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DETERMINATION OF TROPANE ALKALOIDS IN GOLDEN HENBANE (*HYOSCYAMUS AUREUS*) *IN VITRO*

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Aim. *Aim of the work was to study tropane alkaloids in Hyoscyamus aureus plants.* ***Methods****. L-hyoscyamine and (α)-scopolamine were analyzed through GC-MS.* ***Results****. Level of the tropane alkaloids (hyoscyamine and scopolamine) in plants under study late in the vegetative phase of development to be maintained in vitro was shown to depend on ecotypes from which seeds were sampled. In general, hyoscyamine predominated in roots, while leaves demonstrated prevalence only one of the alkaloids with the other being in trace amounts.* ***Conclusions.*** *Amount of alkaloids in roots was found to depend on altitude of plant vegetation as determined by its estimation in offspring generated in vitro. The idea that environment-genotype interaction may affect the amount of biologically active compounds produced by plant organism was confirmed.*

Key words: *Hyoscyamus aureus, tropane alkaloids, culture maintained in vitro, GC-MS, L-hyoscyamine, (α)-scopolamine.*

Introduction. Genus Hyoscyamus is well-known for the production of anticholinergic Tropane alkaloids, which constitute one of the largest groups of pharmaceutically and economically important plant secondary metabolites [1]. These are secondary metabolites produced by a few genera of the family Solanaceae, such as Atropa, Datura, Duboisia, Hyoscyamus and Scopolia [2]. Tropane alkaloids are esters of tropic acid and tropine derivatives. Tropine is derived from ornithine and/or arginine, whereas tropic acid is synthesized from phenylalanine [3]. Scopolamine, a 6,7- epoxide of hyoscyamine, is formed from hyoscyamine by way of 6B hydroxyhyoscyamin [4].

Hyoscyamine and scopolamine, commercially important anesthetic and antispasmodiac drugs, are the two most important *Solanaceae* alkaloids produced in roots and then translocated to the aerial parts of the plant where they accumulate in the cell vacuoles at high levels [5].

Both compounds are used as anticholinergic agents that act on parasympathetic nervous system [6]. Since hyoscyamine has undesirable effects on the central nervous system, scopolamine is preferred over hyoscyamine. The worldwide market for scopolamine is estimated to be about ten times that of hyoscyamine and its racemic form atropine [7] and is preferred for its higher physiological activity and fewer side-effects. Therefore, significant attention has been paid to its commercial production [8].

Tropane alkaloids are mainly produced by extraction from cultivated plant materials. Growing of the plants in the field enables direct, mass production.

However, there are several reasons for considering plant tissue culture as an alternative to field cultivation. It needs a shortened growth period, eliminates the use of herbicides and pesticides and maintains constant growth conditions and product quality [9], during the past few years, considerable efforts have been made to develop economically feasible protocols for *in vitro* production of these compounds.

The major aim of this research was to study the alkaloid content of different parts of *Hyoscyamus aureus* L grown *in vitro* from seeds collected from different locations in southern Syria.

Materials and methods

This work was carried out in the laboratory of Plant Technologies of National Commission for Biotechnology, Damascus, Syria during the period from 2010 to 2012.

Plant Material. Seeds of *H. aureus* were collected during the end of fruit formation (May-June, 2010) from six locations in southern Syria (Fig. 1). Seeds were surface sterilized with ethanol 70% for 1 minute, 1.5% sodium hypochloride for 20 minutes, and washed three times with sterile distilled water. They were cultivated on MS medium [10] where germination occurred after seven days. Seedlings were incubated at temperature 24 ± 2 °C with photoperiod 16 h light/8 h darkness. The *in vitro* plants were harvested in 10th week after germination. Peak of alkaloid production reached at the end of 10th week after seed germination followed by its gradual decrease as the plants entered the generative phase [11].

Chemicals. Pure control products l-Hyoscyamine (H-9002) and Scopolamine (S-8502) were supplied by Sigma Chemicals. Dichloromethane (D-3602), methanol (M-106002), were obtained from Surechem Products LTD.

The methods reported for the determination of alkaloids include official methods [12, 13], high-performance liquid chromatography (HPLC) [14, 15], fluorimetry [4, 16], ion chromatography [17], coulometry [18], gas chromatography [6], and electro chromatography [19].

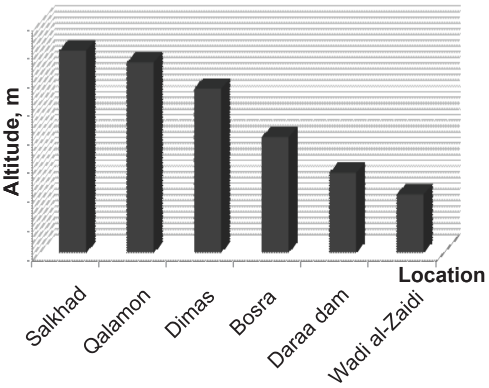

Fig. 1. Altitude of studied locations

Alkaloid Extraction and GC-MS Analysis. For alkaloid extraction from *in vitro* plants the differentiated parts were separately dried at 45°C in an air drying oven until having constant weight and manually grinded to powder. 5g of each powdered sample were soaked in 50 ml H_2SO_4 (0,4N) for 2 hours, filtrated under pressure, the samples were made alkaline (pH=9 by NH_4OH (25%)), and then the alkaloids were extracted twice with 30 ml Cl_2CH_2. After centrifugation (4000 rpm, 10 minutes), the pellucid phase and filtrate were collected, then 4g of sodium sulphate unhydrate were added for each sample, then evaporated under reduced pressure to dryness. The alkaloid extracts were dissolved in 3 ml methanol and 1 µl injected into GC-MS.

GC-MS was carried out on Agilant 7890, a gas chromatograph coupled to Saturn 2000 mass detector. A mass spectrometer with an ion trap detector in full scan (80-325 amu) under electron impact ionization (70 eV) was used. The chromatographic column for the analysis was HP-5-MS capillary column (30 m × 0.25 mm, film thickness 0.20μm). The carrier gas used was helium at a flow rate of 1 mL/min. 1 μL crude alkaloid fractions were injected and analyzed. Column initial temperature was 70°C for 1 min and then increased to 250°C with a 9°C/min heating ramp and subsequently kept at 250°C for 13 min. The injection was performed in splitless mode at 280°C. All the calculations concerning the quantitative analysis were performed with external standardization by measurement of the peak areas.

Standard calibration curves for each of hyoscyamine and scopolamine were constructed by injecting different concentrations of each. Three replicates were performed for each injection and the mean value of the area under the peaks was plotted against the corresponding concentration.

Results and discussion

Tropane alkaloids in roots. The least amount of alkaloids was revealed in plant roots from Wadi Al-Zaidi locality (0.142%), situated at 410 m over sea level while maximal level of tropane alkaloids was recorded in roots derived from Dimas locality (1.134%), situated over 1150 m. Amount of alkaloids in roots was found to rise with locality altitude of seed origin (Fig. 2).

This can be explained by genotype interaction with environment that affects its capacity to produce active compounds. The proportion of each compound may vary between samples; for example, hyosciamin level varied from 0.047% in plant roots from Wadi al-Zaidi to 0.665% in plant roots from Dimas, while scopolamin content varied from 0.055% in plant roots from Al Qalamon to 0.469% in plant roots from Dimas. Generally, both hyosciamin and scopolamin prevailed in roots. These results are consistent with those of Sharma and Sharma (2010), claiming that *Hyoscyamus* plants originated from high-mountain areas, as in our case, are notably richer with alkaloids than plants from lowlands. This also corresponds to Kartal *et al* (2003) findings on *H. reticulatus*, recording that hyosciamin predominates in plant roots.

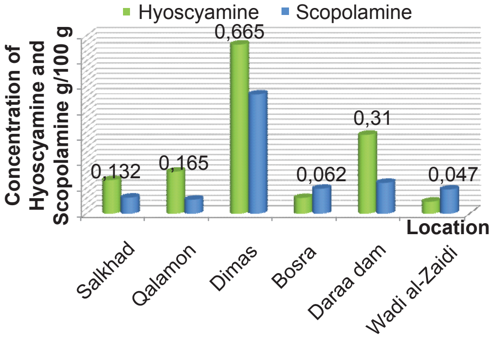

Fig. 2. Concentration of hyosciamin and scopolamin in plant roots grown *in vitro*

Tropane alkaloids in leaves. Most amount of tropane alkaloids exhibited plant leaves from Wadi Al-Zaidi location (0.062%), while those from Salkhad locality displayed the least one (0003%) (Fig. 3).

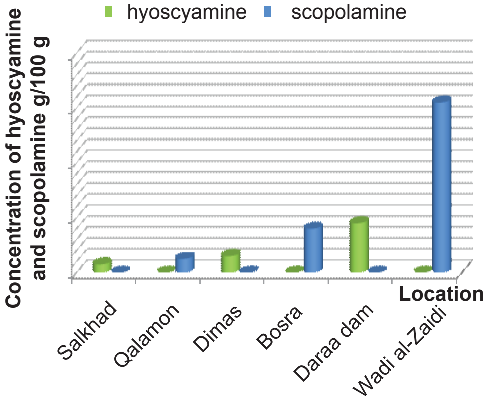

Fig. 3. Hyosciamin and scopolamin concentrations in plant roots, grown *in vitro*

It is remarkable that whereas roots contain both hyosciamin and scopolamin, leaves harbor exclusively one of them. In addition, leaves are richer with alkaloids. This can be attributed to the fact that most of tropane alkaloids is synthesized at early steps of plant development and only later on is transferred to aerial parts of the plant [19]. Therefore, when alkaloid density is increased in roots it will be low in leaves and vice versa. The percentage of each compound may differ from sample to another, where rate of hyoscyamine ranged between a few traces in the leaves of plants from Qalamon, Bosra and Wadi al-Zaidi locations to 0.009% in the leaves of plants from Daraa dam location. While scopolamin level varies from trace amounts in plant leaves from Salkhad, Dimas and Daraa in dam proximity to 0.062% in plant leaves from Wadi al-Zaidi. We also observed that plant leaves may contain only one of the alkaloids, while the other is harbored in trace amounts.

And finally, considering the fact that plants grown *in vitro*, were in comparable conditions, while seeds from which they were generated collected from different localities, distinguished by differing altitudes over sea level, variations in tropane alkaloid level can be assigned to the influence of environment on plant genotype (Fig. 4).

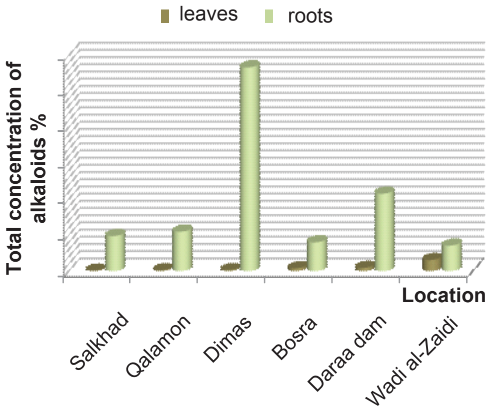

Fig. 4. Total concentration of alkaloids as percentage in leaves and roots of *in vitro* plants

However, higher Dimas area (Qalamon and Salkhad localities) there occurs drop of alkaloid amount both in roots and leaves. This may result from the decreased locality average daily temperature [22].

In general, hyoscyamine predominated in roots, while leaves demonstrated prevalence only one of the alkaloids with the other being in trace amounts.

Though, alkaloid ratio demonstrates differences between various plant organs [23]. And alkaloid composition proved to be more complicated than in aerial part of the plant. This is concurrent with findings obtained for *Atropa belladonna* [24], *Datura innoxia* [25], and *Duboisia myoporoides* [26].

Conclusions

Amount of alkaloids in roots was found to depend on altitude of plant vegetation as determined by its estimation in offspring generated *in vitro*.

The idea that environment-genotype interaction may affect the amount of biologically active compounds produced by plant organism was confirmed.

References

1. *Кунах В. А.* Біотехнологія лікарських рослин. Генетичні та фізіолого-біохімічні основи. – К.: Логос, 2005. – 730 с.
2. *Cardillo A.B., Talou J. R., Giulietti A. M.* Expression of *Brugmansia candida* hyoscyamine 6-beta-hydroxylase gene in *Saccharomyces cerevisiae* and its potential use as biocatalyst, Microb // Cell Fact. – 2008. – P. 717.
3. *Leete E.* Recent developments in the biosynthesis of the tropane alkaloids // Planta Med. – 1990. – Vol. 56. – P. 339–352.
4. *Masatoki K., Hirokazu T.* Fluorometric reactions of purines and determination of caffeine // Talanta. – 2000. – Vol. 36. – P. 1171–1175.
5. *El-Rahman R. A., El-Din A. G., El-Said A., Khalifa H. D.* Production of scopolamine and hyoscyamine in calli and regenerate cultures of *Datura metel* L. // Appl. Sci. Res. – 2008. – Vol. 4. – P. 1858–1866.
6. *Zhang L., Ding R., Chai Y., Bonfill M., Moyano E., Oksman K., Caldentey M., Xu T., Pi Y., Wang Z.,*

Zhang H., Kai G., Liao Z., Sun X., Tang K. Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures // Proc. Natl. Acad. Sci. U.S.A. – 2004. – Vol.101. – P. 6786–6791.
7. *Hashimoto T., Yun D. J., Yamada Y.* Production of tropane alkaloids in genetically engineered root cultures // Phytochemistry. – 1993. – Vol. 32. – P. 713–718.
8. *Palazyn J., Navarro-Ocaca A., Hernandez-Vazquer L., Mirjalili M. H.* Application of metabolic engineering to the production of scopolamine // Molecules. – 2008. – Vol. 13. – P. 1722–1742.
9. *Oksman C., Hiltunen R.* Transgenic crops for improved pharmaceutical products // Field Crops Res. – 1996. – Vol. 45. – P. 57–69.
10. *Murashige T., Skoog F.* A revised Medium for Rapid growth and bioassays with *Tobacco* tissue culture // Plant physiol. – 1962. – Vol.15, №1. – P. 473–479.
11. *Iranbakhsh A., Oshaghi M.A., Majd A.* Distribution of atropine and scopolamine in different organs and stages of development in *Datura stramonium* L. (*Solanaceae*). Structure and Ultrastructure of Biosynthesizing Cells // Acta Biologica Cracoviensia / Botanica. – Vol. 48, № 1. – 2006. – P. 13–18.
12. *Kartal M.* LC method for the analysis of paracetamol, caffeine, and codeine phosphate in pharmaceutical preparations // Pharm. Biomed. Anal. – 2002. – Vol. 26. – P. 857–864.
13. *Levent A. M.* HPLC method for the analysis of paracetamol, caffeine, and dipyrone // Turk. J. Chem. – 2002. – Vol. 26. – P. 521–528.
14. *Tomonari U., Ryuii K., Kazami T., Hiraki H.* Direct injection determination of theophylline and caffeine in blood serum by HPLC using an ODS column coated with zwitterionic bile acid derivative // Analyst. – 1994. – Vol.119. – P. 1767–1770.
15. *Kanazawa H., Kizu J., Matsushima Y.* Simultaneous determination of theophylline and its metabolites by HPLC // Yakagaku Zasshi. – 2000. – Vol. 120. – P. 1051–1060.
16. *Andio M. M., Lima C. G.* Winefordner. Luminescence characteristics of caffeine and theophylline // Spectrochim. Acta A. – 1987. – Vol. 43. – P. 427–430.
17. *Chun C. Q., Wang J.* Simultaneous determination of artificial sweetners, preservatives, caffeine, theobromine, and theophylline in food and pharmaceutical preparations by ion
18. *Qing-qin X., Ming D.L., Wang I.P., Bai A.H.* Direct determination of caffeine and theophylline by gas chromatography // Chromatogr. A. – 2001. – Vol. 937. – P. 57–64.
19. *Qing-qin X., Ming D. L., Wang J. P., Bai A. H.* Direct determination of caffeine and theophylline by gas chromatography // Fenxi Kexue Xuebao. – 2002. – Vol. 18. – P. 520–525.
20. *Hashimoto T., Yamada Y.* New genes in alkaloid metabolism and transport // Current Opinion in Biotechnology. – 2003. – Vol. 14, № 2. – *P. 163–168.*
21. *Sharma Sh., Sharma R.* Long-term storage-dependent changes in seed physiological aspects of *Hyoscyamus niger* from a cold desert region of Trans-Himalaya // International Journal of Plant Biology. – 2010. – Vol. 1. –P. 17.
22. *Kartal M., Kurucu S., Altun L.* Quantitative analysis of L-Hyoscyamine in *Hyoscyamus reticulates* L. by GC-MS. Turk // Chen. – 2003. – Vol. 27. – P. 565–569.
23. http://pharmspravka.ru/farmatsevticheskie-vorosyi-i-otvetyi/kak/kak-rasprostranenyi-alkaloidov-v-rastitelnom-2.html
24. *Constabel F.* Protoplast technology applied to metabolite production // International Review of Cytology Suppl. – 1983. – Vol. 16. – P. 209–217.
25. *Hartmann T., Witte L., Oprach F., Toppel G.* Reinvestigation of the alkaloid composition of *Atropa belladonna* plants, roots and cell suspension cultures // Planta Med. – 1986. – Vol. 52. – P. 390.
26. *Witte L., Muller K., Arfmann H. A.* Investigation of the alkaloid pattern of *Datura inoxia* plants by capillary gas-liquid chromatography-mass spectrometry // Planta Med. – 1987. – Vol. 53. – P. 192–197.
27. *Bachmann P., Witte L., Czygan F.C.* On the Alkaloid Spectrum of the Floral and Postfloral Parts of *Duboisia-Myoporoides R. Br. Solanaceae* // Archiv der Pharmazie. Weinheim. – 1988. – P. 321.

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ОПРЕДЕЛЕНИЕ ТРОПАНОВЫХ АЛКАЛОИДОВ В БЕЛЕНЕ ЗОЛОТИСТОЙ (*HYOSCYAMUS AUREUS*) *IN VITRO*

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Цель. Исследовать тропановые алкалоиды в листьях и корнях растений *Hyoscyamus aureus*, выращенных *in vitro*. **Методы.** Наличие L-гиосциамина и (α)-скополамина проанализировано методом газо-хромато-масс-спектрометрии (ГХ-МС). **Результаты.** Показано, что в изученных растениях белены золотистой, выращенных *in vitro*, в конце вегетативной фазы развития концентрация тропановых алкалоидов (гиосциамина и скополамина) различна. Как правило, в корнях *H. aureus* преобладал гиосциамин. В листьях же превалировал один из алкалоидов (скополамин или гиосциамин), в то время как другой определяли в следовых количествах. **Выводы.** Установлено, что количество алкалоидов в корнях белены золотистой зависело от высоты произрастания исходных растений, от которых были получены семена. Подтверждены представления о том, что взаимодействие окружающей среды и генотипа сказывается на количестве биологически активных веществ, вырабатываемых растительным организмом.

Ключевые слова: *Hyoscyamus aureus,* культура, выращенная *in vitro,* тропановые алкалоиды, ГХ-МС, L-гиосциамин, (α)-скополамин.

ВИЗНАЧЕННЯ ТРОПАНОВИХ АЛКАЛОЇДІВ У БЛЕКОТІ ЗОЛОТИСТІЙ *(HYOSCYAMUS AUREUS) IN VITRO*

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Мета. Дослідити тропанові алкалоїди в листі та коренях рослин *Hyoscyamus aureus*, вирощених *in vitro*. **Методи.** Наявність L-гіосциаміну та (α)-скополаміну проаналізовано методом газо-хромато-мас-спектрометрії (ГХ-МС). **Результати.** Показано, що у вивчених рослинах блекоти золотистої, вирощеної *in vitro*, у кінці вегетативної фази розвитку концентрація тропанових алкалоїдів (гіосциаміну та скополаміну) різна. Як правило, в коренях *H. aureus* переважав гіосциамін. У листі ж превалював один із алкалоїдів (скополамін або гіосциамін), в той час як другий визначали у слідових кількостях. **Висновки.** Встановлено, що кількість алкалоїдів у коренях блекоти золотистої залежала від висоти зростання вихідних рослин, від яких було отримано насіння. Підтверджені уявлення про те, що взаємодія навколишнього середовища та генотипу відбивається на кількості біологічно активних речовин, що виробляються рослинним організмом.

Ключові слова: *Hyoscyamus aureus,* культура, вирощена *in vitro,* тропанові алкалоїди, ГХ-МС, L-гіосциамін, (α)-скополамін.