In vitro Organogenesis and Alkaloid Accumulation in *Datura innoxia*

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The kinetics of tropane alkaloids accumulation in different organs such as roots, leaves, stems, flowers and seeds of *Datura innoxia* was investigated by GC-MS. Twenty-six tropane alkaloids were detected. The ester derivatives of tropine (3α-tigloyloxytropine and 3-tigloyloxy-6-hydroxytropine) are the major compounds. Undifferentiated callus were established from the stem explants of *Datura innoxia* using Murashige and Skoog (MS) medium supplied with 6-benzylaminopurine (BA, 1 mg l^{-1}) and indole-3-acetic acid (IAA, 0.5 mg l^{-1}) in combination for 6 weeks. Callus differentiation was initiated by subculture onto solid MS medium, free from hormones, for more than 10 months. Initially, shoots were formed after four weeks from subculture. Further subculturing in basal MS medium without growth regulators initiated the rooting of a shooty callus after 6 weeks. Investigation of the alkaloid content of the unorganized and organized callus revealed that callus (either green or brown) yielded only trace amounts of alkaloids. On the other hand, re-differentiated shoots contained mainly scopolamine while re-differentiated roots biosynthesized hyoscyamine as the main alkaloid.

Key words: Datura innoxia, *in vitro* Re-Differentiated Callus, Tropane Alkaloids

Introduction

All species of *Datura* and *Brugmansia* contain certain biologically active tropane alkaloids, which have powerful central nervous system effects since they are antagonists at the muscarinic acetylcholine receptor. The main alkaloids present are scopolamine and hyoscyamine. The effects of these alkaloids include stimulation of the central nervous system and simultaneous depression of the peripheral nerves typical for a parasympathomimetic. The medicinal qualities include spasmolytic, antiasthmatic, anticholinergic, narcotic and anesthetic properties (Roddick, 1991).

Plant cell cultures provide an interesting alternative for the production of secondary metabolites. In several *in vitro* studies, it has been demonstrated that this production is higher in differentiated structures than in non-differentiated cells (Lindsey and Yeoman, 1983; Wink, 1987). The accumulation of hyoscyamine and scopolamine by callus, cell, shoot, root and hairy root cultures of different species of *Datura* has been reported before (Parr *et al.*, 1990; Hector *et al.*, 1993; Dupraz *et al.*, 1994; Huang *et al.*, 1996; Lanoue *et al.*, 2002; Berkov *et al.*, 2003).

In the present study we have investigated the accumulation of tropane alkaloids at various stages of growth of intact plants of *D. innoxia*, and compared to undifferentiated callus and organized callus cultures. About 38 tropane alkaloids have been found in *D. innoxia* (Berkov and Zayed, 2003). Hyoscyamine and scopolamine are present in low concentration in all organs whereas the ester derivatives of tropine (3α-tigloyloxytropine and 3-tigloyloxy-6-hydroxytropine) are the major compounds. Examination of the callus and the produced plantlets revealed that there was a distinct relationship between the alkaloid accumulation, organogenesis and morphogenesis. Our results point out that the alkaloid formation is nearly absent or very low in cultured callus and is induced upon differentiation of the callus to roots and shoots.

Experimental

Plant material

Seeds and different germination and developmental stages of *Datura innoxia* were collected from plants cultivated in the experimental garden

of Faculty of Agriculture, Zagazig University, Zagazig, Egypt. After every stage, plant materials were collected and stored in a refrigerator until extraction and analysis. For culture experiments, explants from young *Datura* plants were collected. Plant material was kept under running tap water with soap for 2 h and dissected into 2–4 mm pieces. Surface sterilization was achieved by immersion in fungicide (Benlate, 2 g l^{-1} for 30 min), $HgCl_2$ (0.1% for 10 min) and then with hypochlorite solution (1% for 20 min). The explants were rinsed three times with sterile distilled H_2O after every treatment. The terminal ends of each were cut with a sterilized razor blade and cultured on Murashige and Skoog (MS) solid medium supplemented with 3% sucrose.

Culture media and conditions

Initiation of callus cultures

Initiation of unorganized callus was carried out using auxiliary meristem, petioles and stem as explants. MS basal medium was supplemented by the growth hormones 6-benzylaminopurine (BA, 1 mg l^{-1}) and indole-3-acetic acid (IAA, 0.5 mg l^{-1}) in combination for 6 weeks. Callus was differentiated after 8 weeks when the undifferentiated callus was subcultured on MS medium containing BA (2 mg l^{-1}) and IAA (1 mg l^{-1}).

Initiation of multiple shoots

Multiple shoots callus were obtained when the undifferentiated callus was subcultured in MS medium free from phytohormones. The developed shooting callus was subcultured every 4 weeks and the observed data were used to estimate the morphogenetic response and the biosynthesis of secondary metabolites.

Elongation and rooting of multiple shoots

The previously produced multiple shoots were transplanted in MS medium containing different concentrations of cytokinin and auxins. The experiments were also carried out with MS medium without growth hormones and MS half strength medium. The best shooting and rooting were achieved after incubation for 4 weeks in MS medium without growth hormones.

Extraction and analysis of alkaloid

Extraction

Alkaloids were extracted from the organs at different stages of growth, callus, re-differentiated shoots and roots according to a reported method (Zayed, 2001). The alkaloid contents were analyzed by GLC-MS (Zayed and Wink, 2004).

Gas-liquid chromatography (GLC)

Identification of the alkaloids was carried out by using a Carlo Erba 6000 gas chromatograph equipped with a fused silica capillary column OV1 (15 m × 0.25 mm i.d.); split: 1:5; detector: FID; carrier gas: helium (flow rate 2 ml/min); temperature program: the initial column temperature was 150 °C (3 min isothermal), subsequently the temperature was programmed from 150 °C to 300 °C in two steps, first 15 °C/min up to 250 °C and then 20 °C/min up to 300 °C, finally at 300 °C for 10 min isothermal; injector temperature: 250 °C; detector temperature: 300 °C.

GLC-MS

The above Carlo Erba 6000 gas chromatograph was directly coupled with a Finnigan MAT 4500 mass spectrometer operating at 45 eV. Alkaloids were identified according to their retention indexes and published mass spectra (El-Shazly *et al.*, 1997).

HPLC

A Hewlett Packard (HP) series 1100 instrument (Central Laboratory of Veterinary Medicine, Zagazig University) with a pump 6000 A Waters was used: column: Hypersil BDS-C_{18} (30 cm × 3.9 mm); mobile phase: MeOH/3% aequeous acetic acid (25:75); flow rate: 1 ml/min; UV detector: at 254 nm. The samples were dissolved in MeOH and 20 μl were injected.

Results and Discussion

Establishment of callus cultures, multiple shoots and adventitious root

After different trials, undifferentiated callus was obtained from petioles and stem explants in MS medium supplied with 6-benzylaminopurine (BA, 1 mg l^{-1}) and indole-3-acetic acid (IAA, 0.5 mg l^{-1}). Stem explants showed the best response; they produced a hard, green and brown callus after 3–6 weeks.

Differentiated callus with numerous green small shoots were observed in MS medium containing BA (2 mg l^{-1}) and IAA (1 mg l^{-1}) after 6–8 weeks. Initiation and elongation of the shoots were induced after a series of subcultures of the undifferentiated callus in MS medium without

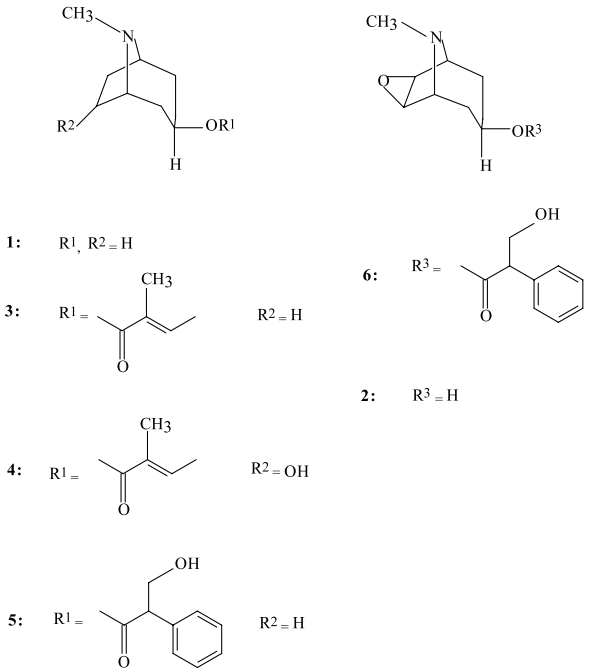

Fig. 1. The major structures of tropane alkaloids investigated in *D. innoxia:* tropine (**1**), scopine (**2**), 3α-tigloyloxytropine (**3**), 3-tigloyloxy-6-hydroxytropine (**4**), hyoscyamine (**5**), scopolamine (**6**).

growth regulator every 4 weeks. When the well grown and elongated shoots were transplanted in MS medium with auxins like naphthalene acetic acid (NAA, 0.5, 1 and 2 mg l^{-1}) for rooting, low and high concentration of NAA didn't initiate the rooting of shoots. The best rooting response was obtained when the elongated shoots were subcultured for 5 weeks in MS medium devoid of growth regulators.

Dynamic of the alkaloid biosynthesis at different germination and developmental stages

Alkaloid profiles were followed through the developmental stages, from seedlings to mature plants with flowers and seeds. Table I illustrates the distribution of 6 major alkaloids, tropine (**1**), scopine (**2**), hyoscyamine (**5**), scopolamine (**6**), 3α-tigloyloxytropine (**3**), and 3-tigloyloxy-6-tropine (**4**) (Fig. 1), in the various organs such as roots, stems, leaves, flowers and seeds.

In roots the free bases scopine and tropine and their tigloyl-esters dominate whereas aerial parts accumulate higher amounts of hyoscyamine and scopolamine. This pattern probably reflects the physiological situation that roots are the site of tropane alkaloid biosynthesis which the alkaloids export to the aerial parts for further derivatizations.

Alkaloid biosynthesis in organized callus

In the present study, the alkaloid content in callus, regenerated shoot and root was analyzed quantitatively by HPLC. This study revealed that levels and profiles varied significantly with respect to the state of differentiation in the cultured tissues. The unorganized callus contained traces of scopolamine (**6**) and hyoscyamine (**5**) and the total yield of alkaloid content was very low. The regenerated multiple shoots contained scopolamine (**6**) and hyoscyamine (**5**) as the major compounds and low percentage of tropine esters. On the other hand, hyoscyamine (**5**) was prominent in the adventitious roots of the regenerated plant which exhibited the highest alkaloid contents (Fig. 2). Also these data are in agreement with the observation, that firstly, differentiated tissues are required for the formation of tropane alkaloids and secondly that roots are the site of biosynthesis.

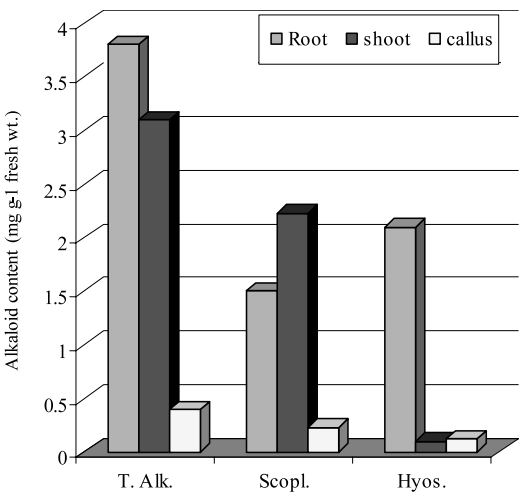

Fig. 2. Tropane alkaloid production in *in vitro* callus, shoots and roots. T. Alk., total alkaloid; Scopl., scopolamine; Hyos., hyoscyamine.

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Table I. Dynamic study of alkaloid accumulation at various stages of germination and seedling development of *D. innoxia* using GLC-MS.

Stage[a]	Organ[b]	T. Alk.[c] [mg g^{-1}]	Alkaloid patterns (%)[d]					
			Tr (**1**)	Sc (**2**)	3-Tr (**3**)	6-OH (**4**)	H (**5**)	S (**6**)
I	R	2.2	18.2	57.2	6.2	tr	tr	10.2
	L	25	40.1	39.1	19.2	1.2	tr	tr
	St	4	5.5	20.4	46.2	5.3	10.8	tr
II	R	11.3	41.2	34.2	17.5	5.3	tr	tr
	L	1.3	17.1	74.2	2.1	tr	tr	5.4
	St	2.4	6.9	63.5	27.1	tr	tr	tr
III	R	2.7	43.2	21	26.2	6.8	tr	tr
	L	2.2	tr	71.7	3.5	tr	tr	13.4
	St	25.6	14.5	81.2	1.3	tr	tr	tr
IV	R	11.9	51.4	38.4	7.5	tr	tr	tr
	L	2.5	5.2	80.5	3.1	tr	tr	5.1
	t	14	8.6	81.9	tr	tr	tr	tr
V	R	19.3	52.8	36.4	7.1	1.4	tr	tr
	L	4.5	tr	84.2	4.2	tr	tr	5.2
	St	14.4	13.2	84.3	tr	tr	tr	tr
	Fl	2.3	32.4	22.7	tr	8.8	17.17	9.3
VI	R	2.7	51.7	18.9	1.2	1.2	10.2	tr
	L	4	2.4	tr	1.2	3.1	tr	81.1
	St	1.5	25.6	10.2	tr	19.5	11.8	23.7
	Se	12.4	2.4	1.3	2.4	3.3	28.4	57.2

[a] Germination stages I, II, III and IV (the collection interval was one week), flowering stage V and mature plant VI.
[b] R, roots; L, leaves; St, stems; Fl, flowers; Se, Seeds.
[c] T. Alk., total alkaloids.
[d] Tr, tropine; Sc, scopine; 3-Tr, 3α-tigloyloxytropine; 6-OH, 3-tigloyloxy-6-hydroxytropine; H, hyoscyamine; S, scopolamine; tr, traces $<1\%$.
Note: Total alkaloids = 100%, the abundance of individual alkaloids is expressed as % of the total alkaloids (Wink *et al*., 1983; El-Shazly *et al*., 1997).

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