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Organization and Alkaloid Production in Tissue Culture of *Datura innoxia* Mill

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ABSTRACT. Cell suspension of *Datura innoxia* Mill, was investigated for growth and production of tropane alkaloids. Study of the *in vitro* plant culture resulted in obtaining high percent of alkaloids more than in the intact plant. Acclimatization of the *in vitro* plant to the conditions of Makkah area was carried out. The qualitative picture of the individual formed alkaloids was obtained by TLC.

Introduction

Tropane alkaloids, found in certain genera of the solanaceae family, include some medicinally important secondary plant products. Atropine and scopolamine have spasmolytic and anaesthetic properties. Datura species are widely distributed in tropical and temperate regions of both hemispheres[1]. *Datura innoxia* is represented in the flora of Saudi Arabia, in addition to other three species *viz.: D. stramomium. D. metal* and *D. fastuosal*[2].

Since tissue cultures have been shown to produce a great variety of secondary products, there has been a considerable interest in the possibility of using plant tissue cultures for the biosynthesis of commercially-important products. Production of tropane alkaloids from tissue culture of solanaceous plant was interesting to many investigators[3-5].

Biomass formation and alkaloid production in callus tissues from *Datura innoxia* was studied by Verzar-petri, 1980[6]. Many studies were carried out concerning the

biosynthesis of the tropane alkaloids dealing with the callus tissues of *Datura innoxia* Mill[7-10]. These studies indicated that the total alkaloidal content of the callus tissue was smaller than that of the intact plant. The total alkaloid in cell suspension of *Datura innoxia* was far below than that detected in the intact leaves[11].

The present work aims to produce higher percent of tropane alkaloids from cell suspension, *in vitro* plants (originating from calli) as well as acclimatizing some of the *in vitro* plants to the environmental conditions of Makkah region.

Material and Methods

Source of Material

a) Wild plant

Datura innoxia Mill[2] growing naturally, in the garden of Umm Al-Qura University, Makkah, Saudi Arabia, was collected at the seedling stage.

b) Culture method

At the stage of cotyledonary leaf, explants were removed and surface sterilized in 8% (v/v) Commercial Clorox for 20 min, washed five times with sterile distilled water. Three cotyledons of about 4 mm segments were aseptically transferred to a sterilized 175 ml glass jar, containing 40 ml UM medium (Ushima and Murashige)[12], supplemented with 3% (w/v) sucrose, 0.7% (w/v) agar (BDH) and 2 mg/l of 2,4-Dichlorophenoxyacetic acid (2,4-D), 0.25 mg/l of kinetin (k) and 2 g/l casein hydrolyzate. The pH of the medium was adjusted to 5.6 using 1N HCl prior to the addition of agar. Callus cultures were grown as light cream in color and friable after incubation for 8 weeks in the dark at 28 ± 2°C. The calli were maintained by monthly transfer to the same freshly prepared medium in diffuse light (not in dark).

Cell suspension cultures were established from callus and maintained in liquid UM medium on an orbital shaker (125 r.p.m.) at 25 ± 2°C with a continuous illumination of 1000 lux. An inoculum of 30 ml of 12 month old suspension cultures was used to inoculate 100 ml medium in 250 ml Erlenmeyer flask. Each transplantation of the cell suspension was carried out twice at one week interval for 12 week passages. The cited values are means of duplicate analysis.

c) In vitro plant differentiation from callus

The calli induced by the cotyledon cultures were transferred into shoot induction medium, designated MSD3 consisting of MS mineral salts and vitamins (Murashige and Skoog, 1962)[13] supplemented with 3% (w/v) sucrose, 0.7% (w/v) agar (BDH) and 1.0 mg/l 6-benzyl-aminopurine (6-BAP) and 2.0 mg/l 3-indoleacetic acid (IAA) at pH 5.6. All cultures were maintained at 28 ± 2°C under continuous illumination of 3000 lux in 500 ml sterilized glass jars.

After 8 weeks, small leaflets were formed from the clusters of adventitious buds on the surface of callus. These leaflets were isolated and subcultured on fresh MSD3 medium. To promote rooting of the shoots, the developed shoots were dissected

after 4 weeks from the callus and subcultured on a 150 ml solid agar MSO (MS medium lacking the growth regulator). The roots were formed after incubation in 500 ml sterilized glass jars at 28 ± 2°C for 3 weeks under continuous illumination of 3000 lux.

Each *in vitro* transplantation was carried out twice at three weeks intervals for 12 weeks. The cited values are means of duplicate analysis.

d) In vivo transplantation

Some of the rooted *in vitro* plants were transplanted to the soil in pots. The adaptation of the plants in soil were lasted 14 days at 25 ± 2°C in a mist propagation chamber for 12 hour/day illumination of 1000 lux. Plants were then transferred to the field of the Research Unit of Plant Ecology, Fac. of App. Sci., Umm Al-Qura Univ., Makkah, and grown to maturity (flowering stage).

e) Extraction and analysis of alkaloids

(i) From cell suspension culture. The harvested cell culture; grown in 100 ml medium; was dried at 110°C for 15 min and then at 60°C until having constant weight. Each sample was finally powdered and its alkaloidal content was extracted and determined[14].

(ii) From in vitro and in vivo plants. The differentiated parts were separately dried at 60°C in an air drying oven until having constant weight and powdered. The alkaloids were extracted and determined as mentioned before for the different plant parts of the studied samples.

(iii) TLC investigation. The samples of alkaloids for thin layer chromatographic (TLC) investigation of the studied tissues (*viz:* cell suspension, *in vitro* and *in vivo* plants) were prepared through the procedure mentioned before and finally dissolved in $CHCl_3$. The alkaloids as well as the available authentic ones were developed on silica gel G (Merck) plates irrigated with $CHCl_3$, MeOH, 25% NH_4OH (85: 14.5: 0.5). The alkaloidal spots were visualized by spraying with Dragendorff's reagent.

Results and Discussion

1 – Cell suspension culture

The stock suspension grew as a pale green cultures with isolated cell and small aggregates of cells. This stock suspension cultures was used in the present study. The suspension culture was pale green, fine and friable till the end of the third week passages. After that, it became light brown, fine and friable till the end of the seventh week passages. Furthermore, suspension cultured turned brown with small aggregate cells and clumps till the end of the experiment (twelve week passages). Maximum growth was reached at the end of the third week (Fig. 1). The formation of tropane alkaloids started at the end of the first week and reached its peak during the seventh week passages but declined thereafter (Fig. 1). Many reports suggested that, in undifferentiated cells, an inverse relationship between primary metabolism, such as growth, and production of secondary metabolites exists[15].

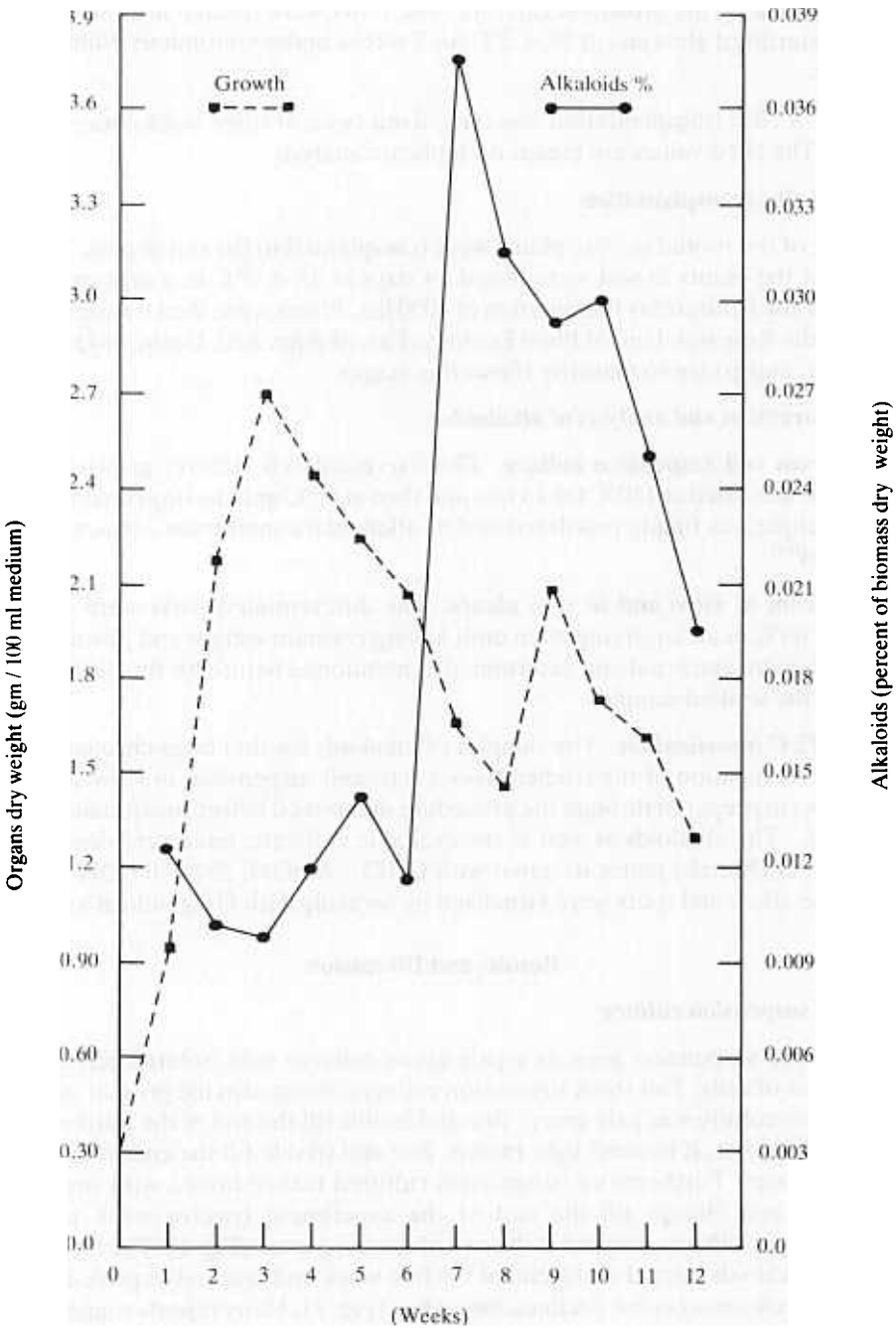

FIG. 1. Growth and alkaloidal formation in cell suspension of *Datura innoxia* at different passages.

The detected alkaloids of the cell suspension; Dragendorff – positive spots; were present in the intact plant and included: atropine, L-hyoscyamine and scopolamine in addition to unknown alkaloid. The formation of alkaloids was followed up by TLC in the cell suspension culture of different passage generation (Table 1). The complete alkaloidal pattern of the intact plant was observed at the end of the sixth week passages with variable relative ratios in the earlier and further passages. No tropane alkaloids were detected in the media used for growing the cells at any of the studied passages. This finding coincided with our previous report[14].

TABLE 1. Distribution of individual alkaloids in the cell suspension cultures at different growth passages.

Alkaloid (R_f) / Cell suspension age (weeks)	Atropine (0.23)	L. Hyoscyamine (0.26)	Scopolamine (0.8)	Unknown (0.92)
0	+	+	+	
1			+	
2		+	+	
3		+	+	
4		+	+	
5	+	+	+	
6	+	+	+	+
7	+	++	++	+
8	+	++	++	+
9	+	+	++	+
10	++	+	++	+
11	+	+	+	+
12	+	+	+	+

2 – In vitro plant differentiated from callus

At the end of the third week of shoot culture, the root formation started (Fig. 2). Maximum growth of the different organs, (leaf, stem and root) was observed at the end of the ninth week (Fig. 3). During the twelfth week, some of the leaves turned yellow and detached from the plant. This changing in leaf character as well as the growth decay of the plant may be attributed to the consumption of the nutrients of the solid medium.

Alkaloid formation started at the end of the third week in different organs. Previous studies[16] indicated that de-differentiated tissues or cells propagated in synthetic culture media produced little or no alkaloids. Fig. (3) indicated that maximum alkaloidal formation was obtained during the twelfth week reaching a percent of 1.2 in roots, 0.75 in stems and 0.18 in leaves. Now, it is clear that alkaloid content depends on the development stage of the plant[17]. These percentages are higher than that found in the intact plant (Table 2). *D. innoxia* regenerated from the cell cultures recover the patterns and overcome the levels of alkaloids characteristic of the plant. Thus, it appears that the capacity of synthesis of specific compounds is usually retained during culture[9]. This high yield of tropane alkaloid reaching (1.2%) is the first record by using *in vitro* shoot formation.

FIG. 2. Organization of *in vitro Datura innoxia.*

The study of Hiraoka and Tabata[9], with *D. innoxia* suggested that cultured cell lines develop in cell cultures and that a selection for "normal" cells may operate during the regeneration of plant.

The high yield of alkaloidal formation from *in vitro D. innoxia* plant may be attributed to the successful selection of cell producing high amounts of the secondary metabolites (tropane alkaloids) because of the heterogenity which is associated with

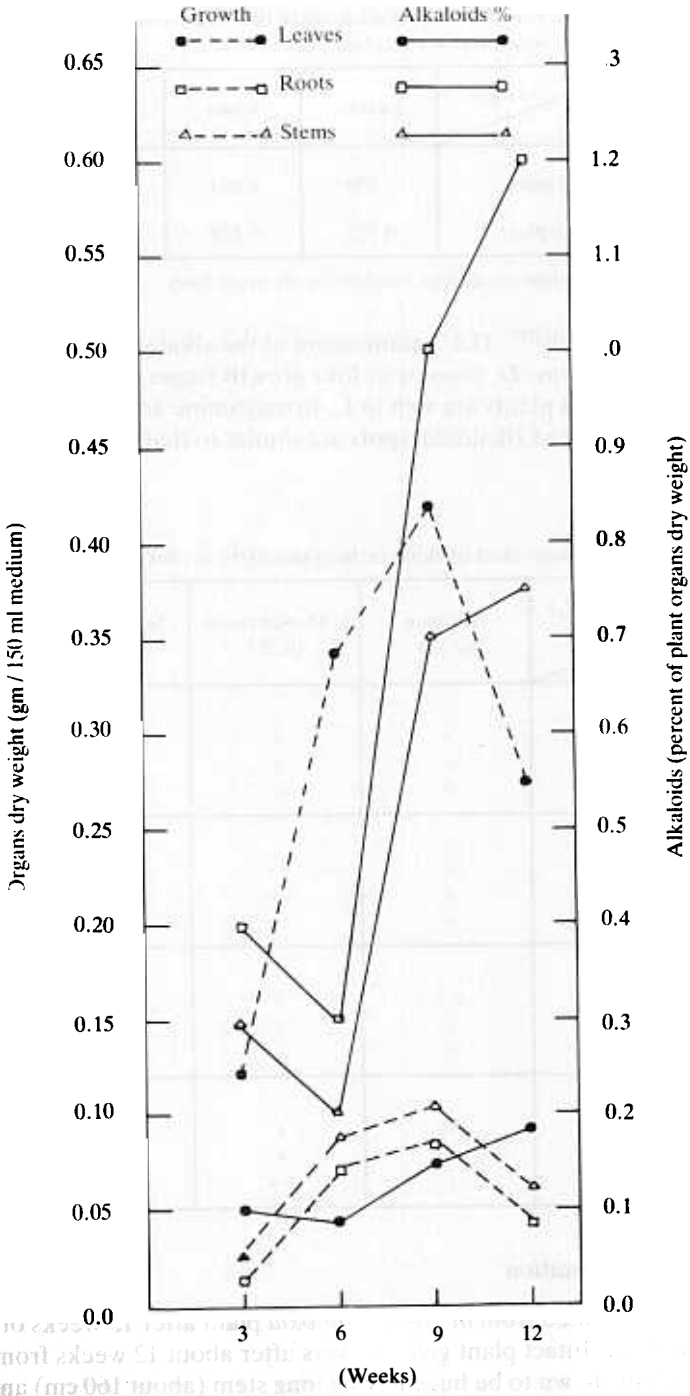

FIG. 3. Effect of plant age on the growth and alkaloidal content of *in vitro Datura innoxia*.

TABLE 2. Percent* of total alkaloids in the different organs of the intact and *in vivo* plant.

Organ / Plant	Leaves	Roots	Stems
Intact plant	0.038	0.064	0.052
In vivo plant	0.123	0.138	0.072

*Mean of duplicate analysis, calculated on dry weight bases.

the cultured plant cells[18]. TLC examination of the alkaloidal pattern formed at different organs of *in vitro D. innoxia* at four growth stages indicated that both of the 6th and 9th week old plants are rich in L. hyoscyamine and scopolamine (Table 3). Generally, the detected alkaloidal spots are similar to that of the intact plant pattern (Table 4).

TABLE 3. Distribution of individual alkaloids in the organs of the *in vitro* plants at different growth stages.

Alkaloid (R_f) / *In vitro* Plant (age)	Atropine (0.23)	L. Hyoscyamine (0.26)	Scopolamine (0.8)	Unknown (0.92)
3 weeks				
leaves	+	+	+	
stems	+	+	+	
roots	+	+	+	+
6 weeks				
leaves	+	++	++	+
stems	+	++	++	
roots	+	++	++	
9 weeks				
leaves	++	+	++	+
stems	+	+	+	+
roots	+	+	+++	+
12 weeks				
leaves	++	+	++	+
stems	+	+	+	+
roots	+	++	+++	+

3 – In vivo transplantation

Flowers were formed from *in vivo D. innoxia* plant after 15 weeks of being transferred to the field. Intact plant gives flowers after about 12 weeks from plantation. The *in vivo* plant shown to be huge having long stem (about 160 cm) and with many branches of large leaves as compared with the intact plant.

TABLE 4. Distribution of individual alkaloids in the organs of the intact and *in vivo* plants

Alkaloid (R_f) / Plant Sample	Atropine (0.23)	L. Hyoscyamine (0.26)	Scopolamine (0.8)	Unknown (0.92)
Intact plant				
leaves	++	++	++	+
stems	+	+	+	+
roots	+	+	+	+
In vivo plant				
leaves	+	++	+++	+
stems	+	+	+	+
roots	+		+	+

Table (2) indicated that the formation of tropane alkaloids is higher in the different organs of the *in vivo* plant as compared with the intact plant, but still less than the percentages observed in case of the *in vitro* plant culture. Investigation of the individual alkaloids formed at different parts of the *in vivo D. innoxia* by TLC resulted in having the same alkaloidal spots. Moreover, the intact plant is rich in atropine while the *in vivo* plants is richer in scopolamine.

Variation of the percent and pattern of formed alkaloids in the different studied plant organs in both of the *in vivo* and *in vitro D. innoxia* coincided with the previous report[19]; that some secondary products can accumulate in specific morphological structures. The high percent of alkaloids observed using the *in vitro* plant culture may be due to supplying the plant with the essential nutrients and by growing the plant in the suitable environmental conditions.

Finally, it is our belief that *in vitro* plant cultures of *D. innoxia* is the suitable technique for producing tropane alkaloids for industrial production of these compounds in the near future.

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تشكل الأعضاء وإنتاج القلويدات في مزارع الأنسجة لنبات الداتورا إنوكسيا

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قسم الأحياء ، كلية العلوم التطبيقية ، جامعة أم القرى ،
مـــكة المكرمـــة ، المملكة العربية السعودية

المستخلص . أجرى قياس النمو وإنتاج قلويدات التروبان في مزارع المعلق الخلوي لنبات داتورا إنوكسيا ، كما تمت دراسة النمو لنفس النبات في البيئة المعقمة ، وقد أظهرت الدراسة الحصـول على نسبـة عالية من قلويدات الـتروبان (بلغت ١,٢ في الجذور ، ٠,٧٥ في السوق ، ٠,١٨ في الأوراق) ، إذا ما قورنت بالنبات النامي طبيعيًا . وقد نقلت بعض النبـاتـات النـاتجـة من الأوسـاط المعقمة إلى البيئة الطبيعية بمنطقة مكة المكرمة بغرض أقلمتها . هذا بالإضافة إلى أنه قد تم فحص كيفي للمحتوى القلويدي لكل الأعضاء وفي أعمار مختلفة للنبات موضوع الدراسة .