

Multiple shoot regeneration and effect of sugars on growth and nitidine accumulation in shoot cultures of *Toddalia asiatica*

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ABSTRACT

Background: *Toddalia asiatica* (Rutaceae) is an important medicinal plant in traditional medicinal system of India and China. Nitidine production from callus cultures of the plant had been investigated, but *in vitro* multiplication and secondary metabolite production from shoot cultures is not reported. **Objective:** The aim of the present work is to establish protocol for *in vitro* multiple shoot regeneration of *T. asiatica* and to investigate the secondary metabolite, nitidine production from the shoot cultures. **Materials and Methods:** Different explants were used for shoot regeneration on MS supplemented with benzyl adenine (BA) either alone or in combination with naphthalene acetic acid (NAA) in different combinations. Effect of different sugars and different concentrations of sucrose on biomass accumulation in shoot cultures in liquid medium was investigated. For *in vitro* rooting, shoots culture were inoculated to half strength MS medium supplemented with different concentrations of indole butyric acid. Quantitative analysis of shoot culture extracts was done for estimation of nitidine by HPTLC. **Results:** Shoot cultures were successfully initiated and established from nodal and shoot tip explants on MS medium supplemented with benzyl adenine and sucrose (3% w/v). Sucrose at a concentration of 3 % w/v was found to be optimum for growth and biomass accumulation. *In vitro* rooting of shoots was achieved on half strength MS medium supplemented with indole butyric acid 3 mg/l. Investigation of secondary metabolite production ability of the *in vitro* regenerated shoot cultures revealed their ability to biosynthesize nitidine. **Conclusion:** Shoot cultures were established and nitidine production has been observed.

Key words: Benzyl adenine, nitidine, shoot cultures, sugars, *Toddalia asiatica*

INTRODUCTION

Toddalia is a monotypic genus consisting of species *Toddalia asiatica* (Linn.) Lam. (Rutaceae), well-known as Lopez root or Wild orange tree. In Indian systems of medicine such as Ayurveda and Siddha, it is commonly used for treatment of malaria, rheumatism and fever. Also used for the treatment of cough, indigestion, lung diseases, stomach ailments, cholera, and diarrhea.^[1,2]

Several chemical constituents with varied chemical nature like benzophenanthridine, quinoline, and protoberberine alkaloids, coumarins, terpenoids, cyclohexylamines

and others were isolated from this plant. The plant has been reported to possess pharmacological activities such as anticancer, antimalarial, anti-HIV activities, antiplatelet aggregation, antipyretic, anti-inflammatory, analgesic, wound healing and antimicrobial activities.^[3] Benzophenanthridine alkaloid, nitidine possess anti-HIV, antimalarial activity.^[4,5] Dihydroneitidine isolated from stem chips exhibited cytotoxic activity toward human lung carcinoma.^[6]

Production of nitidine from callus cultures was reported.^[7] However to the best of our knowledge, *in vitro* shoot multiplication and alkaloid production in shoot cultures was not studied. The objective of the work was to establish simple and reliable *in vitro* shoot multiplication of the plant and to investigate the effect of sugars on biomass accumulation. Further, the biosynthetic ability of well-established shoot cultures was assessed for nitidine.

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MATERIALS AND METHODS

Plant material

Tender twigs were collected from mature field grown *T. asiatica* plant and dissected into leaves (1 cm²), internodal, nodal and shoot tip segments and were washed under running tap water for 3–5 min, followed by surfactant, Tween-20 (10% v/v) for 2–4 min and then washed thoroughly with distilled water. Explants were treated with antifungal agent griseofulvin (0.2% w/v) for 2–3 min and rinsed with distilled water. Then, surface sterilized with 70% ethanol for 30–40 s and finally with 0.1% w/v mercuric chloride for 1–2 min, followed by thorough washing with sterile distilled water for 3–4 times.

Culture medium and culture condition for adventitious shoots development

The surface sterilized explants were cultured on Murashige and Skoogs (MS) medium supplemented with sucrose 3% w/v and phytohormone benzyl adenine (BA-0.5, 1 and 2 mg/l) either alone or in combination with naphthalene acetic acid (NAA-0.1, 0.5 mg/l) [Table 1]. The pH of the medium was adjusted to 5.8 ± 0.2 with 0.1N NaOH/HCl before autoclaving and solidified with 1.1% w/v agar. All the culture flasks were maintained at 25°C ± 2°C, with a 16 h photoperiod and sub cultured onto fresh solid media at 30 days intervals. The MS medium devoid of plant growth regulators (PGR) was used as control. Effectiveness of the PGR and their concentrations was recorded based on the visual observation and was articulated as the percentage of explants either responding for shoot induction or not and number of shoots per explant.

Liquid cultures were established from shoot cultures that showed good growth on solid medium by inoculating ~ 0.5 g (Fresh weight) of shoots into 100 ml conical flask containing 25 ml liquid MS medium

supplemented with 1 mg/l BA and sucrose 3% (w/v). Cultures were grown on a gyratory shaker at 120 rpm under a 16 h photoperiod at 25°C ± 2°C. The fresh weight of the shoots was recorded at weekly intervals up to 4 weeks for growth kinetics. Growth index of shoot cultures was calculated as the ratio of final fresh weight of regenerated shoots to the fresh weight of inoculum.

Effect of different sugars on biomass accumulation of shoot cultures in the liquid medium

Different sugars such as glucose, fructose and maltose at 3% (w/v) concentration were incorporated into the medium besides a positive control containing 3% (w/v) sucrose and a negative control without sucrose. Aqueous stock solutions of glucose, fructose and maltose were prepared and filter-sterilized and added to the autoclaved media under aseptic conditions to attain a concentration of 3% (w/v). The incubation conditions were same as mentioned above for shoot cultures. Fresh weights of shoot cultures were recorded at 7 days intervals up to 21 days.

Effect of different concentrations of sucrose on biomass accumulation of shoot cultures in the liquid medium

Shoot cultures established on MS medium supplemented with BA (1 mg/l) were further investigated to study the effect of different concentrations of sucrose 1, 2, 3, 4, 5% (w/v) on growth of shoot cultures. Aqueous stock solution of sucrose were prepared, filter-sterilized and added to the autoclaved 25 ml of liquid media in 100 ml flask in triplicate, under aseptic conditions to attain the above mentioned concentrations. Fresh weights of shoot cultures were recorded at 7 days intervals up to 21 days.

Rooting and transplantation

Individual shoots of 3–5 cm with 2–3 internodes were separated and cultured in MS medium devoid of PGR and half strength MS medium fortified with different concentrations of IBA (0.5, 1, 2, 3 mg/l).

Extraction

Shoot cultures from MS liquid media fortified with different sugars and different concentrations of sucrose and rooted shoots from solid medium were extracted and analyzed to assess the ability to biosynthesize nitidine. After harvesting, shoots were blotted dried, and dry weights were recorded, made into a coarse powder and refluxed thrice with 70% methanol for 20 min. The extracts were combined and evaporated at room temperature. The dried extracts were dissolved in 70% methanol and were subjected to thin layer chromatography (TLC) and high-performance thin layer chromatography (HPTLC) for identification and

Table 1: Effect of BA alone or in combination with NAA on direct shoot organogenesis from nodal and shoot tip explants of *T. asiatica* after 30 days of incubation

Phytohormone combination		Regeneration frequency (%)		Number of shoots per explants	
BA	NAA	Nodal segments	Shoot tips	Nodal segments	Shoot tips
0.5 mg/l	-	87.5	79.16	2.125±0.125	1.333±0.072
1 mg/l	-	95.83	83.33	3.083±0.381	2.333±0.190
2 mg/l	-	95.83	87.5	3.541±0.190	3.208±0.260
0.5 mg/l	0.5 mg/l	91.66	79.16	2.166±0.144	1.458±0.190
1 mg/l	0.5 mg/l	95.83	91.66	3.375±0.25	2.875±0.25

Values are mean±SD of three independent experiments consisting of 8 explants per each experiment. SD: Standard deviation; BA: Benzyl adenine; NAA: Naphthalene acetic acid

quantification of nitidine by comparing with standard nitidine.

Analysis

Thin layer chromatography

The 70% methanolic extract was analyzed by using precoated TLC silica gel 60 F₂₅₄ plates of 250 μ m layer, (Merck) by co-chromatography with an authentic sample of nitidine purchased from Chengdu Biopurify Phytochemicals Ltd., China. The plates were developed in the mobile phase composed of chloroform: Methanol (7:1, v/v) and detected by observing in ultraviolet chamber.^[7]

High performance thin layer chromatography

High-performance thin layer chromatography analysis of the extract was carried out on Camag HPTLC system consisting of Linomat V semi-automatic applicator, Camag syringe of 100 μ L capacity, Camag twin trough chamber (20 \times 20 cm), Camag TLC scanner 3, equipped with win CATS software (version 1.4.3, Camag, Muttenz, Switzerland). Aluminum backed silica gel 60 F254 TLC plates (10 \times 10 cm, 0.2 mm thickness, E-Merck) were used for separation and identification of nitidine. The mobile phase was chloroform: Methanol (7:1, v/v). Densitometric scanning at 332 nm was performed.^[8]

Statistical analysis

Data were analyzed by analysis of variance followed by Tukey's/Dunnett's multiple comparison test using GraphPad Prism 6 (Graph pad software Inc., USA) for determining the significance of treatment effects.

RESULTS AND DISCUSSION

Establishment of shoot cultures

Among the various explants used for shoot initiation, nodal explants and shoot tips exhibited shoot initiation while leaf and internodal explants did not form shoot buds or shoots on any of the media combinations employed in the study. Nodal segments and shoot tips were able to induce shoots on all the combinations of media. Emergence of young putative shoots from both the explants in most of the treatments was observed within 7-10 days from the day of inoculation [Figure 1a and b]. The growth of shoots was very slow and took 2-3 weeks to attain a minimum height of 3-6 cm after appearance of shoot buds depending on the concentration of BA and NAA in medium [Figure 1c]. It has been documented in several reports that shoot induction in many medicinal plants was achieved by culturing nodal segments and shoot tips and nodal explants being more competent than shoot tips.^[9] In the present study, nodal explants produced more number of shoots per explant than that of shoot tips on all the combinations [Table 1]. It was also observed in many studies that BA either alone or in combination with NAA was found to be effective for shoot initiation and elongation.^[10] However, in the present study NAA had no significant effect on shoot induction frequency in comparison to medium supplemented with BA alone, but it influenced the shoot length. Shoot cultures on media with BA + NAA were elongated than media supplemented with BA alone. It has been observed in many studies that cytokinins promoted shoot bud induction and shoot proliferation but inhibited elongation.^[11]

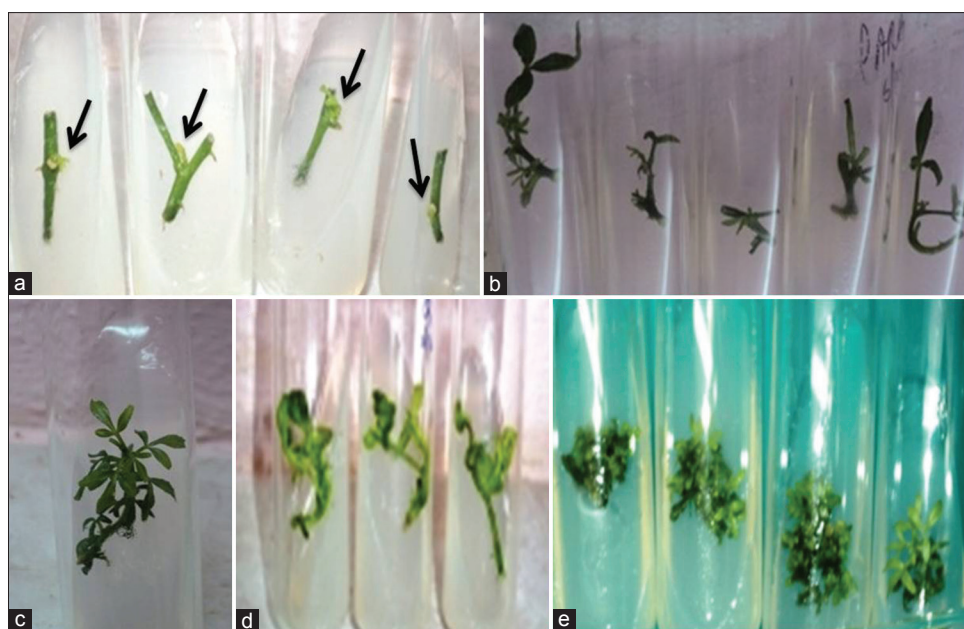


Figure 1: (a) Shoot buds induction from axils of nodal segments; (b) regenerated shoots after 3 weeks of inoculation on different media; (c) individual shoot after two subcultures on medium fortified with benzyl adenine (BA) (1 mg/l); (d) explants cultured on medium fortified with BA (2 mg/l); (e) profuse multiplication of shoots on medium fortified with BA (1 mg/l) after three subcultures

There was no significant difference in percentage response in MS medium fortified with BA 1 mg/l or 2 mg/l, but influenced the shoot length and morphology of leaves. Lower concentration of BA (0.5 mg/l) resulted in increased length (6-7 cm), while higher concentrations reduced the shoot length (3-5 cm). Moreover, leaf morphology in high concentration of BA (2 mg/l) was found to be wrinkled [Figure 1d]. It implies that, increased BA concentration resulted in an increase in the number of shoots per explants, however shoot length decreased and shoot morphology altered. Hence, MS medium supplemented with BA (1 mg/l) was used in further investigations. Shoot cultures were maintained on solid medium by sub culturing at an interval of 4 weeks [Figure 1e].

Subsequently, the well-established shoot cultures on solid medium after 3 subcultures were transferred to the liquid media of the same composition. From growth kinetics of shoot culture, it was found that subculture should be done at an interval of 3 weeks, as at the end of 3rd week they reached stationary phase of growth. In general, the cultures exhibited an initial lag phase before entering an active growth phase around day 7 and reached a maximum of stationary phase after 21 days of inoculation [Figure 2]. It was noteworthy that shoot biomass accumulation in liquid media was significantly more than those grown on solid media (about 1.54-fold) on fresh weight basis. This may be because shoots were submerged in the liquid media which enables large surface area for the uptake of nutrients and PGR. Morphological changes in shoots were also observed. Shoots were more elongated with much internodal distance and stems were much thicker. In *Picrorhiza kurroa*, there was 3-fold increase in shoot length and leaf size in liquid medium.^[12]

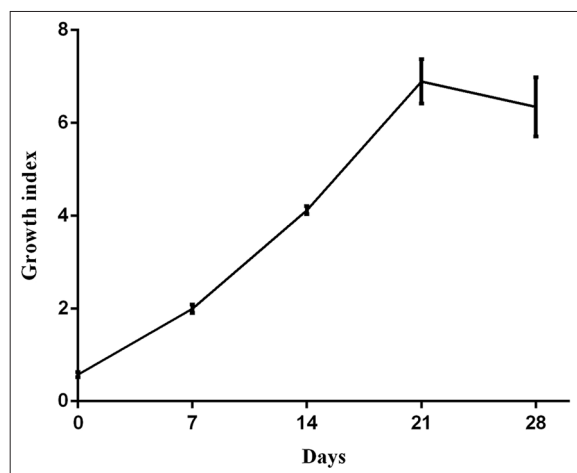


Figure 2: Growth kinetics of shoot cultures of *Toddalia asiatica* ($n = 3$)

Effect of different sugars

The shoots grew well in the presence of different sugars but in medium devoid of sugar, cultures became brown progressively leading to the death of culture. Though all sugars promoted growth, high-biomass accumulation was noted with sucrose, followed by maltose, glucose and fructose [Figure 3]. Biomass accumulation was found to be hindered in the presence of fructose in comparison to sucrose. The highest biomass yield was seen with sucrose, which was about 1.18 and 1.19-fold than that obtained with maltose and glucose respectively. Fructose did not show a significant effect on the biomass accumulation of shoots in comparison to sucrose and was 1.22-fold lower than that obtained with sucrose supplementation.

Effect of different concentrations of sucrose

Growth kinetics of shoot cultures supplemented with different concentrations of sucrose followed more or less the same pattern [Figure 4]. However, maximal biomass accumulation was achieved with 3% level of sucrose. There was a significant difference in biomass accumulation at the end of 21 days in medium supplemented with 3% w/v sucrose in comparison to 1, 2 and 5% w/v. However, there was no significant difference with 4% w/v sucrose. The concentration of sucrose in the medium influenced the biomass accumulation. Highest sucrose concentration might have repressed culture growth contributing to lower biomass yields. Lower concentration of sucrose may not be sufficient for growth. High concentration might suppress the growth by osmotic stress. It has been reported that dry weight of plants is reduced due to high-sugar concentration.^[13] As growth profiles of shoot cultures with 3 and 4% w/v sucrose were almost similar without significant difference in growth indices, the former concentration was preferred for further studies.

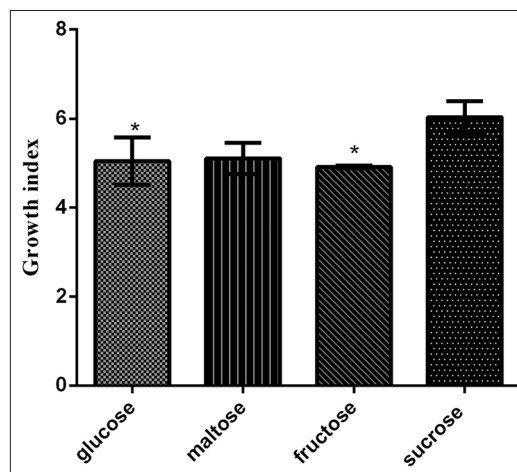


Figure 3: Effect of different sugars on biomass accumulation in liquid medium. Data were analyzed by one-way analysis of variance followed by Tukey's multiple comparisons test ($n = 3$). * $P < 0.05$ as compared to sucrose

Of the nutritional requirements, carbohydrates are important carbon and energy sources for most of the plant cell lines. Incorporation of carbohydrates to plant tissue culture media is essential because, the photosynthetic activity of *in vitro*-grown tissues is usually low. They also play a role as osmotic agents in the culture media. Continuous supply of carbohydrates to plants cultured *in vitro* is essential because, the photosynthetic activity of *in vitro*-grown tissues is usually low. These compounds are also necessary as osmotic agents in the culture media. Hence, sugars have a potential effect on the physiology, growth, and differentiation of cells.^[14] Therefore, the optimal carbon source needs to be considered.

In the present study, sucrose at 3% w/v was the best carbon source for shoot multiplication. And also our results are consistent with earlier reports where in sucrose had been proved to be a better source of carbon than other sugars in shoot multiplication.^[15-17]

Rooting and transplantation

In vitro regenerated shoots were transferred to half strength MS medium fortified with different concentrations of IBA for rooting. Rooting response was achieved with a high concentration of IBA (3 mg/l) after 20–25 days of culturing. The success of rooting by IBA is reported for many medicinal plants. Rooted shoots were further cultured for 8 weeks.^[18,19] After the development of roots, plantlets were removed out from the culture vessel and washed with sterile water to remove agar and then transferred to sterile garden soil in a plastic pot. These were maintained for 3 weeks under greenhouse conditions and transferred to the natural environment.

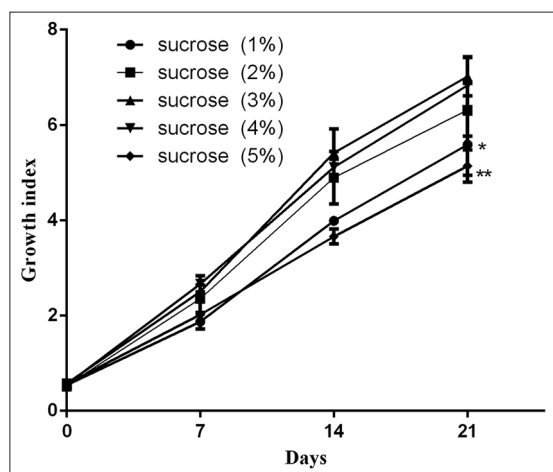


Figure 4: Effect of different concentrations of sucrose on biomass accumulation in liquid medium. Data were analyzed by two-way analysis of variance followed by Tukey's multiple comparisons test ($n = 3$). * $P < 0.05$. ** $P < 0.005$ in comparison to sucrose (3% w/v)

Extraction and analysis

Analysis of shoot cultures from the liquid medium supplemented with different sugars and different concentrations of sucrose after 3 subcultures in the liquid medium and 8 weeks old *in vitro* regenerated complete plantlets on solid medium were extracted and analyzed by HPTLC.

Nitidine production pattern from shoot cultures supplemented with different sugars resembled that of biomass accumulation. Highest production was achieved with sucrose 1.643 $\mu\text{g}/\text{gm}$ d.w. followed by glucose 1.454 $\mu\text{g}/\text{gm}$ d.w. Significant change in nitidine accumulation in shoot cultures supplemented with maltose and fructose 3% w/v was observed in comparison to sucrose 3% w/v. Maltose and fructose fortified cultures produced 1.399 and 1.27 $\mu\text{g}/\text{gm}$ d.w. of nitidine that were 1.17 and 1.29-fold less than sucrose 3% w/v respectively. This implies that the sucrose has been utilized more effectively for growth and thereby influencing the secondary metabolism.

Though sucrose has been utilized more effectively than other sugars, the concentration of sucrose also influenced the growth as well as nitidine production. The highest yield of nitidine 1.643 $\mu\text{g}/\text{gm}$ d.w. was obtained from shoot cultures supplemented with sucrose 3% w/v followed by 1.516 $\mu\text{g}/\text{g}$ d.w. with sucrose 4% w/v. Sucrose at concentrations 1, 2 and 5% w/v produced nitidine which was 1.301, 1.314 and 1.148-fold less than that obtained with sucrose 3% w/v [Figure 5]. Nitidine biosynthesis in shoot cultures supplemented with different sugars and different concentrations of sucrose was in parallel to biomass accumulation, which implies that it could be

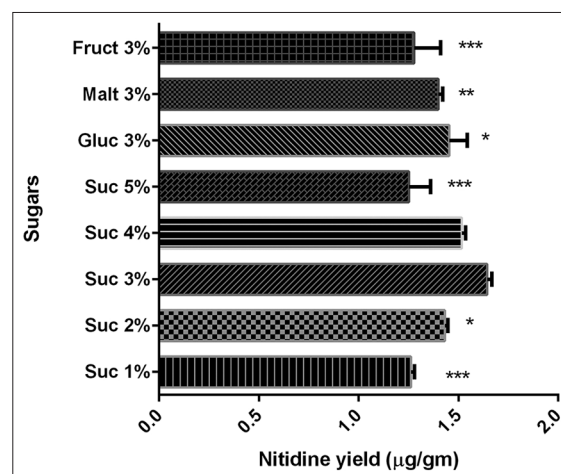


Figure 5: Nitidine yield from shoot cultures supplemented with different sugars and different concentrations of sucrose. Data were analyzed using one-way analysis of variance followed by Dunnett's multiple comparisons test. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.0005$ as compared to sucrose 3% w/v ($n = 3$)

growth associated molecule. Nitidine might be synthesized *in situ* by shoot cultures because of structural similarity to dihydronitidine isolated from the stem of the plant.^[6] Sugars not only influence the physiology of plants, but also provide carbon necessary for the synthesis of primary metabolites, which in turn form bioactive secondary metabolites through intermediary metabolism. Though sucrose is the favored and most commonly used carbon source but other carbohydrate sources have also been used in plant tissue cultures.

The type of sugar and its concentration may also have an impact on both growth and secondary metabolite production^[20] which implicates that type and concentration of sugar will influence the growth as well as the metabolite production. Steviol glycosides production in shoot cultures of *Stevia rebaudiana* was increased when the sucrose level was elevated from 2% to 3%, but a further increase to 5% resulted in lower yields.^[21]

Analysis of 8 weeks old *in vitro* regenerated complete plantlets resulted in the production of 10.617 µg/gm d.w. of nitidine. The yield of nitidine from roots of intact plant has been reported as 0.002% d.w.^[5] which is 10 times more than from the present study. Though the yield of nitidine in the present study is less than intact plant, *in vitro* regeneration can be potential alternate for nitidine production because, root systems of higher plants generally exhibit slower growth and are difficult to harvest. Continuous harvesting of roots will cause plants to become endangered. Therefore, alternative methods have to be found for medicinally important root-derived compounds. Plant tissue culture techniques have been proved to be a potential alternate source for secondary metabolite production.

These preliminary studies also reinforce the earlier findings that morphological differentiation will influence the production of secondary metabolite synthesis as evident from the literature. *In vitro* shoots raised by adventitious shoot formation from internodal segments of *Cephalis ipecacuanha* contained 0.04-0.07% d. wt. emetine and 0.4-0.5% d. wt. cephaeline and complete regenerated plants shown 11.7 and 4.32-fold emetine and cephaeline respectively.^[22] Tropane alkaloid content of rooted shoots of *Datura innoxia* was about 1.5 times than nonrooted shoots and in turn non rooted shoots shown higher amounts than callus cultures.^[23] The content of hyoscyamine and scopolamine in the nonrooted shoots of *Duboisia myoporoides* was about 19.02 and 28.26 times more than nonorganogenic calli respectively but was less than roots regenerated from calli.^[24] Tropane alkaloid content in calli derived from leaf explants of *Datura metal* was higher than regenerated shoots from leaf explants.^[25] All these reports and present study support

that the different stages of tissue organization will effect production of secondary metabolite production. Hence further, influence of age of differentiated cultures and manipulation of culture conditions has to be carried out.

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