Original Research Article

Comparative analysis of hyoscine in wild-type and in vitro-grown Datura innoxia by high performance liquid chromatography

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Abstract

Purpose: To compare the hyoscine contents of Datura innoxia plant grown in the wild and that grown in vitro.

Methods: For callus induction from leaf explants, 15 combinations of 2,4-dichlorophenoxy acetic acid, gibberellic acid, benzyl amino purine and kinetin were used. For regeneration of shoots, 8 combinations of BAP, kinetin and indole butyric acid (IBA) were tested. Different concentrations of IBA were used for rooting. The hyoscine contents of the wild and in vitro samples was initially identified by TLC and subsequently quantified by high performance liquid chromatography using hyoscine N-butyl bromide as standard.

Results: For callus induction, 2,4-D at 2 mg/L produced maximum calli (96 %). Maximum shoots regeneration was obtained with 2 mg/L BAP and 1 mg/L kinetin. IBA at 0.5 mg/L induced good rooting. Substantial amount of hyoscine was detected in the extracts of in vitro grown plants through TLC and HPLC. Wild root, stem and leaves exhibited higher amounts (approx. 2 µg/mL) of hyoscine than the parts of in vitro grown plants. Green callus grown in vitro contained a maximum concentration of hyoscine (1.01 µg/mL) followed by leaf (0.82 µg/mL) and brown callus (0.432 µg/mL).

Conclusion: The callus cultures of D. innoxia are capable of yielding good amounts of hyoscine, and therefore can be exploited to increase hyoscine production using chemical/hormone treatments on a large scale. Thus, this study provides a sustainable and efficient way for the sustainable production of a natural hyoscine product via in vitro-grown D. innoxia cultures.

Keywords: Datura innoxia, Hyoscine, Callus, Hyoscine, Explant, Gibberellic acid, Benzyl amino purine, Kinetin

INTRODUCTION

A number of plant-derived natural products are isolated from plants which are used to cure several ailments and infections in humans. *Datura innoxia* is an attractive medicinal plant [1] which is a rich source of a variety of natural products and secondary metabolites. Among these tropane alkaloids, hyoscyamine and hyoscine are clinically more significant [2-5]. Different plant parts or their extracts have been used traditionally for the cure of common diseases and ailmentssuch as asthma, cough, convulsion, Parkinson disease, catarrhal inflammation, epilepsy, rheumatism, piles, dysmenorrhea, various skin problems and infected wounds [6]. The leaves and seeds of

Datura spp., in particular, are used as anesthetic, anodyne, antispasmodic and bronchodilator [7].

Despite several medical benefits offered by different *Datura spp.*, it has not been practically feasible to grow this medicinal plant at a large scale to harness its medicinal potentials. The main reasons are seed dormancy, thermophilic plant habitat and lack of available research for optimal cultural practices to maintain this non-traditional plant like other crops [8]. In order to explore further opportunities for sustainable hyoscine production, *in vitro* culturing techniques are proposed as a good alternate. These techniques can be employed not only to produce multiple plant nurseries for field propagation but also *in vitro* grown callus cultures could be a direct and sustainable source of hyoscine. This can be achieved through *in vitro* culturing techniques aiming at micro-propagation or somatic embryogenesis.

In vitro culturing of *D. innoxia* was first reported in 1973 by Engvild, where different growth regulators (2,4-D, kinetin and Gibberellic acid (GA_3)) were trialed to evaluate their potential to induce regenerated shootlets from callus cultures [9]. Importantly, for other *Datura spp. D. metel* or *D. stramonium*, callogenesis [3,10,11] and organogenesis [12-15] trials have also been reported.

Utilizing the *in vitro* methods particularly cell or callus cultures to produce hyoscine and other alkaloids can be instrumental in enhancing their production. *D. innoxia* and *D. metel* are reported to contain hyoscine in all parts of plants suggesting these are more suitable for commercial hyoscine production compared to other *Dature spp* [3]. Only a few reports are available on the production of hyoscine/hyoscaymine through *in vitro* grown callus cultures. Interestingly, hyoscamine in hairy root cultures varies in different *Datura spp,* but *D. innoxia* was found to contain higher amount of alkaloids than other speices [15,16].

This study was aimed to mark the best hormonal concentrations for an efficient and reproducible callus formation and *in vitro* regeneration of *D. innoxia* explants by somatic embryogenesis. The hyoscine concentration between wild-type and tissue culture grown plants was compared by employing high-pressure liquid chromatography (HPLC). Our data establishes a novel comparative relationship for hyoscine contents in calli as well as in different parts of *in vitro* regenerated plantlets. Apart from varying hyoscine concentrations noticed, many new peaks have also been identified in brown and green calli opening new avenues for more investigation into the importance of these plant-derived natural products. This study explores further possibilities for the production of hyoscine in *in vitro* grown callus cultures and subsequently regenerated plants.

EXPERIMENTAL

Preparation of plant material for *in vitro* growth

Fresh and healthy leaves of *D. innoxia* plants at flowering stage were collected in the month of May in 2015 from fields in Islamabad; Latitude 33° 41' 24.7" (33.6902°) north, Longitude 73° 7' 52.3" (73.1312°) east. Plants were identified and described by Dr Humaira Shaheen (a plant taxonomist). After assigning voucher number CIITB-2015022 to the specimens, they were kept in CIIT Bioscience Herbarium for future reference. Tap water with mild detergent was used to wash the whole leaves. For leaf surface sterilization, 0.1 %(*w/v*) $HgCl_2$ solution (Sigma-Aldrich) was used. After 2-3 min, the solution was decanted and leaves were washed three to five times with autoclaved distilled water and dried on sterile filter paper. Finally, the leaves were cut into small pieces (1.5 × 1.5 cm) and then shifted to callus induction media.

Media for callogenesis and organogenesis

MS (Murashige and Skoog, 1962) basal medium with Gamborg's basal salts with vitamin (Phytotechnology Laboratories) and various combinations of hormones (**Error! Reference source not found.**) were used to induce calli and subsequent organogenesis. The pH of the media was setat 5.8 with 0.5 N HCL before autoclaving. For solidification, 8 g/L agar was used prior to autoclaving for 15 min at 1.1 kg/cm^2 (121 °C). The cultures were incubated at 25 ± 2 °C with a 16/8 h photoperiod. The photon flux density of 50 µmol m^{-2} s^{-1} was provided by cool-white electric fluorescent lamps.

For shoot regeneration, MS with varying concentrations of 6-benzylaminopurine (BAP), kinetin and Indole-3-butyric acid (IBA) were used. For rooting, small shoots of an average size of 2 - 3 cm were transferred to MS with four different concentrations of IBA (**Error! Reference source not found.**) and sub-cultured after every three weeks. All cultures were maintained at similar conditions of photoperiod and temperature at described earlier.

Hardening of regenerated plantlets

The roots of regenerated plants were carefully washed with distilled water in order to remove solidified media. These plantlets were shifted to plastic pots containing equal amounts of vermiculite, garden soil and farmyard manure. Polythene bags were used to cover the pots to maintain >90 % humidity for tender plantlets which was gradually lowered by creating holes in the polythene bags for hardening. These bags were removed after 10 days and soil established plants were shifted to larger pots filled with a mixture of soil and sand in equal ratio. Initially, the plants were grown in a glasshouse and later moved to a greenhouse conditions.

Extraction of alkaloids

The plant samples for wild-type leaves, stems, roots, fresh as well as brown calli and *in vitro* propagated leaves and stem were shade-dried for 10 - 15 days and ground into fine powder in liquid nitrogen. The powdered plant material (10 g) was mixed with 100 mL of pure methanol. The mixture was kept for 12 h and the suspension was filtered through a Whatman no. 1 filter paper. The filtrate was evaporated using a speed vacuum concentrator at 80 ˚C for 2 h to obtain the powder. Finally the extracted powder was resuspended in the dimethyl sulphoxide (DMSO) @ 100 mg/mL and stored at 4 °C in airtight vials [17].

Detection of hyoscinefrom alkaloid extracts through TLC

The initial screening of hyoscine from all samples (about three months old *in vitro* grown plants, three weeks old green calli, two months old brown calli and mature wild-type plants) was done by employing thin layer chromatography (TLC) procedure. The silica gel was used as stationary phase. The solvent systems were, chloroform: ethanol: water (81:11:8), chloroform: ethanol: water (70:21:9) propanol: DMSO: water (81:11:8), and chloroform: ethanol (3:1). Hyoscine-N-butyl bromide was used as standard. The chromatogram was stained with Dragendorff's reagent (specific for staining alkaloids) and the resulting spots were compared with the standard [18].

Detection of hyoscine from alkaloid extracts through HPLC

The HPLC system of Perkin Elmer, Series 200, USA was used to analyze samples. Hyoscine was separated on a C18 column (4.6 mm x 250 mm, 5µ). The HPLC column was washed with water for 20 min. Mobile phase of 0.02 mol/L sodium acetate buffer containing 0.02 % triethanolamine at pH 6.0 with sodium hydroxide-methanol (60:40) mixture and a detection wavelength of 215 nm were used. A flow rate of 1.0 ml/min (-1) was used. The retention time of hyoscine was approximately 2.3 min. The pressure during experimentation was 3210 psi [19]. For relative quantification various concentrations (0.5, 1, 1.5, 2, 2.5, and 3 µg/µL) of standard, hyoscine N-butyl bromide dissolved in DMSO were used to define the peak area to prepare the standard curve. The concentration of hyoscine in the sample was measured by establishing a linear relationship between the hyoscine concentrations and corresponding peak area. The readings for all samples were taken in three replicates. The regression curve was drawn for quantification of hyoscine in samples.

Statistical analysis

The data were statistically analyzed with the help of SoftwareSAS® version 9.2.Means were compared by performing the analysis of variance (ANOVA) followed by mean comparison using least significant difference (LSD) test at 95 % significance level.

RESULTS

Callogenesis

Among all the treatments tested, those containing 1 mg/L kinetin in combination with 2 mg/L BAP (treatment 14 and 15) initiated calli11-15 days earlier than other treatments (**Error! Reference source not found.**). The highest callus induction rate (96 %) was obtained with 2 mg/L 2,4-D (**Error! Reference source not found.**, Figure 1). Callogenesis was not observed with treatment 1 and 6 (**Error! Reference source not found.**) having 0.5 mg/L 2,4-D and 0.5 mg/L GA$_3$, respectively. In addition, the rate of callus proliferation was highest (11-15 days, **Error! Reference source not found.**) with kinetin + BAP and lowest (16-25 days, **Error! Reference source not found.**) with 2, 4-D.

Organogenesis

Shoot formation from calli was successfully achieved by BAP alone as well as in combination with kinetin and IBA. Moreover, media supplemented with both BAP and kinetin (Treatment 5 and 6) were found best not only for callogenesis (Treatment 14 and 15), but also for getting a maximum number (10-13) of lengthy shoots in a least period of time (**Error! Reference source not found.**). The rate of organogenesis was much faster from calli

produced with BAP and kinetin (treatment 14 and 15, **Error! Reference source not found.**) as compared to other combinations.

Root formation and hardening of plants

Maximum number of roots (2 - 3) in a relatively shortest period of time was obtained with 0.5 mg/L and none with 2 mg/L

Table 1: Different combinations of growth regulators for callogenesis and organogenesis in *Datura innoxia*

| Stage of *in vitro* culture	Growth regulator	Concentration used (mg/L)	Code
Callus induction	2,4-Dichlorophenoxyacetic acid (2,4-D)	0.5	1
		1	2
		1.5	3
		2	4
		3	5
	Gibberellic acid (GA$_3$)	0.5	6
		1	7
		1.5	8
		2	9
		3	10
	GA$_3$ + benzyle amino purine (BAP)	1+1	11
		1+1.5	12
		1+2	13
	Kinetin + BAP	1+2	14
	Kinetin + BAP (in ½ MS)	1+2	15
Shoot formation	BAP	10	1
	BAP + IBA	4+2	2
		10+0.5	3
		10+1	4
	BAP + kinetin	1+2	5
	BAP + kinetin (in ½ MS)	1+2	6
	BAP + kinetin + IBA	2+ 0.5+1	7
	BAP + kinetin +IBA	3+0.5+5	8
Root formation	IBA	0.5	1
		1.0	2
		1.5	3
		2.0	4
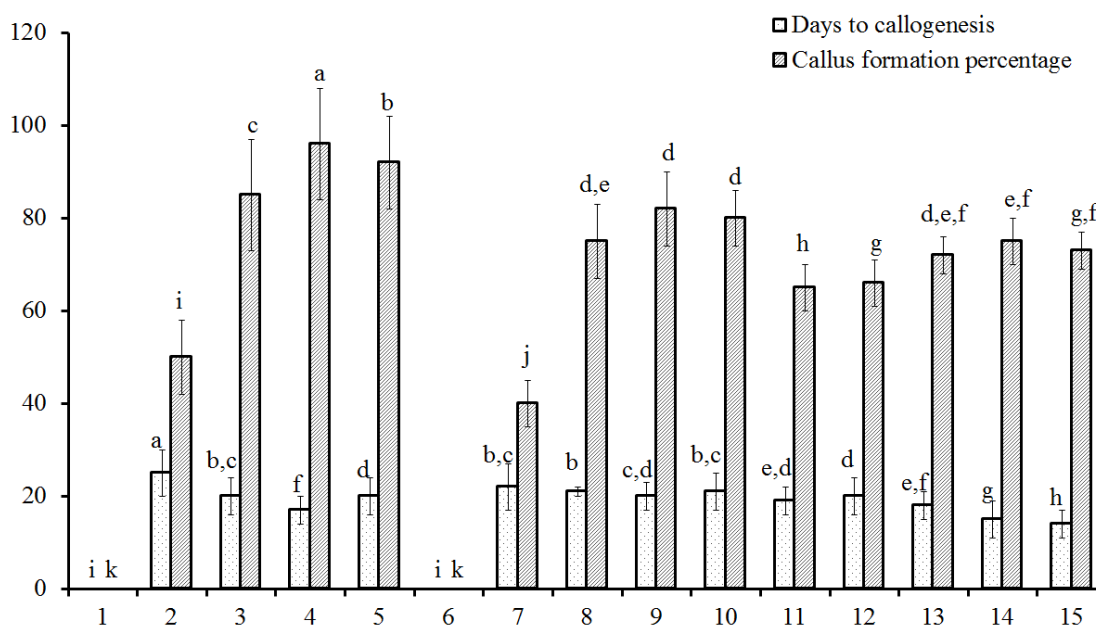
Figure 1: Effect of different combinations of growth regulators on callus induction frequency (%) and days to callogenesis in *Datura innoxia*. Treatment: (1) 0.5 mg/L 2, 4-D, (2) 1 mg/L 2, 4-D, (3) 1.5 mg/L 2, 4-D, (4) 2 mg/L 2, 4-D, (5) 3 mg/L 2,4-D, (6) 0.5 mg/L GA3, (7) 1 mg/L GA3, (8) 1.5mg/L GA3, (9) 2 mg /L GA3, (10) 3 mg/L GA3,

(11) 1 mg/L GA3+ 1 mg/L BAP, (12) 1 mg/L GA3+ 1.5 mg/L BAP, (13) 1 mg/L GA3+ 2 mg/L BAP, (14) 1 mg/L kinetin + 2 mg/L BAP, (15) 1 mg/L kinetin + 2 mg/L BAP (half-strength). (a-g represent means calculated by ANOVA, where mean ± SD with the same letter are not significantly different at a $p \leq 0.05$); $n = 3$, where "n" denotes the number of replicates
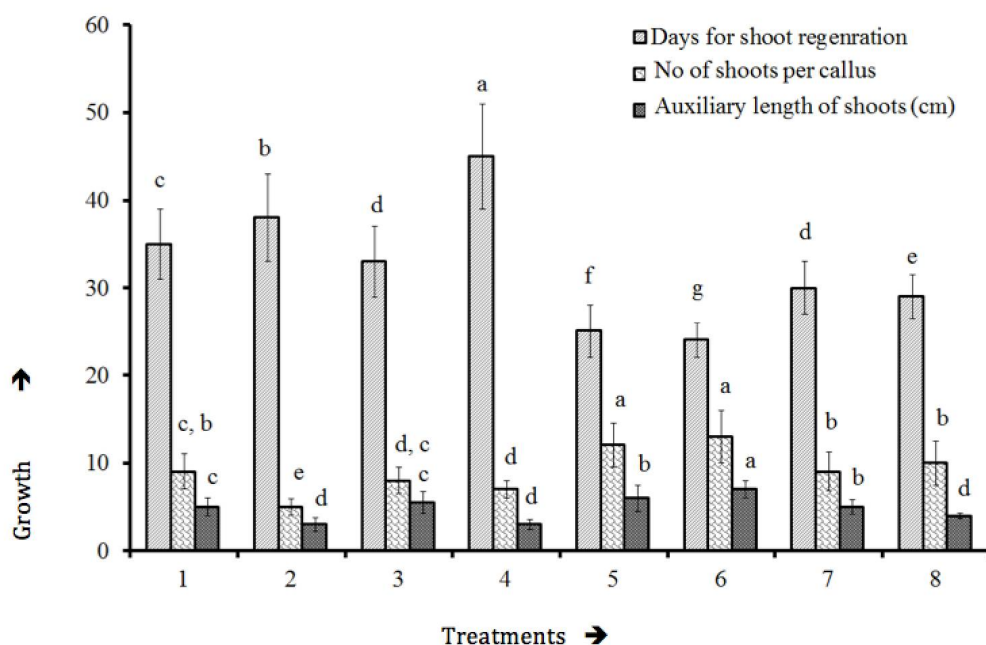

Figure 2: Effect of different hormone combinations on shoot regeneration. Treatment: (1) 10 mg/L BAP, (2) 4 mg/L BAP + 2 mg/L IBA, (3) 10 mg/L BAP + 0.5 mg/L IBA, (4) 10 mg/L BAP + 1 mg/L IBA, (5) 1 mg/L BAP + 2 mg/L kinetin, (6) 1 mg/L BAP + 2 mg/L kinetin (half strength), (7) 2 mg/L BAP + 0.5 mg/L kinetin + 1 mg/L IBA, (8) 3 mg/L BAP + 0.5 mg/L kinetin + 5 mg/L IBA. (a-g denote mean ± SD calculated by ANOVA, where means with the same letter are not significantly different ($p \leq 0.05$); $n = 3$, where "n" shows the number of replicates
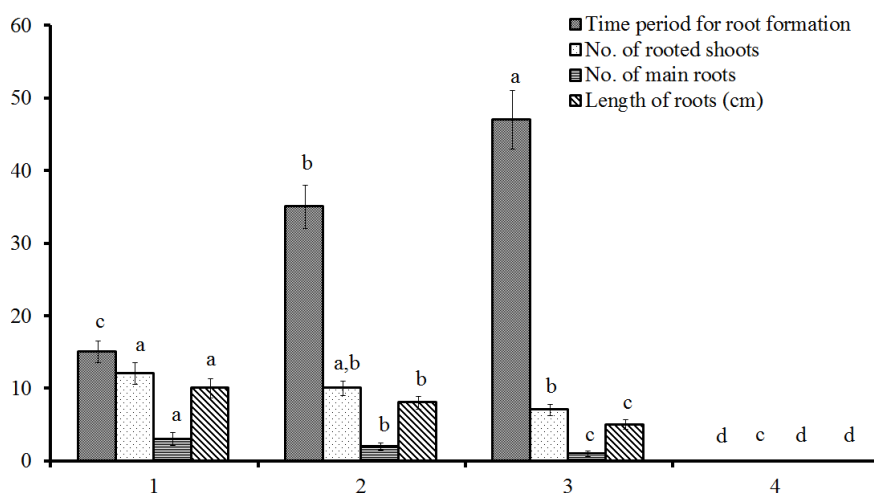

Figure 3: Root formation from *in vitro* regenerated shoots of *Daturainnoxia*. (1) 0.5 mg/L IBA, (2) 1 mg/L IBA, (3) 1.5 mg/L IBA, (4) 2 mg/L IBA. (a-d denote means calculated by ANOVA, where means ± SD with the same letter are not significantly different ($p \leq 0.05$); $n = 3$, where "n" shows the number of replicates

of IBA (Error! Reference source not found.). After successful in vitro culture, one-month old regenerated plantlets (Error! Reference source not found.) with sufficient roots were shifted to soil with 90 % survival rate. All these plants were morphologically similar to field grown plants and exhibited a normal growth pattern.

TLC chromatogram of hyoscine

Thin layer chromatography was performed to detect the presence of hyoscine in different types

of extracts using hyoscine-N-butyl bromide, as standard. After staining with Wagner's reagent, the chromatograms of the wild-type plant organs (root, stem, and leaf) and *in vitro* propagated (green and brown calli as well as *in vitro* propagated leaves) showed presence of

hyoscine as indicated by Rf values (0.81). The representative chromatogram of leaf sample was compared with standard (hyoscine-N-butyl bromide).
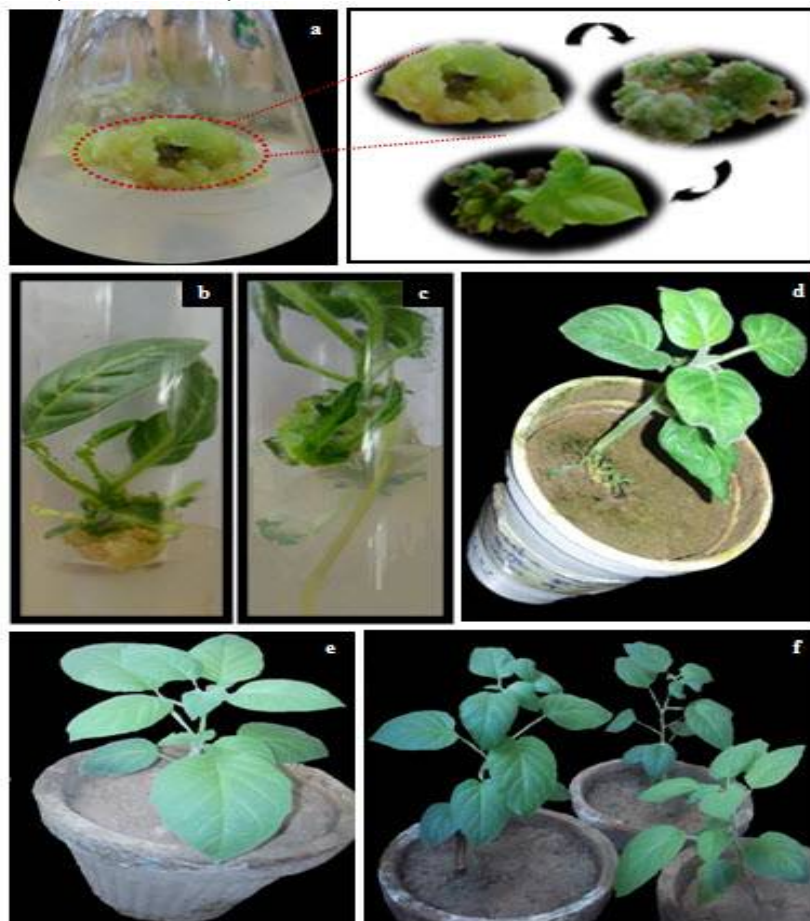
Figure 4: Different stages of *in vitro*-regenerated plants of *Datura innoxia.*(a) multiple steps of shoot regeneration from leaf derived calli after 3 weeks (b) shoot formation one month after callus formation, (c) root formation from shoots after 2 weeks of transfer to rooting media (d, e and f) multistep hardening and acclimatization of plants

HPLC chromatogram of hyoscine

Hyoscine contents of the wild-type and tissue cultured samples were analyzed quantitatively through HPLC. Hyoscine N-butyl bromide was used as standard having retention time of 2.27 min (Figure 5). Standard curve for relative quantification of hyoscine in different samples was developed using six different concentrations of hyoscine N-butyl bromide ranging from 0-3 µg/µL. Linearity of peak areas at 210 nm wavelength was marked with corresponding concentrations of Hyoscine-N-butyl bromide (0.5-3 µg/µL) using HPLC. All test samples (both wild-type and *in vitro*) contained significant concentration of hyoscine as their respective chromatograms were analyzed. All samples exhibited almost similar retention time when

compared with standard (Figure 6). The *in vitro* grown samples showed significant amounts of hyoscine but the amounts were significantly lower than the samples obtained from wild-type plants. Green calli contained 1.01 µg/µL hyoscine followed by tissue cultured leaf (0.82 µg/µL) and brown callus (0.43 µg/µL) (Table 2, Figure 6).

DISCUSSION

There is an ever increasing demand for herbal drugs today than before. The hyoscine derived from *in vitro* plant cultures would meet a high product standard and market value as there are no contaminants, environmental pollutants, pests and diseases involved. Although plant derived natural products are produced in substantial

amounts in the field by the wild-type grown plants,
there is a significant developing interest of pharmaceutical companies to use *in vitro* culture systems for plant natural product production because of clean and controlled environment and ease of recovery. Moreover, products produced this way are free of environmental contaminants, insect pest and diseases and don't rely on the season and climate in which they can be produced on the soil-grown plans under open field conditions.
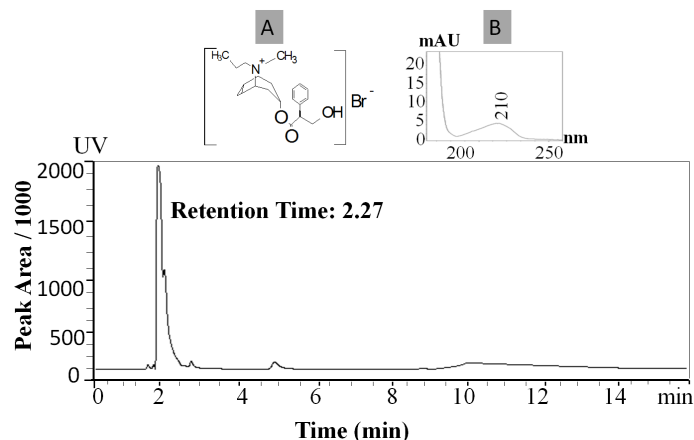

Figure 5: Standard hyoscine-N-butyl bromide detected through HPLC at a retention time of 2.27. (a) Represents the structure of hyoscine-N-butyl bromide. (b) Represents the absorbance spectrum of hyoscine-N-butyl bromide
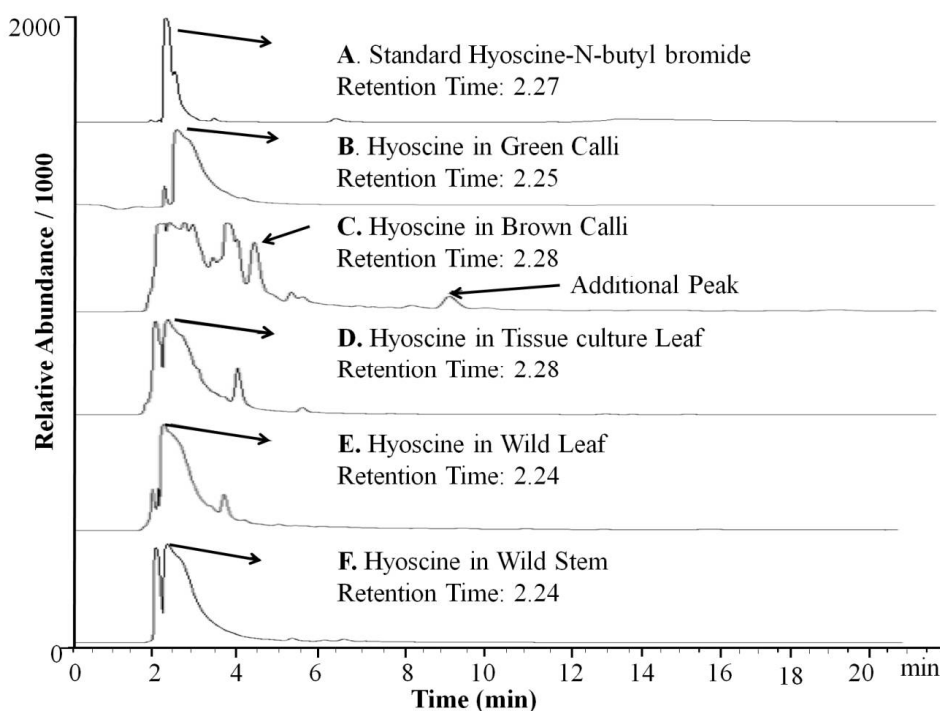

Figure 6: Comparison of hyoscine and additional peaks observed for different samples in *D. innoxia*. (a) standard hyoscine-N-butyl bromide, (b) hyoscine in green calli, (c) Hyoscine in Brown Calli, (d)Hyoscine in Tissue culture Leaf, (e) hyoscine in wild leaf, and (f) hyoscine in wild stem

Table 2: Detection of hyoscine by HPLC

Plant extract type	Hyoscine	Number of major peaks	Relative quantities of hyoscine in samples (µg/µL)
Standard	Present	1	1
Wild stem	Present	2	2.22

Wild leaf	Present	2	2.00
Wild root	Present	2	2.24
Tissue cultured leaf	Present	4	0.82
Green callus	Present	3	1.01
Brown callus	Present	5	0.43

Hence, *in vitro* plant cultures offer an attractive opportunity to produce and extract their natural products on sustainable basis. However, the *in vitro* culture procedures demand optimization of culture conditions, hormone concentration and media combinations. In this study, *Datura innoxia* plants were developed stably in *in vitro* cultures through somatic embryogenesis and a comparative analysis for hyoscine accumulation was undertaken. Calli were initiated from leaf explants using different hormone combinations. It is established that the rate and extent of callogenesis was significantly different for the treatments. Initially, the callogenesis was observed from the excised edges of the cultured leaves which later expanded throughout the whole leaves. Treatments containing 1 mg/L kinetin in combination with 2 mg/L BAP initiated callus formation relatively earlier than other treatments. Previously, for another *Datura* spp (*D. stramonium*), 0.25 - 0.5 mg/L kinetin and 1 - 2 mg/L 2,4-D have been reported as a suitable combinations for callus initiation [14].

Our findings suggest that the highest amount and fresh weights of calli were obtained with 2 mg/L2,4-D. The role of 2,4-D as an inducer of cell expansion and cell division in callus formation is well established [20]. However, the optimum concentration required for callogenesis is strictly genotype specific.

Callogenesis was not observed with treatments having 0.5 mg/L 2,4-D and 0.5 mg/L GA_3, indicating that these concentrations are sub-optimal for inducing calli from the leaf explants of *D. innoxia* under given culture conditions. This notion is also supported by the fact that significant amounts of calli were produced with different combinations of GA_3, BAP and Kinetin. Previously, media containing different combinations of 2,4-D, kinetin, and IAA produced calli in *D. metel* from leaf and anther explants [10]. We have obtained comparable amounts of calli from the media combinations with and without 2,4-D suggesting that 2,4-D is not essential for callus induction from leaf explants in *D. innoxia*. Thus callus formation is not limited to selective growth regulator rather a variety of combinations could be utilized in *D. innoxia*. In addition, the rate of callus proliferation was highest with kinetin + BAP and lowest with 2,4-D alone. This increased dedifferentiation and high rate of callogenesis seems to be due to synergistic effects of cytokinins. This can be attributed to the multiple roles of cytokinins in plant development, such as cell division, cell expansion, stimulation of protein synthesis and enhancement of certain enzyme activities [21].

Calli transferred to regeneration media produced shoots in 20 - 45 days depending upon the type and concentration of hormone used. Initially, after a week of calli sub-culturing, green spots appeared at the surface of calli which then transformed into shoot primordia and later developed into complete shoots. Shoot formation from calli was also observed with BAP alone as well as in combination with kinetin and IBA. Moreover, media supplemented with both BAP and kinetin were found most suitable not only for callogenesis but also for maximum number of fully expanded shoots in a minimum period of time. The effectiveness of BAP for shoot regeneration from callus in other plants such as *Acorus calamus* has been reported [22]. Nevertheless, our data support that both kinetin and BAP hold a pivotal role in shoot formation of *Datura innoxia*.

The time taken for the induction of roots from regenerated shoots was found strictly dependent on IBA concentration in the rooting media. The earliest obtained maximum number of roots was noticed at 0.5 mg/L and no root formation was observed at 2 mg/L IBA. In *D. stramonium*, no rooting was reported at 1.5 mg/L IBA [14]. In contrast, we noticed a relatively delayed and less extensive rooting for this concentration.

TLC has been reported as a reliable way to separate and quantify alkaloids in *Datura spp.* [23]. TLC revealed the presence of hyoscine in all tested samples from wild-type as well as *in vitro* grown plants. However, our target alkaloid hyoscine was quantified through HPLC which revealed that all plant parts of *Datura innoxia* contain considerable amount of hyoscine. Importantly, hyoscine concentration was dependent not only on the part of the plant used for analysis but also the plant growth stage. We have reported for the first time that tissues obtained from the wild-type plants accumulated more hyoscine contents when compared to *in vitro*-cultured plants.

Since there has not been any comparison made for hyoscine contents in tissue cultured grown

plants or calli in *D. innoxia* in previous studies by others. Among *in vitro*-grown samples, differential hyoscine concentration was observed for different type of cells. Dedifferentiated green calli contained the highest amount of hyoscine followed by leaves and brown calli. Our data support the findings from previously published reports that culture conditions are important determinants in the development of secondary metabolites in *Datura spp* [25]. Lower concentrations of hyoscine exhibited by *in vitro* grown cultures could be attributed to the developmental phase difference of two plant types. The wild-type plants were at maturity stage whereas the *in vitro* grown were juvenile.

The display of several additional peaks in chromatograms, particularly, for the brown calli suggests the presence of unexplored isoforms of hyoscine or other alkaloids in the tissue cultured derived calli and plants which need future investigations for their medicinal use. These undifferentiated mass of cells could be a potential sustainable source for the production of many useful compounds either directly or through cell cultures.

CONCLUSION

The findings of this study indicate that *D. innoxia* tissue culture grown calli and plants accumulate substantial amount of hyoscine contents comparable to wild-type plants. The natural product produced is clean, environment-friendly and does not rely on climatic conditions. Thus, high hyoscine production can be achieved in cultured *D. innoxia* tissues forming calli without reduction in quantity. The highest amounts were obtained from fresh green calli followed by leaves and old brown calli.

DECLARATIONS

Acknowledgement

This research was partly supported by grant no. 1-42/ILS-UITSP/HEC/2008 from Higher Education Commission to Dr.Asrar Muhammad Khan. The authors are very grateful to Dr. Mustafa Shafaqat, Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan for providing assistance with the statistical analysis. The authors are also thankful to the English Languish Center team, Department of Humanities, CIIT Islamabad for proofreading and editing the manuscript.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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