterilized using 0.1 % $HgCl_2$ for 7 min and washed well with double distilled sterile water for three times, each wash for 5 min. Fruits were then flame sterilized in 70 % alcohol for 1 min. Seeds were aseptically isolated and inoculated on ½ strength Murashige and Skoog (1962; MS) basal medium. The pH of the medium was adjusted to 5.8 with 0.1 M HCl or 0.1 M NaOH prior to autoclaving (121 °C, 20 min). After 14 d cotyledons were aseptically isolated from *in vitro* seedlings and inoculated on MS medium with 87.9 mM sucrose supplemented with 11.41 μM of indole 3-acetic acid (IAA) and 18.6 μM kinetin (KIN). After 30 d, the whole biomass per tube were divided into 6 pieces and inoculated on MS basal medium with 43.95, 87.90, 131.85, 175.80, 219.75 and 263.70 mM sucrose. The cultures were incubated at 25 ± 2 ºC and 16-h photoperiod (irradiance of 65 μmol m^{-2} s^{-1}); subcultured in 30 d interval for 90 d and then plantlets were harvested for analysis.

The number and height of plantlets, number of leaves, leaf length, breadth, leaf length/width (L/W) ratio, number and length of roots were recorded and chlorophyll content of the leaves was estimated in acetone extract according to Arnon (1949) by spectrophotometer (*Shimadzu Pharmasec UV-1700,* Tokyo, Japan). Scanning electron micrographs were taken with scanning electron microscopy (*Philips*

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Abbreviations: Chl - chlorophyll, 2,4-D - 2,4-dichlorophenoxyacetic acid; d.m.- dry mass; GI - growth index; IAA - indole-3-acetic acid; KIN - kinetin; L/W - length/width ratio; RP-HPLC - reverse phase high pressure liquid chromatography; SEM - scanning electron microscopy.

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XL30CP SEM, The Netherlands) at 50 - 300× magnification from the third nodal leaves from field grown plant (control), *in vitro* grown plant established in a soil and *in vitro* plants on the medium with different concentrations of sucrose. Growth index (GI) based on fresh mass increase was measured according to Jacob and Malpathak (2004). Extraction, isolation and quantification of solasodine were done according to Kittipongpatana *et al*. (1999). The precipitated residue with ammonium hydroxide solution was dried and repeatedly washed with double distilled sterile water 4 - 5 times to remove the traces of ammonium chloride. The washed suspension was filtered through *Whatman No. 1* filter paper. The residue was dried, re-dissolved in 10 cm^3 of 30 % HPLC grade methanol (*SRL,* Mumbai, India) and filtered using 2 μm filter. Solasodine was quantified by using reverse phase high performance liquid chromatography (RP-HPLC; *Shimadzu SPD-10A*, column C-18, pore size 2 μm) using the mobile phase methanol:water (70:30), flow rate 1 cm^3 min^{-1}, injection volume 0.02 cm^3. Solasodine was detected by UV absorption at 205 nm. The isolated fraction was also co-chromatographed with standard solasodine (*Sigma- Aldrich*, St. Louis, USA).

All the experiments were arranged in a completely randomized design. Each experiment consisted of 10 replicates. Two sets of experiments were conducted and statistical analysis was performed with software *SPSS/PC* version *4.0* (*SPSS Inc*., Chicago, USA). The standard error of means (SE) were calculated and differences between means were tested using Scheffe's multiple comparisons at $P = 0.05$.

Callus initiation and shoot regeneration was observed within 14 d in the cotyledons inoculated on MS medium supplemented with 11.41 μM IAA, 18.6 μM KIN and 87.9 mM sucrose. Numerous microshoots were developed within 30 d. After three sub-cultures on MS medium supplemented with different concentration of sucrose, maximum number of plantlets per explants was obtained from MS medium supplemented with 131.85 μM sucrose. Maximum chlorophyll content was also observed in these plantlets (Table 1).

SEM analysis of the leaves from field grown plant revealed only presence of stellate trichomes, however in the leaves of *in vitro* plants glandular, stellate and acicular trichomes were observed (Fig. 1). The *in vitro* plants established in soil showed maximum number of glandular trichomes along with a few stellate and acicular trichomes. In the leaves from *in vitro* plantlets on the medium, the frequency of glandular trichomes varied with respect to the sucrose concentration. High frequency of glandular trichomes was observed in the plants maintained on MS medium supplemented with 131.85 mM of sucrose. The number of glandular trichomes decreased in the leaves obtained from the medium with lower or higher concentration of sucrose (Table 2).

The maximum GI was observed in the plantlets maintained on the medium supplemented with 175.8 mM sucrose but maximum solasodine content was observed in the plantlets from the medium supplemented with 131.85 mM sucrose (Table 3). Thus the high GI was not correlated with the increased amount of solasodine in the plant tissues. Only 11.30 mg g^{-1}(d.m.) of solasodine was obtained from the plantlets maintained on MS medium supplemented with 43.95 mM sucrose whereas 35.97 mg g^{-1}(d.m.) solasodine was obtained from the plantlets maintained on MS medium with 131.85 mM

Table 1. Morphological variations of *in vitro* regenerated plants on MS medium supplemented with different concentrations of sucrose. Means ± SE, $n = 10$; means with same superscripts are not significantly different at $P = 0.05$

Sucrose [mM]	Number of plantlets	Height [cm]	Leaf number	Leaf length [cm]	Leaf width [cm]	L/W ratio	Root number	Root length [cm]	Chl [mg g^{-1}(f.m.)]
43.95	17.40±1.20^{a}	1.86±0.35^{a}	8.20±0.73^{ab}	1.83±0.14^{b}	1.94±0.18^{a}	0.94±0.06^{a}	1.20±0.11^{a}	11.14±1.03^{a}	4.22±0.21^{b}
87.90	22.80±0.40^{a}	3.28±0.39^{ab}	8.33±0.57^{b}	2.40±0.24^{c}	2.32±0.19^{a}	1.03±0.05^{b}	1.16±0.21^{a}	18.92±1.42^{a}	4.39±0.33^{c}
131.85	31.20±0.46^{a}	3.45±0.70^{ab}	8.47±0.86^{b}	2.29±0.20^{bc}	1.96±0.16^{a}	1.17±0.04^{ab}	1.17±0.15^{a}	21.91±1.96^{a}	8.81±0.73^{f}
175.80	25.20±0.86^{a}	1.60±0.37^{a}	5.10±0.37^{ab}	1.23±0.23^{a}	1.13±0.22^{a}	1.08±0.07^{a}	1.20±0.32^{a}	7.66±3.29^{a}	6.53±0.51^{e}
219.75	12.60±0.60^{a}	1.28±0.18^{a}	4.32±0.44^{b}	1.32±0.14^{a}	1.26±0.13^{a}	1.04±0.09^{a}	1.50±0.16^{a}	8.15±1.25^{a}	6.01±0.71^{d}
263.70	19.80±1.38^{a}	1.42±0.41^{a}	1.19±0.21^{b}	1.19±0.21^{a}	1.12±0.20^{a}	1.06±0.08^{ab}	1.00±0.00^{a}	17.53±3.35^{a}	4.17±0.42^{a}

Table 2. Trichome number [cm^{-2}] observed in the leaves of *in vitro* regenerated plants on MS medium supplemented with different concentrations of sucrose. Means ± SE, $n = 10$; means with same superscripts are not significantly different at $P = 0.05$

Types of trichomes	Control	Plants in field	Concentration of sucrose in medium [mM] 43.95	87.90	131.85	175.80	219.75	263.70
Glandular	-	86.37±6.60^{d}	33.75±3.25^{ab}	33.87±1.20^{ab}	103.77±10.39^{cd}	79.50±7.91^{cd}	57.67±2.82^{d}	48.87±6.89^{bc}
Stellate	3.87±0.89^{a}	4.50±0.53^{a}	11.37±0.65^{ab}	20.38±1.19^{b}	18.13± 1.39^{b}	15.22±1.87^{b}	15.00±1.86^{b}	16.88±3.40^{b}
Acicular	-	0.87±0.29^{ab}	3.50±0.42^{bc}	3.25±0.16^{bc}	1.38± 0.45^{ab}	4.37±0.71^{c}	2.00±0.37^{abc}	4.37±0.09^{c}

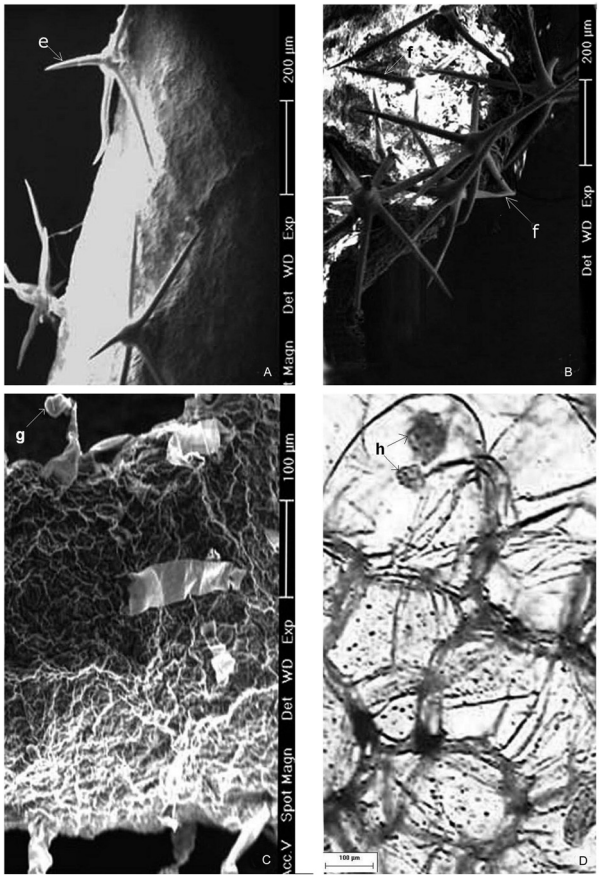

Fig. 1. Scanning electron micrographs of stellate trichomes of field plant (*A*), of stellate, acicular and glandular trichomes of *in vitro* grown plant transferred to field (*B*) and of glandular trichomes of *in vitro* plants grown in 131.85 mM sucrose (*C*). Histochemical localization of solasodine crystals (h) from *in vitro* regenerated plants by using Dragendorff's reagent (*D*).

sucrose. The field grown plants yielded only 2.8 mg g^{-1}(d.m.) solasodine.

Sucrose concentration in the culture medium affected the morphological parameters such as growth, primary metabolism and yield of secondary products in the plants (Indrayanto *et al*. 1995, Yu *et al*. 1996, Jacob and Malpathak 2004). Kim *et al.* (1995) suggested the role of sucrose concentration in the culture medium for biomass accumulation and taxol production. Nigra *et al.* (1987) reported that plants may have similar receptors and/or signal transduction pathways for the synthesis of photosynthetic pigments and some alkaloids. The possibility of chloroplast associated key enzymes in biosynthetic pathway of withanolide formation was also reported in *Withania somnifera* (Sharada *et al*. 2007). High chlorophyll content in the plantlets was correlated with solasodine production as suggested previously in *S. laciniatum* (Bhatnagar *et al*. 2004) and in *S. aviculare* (Jacob and Malpathak 2004). In *S. trilobatum* the morphological parameters showed only slight variation with respect to the sucrose concentration in the medium. The content of pharmacologically active compounds in medicinal species is influenced both genetically and environmentally. Thus enhancement in solasodine production in the present study may be correlated with the increase in sucrose concentration in the medium. However, as Aziz *et al*. (2007) observed in *Centella asiatica*, the limits of such biosynthesis cannot exceed specific thresholds. Also Bonfill *et al.* (2007) observed that *Taxus* cells excrete taxanes in the culture medium and this accumulation may limit biosynthesis possibly by feedback inhibition. There is no significant difference in GI of the plantlets grown in MS medium supplemented with higher concentration of sucrose. This may be due to the deposition of cellulose materials in the cell wall rather than enhanced cell division in higher concentrations of sucrose in the medium as suggested by Gollagunta *et al*. (2004).

Table 3. Growth indices and solasodine content [mg g^{-1} (d.m.)] of *in vitro* regenerated plants on MS medium supplemented with different concentrations of sucrose. GI = (final f.m.)/(inoculum f.m). Means ± SE, n = 10; means with same superscripts are not significantly different at P = 0.05

Sucrose [mM]	Fresh mass	Dry mass	GI	Solasodine content
43.95	0.13 ± 0.04^{a}	0.04 ± 0.01^{a}	1.58 ± 0.04^{a}	11.30^{c}
87.90	0.55 ± 0.20^{b}	0.13 ± 0.04^{a}	6.77 ± 0.28^{b}	12.19^{c}
131.85	0.43 ± 0.16^{b}	0.15 ± 0.05^{a}	5.21 ± 0.96^{b}	35.97^{e}
175.80	0.81 ± 0.16^{a}	0.13 ± 0.05^{a}	9.61 ± 0.49^{c}	5.79^{b}
219.75	0.24 ± 0.09^{a}	0.08 ± 0.01^{a}	3.08 ± 0.40^{a}	5.34^{b}
263.70	0.39 ± 0.14^{a}	0.14 ± 0.05^{a}	3.71 ± 0.11^{a}	0.19^{a}

Plant glandular trichomes function either as repositories or as releasing sites of various chemicals (Peter and Shanower 1998, Raina *et al*. 2005). The development of glandular trichomes in *in vitro* grown plants may be an adaptive mechanism to overcome the osmotic stress in culture with high sucrose concentration. *In vitro* plants remain metabolically active for a longer period compared to field plants yielding increased amount of secondary products. Sucrose concentration in the medium might affected the metabolic profile and modified the endogenous pathways to increase the flux towards the production of a particular desirable compound. In previous reports 7 mg(solasodine) g^{-1}(d.m.) was reported from *in vitro* cultures of *S. laciniatum* (Bhatnagar *et al*. 2004). In the present study, 12 fold increase of solasodine content was obtained from *in vitro* plantlets of *S. trilobatum* on the medium with 131.85 mM sucrose. The increase in solasodine content indicates the possibility to use this system for commercial large scale production of solasodine.

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