In vitro androgenetic cultures of *Hyoscyamus niger* L., *H. albus* L. and alkaloid content assay

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Abstract

In vitro cultures of *Hyoscyamus niger* L. and *H. albus* L. anthers were initiated which resulted in obtaining androgenetic plants and callus cultures. The leaves of these plants and the callus cultures were subjected to analysis (TLC, GC) for the presence of alkaloids, derivatives of tropane. In the studied material, alkaloids of different qualitative and quantitative composition from that of ground-grown plants were found.

Key words: *in vitro*, androgenesis, tropane alkaloids, *Hyoscyamus*

INTRODUCTION

Many haploid species from the *Solanaceae* family have been obtained so far from *in vitro* anther cultures (Zenkteler 1972, Vasil et al. 1979). Raghavan (1975, 1978), Corduan (1975), Wernicke and Kohlenbach (1977), Wernicke et al. (1979) have worked in this field with the genus *Hyoscyamus* L. The accumulation of secondary metabolites, in particular, of tropane alkaloids which determine the medicinal value of plants from the genus *Hyoscyamus* L., has not been followed yet. The aim of our studies was to obtain callus tissue and regenerated plants from the species *H. niger* L. and *H. albus* L. through androgenesis, in the search for new sources of therapeutic compounds. We intended to see if callus tissue and leaves from both species contained tropane alkaloids. In case the composition of these compounds was found to be desirable, these cultures could form a line with a defined content of active substances.

Abbreviations: 2,4-D — 2,4-dichlorophenoxyacetic acid; BAP — 6-benzylaminopurine; IAA — β -indolyl-3-acetic acid; KIN — kinetin; TLC — thin layer chromatography; GC — gas chromatography

MATERIAL AND METHODS

In vitro cultures. Androgenetic *in vitro* cultures were run according to generally accepted methods (Corduan 1975, Raghavan 1975, 1978). Black henbane (*Hyoscyamus niger* L.) and white henbane (*Hyoscyamus albus* L.) anthers were isolated from buds gathered in June and July (1979-1982) from experimental fields belonging to the "Herbapol" Botany Department of Institute of Medicinal Plants in Poznań and the Department of Medicinal Plants of the Poznań Medical Academy.

Anthers in the microspore development stage — uninucleate and two-celled, were placed on the following media: Murashige and Skoog's (1962, MS), Theiler's (1977) and Nitsch and Nitsch's (1969, NN) which were enriched with auxins (2,4-D, IAA, NAA) and cytokinins (BAP, KIN). The process of androgenesis initiation and development of buds from anthers was run on a total of 24 modifications of the above-mentioned media. All of the media containing 3% sucrose were gelled with agar ($9 \text{ g} \cdot \text{dm}^{-3}$) at pH 5.8-6.0 before sterilization (0.1 MPa, 20 min). The cultures were run under the following conditions: illumination either 800 or 2000 lx or continuous darkness, 24-28°C, relative humidity 60-70%.

Callus cultures were transferred (1981-1983) on MS medium with the addition of 2,4-D ($0.25 \text{ mg} \cdot \text{dm}^{-3}$) and KIN (0.5 or $1.0 \text{ mg} \cdot \text{dm}^{-3}$) under a constant illumination of 2000 lx. The androgenetic plantlets which were formed were planted in earth-filled pots and then in the ground.

Chromatographic analysis. The alkaloid content was studied in callus cultures after 20 transfers and in leaves collected from blooming specimens of *H. niger* L. and *H. albus* L. regenerated through *in vitro* anther cultures. Callus tissue and leaves were dried at a temperature of 50-60°C and were then powdered. The alkaloid fraction was obtained by the method used by Eapen et al. (1978a).

Qualitative analysis was done by thin layer chromatography (TLC) using two types of adsorbents and five solvent systems (Table 1). Reagents (pure for analysis grade) were used in volumetric proportions. Unless stated otherwise, ammonia was used at a concentration of 25%. The chromatograms were developed first with Dragendorff's reagent, and then with a 10% aqueous solution of sodium nitrite (Anderson et al. 1977) and a potassium iodoplatinate solution (Harborne 1973). For fuller documentation of the presence of alkaloids, an analysis was also run on plates with 60 F₂₅₄ gel (Merck, with a fluorindicator) which were sprayed with TCNQ — tetracyanoquinodimethane (Rücker and Taha 1977). Standard alkaloids were chromatographed simultaneously with the extracts. These were: atropine, hyoscyamine, scopolamine, apoatropine and tropanol. Before quantitative measurements were done, alkaloid extraction parameters were determined (Wesołowska 1981).

Quantitative determinations were done by gas chromatography (GC) with the following parameters: Chrom-4, flame ionization detector, glass column 150 cm in length filled with 3% SE-30 on chromosorb W (80/100 mesh), N_2 as carrier gas, sample temperature 230°C, thermostat temperature 202°C, tape speed 4 mm per min. The identities of atropine and scopolamine peaks were determined on the basis of comparison of GC analysis results with standards. Quantities were calculated from atropine and scopolamine calibration curves.

RESULTS

The callus tissue and leaves from *H. niger* L. and *H. albus* L. in which the tropane alkaloid composition was determined, were obtained through *in vitro* anther cultures under conditions of constant illumination (2000 lx). From the 24 modifications of media checked in the process of androgenesis initiation, the following results were found. *H. niger* L. buds were obtained directly or through callus from anthers after 4-8 weeks of culture on NN medium without any additions, and also on the same medium supplemented with 2,4-D ($2 \text{ mg} \cdot \text{dm}^{-3}$) or BAP ($2 \text{ mg} \cdot \text{dm}^{-3}$). Leafly shoots from this species, developing from buds, were rooted on MS medium with IAA ($2 \text{ mg} \cdot \text{dm}^{-3}$). Androgenetic *H. albus* L. plantlets were obtained on pure NN medium or supplemented with BAP ($2 \text{ mg} \cdot \text{dm}^{-3}$). Plants from both species of *Hyoscyamus* developed very well after being planted in earth-filled pots, and reached the blooming stage after a few weeks (Figs. 1 and 2). Those transplanted to the ground before blooming survived and reached full development. Callus cultures from anthers under the conditions described here were obtained only for *H. niger* L. *H. albus* L. callus degenerated after a sequence of transfers. The best growth of callus cultures was observed on MS medium with the addition of KIN and 2,4-D (in the proportions of 2:1 and 4:1) under constant illumination (2000 lx). The presence of alkaloids was tested in green callus after 20 transfers (Fig. 3).

The qualitative and quantitative evaluation of tropane alkaloids in the studied material is presented in Table 1. A relatively good separation of alkaloids was obtained with the A (Fig. 4) and E developing systems, in which the R_f values for hyoscyamine and atropine are different.

On the basis of the analyses carried out it can be concluded that biosynthesis of tropane alkaloids is taking place in *H. niger* L. callus cultures from anthers and in the leaves of *H. niger* L. and *H. albus* L. obtained from androgenetic cultures *in vitro*. On the basis of different colors obtained after spraying the plates with Dragendorff's reagent and

Table 1

Analysis of alkaloids in callus tissues and leaves from *in vitro* regenerated species of *Hyoscyamus* L.

Standards	R_f values $\times 100$					TLC			Quantitative GC* analysis, %	
	developing systems					leaves from species		callus	<i>H. albus</i>	<i>H. niger</i>
	A	B	C	D	E	<i>H. albus</i>	<i>H. niger</i>	<i>H. niger</i>	leaves	
Atropine	16	44	45	50	37	+	—	—	0.033	trace
Hyoscyamine	14	44	45	50	40	+	trace	+	0.044	0.013
Scopolamine	60	55	83	59	89	+	+	—		
Apoatropine	38	43	45	57	83	+	—	—		
Tropanol	6	9	11	70	23	+	—	—		
Others**						+	+	+		

A — silica gel 60F₂₅₄ — CHCl₃-EtOH-NH₄OH, 85:14:1 (Eapen et al. 1978b); B — silica gel 60F₂₅₄ — Me₂CO-C₆H₅C-H₃-MeOH-NH₄OH, 9:8:2:1 (Rücker i Taha 1977); C — silica gel 60F₂₅₄ — Me₂CO-H₂O-NH₄OH, 90:7:3 (Stahl 1973); D — silica gel G60 — n-C₆H₁₄-NH₄OH, 3:2 (Wesołowska 1981); E — silica gel G60 — NH₄OH 10%-MeOH, 1:3 (Wesołowska 1981), where CHCl₃ — chloroform, EtOH — ethanol, NH₄OH — ammonia, Me₂CO — acetone, C₆H₅CH₃ — toluene, MeOH — methanol, n-C₆H₁₄ — hexane, H₂O — water. "+" — present, "—" — absent.

* — Average values from at least three determinations.

** Compounds staining with Dragendorff's and Zaffaroni's reagent (potassium iodoplatinate).

Figs. 1, 2. Blooming androgenetic *Hyoscyamus niger* L. (1) and *H. albus* L. (2) plants, x 0.47.

Fig. 3. The green callus line derived from *Hyoscyamus niger* L. anthers transferred from the basic culture, x 1.25

Fig. 4. The chromatographic separation of alkaloids on silica gel 60, $\text{CHCl}_3\text{-EtOH-NH}_4\text{OH}$ (85:14:1) stained with Dragendorff's reagent. A — atropine, H — hyoscyamine, S — scopolamine, Ap — apoatropine, T — tropanol, H.a. — extract from leaves of an androgenetic *H. albus* L. plant, H.n. — extract from leaves of an androgenetic *H. niger* plant, K — extract from *H. niger* callus

next, after spraying them with a solution of sodium nitrite, it can be stated that atropine and hyoscyamine are found in the leaves of *H. albus* L., whereas hyoscyamine alone is found in callus tissue and trace amounts of it are found in the leaves of *H. niger* L.

The presence of tropanol was shown only in *H. albus* L. Scopolamine, however, is found in leaves but lacking in the callus tissue. The apotropane spot arising immediately after spraying the chromatograms with reagents, was found only in the leaves of *H. albus* L. Both in the callus tissue and in the leaves of both studied species, further alkaloid spots were found at the starting points of the chromatograms. The chromatograms of the callus tissue and white henbane leaf extracts showed the presence of an additional alkaloid spot with an R_f value=0.17 (Fig. 4). Quantitative determinations made by GC show a higher alkaloid content in the leaves of *H. albus* L. than in *H. niger* L. In both species, scopolamine dominates (Table 1).

DISCUSSION

On the basis of our own experiments, changed conditions of *in vitro* *Hyoscyamus* L. anther culture were employed. It was found that 5 and 10 mg·dm⁻³ of 2,4-D added to media as advised by Cordaun (1975) inhibited the development of anthers. Positive effects of callus initiation in the dark were also not observed. Results similar to those obtained using photoperiods of L:D=12:12 or L:D=16:8 (Raghavan 1975, 1978, Wernicke and Kohlenbach 1977, Wernicke et al. 1979) were reached using continuous illumination (2000 lx) of the culture. The stimulatory effect of BAP in the androgenetic culture of species of *Hyoscyamus* L. was in agreement with the results of studies by Wernicke et al. (1979). It seems that the differences in the alkaloid content in the leaves of the studied plants are not determined by the use of 2,4-D in the initiation of androgenesis in *H. niger* L.

Callus cultures from *H. niger* L. seedlings were derived by Dhoot and Henshaw (1977) using first the MS medium, then SH enriched with different amounts of KIN and 2,4-D. In our studies, these same growth regulators stimulated the growth of anther-derived callus tissue. However, the observations made by these authors on the earlier disappearance of hyoscyamine than scopolamine in successive subcultures, differ from the results of our studies.

It was interesting to compare the results of our experiments with those of Yamada and Hashimoto (1982), who ran studies on callus cultures from leaf fragments of plants belonging to species known as rich sources of tropane alkaloids. They studied *Atropa belladonna* L., *Datura stramonium*

L. and *Hyoscyamus* L. They used Linsmaier and Skoog's medium containing NAA and BA in the initiation of callus. Yamada and Hashimoto (1982) found that only the callus tissues from *H. niger* L. contained alkaloid compounds. In addition, they showed the presence of hyoscyamine and scopolamine in the callus tissue as well as the presence of an unidentified substance which gave a positive reaction with Dragendorff's reagent and migrated on the chromatograms in the vicinity of hyoscyamine. In callus cultures of *H. niger* L. derived from anthers, after 20 transfers, we found the presence of hyoscyamine ($R_f=0.14$) and an alkaloid with an $R_f=0.17$. The presence of scopolamine was not found however, although it was demonstrated after 10 transfers.

Karyological analyses and alkaloid biosynthesis studies on the *Hyoscyamus* L. callus tissue arising through androgenesis are underway. This seems justified due to the presence of alkaloids in the early stages of tissue cultures from this genus.

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*Androgenetyczne kultury in vitro Hyoscyamus niger L. i H. albus L. —
— oznaczanie zawartości alkaloidów*

Streszczenie

Zainicjowano kulturę *in vitro* pylników *Hyoscyamus niger* L. i *H. albus* L., w wyniku której otrzymano androgenetyczne rośliny oraz kultury kalusowe. Liście tych roślin i tkanki kulusowe poddano analizie (TLC, GC) na obecność alkaloidów, pochodnych tropanu. W badanym materiale stwierdzono obecność związków alkaloidowych, których skład jakościowy i ilościowy różni się od surowca z roślin gruntowych.