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In vitro Production of Diosgenin under Varying Concentrations of Sucrose and Cholesterol in *Solanum trilobatum* Linn.

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ABSTRACT

Solanum trilobatum Linn. belongs to the family Solanaceae. The plant has been used as folk and Ayurvedic medicine for respiratory diseases. The decoction of the plant is used for asthma, chronic bronchitis and other lung diseases. Leaf explants from *in vitro* seedlings inoculated on MS medium augmented with 0.1 mg/l of IAA and 2 mg/l of kinetin showed shoot regeneration from the petiole and leaf margins. The shoots were subcultured in the same medium, maintained for two months developed into plantlets. These plantlets were transferred to MS medium with varying light environment or (2%, 3%, 4% and 5%) of sucrose concentrations or MS medium with (0.1, 0.5, 1 and 2 mg/l) cholesterol. After one month the plantlets were used for estimating diosgenin content. Higher percentage of sucrose or cholesterol in the medium increased the diosgenin content in the *in vitro* plantlets.

INTRODUCTION

Plants have been the cornerstone of medicine therapies for thousands of years and continue to be an essential part of health care for much of the world. *Solanum trilobatum* Linn. (Solanaceae) is a spiny under shrub of 1.8-3.6 m long, trailing or subscandent common in South India (**Fig. 1**). The whole plant is considered medicinal. The decoction of the entire plant is administered in case of acute and chronic bronchitis. It is considered as a home remedy for all kinds of cough. The leaves are consumed as vegetable [1]. The antitumour activity of the plant extracts have also been reported and is due to the presence of steroid rich compounds [2].One of the steroids was identified as diosgenin [3]. Diosgenin is anti-inflammatory, antistress, hepatoprotective, hypocholestrolemic and mastogenic.

It is given as a treatment for labour pains, gastrointestinal irritations, morning sickness, asthma, spasmodic hiccough and rheumatism. It is also used for treatment of dysmenorrheal, testicular deficiency, menopause, premenstrual syndrome, impotence and prostrate hypertrophy. Diosgenin is used

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as a starting material for synthesis of oral contraceptives, sex hormones and other steroids [4]. Diosgenin has been shown to suppress inflammation and proliferation and to induce apoptosis in a variety of tumour cells [5,6,7,8].

Fig1: *Solanum trilobatum* Habit

In the present study, *in vitro* raised plantlets were inoculated in different light environments and in medium with varying concentrations of sucrose or cholesterol and diosgenin content was estimated.

MATERIALS AND METHOD

Solanum trilobatum plantlets were collected from Ayurveda Research Centre, Poojapoora, Thiruvananthapuram, Kerala and maintained in the green-house at Department of Botany, Kariavattom Campus, Thiruvananthapuram, Kerala. Healthy and ripe fruits were plucked and sterilized in 0.1% mercuric chloride (w/v) for 10 minutes. The seeds were inoculated in basal MS medium, incubated at 25 ± 2°C, 55-65% humidity and 2500 lux light intensity, provided by fluorescent lamps (Philips, India). The culture room was maintained at 16 hours photoperiod.

After two weeks *in vitro* leaves were selected from the seedlings and were inoculated on MS medium augmented with 0.1 mg/l IAA and 2 mg/l kinetin. The *in vitro* shoots were multiplied for a period of two months through sub culturing in the same medium. After two months the roots developed. Sixty days old *in vitro* shoots/plantlets were selected for the present study. These were inoculated in varying medium composition or physical environment. Three parameters were taken into consideration for the present study: a. different light environment, b. varying percentage of carbohydrate (sucrose) source and c. addition of precursor – cholesterol (Sigma Chemicals Company, USA)

Different light environment

The sixty day *in vitro* shoot/plantlets were inoculated on MS medium augmented with 0.1 mg/l IAA and 2 mg/l kinetin. These were incubated in two different light environments, for one month. Interference between light treatments was avoided by separating each chamber by a black cloth. All cultures were maintained at 16 hours photoperiod at 25±2°C. The shoots were kept alternatively in 24 hours red/ blue light (10 lux) followed by 72 hours in white fluorescent light (1500 lux) for one month. The regenerated plants

maintained in varying light environment were grouped into four sets based on the light condition they were last kept.

- 72 hours white fluorescent light followed by 24 hours red light (R)
- 72 hours white fluorescent light followed by 24 hours blue light (B)
- 24 hours red light followed by 72 hours white fluorescent light (RL)
- 24 hours blue light followed by 72 hours white fluorescent light (BL)

Varying percentage of carbohydrate (Sucrose) source

The sixty day *in vitro* shoots were inoculated in MS medium augmented with 0.1 mg/l IAA and 2 mg/l kinetin with (2%, 3%, 4% and 5%) of sucrose.

Addition of precursor – cholesterol.

Another set of *in vitro* plantlets were transferred to MS medium augmented with 0.1 mg/l IAA and 2 mg/l kinetin with (0.1, 0.5, 1 and 2 mg/l) cholesterol.

The plantlets transferred to MS basal medium with 3% sucrose were used as the control.

Phytochemical Analysis After one month the plantlets were removed from the medium, weighed, dried in a hot air oven at 60 °C and powdered. The dried samples were weighed and petroleum ether extracts were taken by means of a soxhlet extractor.

The extracts were evaporated to dryness, weighed and saponified by refluxing in 0.5 N alcoholic KOH for one hour, cooled and diluted with distilled water. The resultant sample was further extracted with diethyl ether (five to six times) in a separator funnel and transferred to a beaker containing anhydrous sodium sulphate.

The sample was thus kept overnight to make it free from water. This was filtered repeatedly with diethyl ether, concentrated by evaporation and weighed. The resultant extract (1 mg) was used for TLC analysis (85 hexane: 15 diethyl ether: 2 acetic acid, v/v/v). The steroidal bands developed when exposed to iodine vapour. Diosgenin was identified by use of standard (Sigma Chemical Co.) and marked. The marked spots were scrapped, eluted with diethyl ether, centrifuged and the supernatant was concentrated in a vacuum evaporator. The supernatant was collected in a pre-weighed tube. The supernatant was concentrated to dryness and the final weight was taken. Difference between the initial and final weight gave the amount of diosgenin.

RESULTS AND DISSCUSION

Ripe, red-scarlet coloured fruits were collected (**Fig. 2**) from the plant as explants. In vitro seedlings were raised on MS basal medium. Leaves of in vitro seedlings inoculated on MS medium supplemented with 0.1mg/l IAA and 2 mg/l kinetin showed shoot regeneration from the petiole and leaf ends (**Fig. 3**). These shoots were sub-cultured into the same medium composition for two months (**Fig. 4**). Root development also occurred. These plantlets were subjected to varying light, sugar or cholesterol concentrations for one month.

Fig 2: Fruits of *S. trilobatum*

Fig 3: Regeneration of shoots from petiole and leaf margin of *S trilobatum*

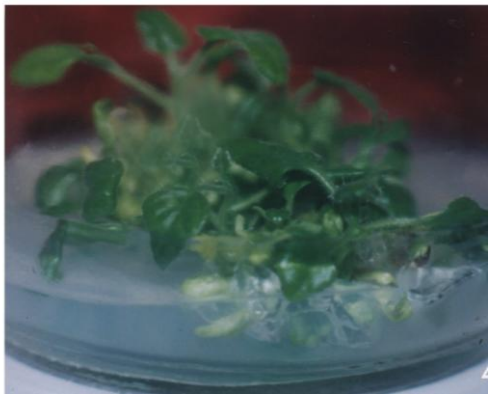

Fig 4: Two month old plantlets of *Solanum trilobatum* used for the present study

Exposure of plantlets to the red light for 24 hrs reduced the shoot multiplication rate (60%) and the shoots formed were also shorter in length when compared to those kept in blue light (**Fig. 5a**). Prolonged exposure to red light turned the leaves yellow (**Fig. 5b**), ultimately leads to drooping of leaves and senescence. The shoots regained its vigour when transferred to white fluorescent light for 72 hrs. Plantlets kept in blue light showed high multiplication rate (**Fig. 5c**).

The plantlets were healthy with green leaves and small, thin, white unbranched roots. Plant biomass was found to be more in blue light but lipid extract and steroid 'rich' extract was favoured by red light. Since the amount of unsaponified fatty acid was very less for qualitative analysis by TLC it was excluded from the present study. Sucrose as a carbon source is one of the mportant nutrition
factor affecting plant growth in cultures. An important role of carbohydrates for diosgenin synthesis has been reported by Ciura *et al.*,[9]. Increase in sucrose concentration facilitated multiplication of shoots (**Fig 6-a, b & c**). Maximum level of shoot multiplication was obtained in 4% sucrose concentration (**Fig 6c**). Further increase in sucrose concentration to 5% reduced the rate of shoot proliferation. The shoots showed occurrence of spines (**Fig 6d & 7**). The shoots could be directly transferred to the soil without hardening. Sucrose concentration in the medium increased the biomass of the plantlets.

Increase in concentration of sucrose also favoured secondary metabolite synthesis in regenerated plantlets of *Solanum trilobatum* (**Table 1**).

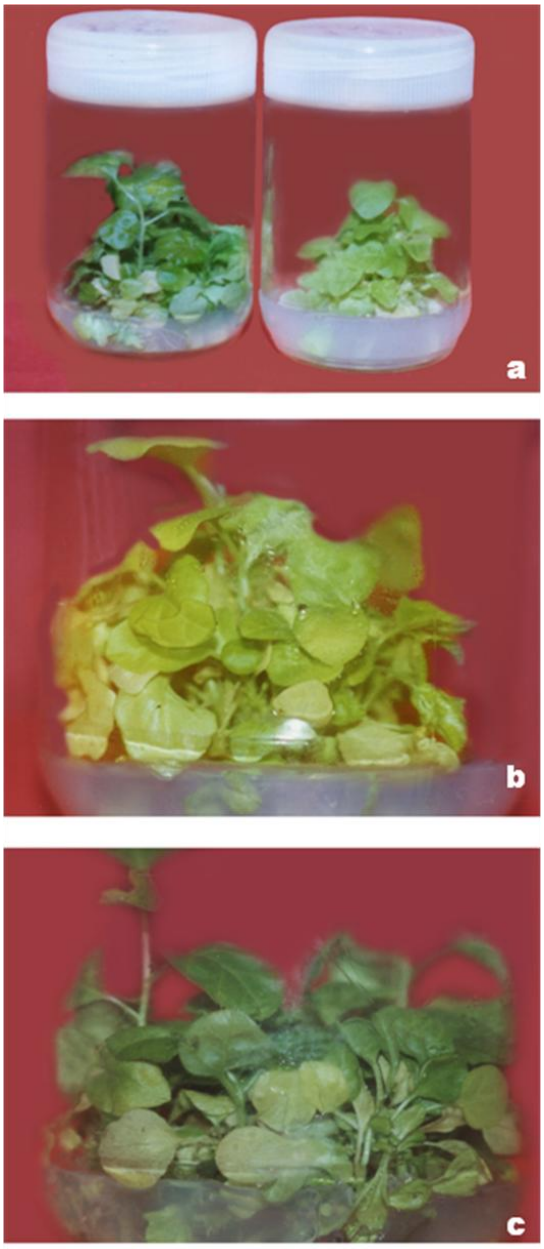

Fig 5: *S. trilobatum* *(a)* Growth of plantlets in red and blue light (b) Yellowing of leaves in red light (c)Shoot multiplication in blue light

It is a common phenomenon in plant cell cultures. The TLC chromatogram showed the presence of seven fractions with Rf values 0.99, 0.151, 0.258, 0.318, 0.497, 0.549 and 0.987, as in control plants. Diosgenin and β sitosterol (fraction four and five) were identified at Rf 0.318, 0.497, respectively, using standards (**Fig. 10**). Diosgenin content increased with increase in sugar concentration (**Table 1**).

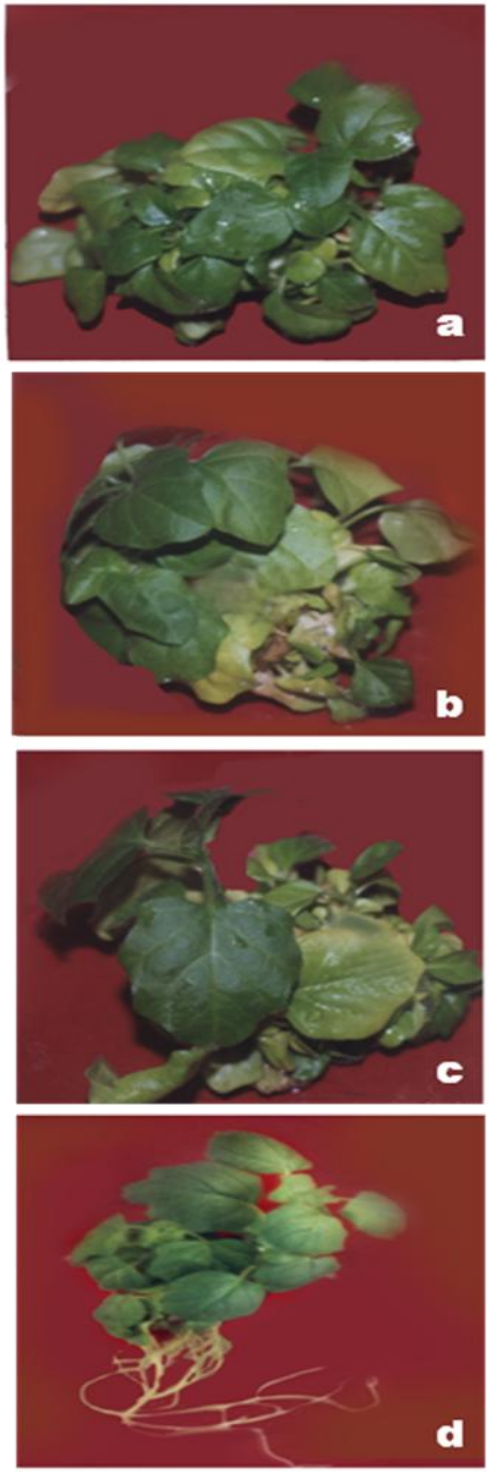

Fig 6: *S. trilobatum* plantlets from: (a) 2% sucrose (b) 3% sucrose (c) 4% sucrose (d) 5% sucrose with long roots.

Sample		Dry weight %	Lipid Extract %		Unsaponified Fatty Acid %		Amount of Diosgenin (mg/g)
			In Fresh Weight	In Dry Weight	In Fresh Weight	In Dry Weight	
Control	**Field Grown**	**12.38**	**0.25**	**3.52**	**3.65**	**1.64**	**1.11**
	In vitro	**7.91**	**0.16**	**2.18**	**2.09**	**0.20**	**0.99**
Sucrose	**2%**	**7.38**	**0.14**	**1.92**	**1.92**	**0.04**	**0.83**
	4%	**8.24**	**0.19**	**2.25**	**2.25**	**0.52**	**2.50**
	5%	**9.28**	**0.27**	**2.74**	**0.07**	**0.74**	**3.10**
Cholesterol	**0.1 mg**	**8.77**	**0.19**	**2.22**	**0.03**	**0. 30**	**1.03**
	0.5 mg	**7.66**	**0.17**	**2.14**	**0.02**	**0.31**	**1.51**
	1.0 mg	**7.44**	**0.16**	**2.10**	**0.02**	**0.32**	**3.94**

Table 1: Amount of Diosgenin in Steroid Fraction of *Solanum trilobatum*

A maximum amount of 3.1 mg/g diosgenin was obtained at 5% sucrose concentration. A positive correlation between sucrose and secondary metabolite synthesis has been cited in *Taxus* [10], *Rhodiola sachalinesis* [11] and *Costus speciosus* [12]. Tal *et al*., [13] reported a 2.6 fold increase in diosgenin production by fourfold increase in sucrose content in *in vitro* system. Similar results were obtained in other plants [14, 15] and it was assumed that additional carbohydrate pool was used for biomass formation.

Shoot multiplication increased with increase in cholesterol concentration. The shoots obtained in higher concentrations of cholesterol were dark green in colour with thick leaves and well developed root system (**Fig 8**). The plantlets survived in soil without any

Cholesterol at 2 mg/l showed the presence of hairs as well as spines on the stem (**Fig 9**). Increase in cholesterol

Fig 7: *S. trilobatum* plantlets obtained in

level decreased the dry weight of plantlets but the amount of steroid

contents increased (**Table 1**).TLC analysis showed the presence of seven fractions. Diosgenin was identified by the use of standard (**Fig 10**). Diosgenin content increased from 1.032 at 0.1 mg/l to 4.21 at 2 mg/l cholesterol.

Cholesterol is an important intermediate in the synthesis of the steroids in plants and animals. The conversion of cholesterol to diosgenin has been shown by feeding experiments [16, 17]. Feeding of precursors has been employed with variable success to increase *de novo* synthesis of metabolites. Increase in diosgenin content with increasing concentrations of cholesterol has been reported in suspension cultures [18, 19, 20].

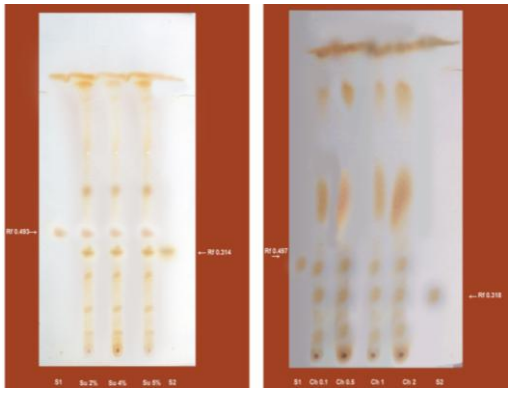

Fig10: TLC Chromatogram of plantlets obtained from varying percentage of sucrose and cholesterol (S1- S2- Su- Sucrose, Ch – Cholesterol). Rf value: Diosgenin-0.318 and β sitosterol- 0.497

Isolation of diosgenin has been reported in a few Solanaceae members [21,22]. Rejitha *et al.* [3] has reported the presence of diosgenin in the plant extract of *Solanum trilobatum*. Earlier *Dioscorea sp*. was considered as a sole source of diosgenin. However, now alternate precursors like sitosterol, cholesterol, stigmasterol and hecogenin are used for the production of diosgenin. Jain *et al*., [23] also reported the presence of diosgenin, sitosterol and solasodine from *Solanum jasminoides* and *S. verbascifalium.*

Natural compounds of

Fig 8: *S. trilobatum* plantlets (Shoot multiplication) in 1mg/l and 2mg/l cholesterol

Fig 9: *S. trilobatum* plantlets obtained 2 mg/l cholesterol with thick stem and small, thick roots

pharmaceutical importance that were once obtained from higher plant sources are now produced largely by chemical synthesis. However, the complex stereochemical structure of many compounds precludes its economical synthesis on a large scale. Here comes the significance of production of biochemicals using plant cell and tissue culture techniques. In the intact plant the secondary metabolites are thought to be protective compounds. In *in vitro* conditions the production can be induced by giving stress. Manipulation of plant cell culture environment and media can affect the rates of both cell growth and accumulation of secondary metabolites. In view of economic constraints candidates for commercial production via plant cell culture are limited to a few types of high value plant specific compounds [24]. This includes diosgenin derived steroid hormone precursors. Future advances in our understanding of immunology and related areas should permit the development of new selective and sensitive bioassays to guide the isolation of bioactive natural products.

CONCLUSIONS

The present study revealed that growth and secondary metabolite synthesis is highly dependent on the culture medium and environmental conditions. The production of steroids could be enhanced by the use of physical or chemical agents however standardization is required for large scale production. Thus, *Solanum trilobatum* cultures can prove to be an efficient source for the pharmaceutical industry for production of valuable biochemicals.

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ABBREVIATIONS

IAA- Indole 3 Acetic Acid, MS- Murashige and Skoog's medium, TLC- Thin Layer Chromatography

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