Inorganic ions in the medium modify tropane alkaloids and riboflavin output in *Hyoscyamus niger* root cultures

Katrin Pudersell[1], Tõnis Vardja[2], Rael Vardja[2], Vallo Matto[1], Elmar Arak[1,3], Ain Raal[1,3]

[1]Department of Pharmacy, University of Tartu, Tartu, Estonia, [2]Institute of Agricultural and Environmental Sciences, Estonian University of Life Sciences, Harku, Estonia, [3]Estonian Academical Society of Pharmacy, Tartu, Estonia

Submitted: 12-04-2011 Revised: 18-05-2011 Published: 28-02-2012

ABSTRACT

Context: *Hyoscyamus niger* L. (Solanaceae) roots are rich of tropane alkaloids, such as hyoscyamine and scopolamine are used as the source of raw material for the pharmaceutical industry. **Aims:** The aim of the present study was to investigate the effect of calcium, magnesium, and iron ions on the production of tropane alkaloids and excretion of riboflavin in *H. niger* root cultures. **Materials and Methods:** The calcium, magnesium, or iron enriched/deprived Murashige and Skoog (MS) growth medium were used for culture of *H. niger* root tissues. The secondary metabolites were quantified using high performance liquid chromatography with ultraviolet detector (HPLC-UV) and fluorimetry techniques. **Results:** An increased calcium content in the medium unidirectionally reduced hyoscyamine, while increasing scopolamine production with only a moderate impact on riboflavin excretion. Manipulations with magnesium and iron contents in the medium resulted in divergent changes in hyoscyamine, scopolamine, and riboflavin concentrations. **Conclusions:** Our results show that increased calcium ion content in the Murashige and Skoog medium may be used for the intensification of the scopolamine production in *H. niger* root cultures.

Key words: Cations, *Hyoscyamus niger* L., *in vitro* root culture, riboflavin, scopolamine, tropane

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.93330

Quick Response Code:

INTRODUCTION

The primary metabolites of plants maintain the essential life functions of plants, such as the cell division and growth, respiration, storage, and reproduction. In contrast, the secondary plant metabolites play a major role in the adaptation of plants to their environment.[1] Also, the secondary metabolites are usually defined by their low abundance – often less than one percent of the total plant carbon. However, for centuries, the secondary metabolites of plants have been used for preparation of diverse remedies.

Though the contemporary pharmaceutical industry relies on chemical synthesis, a significant proportion of raw materials are still derived from natural sources. Among others, a considerable amount of compounds acting at m-cholinoreceptors is of a natural origin. *Hyoscyamus niger* L. (Solanaceae) has been regarded as a rich source of pharmaceutically-important tropane alkaloids, such as the m-cholinoreceptor blockers, hyoscyamine, and scopolamine. These alkaloids are synthesized in the roots of the plant, and accordingly, *in vitro* root cultures are the alternative sources of these alkaloids.[2] Today, the use of the plant cell and tissue culture techniques for the multiplication and extraction of secondary metabolites is a routine method.[3] On the other hand, due to the low content of the secondary metabolites in the overall cell culture biomass, various strategies to enhance the production of the secondary metabolites have been considered. During the last decades, substantial biotechnological advance in elucidation of factors contributing to increased hyoscyamine and/or scopolamine production from *H. niger* roots has been done.[4,5] Despite the classic finding of Pinol and collaborators[6] that hyoscyamine production in *Datura stramonium* L. hairy root cultures is calcium sensitive, the influence of ion content in cell culture media for tropane production is so far not well understood and the validity of this finding for *H. niger* remains unknown. Our previous works have shown that nutritional elements may affect hyoscyamine and scopolamine content[7,8] and riboflavin excretion[9,10] in *H. niger* root cultures. However, we were not able to define the exact impact of selected ion contents in the mechanisms of hyoscyamine, scopolamine, and riboflavin output.

Address for correspondence:
Dr. Vallo Matto, Department of Pharmacy, University of Tartu, Nooruse 1, 50411 Tartu, Estonia. E-mail: vallo.matto@ut.ee

The aim of the present study was to investigate the effect of calcium, magnesium, and iron ions on the production of tropane alkaloids and excretion of riboflavin in root cultures of *H. niger*. The experimental set-up of the present study differs from the reference work[3] as it focuses primarily on the augmentation of the production of the secondary metabolites via the optimization of the medium, but not on the best growth of the plant. The research outcome of the present study can also be applied for the maximization of the large scale pharmaceutical production of tropane alkaloids and riboflavin from *H. niger*.

MATERIALS AND METHODS

In vitro plant materials and maintenance of tissue cultures

Seeds of *H. niger* (obtained from the Biocenter of Viikki, University of Helsinki, Finland) were used as the starting material for the root cultures. Nine percent chlorinated lime with some drops of Tween 80 were used to sterilize the seeds, then the seeds were placed to a solid medium that contained ¼ strength Murashige and Skoog (MS) medium[11] salts and vitamins, 1% (w/v) sugar and 8% (w/v) agar. The temperature in the growth room was 22 ± 2ºC, day/night cycle was 16/8 h and the cool white fluorescent lamps (20 μmol m^{-2} s^{-1}) were used for the illumination. The seeds germinated during 10 days at the rate of 90% and vital plantlets (approximately three cm in length) were derived after three weeks. Following 21 days of cultivation, the root was separated from the plantlet and subsequently the separated roots were further cultivated for 28 days in a liquid MS medium. Sterile root material was maintained in respective media (listed below) in the dark phytotrone at 25 ± 2°C on a shaker at 90 rpm up to 28 days or until 10 parallel experiments was carried out. The quantity of the explant was 100 ± 50 mg. The root cultures of *H. niger* were maintained in the media of following composition: full strength MS medium, modified MS medium with iron-deficient and halved iron content (2.8 mg/l), modified MS medium with double (239.4 mg/l) and halved (59.9 mg/ml) calcium content, and modified MS medium with double (72.2 mg/l) and halved (18.1 mg/ml) magnesium content.

Determination of tropane alkaloids

The content of tropane alkaloids was determined using the high performance liquid chromatography with ultraviolet detector (HPLC-UV). The experimental material was dried at a room temperature and tropane alkaloids were extracted from the plant material with ethyl ether as follows. Exactly weighed (±0.1 mg) ground plant material with particle size < 1 mm was placed in the 100 ml flask and 4 ml 25% ammonium hydroxide solution and 20 ml ethyl ether were added. The flask was covered with aluminum foil and the mixture was shaken on a rotary shaker for 1 h. The extract was filtered through the Whatman grade 4 filter paper (Whatman Ltd, Maidstone, UK) and medical cottonwool into the distribution flask. The funnel was covered with the glass plate during the filtering process. The flask was flushed three times with 5 ml ethyl ether which was also added to the extract. Followed by the addition of 5 ml water to the filtrate, the mixture was shaken and stratified, and water layer was discarded. The procedure was repeated three times. The ethyl ether layer was placed in a dry bottle. The distribution flask was flushed three times with 5 ml ethyl ether that was also added to the extract. Finally, ethyl ether was evaporated on the sand bath under the suction until the sample and the bottle were dry. The 5 ml mixture of phosphate buffer (consisting of dipotassium hydrogen phosphate and potassium dihydrogen phosphate) and 96.6% ethanol (1:4) were added to the desiccated extract in the bottle.

The procaine hydrochloride was used as the internal standard. The chromatographic conditions were adjusted with the hyoscyamine hydrochloride and scopolamine hydrobromide. All chemicals and standard compounds used were obtained from a local distributor (Sigma-Aldrich Co., St. Louis, MO) or a local pharmacy of the University of Tartu (ethanol).

The HPLC system consisted of a pump (SP 8 700, Spectra-Physics, Santa Clara, CA), a 3 mm × 150 mm (5μm) column (C18, Tessek Ltd., Praha 10, Czech Republic) and a UV-detector (Millichrom Ltd., Sofia, Bulgaria) was set at 210 nm detection. The HPLC system was coupled with an auxiliary data recording and handling set (LKS 4-003, ZAO NauchPribor, Oryol, Russian Federation).

Ten microliter sample was manually injected into the HPLC system where the flow rate of the mobile phase was kept constant at 0.25 ml/min. The mobile phase consisted of a phosphate buffer solution (pH 6.0): 96.6% ethanol (1:4) and 0.0033% of triethylamine, all chemicals were of a HPLC grade and purchased from a local distributor (Sigma-Aldrich Co., [YA Kemia Oy] Helsinki, Finland). The actual hyoscyamine and scopolamine concentrations were calculated from the principal peak areas of the sample investigated in reference to the principal peak areas of the high purity reference compounds.

Isolation and determination of riboflavin

The riboflavin content was evaluated in the growth medium. The growth medium (40 ml) was filtered through a sheet of filter paper (pore size 20-25 μm, Filtrak, VEB Spezialpapierfabrik, Niederschlag, Germany) and passed through a proprietary column (5 mm × 2.3 mm, C10, Pharmacia, Uppsala, Sweden). The yellow substance

(i.e., riboflavin) was retained at the top of the column during application and washing with water. The column was washed with 40 ml water to remove growth medium components and other substances. The riboflavin was eluted with ethanol of various concentrations in the effluent volume which depended on the ethanol concentration. The effluent obtained with 30% (v/v) ethanol was routinely used in the spectrophotometric riboflavin concentration determination.

Concentration of riboflavin was determined using a fluorimeter (Analiz, Geologorazvedka, Saint Petersburg, Russian Federation). The maximum of excitation wavelength was 436 nm and emitted light was passed through a 500 nm cutoff filter. Riboflavin concentration was determined using a standard curve established with a high purity reference standard of riboflavin (Chemapol, Praha, Czech Republic). All riboflavin assay procedures were carried out in darkness or with dim red safe light.

Statistical analysis

The raw data were subjected to one-way analysis of variances (ANOVA) and when appropriate, for *post hoc* comparisons, the Bonferroni's Multiple Comparisons Test (Bonferroni test) for selected data groups (modified ion content groups *versus* full ion content group) was used. All data are given as average ± SEM of 10 samples.

RESULTS

There were no gross morphological differences in any of the root cultures of *H. niger* maintained in the media of different composition. The absolute values of the hyoscyamine and scopolamine output and riboflavin excretion are given in Table 1. The growth of the root cultures maintained in the media of different calcium content was similar (F [2,27]=0.113, NS). The content of hyoscyamine in the root cultures decreased by increasing calcium concentration (F [2,27]=9.026, P=0.001). For the scopolamine content, an opposite phenomenon was observed: the lower the calcium content, the less scopolamine content (F [2,27]=204, P<0.0001). The increase of the calcium content of the medium had an overall effect on the riboflavin excretion by the root cultures (F [2,27]=38.96, P<0.001); however, only the double calcium concentration decreased the riboflavin excretion statistically significantly. The low magnesium concentration in the growth medium tended to decelerate the growth of the root culture, but this effect did not reach the statistical significance (F [2,27]=2.461, NS). The low or nonexistent iron concentration in the growth medium accelerated (F [2,27]=11.30, P<0.001) growth of the root cultures.

Table 1: Growth, tropane alkaloid content, and riboflavin excretion in root cultures of *H. niger* cultivated in the media with different ion contents

Growth and content in root cultures	Calcium (mg/ml)			Magnesium (mg/ml)			Iron (mg/ml)		
	59.9	119.7	239.4	18.1	36.1	72.2	0	2.8	5.6
Growth (g/flask)	0.1326 ± 0.0463	0.1148 ± 0.0297	0.1390 ± 0.0341	0.0898 ± 0.0188	0.1309 ± 0.0104	0.1361 ± 0.0098	0.2060 ± 0.0200***	0.2130 ± 0.0160***	0.1160 ± 0.0110
Hyoscyamine (mg/g)	7.8 ± 1.6*	5.7 ± 0.1	2.2 ± 0.3***	b.d.l.	4.5 ± 0.9	b.d.l.	b.d.l.	b.d.l.	5.5 ± 0.3
Scopolamine (mg/g)	1.5 ± 0.2***	17.7 ± 3.6	24.8 ± 0.6***	9.74 ± 0.12***	25.64 ± 0.51	6.19 ± 0.12***	3.42 ± 0.07***	15.65 ± 0.30***	29.97 ± 0.60
Riboflavin [in the medium (mg/l)]	3.29 ± 0.12	3.47 ± 0.14	2.08 ± 0.10***	3.69 ± 0.10	3.66 ± 0.12	4.11 ± 0.18	3.83 ± 0.12	1.72 ± 0.13***	3.84 ± 0.18

Values are average of 10 replicates ± SEM; b.d.l. – below detection limit; *p<0.05; ***p<0.001, selected data group versus control group (Bonferroni test for selected data groups). The control groups were the 119.7 mg/ml, 36.1 mg/ml, and 5.6 mg/ml groups of calcium, magnesium, and iron, respectively

DISCUSSION

For almost half of the century, the Murashige and Skoog (MS) medium has been used to grow plant cultures and the modification of the MS medium ionic content is a routine procedure.[12,13] The result of our study shows for the first time using a root culture technique that the content of the calcium ions is critical for the optimal tropane production in *H. niger* root cultures. Although the role of the growth conditions has been addressed earlier,[14-16] but with controversial results. In our previous study, we reported that the MS medium with variations seems to be the most optimal medium for the *H. niger* growth.[4] In the present study, we found that there was a clear positive relationship between the calcium concentration in the growth medium and the scopolamine production. Since hyoscyamine is a precursor of the scopolamine,[17] it is not surprising to see that the hyoscyamine production was conversely abolished by the simultaneously increased hyoscyamine production. In a recent study by Amdoun and colleagues,[18] it was found that in the B5 medium, Ca^{2+} and Mg^{2+} had no significant effect on biomass production by *Datura stramonium* L., but K^{+} and Ca^{2+} had a positive effect on alkaloids production. In 1994 Gontier *et al.*,[19] reported that calcium was beneficial for tropane alkaloid synthesis in *Datura inoxia* P. Mill. cell line suspension, and the results of our study expand this idea to *H. niger* root cultures. However, the differences in plant species, growth medium, and the fact that no scopolamine content was estimated have to be considered when comparing the outcome of that research with that of our research. Furthermore, genotype changes due to the genetic engineering of the tropane-rich plants, including *H. niger*[4,20,21] seem to be strongly associated with the discrepancies of the results obtained.

The industrial scale production of riboflavin is mainly carried out using different microorganisms, including filamentous fungi[22] and bacteria.[23] Therefore, from a point of view of the active pharmaceutical ingredient production the riboflavin excretion to the growth medium by the *H. niger* root cultures has to be considered as a secondary phenomenon. The variation of ion content in medium did not cause tremendous alterations in the riboflavin excretion. This result reinforces the result of our previous work which demonstrated that in the *H. niger* root cultures the growth medium composition rather the pH or the ionic content is the crucial determinant of the magnitude of the riboflavin excretion.[9]

The role of magnesium and iron ions in the growth medium has not been previously addressed in the context of production of the tropane alkaloids by the *H. niger* root cultures. As indicated above, the manipulation of the calcium content in growth medium unidirectionally affects the tropane production, but the alterations of magnesium and iron content do not elicit such a unidirectional effect. The magnesium content change caused decrease in production of both hyoscyamine and scopolaminein both directions. Though out of the scope of the present study, it is likely that the manipulations with the magnesium content in the growth medium caused a switch from the "putrescine to tropane" pathway to the "putrescine to spermidine" pathway. Additional factors caused by the ion content change such as the pH of the medium and intensity of the plant growth have to be taken into account, too. Indeed, the low magnesium content reduced the growth of the *H. niger* root cultures, while the iron deprivation had an opposite effect and compensatorily increased the growth of the culture. Hermans and coworkers[24] reported that magnesium deprivation caused decrease in green mass production in sugar beet (*Beta vulgaris* L.) shoots kept in a hydroponic condition and they found that this phenomenon is likely related to very basic mechanisms of the electron transfer in the photosynthesis. Whether this phenomenon is the biological basis of the decelerated growth of *H. niger* root cultures observed in our magnesium-deprived medium group remains to be elucidated.

There is a widely accepted view that iron deficiency in plants leads to chlorosis and further to necrosis.[25] Our experiments, however, show that in an optimal MS medium the *H. niger* root cultures oppose the iron deprivation with accelerated growth as all other nutrients are available *ad libitum*. On the other hand, our study also unveiled that despite the abnormal growth, production of tropane alkaloids and excretion of riboflavin is divergent. In *H. albus* hairy root cultures cultivated in the iron-deficient MS medium, the riboflavin excretion retained, but this phenomenon was strongly dependent on root clone used and other experimental conditions.[26] The latter indirectly supports our view that the changes in secondary metabolites of the *Hyoscyamus* species caused by the iron deprivation are much more complex as believed so far. Consequently, our tentative results suggest that the changes in magnesium or iron ion contents of the MS medium should be undertaken with precaution. Further experiments are needed to elucidate the exact mechanisms related to the diverged tropane alkaloid production or riboflavin excretion in *H. niger* root cultures.

CONCLUSIONS

Our present study unveils that increase of the calcium content in the MS medium augments the scopolamine production and correspondingly abolishes the hyoscyamine production with only a moderate impact on riboflavin excretion in *H. niger* root cultures. This result can be utilized

for the intensification of the scopolamine production in *H. niger* root cultures. Manipulations with magnesium and iron contents in the MS medium do not cause unilateral changes in tropane production or riboflavin excretion, and thus are not recommended for MS medium optimization.

REFERENCES

1. Kossel A. On the chemical composition of the cell. Arch Anat Physiol 1891;181:181-6.
2. Hashimoto T, Yamada Y. Hyoscyamine. 6-beta-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, in alkaloid-producing root cultures. Plant Physiol 1986;81:619-25.
3. Karuppusamy S. A review on trends in production of secondary metabolites from higher plants by *in vitro* tissue, organ and cell cultures. J Med Plants Res 2009;3:1222-39.
4. Zhang L, Kai GY, Lu BB, Zhang HM, Tang KX, Jiang JH, *et al*. Metabolic engineering of tropane alkaloid biosynthesis in plants. J Integr Plant Biol 2005;47:136-43.
5. Srivastava S, Srivastava AK. Hairy root culture for mass-production of high-value secondary metabolites. Crit Rev Biotechnol 2007;27:29-43.
6. Pinol MT, Palazon J, Cusido RM, Ribo M. Influence of calcium ion concentration in the medium on tropane alkaloid accumulation in *Datura stramonium* hairy roots. Plant Sci 1999;141:41-9.
7. Pudersell K, Vardja R, Vardja T, Raal A, Arak E. Plant nutritional elements and tropane alkaloid production in the roots of henbane (*Hyoscyamus niger*). Pharm Biol 2003;41:226-30.
8. Pudersell K, Vardja R, Vardja T, Arak E, Raal A. Influence of calcium on the tropane alkaloid production and riboflavine excretion in the root cultures of *Hyoscyamus niger* L. Eur J Pharm Sci 2007;32 Suppl:S33-4.
9. Vardja T, Vardja R, Pudersell K, Tohver A. Riboflavin excretion from the excised roots of *Hyoscyamus niger*. Pharm Biol 2004;42:353-9.
10. Vardja R, Pudersell K, Vardja T, Raal A, Arak E. Tropane alkaloid production and riboflavin excretion by *Hyoscyamus niger* L. hairy root cultures. Proc Estonian Acad Sci Biol Ecol 2004;53:14-24.
11. Murashige T, Skoog F. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 1962;15:473-97.
12. Kothari SL, Agarwal K, Kumar S. Inorganic nutrient manipulation for highly improved *in vitro* plant regeneration in finger millet - *Eleusine coracana* (L.) Gaertn. *In Vitro* Cell Dev Biol Plant 2004;40:515-9.
13. Jain R, Sinha A, Jain D, Kachhwaha S, Kothari SL. Adventitious shoot regeneration and *in vitro* biosynthesis of steroidal lactones in *Withania coagulans* (Stocks) Dunal. Plant Cell Tissue Organ Cult 2011;105:135-40.
14. Mulabagal V, Tsay HS. Plant cell cultures--An alternative and efficient source for the production of biologically important secondary metabolites. Int J Appl Sci Eng Tech 2004;1:29-48.
15. Boitel-Conti M, Laberche JC, Lanoue A, Ducrocq C, Sangwan-Norreel BS. Influence of feeding precursors on tropane alkaloid production during an abiotic stress in *Datura innoxia* transformed roots. Plant Cell Tissue Organ Cult 2000;60:131-7.
16. Roberts SC, Shuler M. Large-scale plant cell culture. Curr Opin Biotechnol 1997;8:154-9.
17. Sato F, Hashimoto T, Hachiya A, Tamura K, Choi KB, Morishige T, *et al*. Metabolic engineering of plant alkaloid biosynthesis. Proc Natl Acad Sci U S A 2001;98:367-72.
18. Amdoun R, Khelifi L, Khelifi-Slaoui M, Amroune S, Benyoussef EH, Thi DV, *et al*. Influence of minerals and elicitation on Datura stramonium L. tropane alkaloid production: Modelization of the *in vitro* biochemical response. Plant Sci 2009;177:81-7.
19. Palazon J, Navarro-Ocana A, Hernandez-Vazquez L, Mirjalili MH. Application of metabolic engineering to the production of scopolamine. Molecules 2008;13:1722-42.
20. Oksman-Caldentey KM. Tropane and nicotine alkaloid biosynthesis-novel approaches towards biotechnological production of plant-derived pharmaceuticals. Curr Pharm Biotechnol 2007;8:203-10.
21. Gontier E, Sangwan BS, Barbotin JN. Effects of calcium, alginate, and calcium-alginate immobilization on growth and tropane alkaloid levels of a stable suspension cell line of *Datura-Innoxia* Mill. Plant Cell Rep 1994;13:533-6.
22. Tajima S, Itoh Y, Sugimoto T, Kato T, Park EY. Increased riboflavin production from activated bleaching earth by a mutant strain of *Ashbya gossypii*. J Biosci Bioeng 2009;108:325-9.
23. Tännler S, Zamboni N, Kiraly C, Aymerich S, Sauer U. Screening of *Bacillus subtilis* transposon mutants with altered riboflavin production. Metab Eng 2008;10:216-26.
24. Hermans C, Johnson GN, Strasser RJ, Verbruggen N. Physiological characterisation of magnesium deficiency in sugar beet: Acclimation to low magnesium differentially affects photosystems I and II. Planta 2004;220:344-55.
25. Abadia J, Lopez-Millan AF, Rombola A, Abadia A. Organic acids and Fe deficiency: A review. Plant Soil 2002;241:75-86.
26. Higa A, Miyamoto E, Rahman LU, Kitamura Y. Root tip-dependent, active riboflavin secretion by *Hyoscyamus albus* hairy roots under iron deficiency. Plant Physiol Biochem 2008;46:452-60.

Cite this article as: Pudersell K, Vardja T, Vardja R, Matto V, Arak E, Raal A. Inorganic ions in the medium modify tropane alkaloids and riboflavin output in *Hyoscyamus niger* root cultures. Phcog Mag 2012;8:73-7.

Source of Support: Nil, **Conflict of Interest:** None declared.