Data for characterizing genetically transformed cultures of *Atropa belladonna* L.

Ph.D. Thesis

Hajnalka Hank

Semmelweis University
Pharmaceutical Sciences Doctoral School

Theme Leader: Prof. Éva Szőke M.Sc., Ph.D., D.Sc.

Consultant: László Kursinszki assistant professor, Ph.D.

Budapest
2007

Ph.D. Thesis
Prepared under the guidance of Prof. Éva Szőke, D.Sc.,
in the Institute of Pharmacognosy, Semmelweis University, Budapest, 2007

Hajnalka Hank

Data for characterizing genetically transformed cultures of *Atropa belladonna* L.

Summary

Atropa belladonna L. (deadly nightshade) contains several agents with pharmacological importance; it is an official drug in the VIIIth Hungarian Pharmacopoeia. The plant contains tropane- and pyrrolidine-base alkaloids; its main alkaloid is the hyoscyamine which has parasympatholytic effect. Nowadays gene technological methods are widely used to increase the production of active ingredient beside the conventional tissue culturing procedures.

During our experiments we investigated the growth of the genetically transformed and non-transformed cultures of *in vitro* and *in vivo A. belladonna*, and its tropane alkaloid composition, specifically its hyoscyamine, scopolamine and 6OH-hyoscyamine content (LC-MS-MS, HPLC). Genetical transformations mediated by *Agrobacterium rhizogenes* (R1601) in cultures was demonstrated by using polymerase chain reaction (PCR) and opine detection.

This method developed by us is simple, reproducible (RSD%=3.3-8.4) and sensitive, it results in a proper separation of tropane alkaloids, therefore during alkaloid production studies it can be perfectly used for the analysis of hairy root clones synthesising tropane alkaloids. [1.,2.].

Alkaloid contents (0.52%, 0.40%, 0.35%) of the investigated hairy root clones (#K_4, #K_5 and #K_8) reached or exceeded that of the root of the *in vivo* plants. According to our results in the universal and special metabolism and alkaloid production of the genetically transformed cultures, considerable differences could be observed among clones with different genetical structure that formations resulted by random bacterial tDNA infiltration. Several additional factors can be beneficial for the formation and alkaloid production of the hairy root cultures; therefore we studied in details the effect of light and the carbohydrate content, nitrogen-source, and macroelement content of B5 culture medium on the formation, tropane alkaloid content and production of the genetically transformed tissues [3.]

All three hairy root clones spontaneously organized using liquid B5 culture medium. The *in vitro* transgenic plants were cultivated *in vivo* after micropropragation. Phenotypes of #K_4 and #K_8 clones differed from that of the mother plant and their morphological characteristics were typical for the transgenic plants. It has been established that the plants produced more alkaloids *in vivo*. Among *in vitro* cultured clones the #K_4 (3.59mg/g) clone, while among *in vivo* cultured clones the #K_5 (5.76mg/g) clone had the highest hyoscyamine content.

In order to know more about the metabolism of tropane-base alkaloids, and to understand more accurately the changes during abiotic stress effects, the measurable endogenous HCHO-content of the samples has been determined (OPLC, HPLC) [4.]. Our biological experiments that have been performed in BioArena-system demonstrated that the investigated components of *A. belladonna* most likely affects through HCHO.

1. Hank H., Szőke É., Tóth K., László I., Kursinszki L. (2004): Investigation of Tropane Alkaloids in Genetically Transformed *Atropa belladonna* L. Cultures. Chromatographia 60. 55-59.

2. Kursinszki L., Hank H., László I., Szőke É. (2005): Simultaneous analysis of hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine and apoatropine in Solanaceous hairy roots by reversed phase high-performance liquid chromatography. Journal of chromatography A 1091, 32-39.

3. Hank H., László I., Bálványos I., Kursinszki L., Kovács Gy., Tóth K., Szőke É. (2003): Effect of Magnesium on the Growth and Alkaloid Production of Hairy Root Cultures. Acta Horticult.597, 271-274.

4. Hank, H., Tyihák, E., Kátay, Gy., Kursinszki, L., Szőke, É. (2005): Tropane alkaloids and endogenous formaldehyde levels in vivo and in vitro in genetically modified *Atropa belladonna* L. European Journal of Pharmaceutical Sciences 25S1. 110-111.

1. INTRODUCTION AND AIM

Special metabolites of the flora are the unique sources of several active agents applied in the therapy, however in many cases these compounds can be found in plants only in slight amount and due to their complex structure their production – regarding few exceptions – remains unsuccessful in industrial scale. The expansion of the biotechnological and molecular biology information provided the opportunity to investigate the industrial production of some plantal compounds by using cell- and tissue cultures. Plants belonging to the Solanaceae group – beside many other special metabolites – synthesize pharmacologically substantial tropane base alkaloids that have parasympatholytic effects. The biosynthesis of tropane alkaloids and most of the enzymes taking part in the process have been already identified. Methylizing reactions mediated by S-adenozil-methionine (SAM) take an essential part in biosynthesis, and the endogenous HCHO produced within the cells can also be linked to these processes. At the same time the biosynthesis of tropane alkaloids is in a close relationship with the biosynthesis of polyamines that are responsible for – among others – combating stress effects.

Nowadays the gene technological methods are also widely used beside the conventional tissue culturing methods. Among all the methods applying *Agrobacterium* gene transferring mechanisms our interest primarily aimed the hairy root cultures. Among their beneficial effects it should be highlighted that they are genetically stable, they can intensively grow on a culture media free of hormone, and their active agent content exceeds that of the intact plant in many cases.

During our experiments we would like to study the biomass and tropane alkaloid production of the genetically modified tissues (so called hairy root) originated from *in vitro Atropa belladonna* L. In order to increase the production of active agents, it is important to cultivate tissues, cell-lines having beneficial properties in optimal circumstances. Growth and alkaloid biosynthetic capacity of tissues can be affected by a variety of circumstances, thus we would like to investigate the effect of light, the composition of the culture medium, such as carbon-, nitrogen-source and macroelements on the growth, biomass and alkaloid production of the tissues. In addition our aim was to study the characteristic morphological features and biosynthetic potential of transgenic plants organised from hairy root cultures.

Our aim was also to study the relationship between measurable endogenous HCHO level and the growth of cultures, their alkaloid metabolism and different biotic and abiotic stress effects and the application of special molecules in the culture medium influencing transmethylation reactions. It is known that HCHO has also a determinant role in the effect of different

biologically active (antibiotic, toxic) compounds. Therefore we thought that it could be interesting to investigate special metabolites of *A. belladonna* by using BioArena system.
We hope that our results will deepen the knowledge about the growth of *A. belladonna* tissue cultures, tropane alkaloid metabolism, and the changes related to abiotic stress.

2. MATERIALS AND METHODS

2.1. *Atropa belladonna* L. *in vitro* plants

The *in vitro* plants were obtained from Stejarul Research Centre located in Romania. The maintenance and reproduction of *A. belladonna* L. sterile plants were performed by vegetative micropropagation, and then they were transferred to Murashige-Skoog solid medium (MS), in light (2500Lux, 12 h/day), at a standard temperature (24±1^{o}C).

2.2. Establishment and maintenance of genetically modified cultures

- **Hairy root cultures**

A. belladonna in vitro plants were infected by microinjection using *Agrobacterium rhizogenes* R1601 strain. Following infection, 9 hairy root clones were isolated and transferred to MS solid medium supplemented with cefotaxim and ampycillin in order to eliminate Agrobacterium. The hairy root clones free of bacterium were transferred to liquid Gamborg B-5 medium containing 2% sucrose (100rpm, 24±1^{o}C) and cultivated both in dark and in light (2500Lux, 12 h/day). 3 clones (#K4, #K5 and #K8) were selected for further investigations according to their growth and tropane alkaloid production.

- **Organised cultures**

Following the spontaneous organisation of *A. belladonna* #K_4, #K_5 and #K_8 cultures, the seedlings were cultivated *in vitro* in light (on B5 solid medium). After 2.5 weeks the cultures were transplanted to a mixture of soil, perlite and peat and were cultivated further in a green house under 12/12 light per dark photo period. Nylon net was stretched out above the seedlings. Samples were collected after three months of cultivation.

2.3. Studies on genetically transformed characteristics of hairy roots

- Investigation of hairy roots and organised cultures by PCR: following plant DNA isolation the polimerase chain reaction was performed in iCycler Thermal Cycler PCR apparatus. DNA fragments obtained by PCR were separated on agarose gel plates. Evaluation was performed under UV light (λ=302nm).
- Opine synthesis of the tissues was studied by paper electrophoresis according to Petit. Agropine, mannopine, agropinic- and mannopinic acids were identified in genetically transformed plant tissues.

2.4. Measurement of growth and biomass production of the cultures

Fresh and dry weight, dry matter content and growth value (referring to dry weight) were measured or calculated in order to describe the growth of *in vitro* cultures.

2.5. Changes in the composition of B5 culture media

After 4 weeks the effect of medium composition on the growth and tropane alkaloid production was investigated in case of *A. belladonna* hairy root clones #K_4, #K_5 and #K_8 that were cultivated in liquid B5 medium, in dark and at a standard temperature.

- carbohydrate (sucrose, maltose, glucose) concentration (0% - 12%)
- nitrogen [$(NH_4)_2SO_4$] content (0 mg/l - 536 mg/l, along with stabile nitrate (KNO_3))
- change of macroelement content: - $MgSO_4$ (0 mg/l - 1000 mg/l)
  - $CaCl_2$ (0 mg/l - 900 mg/l)
- aminoguanidine and hydralazine treatment (0ppm - 1000ppm) in addition to the growth of cultures (#K_4) and tropane alkaloid content, the endogenous HCHO level was also analysed.

2.6. Analytical examinations

- **Identification and determination of tropane alkaloids**

Following extraction (chloroform: methanol: 25% ammonium 15:5:1 *v/v/v*) the samples were cleaned by Extrelute column and by solid phase extraction (SPE) (SUPELCO Supelclean LC-18 SPE). Our experiments confirmed that the effectiveness of the extraction and the reproducibility and recovery of both procedures are adequate. Total alkaloid content was determined in accordance with the VIIIth Hungarian Pharmacopoeia.

- **HPLC-MS-MS-method:**

Analysis was performed in positive ion mode using an Agilent 6410 Triple Quad LC-MS-system. HPLC-column: Phenomenex Luna (5µm) C18 (2), mobile phase: 17:83 (*v/v*) acetonitrile: 30mM ammoniumphormiate, pH 2.80. LC–MS-MS parameters: vaporizer pressure 45.0 psi, capillar potential 3500V, scanning range 50-700m/z, collision energy 35Ev.

- **HPLC determination**

- Separation on *Luna C8 column:* Spectra Physics P4000 HPLC-apparatus (Fremont, USA), Luna C8 (5 µm) (Phenomenex, USA) reversed-phase column (250x 4,6mm I.D.), fitted with a Phenomenex Security Guard C8 guard column (8x 3mm I.D.). solvent system composed of acetonitrile - 30mM phosphate buffer (pH 6.2) - methanol 12.2: 79.7: 8.1 (*v/v/v*) supplemented with 0.1% triaethanolamine.

- Separation on *Luna C18 column:* Qualitative determination was performed using a Surveyor modular HPLC system (Thermo Finnigan, USA). The column applied was a LUNA

C18 reversed-phase column (250x 4,6mm I.D., Phenomenex, USA) fitted with a Phenomenex Security Guard C18 guard column. The mobile phase consisted of acetonitrile - 30mM KH_2PO_4-K_2HPO_4 (815:185, *g/g*) buffer solution pH 6.00 - methanol (12: 80.1: 7.9, v/v/v, 1.00 ml/min). Peaks were identified by retention time of the appropriate authentic standard (λ=210nm) and by UV spectral data.

Values of alkaloid contents were given in μg/g, and mg/g (referring to dry weight).

- **Qualitative and quantitative determination of endogenous HCHO**

- OPLC method: endogenous HCHO content was measured in genetically transformed root cultures and plants. The adequately prepared samples were treated with 0.2% solution of demethone in methanol. The separation of formaldemethone was carried out using chloroform – methanol eluent mixture (25:60, v/v) in an OPLC instrument (OPLC-NIT, Budapest). The quantitative analysis was performed densitometrically (Shimadzu scanner, Japan), λ = 260nm.
- HPLC method: Shimadzu (Japane) HPLC-system, Hypersil C18 (200× 4,6mm I.D., 5μm) column, mobile phase methanol: water (75: 25, *v/v*), detection was performed at λ=258 nm.

2.7. Investigation of antimicrobial effect in BioArena-system

During our investigations in BioArena-system, we applied the *Pseudomonas savastanoi* pv. *phaseolicola* bacterium strain (London University). Different amounts of the extracts and test compounds (hyoscyamine, scopolamine, scopoletine) were applied to Kieselgel $60F_{254}$ (Merck 0,2mm) layer, for further influence of effectiveness L-arginin, reduced gluthation, and Cu(II)-sulphate were used. The eluent was chloroform – methanol - concentrated ammonia (70:18:2, *v/v/v*). The densitogram was measured by using densitometry method before (λ=210, 300nm) and after (λ=590) biological development (Simadzu CS-9301PC, Kyoto).

2.8. Statistical evaluation of the experimental results

Data of the cultures represented by n=6 individuals were evaluated and summarised each time. For the statistical evaluation of data, we calculated distribution, standard deviation (± SD, confidence limits were added at $p<0.05$), and we performed T-test.

3. NEW SCIENTIFIC RESULTS

3.1 Separation and identification of tropane alkaloids (method development)

During our experiments there was a need for a quick and accurate method for the qualitative analysis and quantitative determination of numerous and complex samples. This method is simple, reproducible (RSD% = 3.3-8.4) and sensitive, it results in a proper separation of tropane alkaloids, therefore during alkaloid production studies it can be perfectly used for the analysis of hairy root clones synthesising tropane alkaloids. Composition of the eluent is

simple, considering that there was no need for the application of neither triethylamine nor ion-pair reagent for the appropriate peak shape and selectivity. Absolute limit of detection was 0.6ng for hyoscyamine and 6OH-hyoscyamine, 0.8ng for scopolamine, and 0.3ng for apoatropine. Mean recoveries for the global method were 94.2% for hyoscyamine, 95.0% for scopolamine, 85.2% for 6OH-hyoscyamine, and 85.4% for apoatropine. Additional benefit of this method is the possibility of further analysis of the sample (e.g. LC-MS-MS), since the amendment of the buffer – at the same pH – does not cause any change in selectivity. By using LC-MS-MS method, hyoscyamine, 6OH-hyoscyamine and scopolamine were identified in all three clones.

3.2. *Atropa belladonna* L. *in vitro* plants

Sterile plants were maintained and reproduced on solid MS medium, in light by vegetative micropropagation. Similarly to the intact plant hyoscyamine in greater quantities (2.34mg/g), scopolamine (0.26mg/g) and 6OH-hyoscyamine (0.76mg/g) in lower quantities were identified in the root. The steam contained also hyoscyamine in greater quantities (2.12mg/g) while its scopolamine (0.39mg/g) and 6OH-hyoscyamine (0.91mg/g) contents were lower, however they were slightly higher than that of the root. The alkaloid levels of steams and roots of the *in vitro* plants reached that of the *in vivo* plants.

3.3. Genetically modified root cultures

- **Hairy root cultures**

The hairy roots appeared at the site of microinjection on the shoot of *A. belladonna in vitro* plants were cultured in solid then in liquid B5 culture medium supplemented with 2% sucrose 3 clones (#K_4, #K_5 and #K_8) had been selected to study their transformation characteristics, growth and alkaloid production of the tissues. In all three cultures the predominant alkaloid was hyoscyamine, similarly to the mother plant.

- ***Confirmation of genetical modification***

Polimerase chain reaction (PCR) was performed to identify specific sequences of bacterial T-DNA in plant genome containing *A. rhizogenes* R1601. In the presence of negative (*A. belladonna in vitro* root) and positive (*A. rhizogenes* R1601 plasmid DNA genome) controls we confirmed the presence of adequate *rolB* sequences in the cells of the investigated clones. The activity of T-DNA in plant cells was demonstrated by the detection of the special metabolites, the so called opines produced by hairy roots, using paper-electrophoresis. Applying the above methods we clearly evidenced that the investigated hairy root clones contain bacterial T-DNA genes and these genes work properly in *A. belladonna* cells.

- ***Growth characteristics and alkaloid content of hairy root clones in dark and in light***

Analysing the growth characteristic of *A. belladonna* hairy root #K_4 and #K_8 clones it has been established that the fresh weight and dry weight consistently thrived and resulting a maximum biomass at clones of 4-5 weeks old. In dark the #K_8, while in light the #K_4 clone thrived more intensively. The #K_5 clone thrived less, however its biomass production considerably improved for light. Determining each clone's alkaloid content, it can be established that the total alkaloid (5.16mg/g) and at the same time the hyoscyamine content (3.91mg/g) of the #K_4 clone cultured in dark was the highest, and it remained the same even for light, however due to the more intensive growth its alkaloid production (3.62mg/culture) has been increased. The #K_5 clone responded most intensively for light, and both biomass production, and hyoscyamine and scopolamine content considerably increased resulting in threefold higher hyoscyamine production and sevenfold higher scopolamine production.

According to our results it seems that the accidental incorporation of bacterial T-DNA resulted in genetically unique hairy root clones possessing similar biomass production capacity but significantly different secondary metabolism (e.g. tropane alkaloid production).

- ***Effects of medium components on hairy root cultures***

Proper selection of medium components is essential in case of the different hairy root clones.

- *Effects of carbohydrates – sucrose, glucose and maltose*

The *in vitro* cultures usually require external carbon-source for their growth, therefore the sucrose, glucose, maltose content of the medium has been investigated in more details. According to our results it was evident that all three clones are able to utilize the sucrose best. The higher sucrose concentration (#K_4 3-6%; #K_5, #K_8 3-9%) was beneficial for the growth of the clones while lower sucrose concentrations (2-3%) were beneficial for alkaloid synthesis. Increasing the sucrose concentration of the medium from 2% to 3%, the hyoscyamine production of the #K_4 clone was increased 1.6-fold (4.02mg/culture) compared to the hairy root clone cultured on the basic medium. Production of scopolamine, 6OH-hyoscyamine are also increased nearly 1.5-fold. Maltose and glucose applied in any concentration resulted in considerable decrease of alkaloid production of the cultures.

- *Effect of ammonium [$(NH_4)_2SO_4$]*

Analysing the effect of nitrogen required for the development of hairy root cultures, it has been determined that both three clones grew most intensively in the medium containing 134mg/L $(NH_4)_2SO_4$ (equivalent to B5 basic medium). By increase the $(NH_4)_2SO_4$ content for twofold, the hyoscyamine concentration of the #K_4 clone increased, while in case of #K_5 and #K_8 clones the synthesis of co-alkaloids increased for two-threefold.

- *Effect of magnesium ($MgSO_4$)*

Previous experiments indicated that $MgSO_4$ has beneficial effects on the growth and active agent production of hairy root cultures, therefore the investigation of the effect of magnesium was also considered to be important in case of *A. belladonna* hairy root cultures. It was evidenced that the growth of all three clones were optimal at the higher (250mg/L) $MgSO_4$ concentration. 500mg/L $MgSO_4$ concentration resulted in significant increase in alkaloid content as well in case of #K_4 and #K_5 clones (hyoscyamine: 4.14mg/g #K_4, 4.49mg/g #K_5).

- *Effect of calcium ($CaCl_2$)*

Calcium has also an important role in the metabolism of plant cells and its importance has been confirmed in case of hairy root cultures as well. $CaCl_2$ added to the medium has not affected significantly the growth of certain clones, while it affected alkaloid biosynthesis. This is partly due to the fact that Ca^{2+}-ion has an important role in the activity of certain enzymes in the tropane alkaloid biosynthesis.

Increased calcium-ion concentration of the medium resulted in an increased biosynthesis of certain alkaloids. The #K_4 clone that was cultivated in a medium whose $CaCl_2$ concentration was doubled (300mg/L) synthesised the greatest quantity of hyoscyamine (4.62mg/g) and 6OH-hyoscyamine (0.73mg/g), while the greatest quantity of scopolamine (0.43mg/g) was measured in case of the #K_5 clone, applying 450mg/L $CaCl_2$.

3.4 Analysis of *Atropa belladonna* plants organised from hairy root clones

All three hairy root clones spontaneously organized using liquid B5 culture medium. Micropropagation was performed on *in vitro* transgenic plants, and then cultures were cultivated into horticultural ground following adaptation. Nylon net was stretched out above the seedlings and the plants were cultivated until maturation.

Phenotypes of both in vitro and in vivo #K_4 and #K_8 clones differed from that of the mother plant. Among its morphological characteristics typical for the transgenic plants, it can be highlighted that the steam has decreased peak dominance, it is significantly lower, bushy and due to genetic transformation its leaves contain high quantity of chlorophyll and they are wrinkled; flowers can be characterised by shortened, rounded corolla, therefore the more powerful anthers heighten from the shorter leaf. Premature blooming could be observed in case of all three clones.

Comparing the alkaloid content of the plants cultivated *in vitro* and *in vivo*, it has been established that *in vivo* plants produced more alkaloids. Among *in vitro* clones the maximum hyoscyamine concentration was confirmed in the roots of #K_4 (3.59mg/g) clone, while among *in vivo* clones the roots of the #K_5 clone (5,76mg/g) contained the highest quantity of

hyoscyamine. While the hyoscyamine content is higher in the roots, the scopolamine and 6OH-hyoscyamine contents are higher in the steams, however their levels did not exceed the level of hyoscyamine. Alkaloid content of the leaves depends on their location on the steam; it is the highest on the top of the plant.

3.5. Determination of endogenous formaldehyde content of *Atropa belladonna* tissues

Active agents of *A. belladonna* are N- and O-methylated and these groups can be easily detached from them, so as these molecules are potential sources of formaldehyde, and certain alkaloids – during production of quaternary derivatives – can be considered as HCHO-recipient molecules. The HCHO content of the *in vivo* plants was several-fold higher than that of the same clone cultivated in vitro in B5 medium. The endogenous HCHO level of the steam and root of the genetically transformed control plant can be considered as almost the same, while in the roots of transgenic plants it considerably increases both *in vivo* and *in vitro*. Among the hairy root clones, the highest endogenous HCHO level (75.9μg/g) was obtained from the #K_8 clone. It gradually decreases during cultivation, then increases again by ageing of the tissues; it has been already published that HCHO might have some role in programmed cell death.

- ***Abiogen stress effect on the metabolism of A. belladonna hairy root tissues***

Aminoguanidine and hydralazine – as abiogen stress factors – had effect on endogenous HCHO content and consequently on the universal and special metabolism of cells and tissues. By the application of lower concentrations (1-100ppm) of these compounds, the biomass production and hyoscyamine synthesis of the #K_4 clone were also increased, its scopolamine and 6OH-hyoscyamine concentrations were significantly increased, they were doubled compared to the control. Investigating the effect of aminoguanidine and hydralazine (10ppm) during cultivation period, it has been established that the biomass and tropane alkaloid production of the cultures increased by the end of week 6 compared to those of the control cultures, thus hyoscyamine production increased almost one and a half fold, while the scopolamine, and 6OH-hyosciamine production nearly doubled.

Aminoguanidine and hydralazine added to the medium – beside the growth and alkaloid production of the cultures – characteristically altered the measurable endogenous HCHO content and its temporal change in the hairy root cultures. The elevated endogenous HCHO level resulted by the lower concentrations may probably be in relation to the increased metabolism of the cultures, while applying high concentrations this growth was completely stopped, since at this time the applied molecules deprived considerable quantity of fixed

endogenous HCHO in the cells; apoptotic processes were dominant, resulting in an elevated endogenous HCHO level.

Investigating the effect of aminoguanidine and hydralazine after 1, 2, 3, 6 days on the alkaloid production of 4 weeks old hairy root cultures, we established that by the application of 1-100ppm hydralazine, the hyoscyamine concentration has markedly increased even after day 1 and it even exceeded the control level throughout the whole investigational period, while the aminoguanidine considerably increases the hyoscyamine concentration only by day 2-3. Scopolamine and 6OH-hyoscyamine contents were increased for both compounds.

- ***Analysis of special metabolites of A. belladonna in BioArena-system***

The presence of N-methyl group in hyoscyamine and scopolamine influences their therapeutical effect; therefore we considered that investigating these compounds in BioArena-system can be important.

Investigating the effect of cumarin and scopoletine beside tropane alkaloids we established that they have marked antimicrobial effect on *Pseudomonas phaseolicola* bacterium cells. Antimicrobial effect has been also confirmed by the comparison of the positive and negative densitograms. L-arginin, and reduced gluthation, existing in biological systems and added to bacterium suspensions caused decreased effectiveness, while copper(II)-sulphate solution resulted in increased effectiveness. Our biological experiments that have been performed in BioArena-system demonstrated that the investigated components of *A. belladonna* most likely affects through HCHO.

Conclusion: our genetically modified *Atropa belladonna* cultures thrive well both *in vitro* and *in vivo* and their active agent concentration is quite high. The selected hairy root clones have high biosynthetic potential beside the intensive biomass production, their total alkaloid content reaches, or exceeds that of the intact plant. Modification of the cultural conditions and the components of the medium resulted in additional, marked increase of the alkaloid content, with respect to hyoscyamine, scopolamine and 6OH-hyoscyamine production.

Analysis of abiogen stress effects indicated that the development, endogenous HCHO level and tropane alkaloid content of the tissues markedly changed. Increase of the biomass and alkaloid production raises the possibility of specific effects such as change in gene expression on the universal and special metabolism. Our experiments performed in the BioArena-system confirm that the methylated tropane alkaloids also affect their cell growth inhibition properties through HCHO resulted from methyl group.

ACKNOWLEDGEMENT

Thank you ever so much Prof. Dr. Éva Szőke for the valuable advice and for all the help with my research work.

And also many thanks to:

Dr. László Kursinszki,

Dr. Katalin Tóth,

Rudolfné Mathuny,

Prof. Dr. Ernő Tyihák,

Dr. György Kátay,

Ágnes Móricz,

Dr. Lehel Hullán,

Dr. János Bariska,

and my co-workers.

for their precious help.

PUBLICATIONS IN THEME OF THE Ph.D. THESIS

Articles:

1. **Hank, H.**, László, I., Bálványos, I., Kursinszki, L., Kovács, Gy., Tóth, E., Szőke, É. (2003): Effect of Magnesium on the Growth and Alkaloid Production of Hairy Roots Cultures. Acta Horticulturae **597**, 271-274.
2. **Hank, H.**, Szőke, É., Tóth, K., László, I., Kursinszki, L. (2004): Investigation of Tropane Alkaloids in Genetically Transformed *Atropa belladonna* L. Cultures. Chromatographia 60. 55-59.
3. Kursinszki, L., **Hank, H.**, László, I., Szőke, É. (2005): Simultaneous analysis of hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine and apoatropine in Solanaceous hairy roots by reversed-phase high-performance liquid chromatography. Journal of Chromatography A. 1091. 32-39.
4. Balázs, A., **Hank, H.**, Buda, I., Isó, I., Szőke, É., Kursinszki, L. (2007): Chemical Analysis on Hallucinogenic of Angel's Trumpet (*Brugmansia Suaveolens* L.). Chromatographia, In Press.

Abstract in journal:

1. Bálványos, I., László, I., Vida, K., **Hank, H.**, Kursinszki, L., Tóth, E., Szőke, É.(2001): Effect of magnesium on the genetically modified *Atropa belladonna* cultures. Magnesium Research 14, 309-310. IF: 0,689
2. **Hank H.,** Bálványos I., Kursinszki L., Tóth E., Szőke É. (2003): A táptalaj összetételének hatása transzgénikus *Atropa belladonna* kultúrák alkaloid produkciójára. Gyógyszerészet, Suppl. 69-70.
3. **Hank, H.**, László, I., Bálványos, I., Kursinszki, L., Kovács, Gy., Tóth, E., Szőke, É. (2003): Effect of Mg^{2+} on the growth and alkaloid production of hairy roots cultures obtained from different plant species. Magnesium Research, 16. 328-329. IF.:0,667
4. Kursinszki L., László I., **Hank H.**, Szőke É. (2003): Tropán alkaloidok nagynyomású folyadékkromatográfiás vizsgálata biotechnológiai mintákban új-generációs állófázison. Gyógyszerészet, Suppl. 79.
5. **Hank, H.**, Tyihák, E., Kátay, Gy., Kursinszki, L., Szőke, É. (2005): Tropane alkaloids and endogenous formaldehyde levels in vivo and in vitro in genetically modified *Atropa belladonna* L. European Journal of Pharmaceutical Sciences 25S1. 110-111. IF.:1,949

Conferences (lecture, poster):

1. Bálványos, I., László, I., Vida, K., **Hank, H.**, Kursinszki, L., Tóth, E., Szőke, É. (2001) Effect of magnesium on the genetically modified *Atropa belladonna* cultures. 7th Hungarian Magnesium Symposium, Program and Abstracts p.13-14. Siófok.
2. **Hank, H.**, Vida, K., László, I., Bálványos, I., Kursinszki, L., Tóth, E., Szőke, É. (2001) Investigation on the tropane alkalid production of genetically modified *Atropa belladonna* L. in vitro cultures. World Conference on Medicinal and Aromatic Plants, Abstracts (PIII/53) p. 265. Budapest.
3. **Hank H.**, László I., Bálványos I., Kursinszki L., Vida K., Kovács Gy., Tóth E., Szőke É. (2002) Influence of culturing conditions on the primary and secondary metabolism of genetically modified *Atropa belladonna* L. tissues. PhD Tudományos Napok, (P34),p.45, ISBN-973-8174-75-9,Budapest.
4. **Hank H.**, Bálványos I., László I., Vida K., Kursinszki L., Kovács Gy., Tóth E., Szőke É. (2002) Effect of magnesium on the growth and alkaloid production of hairy roots

and reorganised plant cultures, P 29. The IIIrd Romanian Magnesium Symposium With International Participation, Iasi (Romania), Abstract book p135.
5. **Hank H.**, László I., Bálványos I., Kursinszki L., Kovács Gy., Tóth E., Szőke É. (2002) A magnézium hatásának vizsgálata *Atropa belladonna* L. géntranszformált gyökér- és reorganizált növénykultúrákra. Gyógynövények kutatása és felhasználása, 10. Magyar Gyógynövény Konferencia, 8. Gyógyszerkutatási Konferencia, 5. Magyar Fitoterápiás Konferencia, Kecskemét (P9), p92.
6. Kursinszki L., László I., **Hank H.**, Szőke É. (2002) Tropán alkaloidok meghatározása genetikailag módosított növényekben kombinált SPE-HPLC módszerrel. Gyógynövények kutatása és felhasználása, 10. Magyar Gyógynövény Konferencia, 8. Gyógyszerkutatási Konferencia, 5. Magyar Fitoterápiás Konferencia, Kecskemét (P-17.) p.100.
7. Kovács Gy., Kuzovkina I. N., Kursinszki L., **Hank H.**, Szőke É. (2002) Scutellaria baicalensis Georgi hairy root kultúrák flavonoid produkciójának vizsgálata. Gyógynövények kutatása és felhasználása, 10. Magyar Gyógynövény Konferencia, 8. Gyógyszerkutatási Konferencia, 5. Magyar Fitoterápiás Konferencia, Kecskemét (P16), p.99
8. **Hank H.**, László I., Bálványos I., Kursinszki L., Kovács Gy., Tóth E., Szőke É. (2003) A magnézium hatása különböző növényfajok géntranszformált kultúráinak növekedésére és alkaloid tartalmára. 8. Magyar Magnézium Szimpózium, Hajdúszoboszló, előadás p.3-4.
9. László, I., Kursinszki, L., **Hank, H.**, Kátay, Gy., Tyihák, E., Szende, B. and Szőke, É. (2003): Analysis of apoptotic processes, tropane alkaloids and endogenous formaldehyde in genetically modified plant tissue cultures. 6th International Conference on Role of Formaldehyde in Biological Systems. Methylation and Demethylation Processes Scientific Program and Abstracts p. 17. Pécs.
10. **Hank, H.**, Kursinszki, L., László, I. and Szőke, É. (2003): Analysis of tropane alkaloids and endogenous formaldehyde in genetically modified *Atropa belladonna* cultures. 6th International Conference on Role of Formaldehyde in Biological Systems. Methylation and Demethylation Processes Scientific Program and Abstracts p. 52. Pécs.
11. **Hank H.**, László I., Bálványos I., Kursinszki L., Kovács Gy., Tóth E., Szőke É. (2003) Transzgénikus *Atropa belladonna* L. kultúrák alkaloid produkciójának vizsgálata. PhD Tudományos Napok 2003, Budapest (Hungary) P43
12. **Hank H.**, László I., Bálványos I., Kursinszki L., Kovács Gy., Tóth E., Szőke É. (2003) A táptalaj összetételének hatása transzgénikus *Atropa belladonna* kultúrák alkaloid produkciójára. Congressus Pharmaceuticus Hungaricus XII., Budapest, Előadás összefoglalók, P43
13. Kursinszki L., László I., **Hank H.**, Szőke É. (2003) Tropán alkaloidok nagynyomású folyadék kromatográfiás vizsgálata biotechnológiai mintákban új-generációs állófázison. Congressus Pharmaceuticus Hungaricus XII., Budapest, Előadás összefoglalók, P66
14. **Hank H.**, Bálványos I., Kursinszki L., Tóth E., Szőke É. (2003) Investigation of genetically transformed *Atropa belladonna* L. cultures by HPLC method. 5th Balaton Symposium on high-performance separation methods, Siófok (Hungary) (P36)
15. **Hank H.**, Bálványos I., Kursinszki L., Tóth E., Szőke É. (2003): A táptalaj összetételének hatása transzgénikus *Atropa belladonna* kultúrák alkaloid produkciójára. Congressus Pharmaceuticus Hungaricus XII. Budapest. Előadás összefoglalók, P-43.

16. Kursinszki L., László I., **Hank H.**, Szőke É. (2003): Tropán alkaloidok nagynyomású folyadékkromatográfiás vizsgálata biotechnológiai mintákban új-generációs állófázison. Congressus Pharmaceuticus Hungaricus XII. Budapest. Előadás összefoglalók, P-66.
17. **Hank H.**, Kursinszki L., Kovács Gy., Tóth E., Szőke É. (2003) Influence of sucrose-concentration in the medium on tropane alkaloid production in *Atropa belladonna* L. hairy root cultures. VIII International Conference, The biology of plant cells in vitro and biotechnology, Saratov (Russia) p388. ISBN 5-901979-1
18. Kovács Gy., Kuzovkina I. N., Kursinszki L., **Hank H.**, Szőke É. (2003) Investigation of flavonoids in the hairy root cultures of Scutellaria baicalensis Georgi. VIII International Conference, The biology of plant cells in vitro and biotechnology, Saratov (Russia) p392. ISBN 5-901979-1
19. **Hank H.**, Kursinszki L., László I., Szőke É. (2004) Analysis of tropane alkaloids and endogenous formaldehyde in genetically modified *Atropa belladonna* L. cultures. PhD Tudományos Napok 2004, Budapest (Hungary) (P-I/9), p100-101.
20. **Hank H.**, Kursinszki L., László I., Tóth K., Szőke É. (2004) Influence of sucrose on tropane alkaloid production in hairy root cultures of Solanaceous plants. Symposium of Pharmaceutical Biotechnology, Contributions to the Symposium, Trieste (Italy) (P5)
21. Kursinszki, L., **Hank, H.**, László, I., Szőke, É. (2004): HPLC-Analysis of Tropane Alkaloids in Hairy Root Cultures. 4th International Symposium on Chromatography of Natural Products, Lublin - Kazimierz Dolny (Poland). L-22.
22. **Hank H.**, Kursinszki L., Hullán L., Szőke É.(2005) Investigatoin of hydralazine on *Atropa belladonna* L. hairy root cultures. 1st BBBB Conference on Pharmaceutical Sciences Siófok (Hungary) (P17) p.143-144.
23. **Hank H.**, Kursinszki L., Hullán L., Bariska J., Szőke É. (2005): Investigation of Hydralazine on *Atropa belladonna* L. Hairy Root Cultures. Pharmacy: Smart Molecules for Therapy. Semi-centennial conference of Semmelweis University, Faculty of Pharmacy, Budapest P-43.
24. Balázs, A., Buda, I., Héthelyi, É., Kursinszki, L., **Hank, H.**, Szőke, É. (2005): Chemical Analysis of Hallucinogenic Components in Plants, Mushrooms and Cacti Grown in Hungary. 6th Balaton Symposium, Siófok.(P-72).
25. **Hank H.**, Kursinszki L., Hullán L., Bariska J., Szőke É. (2006): Nukleofil reagensek hatása géntraszformált *Atropa belladonna* kultúrák hatóanyag képzésére. Congressus Pharmaceuticus Hungaricus XIII. Budapest. Gyógyszerészet, Kongresszusi különszám. Előadás-összefoglalók. p.91.
26. Kursinszki L., **Hank H.**, Szőke É. (2006): Növényi minták alkaloid-tartalmának vizsgálata nagynyomású folyadékkromatográfiával. Congressus Pharmaceuticus Hungaricus XIII. Budapest. Gyógyszerészet, Kongresszusi különszám. Előadás-összefoglalók. p.37.
27. Balázs, A., **Hank, H.**, Buda, I., Isó, I., Szőke, É., Kursinszki, L. (2007): Chemical Analysis on Hallucinogenic of Angel's Trumpet (Brugmansia Suaveolens L.). 7th Balaton Symposium, Siófok. p.65.