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Production of medicinally important secondary metabolites (stigmasterol and hecogenin) from root cultures of *Chlorophytum borivilianum* (Safed musli)

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Abstract
Chlorophytum borivilianum (Liliaceae) commonly known as safed musli is a perennial rhizomatous herb widely distributed in the pan tropical regions containing pharmaceutically important saponins. Root cultures were established from micropropagated plants of *Chlorophytum borivilianum* on Murashige and Skoog media supplemented with 3 mg/l Indole Butyric Acid. Growth kinetics of roots was done under shake flask conditions and maximum biomass was observed after 4 weeks of culture. A twenty four-fold increase of fresh biomass was evident in shake cultures. These *in vitro* propagated roots were tested for the production of pharmaceutically important secondary metabolites stigmasterol and hecogenin. A maximum of 46.4 mg/gDCW of stigmasterol and 685 mg/gDCW of hecogenin was evident in these cultures.

Keywords: *Chlorophytum borivilianum* (Safed musli), stigmasterol, hecogenin, Root cultures, MS liquid media, Shake flask.

INTRODUCTION

Chlorophytum borivilianum is a medicinal plant distributed in eastern part of India (Andhra Pradesh, Bihar, Assam, Eastern Ghats and Eastern Himalayas) [1,2,3]. Fasiculated roots of *Chlorophytum borivilianum* are used as tonic and important ingredient of 20 ayurvedic and unnani preparations [4]. In traditional systems of medicine, which include Ayurveda, Siddha, Unani, its roots are used as the principal component for preparations of various drugs [5]. Thirteen species of *Chlorophytum* have been reported from India which differ in appearance and native species are sold as 'Safed musli' in the Indian drug market. Among these, *Chlorophytum borivilianum* produces the highest yield and highest saponin content [6]. Major chemical constituents of Safed musli are saponins 2-17% [7], among them stigmasterol and hecogenin are given utmost pharmaceutical importance. Its demand is rapidly increasing in Indian and International drug market. Foreign demand has been estimated as 300-700 tonnes annually [7], a quantity which Indian forests cannot sustain. To avoid pressure on the natural habitats, attempts have been made to cultivate Safed musli [8,9]. However, seed germination has been reported to be very poor (14-16%) [10], and there is scarcity of planting materials like tubers. A solution to overcome such situation is the development of biotechnological methods like plant cell and organ cultures in order to obtain valuable metabolites. The rate of metabolism is much higher in cell and organ cultures than field grown plants [11]. Further, plant cell organ cultures are altered by the environmental, ecological and climatic conditions hence cells can proliferate at higher growth rates in comparison to the conventional methods [12]. Although there are a few reports on micro propagation, there are no reports on *in vitro* root cultures of *Chlorophytum borivilianum* so far. Therefore this work was undertaken to study the production of medicinally important phytopharmaceuticals stigmasterol and hecogenin from *in vitro* grown root cultures of *C. borivilianum*.

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MATERIALS AND METHODS

Chemicals and Reagents

MS media, Indole-3-Butyric Acid, agar were from Himedia, India. Methanol of analytical/ HPLC grade, Acetic acid of HPLC grade were procured from Merck. Standard stigmasterol was supplied by Tokyo chemical industry Co. Ltd, Japan and hecogenin by MP Biochemicals, LLC, France.

Plant material

Plant material of *Chlorophytum borivilianum* was collected from Nandan Biomatrix ltd., Agro farms, Hyderabad.

In vitro culture establishment

The root part of the plant was detached leaving the crown attached to the plant portion, leaves were cut down leaving 2-3 cm of length from the crown with the help of a scalpel. The explants were cleaned and surface sterilized with 0.1% mercuric chloride for 7 minutes and then rinsed with sterile distilled water thoroughly. The sterile explants were cultured onto Murashige and Skoog (MS) agar medium supplemented with 2.5 mg/l of BAP and 0.5 mg/l of NAA. The pH of this media was adjusted to 5.88 before autoclaving (121°C for 15 min at 15 lbs pressure). The cultures were incubated under controlled environmental conditions at 25±2° C with 16h light/dark regime. These cultures were subcultured at an interval of 28 days. After six weeks of initiation, the multiple shoots were cut into individual plantlet and sub cultured on to the basal media

supplemented with different concentrations of IBA (Indole Butyric Acid) (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 mg/l), NAA (Napthalene Acetic Acid) (0.5,1.0, 1.5, 2.0 ------ 4.0 mg/l) and IAA (Indole Acetic Acid) (0.5, 1,1.5, 2.0 -------- 4.0) for root induction.

Initiation of root cultures

After 8 weeks of culture, the roots were separated from the explants aseptically and cultured in liquid MS media supplemented with 3mg/l IBA and kept under continuous agitation at 125 rpm in an incubated shaker (Amerax, USA.) under 16/8 h (light/ dark) photoperiod at 25°C

Growth curve studies

Growth study was done with an initial inoculum of 2% w/v on MS media fortified with 3 mg/L IBA. These flasks were kept in the shaking incubator at 125 rpm and 25°C. Root cultures were harvested at an interval of five days and their fresh weight was recorded. The excess water content was removed by blotting to obtain a constant weight. Growth curve was plotted based on the increase in biomass. The experiment was done in triplicate and the mean values were calculated. The roots were dried in lyophilizer and dried roots were used for extraction of saponins.

Phytoconstituent extraction from *Chlorophytum borivilianum*

The dried roots were ground and extracted in methanol using a soxhlet apparatus. The extract was filtered and dried, later the dried powder was extracted with 1ml of HPLC grade methanol. The samples were filtered using (0.22µm) Millipore filters, followed by quantification. The two major saponins stigmasterol and hecogenin were quantified using HPLC.

Quantitative estimation of stigmasterol and hecogenin

The analysis was carried out using the Shimadzu—LC-10AT VP series HPLC system equipped with a Supelco column (250x4.6 mm, C18, ODS with particle size of 5 µm) with a flow rate of 1ml/min.

The mobile phase for stigmasterol analysis was methanol, water and Acetic acid in the ratio of 70:30:1, which was detected at 254nm. Hecogenin was detected at 210nm using methanol and water in the ratio of 90:10.

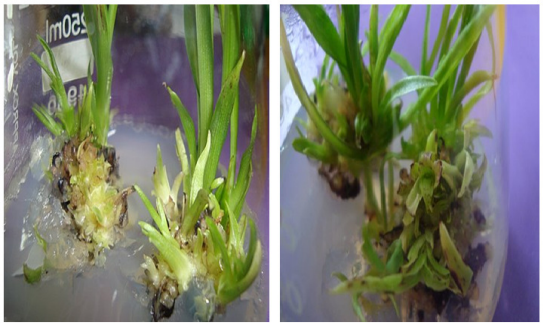
RESULT

***In vitro* culture initiation**

Surface sterilized explants were inoculated on to MS medium supplemented with 2.5 mg/l BA and 0.5 mg/l NAA. After 20 days of transfer to fresh medium, multiple shoot buds developed from the base of explant which on maturation elongated into shoots (fig: 1 a&b).

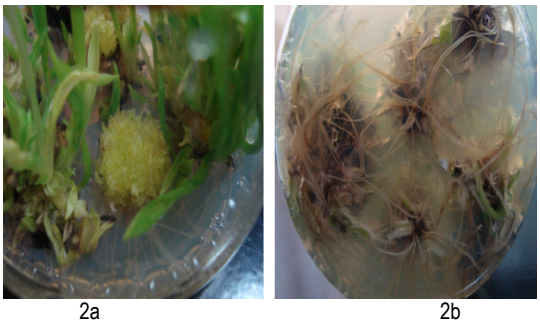
Root induction

The young shoot buds obtained after shoot induction were excised and transferred to MS media containing different concentrations of IBA, NAA and IAA. The best result was obtained on MS media supplemented with IBA at a concentration of 3mg/l. Root induction was seen after 2 weeks of culture (Fig: 2a and 2b)



1a 1b

Fig1(a & b). Multiple shoot formation on MS media supplemented with 2.5mg/l BA and 0.5mg/l NAA. Observations were taken after 28 days of cultures.



2a 2b

Fig 2a and 2b. Induction of roots from shoots of *Chlorophytum borivilianum* in MS media supplemented with 3 mg/l of IBA. Observations were taken after 28 days of cultures.

Table 1. Effect of various plant growth regulators on root induction from shoots of *Chlorophytum borivilianum.* Observations were recorded after 28 days of culture.

Phytohormone	Concentration (mg/l)	Percentage of explants responding (%)
IBA	0.5	0
	1.0	35.1 ± 0.9
	1.5	43.6 ± 0.45
	2.0	46.9 ± 0.30
	2.5	52.3 ± 0.5
	3.0	98.75 ± 0.19
	3.5	84.3 ± 0.50
	4.0	82.1 ± 0.7

Growth Kinetics

The growth study of roots in shake cultures exhibited a maximum biomass increase after 25 days of transfer (fig 4 a &b). A maximum of 12.1± 2.18 mg/gDCW of biomass could be observed after 25 days of cultures.

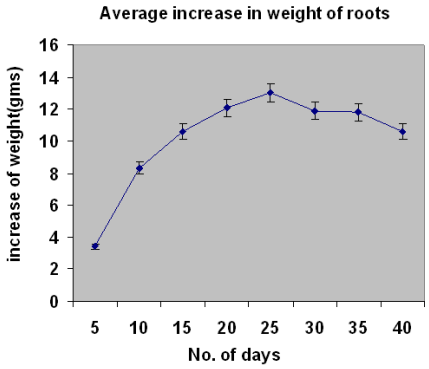


Fig 3. Growth curve of roots in MS media supplemented with 3 mg/l IBA

4a 4b

Fig 4a & 4b. Root cultures of *C.borivilianum* after 25 days on MS media supplemented with 3 mg/l IBA.

Estimation of Stigmasterol from in vitro grown roots of *Chlorophytum borivilianum*

Lyophilized roots were used for extraction of stigmasterol and hecogenin. Among the root extracts tested for the presence of stigmasterol, a maximum of 46.4±0.47 mg/gDCW stigmasterol was observed after 25 days of culture (fig 5).

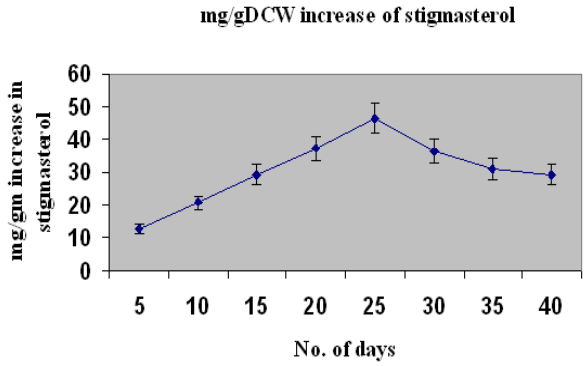


Fig 5. Production of stigmasterol from root cultures of *C. borivilianum*

Production of hecogenin from root cultures of *C. borivilianum*

Maximum amount of hecogenin was observed after 25 days of culture (685.68±0.51 mg/gDCW). This is the first report on production of stigmasterol and hecogenin from *in vitro* grown root cultures of *Chlorophytum borivilianum*.

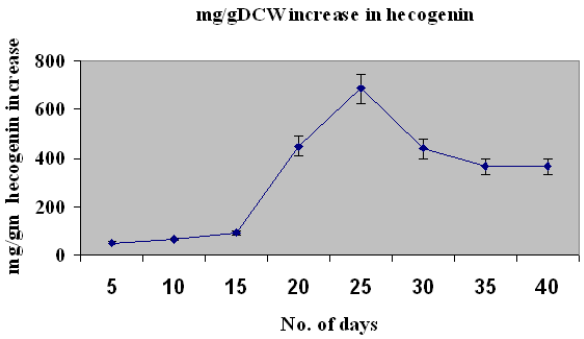


Fig 6. Percentage increase in hecogenin from root cultures of *Chlorophytum borivilianum*

DISCUSSION

Growth regulators are expected to control the production of adventitious roots in plants [13], wherein the auxins play a key role [14, 15]. However, for the production of high quality plant based medicines, the development of efficient and reproducible plant cell and organ culture protocol holds a tremendous potential.

IBA was found to be effective in comparision to NAA and IAA in *C. borivilianum* for root induction, the superiority of IBA is not a general rule though. Leonardi [16] observed that in *Grevilla rosmarinifolia*, the effects of IBA & NAA on *in vitro* rooting were similar at a range of different concentration. In some *Grevillea* species, NAA was more effective than IBA in promoting root formation as suggested by Watad [17], Whereas in *panax ginseng*, adventitious root cultures, MS medium with either NAA or IBA was found to be suitable for enhanced biomass production [18]. Nandagopal [15] reported the effect of auxin on root induction and elongation in *in vitro* root cultures in chicory culture.

According to Praveen [19] the root cultures of *Andrographis paniculata* showed seven-fold increase of fresh biomass in suspension cultures along with 3.5 fold higher andrographolide compared to plants grown in natural habitat. Saponin content was significantly enhanced (75.3-92.7 mg/gDCW) by infection with mycorhizal fungi in field grown plants [20].

In our study we are reporting for the first time the production of pharmaceutically important saponins stigmasterol and hecogenin by *in vitro* root cultures of *C. borivilianum*

CONCLUSION

The result of our study proves that, *in vitro* grown root cultures of *Chlorophytum borivilianum* can be successfully used direct extraction of secondary metabolite in 25-30 days, which saves time and labor compared to field grown plants. The procedure developed for the root cultures of *Chlorophytum borivilianum* provides a tool for rapid propagation of plant biomass and rapid production of valuable secondary metabolites under laboratory conditions. This can pave way for a process development for production of these pharmaceutically important secondary metabolites at commercial level.

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