SEMMELWEIS UNIVERSITY

Ph.D. Thesis

Investigation on primary and secondary metabolism of *Datura innoxia* Mill. tissue cultures

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Summary

Datura innoxia Mill. contains several secondary metabolites with pharmacological and toxicological importance, including more than 30 tropane- and pirrolidin-base alkaloids. Total amount of these alkaloids varies between 0.05%-0.5% in different organs of the plant. Main alkaloids are scopolamine and hyoscyamine which have parasympatholytic effect.

The aim of our experiments was to study different – genetically modified and non-transformed – *in vitro* cultures of *D. innoxia*. The growth (fresh and dry weight, dry matter content, growth value and pH of liquid culture media) and tropane alkaloid content (scopolamine, hyoscyamine and apoatropine, using HPLC technique) of *in vitro* plants, callus tissues and genetically modified, so called hairy root cultures were investigated. The transferred character of hairy root tissues obtained by *Agrobacterium rhizogenes* A4 microinjection was demonstrated by using polimerase chain reaction (PCR) and opine detection (paper electrophoresis). We have established that the total alkaloid content of hairy root clones (#410, #411 and #415, 0.23% hyoscyamine and 0.21% scopolamine) reached that of the root of the *in vivo* plants.

Growth and alkaloid content of *in vitro* cultures has been effected by several circumstances. We studied the effects of culturing in light, the sucrose and Mg^{2+} content of liquid MS basal medium on the growth, biomass production and tropane alkaloid content of the tissues. The presence of apoptotic processes in cells of different tissues (callus and root) accompanied with the shrinkage of the nuclei was investigated by TUNEL reaction.

To study the relationship among abiotic stress, growth, alkaloid metabolism and measurable endogenous HCHO concentration different molecules affecting transmethylation reactions have been administered to the culture media (dimedone, semicarbazide, aminoguanidine and hidralazine, 1ppm-1000ppm) of tissues [1].

The endogenous HCHO concentration of callus (4.6? g-27.0? g) and hairy root cultures (345.0? g) was determined by OPLC and HPLC techniques as formaldemethone [2]. Following administration of dimedone and semicarbazide the apoptotic processes were investigated by TUNEL reaction [3].

The results obtained might help deepening our knowledge about the growth of *D. innoxia in vitro* cultures, tropane alkaloid metabolism, apoptotic processes in plant cells and the effect of abiotic stress (special chemical stress).

1. László, I., Szoke, É. and Tyihák, E. (1998) Relationship between abiotic stress and formaldehyde concentration in tissue culture of *Datura innoxia* Mill. Plant Growth Regulation, **25**, 195-199.

2. László, I., Szoke, É., Tyihák, E., Németh, Zs. and Albert, L. (2003): Identification and measurement of endogenous formaldehyde in *Datura innoxia* Mill. tissues by OPLC, HPLC and MALDI MS techniques. Acta Horticulturae, **597**, 265-270.

3. László, I., Szoke, É., Tyihák, E. and Szende, B. (2001) Programmed cell death in *Datura innoxia* Mill. cultures cultivated in light and in dark. Plant Growth Regulation, **33**, 231-236.

1. INTRODUCTION AND AIMS

Products of plant secondary metabolism are important raw materials of food, cosmetical and pharmaceutical industry. The plants contain these metabolites generally in low quantities. However, these molecules have complex structure in most cases so their production – regarding few exceptions – remains unsuccessful in industrial scale. On the other hand parallel with the development of analytical methods increasing number of plant secondary metabolites can be discovered and investigated.

Biotechnological techniques and molecular biology provided the opportunity to investigate the primary and secondary metabolism of plants under laboratory conditions. Special cell and tissue cultures seem to be good objects for the production of plant metabolites in industrial scale.

Investigation of *in vitro* plant cell and tissue cultures – similarly to human or animal cell and tissue cultures – might help to understand the effect of special molecules on primary/secondary metabolism and on apoptotic processes (e.g. carcinogenic effect). According to the latest experimental evidences concerning programmed cell death (PCD) of plant cells it is supposed that – in spite of basic differences between human/animal and plant cells – some of the regulation processes remained identical during the evolution.

We have studied different, genetically modified and non-transformed tissue cultures of *Datura innoxia* Mill. The plant itself contains medically and toxicologically important tropane alkaloids which are mainly scopolamine and hyoscyamine. Both alkaloids have parasympatholytic effect (e.g. spasmolytic and midriatic effects) so they are widely used in ophthalmology, in cardiac and gastrointestinal disorders.

During our experiments we would like to study the growth and tropane alkaloid production of callus and genetically modified (so called hairy root) tissues originated from *in vitro D. innoxia* plants. Growth and biosynthetic capacity of tissues can be affected by a variety of circumstances, thus we would like to investigate the effect of light, sucrose concentration (carbon source) and Mg^{2+} (macro element) concentration of culture medium on the growth, biomass production, hyoscyamine, scopolamine and apoatropine content of the tissues. The occurrence of typical morphological changes related to apoptotic processes will be also studied.

According to experimental evidences different biological systems (human, animal and plant cells/tissues) contain measurable amount of endogenous formaldehyde (HCHO). The level of endogenous HCHO is strongly affected by different biotic, abiotic stresses. During our experiments the level of endogenous HCHO will be determined as dimedone adduct. Dimedone reacts irreversibly with HCHO and the reaction product (formaldemethone) can be detected and measured by different analytical methods.

Our aim is to study the effects of abiotic stress – administration of special molecules in the culture medium (dimedone, semicarbazide, aminoguanidine and hydralazine) influencing transmethylation reactions – on the growth, tropane alkaloid metabolism and measurable HCHO content of *in vitro* cultures. Following administration of dimedone and semicarbazide we would like to investigate the possible effects of these molecules on apoptotic processes, too.

We hope our results will help deepen the knowledge about the growth of *D. innoxia in vitro* cultures, tropane alkaloid metabolism, the course of apoptotic processes and the changes related to abiotic stress (special chemical stress).

2. MATERIALS AND METHODS

Seeds of *D. innoxia* Mill. to obtain a variety of *in vitro* cultures were obtained from the Botanical Garden of Vácrátót of the Hungarian Academy of Sciences.

2.1. *D. innoxia in vitro* plants

Seeds of *D. innoxia* were sterilised by diocid solution, then washed by distilled water. Seeds were germinated in light. The plantlets were transferred to Murashige and Skoog (MS) solid medium containing Ca-pantotenate (S medium) and cultivated in light.

2.2. Tissue cultures

? Secondary callus tissues were isolated from shoots of *D. innoxia in vitro* plants. Callus pieces were grown in light (2500Lux, 12 h per day) and in dark on solid AMS medium containing 3% of sucrose and growth regulators as well.

? Shoots of *D. innoxia* plants cultivated on S medium were infected by microinjection using *Agrobacterium rhizogenes* A4 strain. Two weeks following infection 26 hairy root clones were isolated and transferred to MS solid medium supplemented with cefotaxim and ampycillin in order to eliminate *Agrobacteria*. Among the cultures cultivated on MS solid medium free of antibiotic clones #410, #411 and #415 were selected for further investigations according to their growth and tropane alkaloid production. The clones were transferred to liquid MS medium (100rpm, 24?1^{o}C) and cultivated both in dark and in light (2500Lux, 12 h/day).

2.3. Studies on genetically transformed characteristics of hairy roots

? Investigation of *D. innoxia in vitro* plants and hairy root cultures by PCR

For the isolation of plant DNA fresh root and hairy root samples were frozen in liquid nitrogen (-196^{o}C) and powered. Plant DNA was isolated and cleaned on QIAshredder columns (2ml, Qiagen).

DNA fragments obtained by PCR were separated on agarose gel plates. Evaluation was performed under UV light (??302nm).

? Detection of opines

Opine synthesis of hairy root clones #410, #411, and #415 obtained following *A. rhizogenes* A4 infection 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 were measured or calculated in order to describe the growth of *in vitro* cultures. Determination of pH values of the liquid MS culture medium of hairy root tissues were performed using an OP-211/1 Radelkis Laboratory Digital pH Meter (23?1°C, in 20ml volume).

2.5. Changes in the composition of AMS and MS culture media

? Changes in sucrose and $MgSO_4$ content of the medium

The effect of the sucrose (0%, 1.0%, 2.0%, 3.0% and 6.0%) and $MgSO_4$ (0 mg/l, 185 mg/l, 370 mg/l, 740 mg/l and 1480 mg/l) content of liquid MS medium on the growth and tropane alkaloid production was investigated in case of hairy root clone #410.

? Administration of dimedone, semicarbazide, aminoguanidine and hydralazine

The effect of dimedone (on *D. innoxia* callus cultures cultivated in solid AMS medium) or dimedone, semicarbazide, aminoguanidine and hydralazine (on hairy root clone #410 cultivated in liquid MS medium) content of culture medium was investigated on the growth, endogenous HCHO content and tropane alkaloid production of tissues. The molecules were applied in the amount of 0ppm, 1ppm, 10ppm, 100ppm and 1000ppm. The tissues were cultivated in dark – following the administrartion of dimedone or semicarbazide – and in light (2500Lux, 12 h/day), too.

2.6. Qualitative and quantitative determination of tropane alkaloids

? Hystochemical investigation of plant tissues by Dragendorff-reagent

In order to examine the localisation of alkaloids within the roots we have used the roots of *D. innoxia in vitro* plants and the tissues of #410 hairy root clone.

? MALDI-MS method

Tropane alkaloids (hyoscyamine and scopolamine) were detected and identified by Finnigan LASERMAT 2000 mass spectrometer. The matrix material applied was ?-cyano-4-hydroxy cinnapic acid (ACH, Sigma).

? HPLC method

Following extraction the samples were cleaned by solid phase extraction (SPA) (SUPELCO Supelclean LC-18 SPE). Qualitative determination was performed using a Surveyor modular HPLC system (Thermo Finnigan, USA). The column applied was a LUNA C18 reversed phase column (Phenomenex, USA) with the application of a precolumn. The eluent mixture was acetonitrile : 30mM phosphate buffer (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 (??210nm) and by UV spectral data.

2.7. Qualitative and quantitative determination of endogenous HCHO

? MALDI-MS method

The MALDI mass spectrometer used in this study was a Finnigan LASERMAT 2000 (Finnigan MAT Ltd., Hemel Hempstead, UK). The fresh callus tissues were frozen in liquid nitrogen, powdered and treated with 0.2% solution of dimedone in methanol. The matrix material was ?-cyano-4-hydroxy cinnamic acid (ACH, Sigma). Formaldemethone was identified using authentic standard.

? OPLC method

The fresh callus and hairy root pieces of *D. innoxia* were frozen in liquid nitrogen, powdered and treated with 0.2% solution of dimedone in methanol. The separation of formaldemethone was carried out using a chloroform – methylenechloride eluent mixture (35:65, v/v) in an OPLC instrument (OPLC-NIT CO., Ltd., Budapest, Hungary). The quantitative analysis was performed densitometrically (Shimadzu CS 930 scanner, Shimadzu Co., Kyoto, Japan) at ? = 260nm.

? HPLC method

The HPLC separation of formaldemethone was performed with the use of a Gynkotec M 480 pump, TOSOH 6040 UV detector (? = 260nm) and Rheodyne 8125 injector (20?l loop). The column used was a Chrompack ChromSpher G-18 (150 x 4.6 mm i.d., 5?m). The applied mobile phase was CH_3OH : 0.01M HCl (76:24, v/v).

2.8. Detection of apoptotic cells by TUNEL reaction

TUNEL reaction was performed by Apop-Tag (Oncor, Gaithersburg, MD, USA). The ratio of apoptotic cells (apoptotic index, A_i) were also determined in tissues.

2.9. Statistical evaluation of results

Data of the cultures represented by n=7 and n=20 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 RESULTS

3.1. INVESTIGATION OF *D. innoxia IN VITRO* CULTURES

✍ ***In vitro* plants**

D. innoxia plants – used to obtain different tissue cultures and were maintained by vegetative micropropagation – grew well on solid S (modified MS) medium containing Ca-pantotenate and casein hydrolisate.

The investigation of alkaloid content in shoots of 8 weeks old plants revealed that apoatropine was the only tropane alkaloid which could be detected in measurable quantities. The roots with the same age contained hyoscyamine (3.0‰) and scopolamine (1.04‰) as well, but their volume did not exceed that of the in vivo plants (0.05-0.5%).

The tissues of the roots of *in vitro* plants were studied following HE staining. The roots of 8 week-old plants were separated from shoots and were fixed before sectioning. The structure of tissues were identical with that of the young roots. Following administration of Dragendorff reagent we could observe yellow-brownish coloured precipitate indicating the presence of alkaloids. The precipitate – according to previous observations in *in vivo* plants – localised close to the cell wall, especially in the cells of bast parenchyma next to the rhyzodermis and hypodermis and in the parenchymatic cells of medullar rays.

✍ **Callus tissues**

Tissues cultivated both in dark and in light grew well till the end of the 6 week long cultivation period on AMS solid medium containing kinetin and 2,4-D (fresh weight became 14/16 times higher and the dry weight became 5 times higher). Fresh weight of 2 week-old tissues cultivated in light already exceeded that of the callus cultures cultivated in dark and this difference between the tissues could be observed till the end of the cultivation period.

The structure of tissues were investigated following HE staining. There were no morphological differences between the tissues cultivated in dark or in light. The cells were parenchymatic with no signs of differentiation. Clusters of small cells with densely staining nuclei could be observed in all slides. The cells containing compact nucleus gave positive TUNEL reaction which proved the presence of apoptotic processes in these cells. The ratio of TUNEL positive cells was high in the 10-20 cell layers close to the surface of tissues (around 30%), but their ratio significantly decrease in deeper cell layers.

✍ **Genetically modified root cultures**

Shoots of *D. innoxia in vitro* plants were infected by *Agrobacterium rhizogenes* A4 strain by microinjection. Following the infection several hairy root clones could be isolated. Three clones (? 410, ? 411 and ? 415) had been selected to study the transformed characteristic, growth and alkaloid content of the tissues.

Polimerase chain reaction (PCR) was performed to identify specific sequences of bacterial DNA (tDNA) in plant genome. Functioning of bacterial transgenes was investigated by determination of opines which are produced by the transformed plant cells and consumed by *Agrobacteria*. Following paper electrophoresis we could demonstrate the presence of special opine molecules in hairy root clones ? 410, ? 411 and ? 415. Clone ? 410 and ? 411 contained agropine (Rf = 0.56) agropinic acid (Rf = 0.25), mannopine and mannopinic acid with the same Rf value (Rf = 0.46). Tissues of hairy root clone ? 415 contained mainly agropinic acid. According to bibliographical data, the opines identified are characteristic of plant cells infected by *A. rhizogenes* A4 strain.

The results of PCR and opine determination clearly demonstrated that the clones investigated contain bacterial genes and these genes were functioning in *D. innoxia* cells.

Summarizing our results concerning the growth of tissues, it is demonstrated that biomass production of hairy root clones ? 410, ? 411 and ? 415 was almost the same. However, some differences could be observed. Fresh weight of tissues cultivated in light – especially the 3 weeks old tissues – was higher and dry weight was lower than in dark. The pH of culture media was also higher in light following the first week of cultivation already.

Determination of tropane alkaloid content of the clones showed that in contrast to the growth of tissues there were great differences among the clones. Clone ? 410 contained hyoscyamine as predominant tropane alkaloid and the highest amount could be measured in 4 weeks old tissues (0.20% in dark and 0.21% in light). The highest scopolamine contents detected were 0.16% and 0.17%. Apoatropine content of the clone was much lower, 0.01%-0.02% in general.

The hyoscyamine content of clone ? 411 was lower both in dark (0.11%) and in light (0.17%), but these values were combined with high scopolamine content in dark (0.11%) and in the light (0.12%), as well during the 6 week-long cultivation period. According to these results clone ? 411 contained scopolamine as predominant tropane alkaloid.

Clone ? 415 – similarly to clone ? 410 – produced hyoscyamine in greatest quantities. However, in this clone there were great differences between the alkaloid content of tissues cultivated in dark and in light. Highest hyoscyamine (0.23%) and scopolamine (0.13%) content was measured in 6 weeks old tissues in dark. The highest alkaloid contents in light were measured following the third and fifth weeks of cultivation (0.18-0.19% of hyoscyamine and 0.11% of scopolamine).

Results of alkaloid production showed that – in spite of slightly lower alkaloid content – the tissues cultivated in light produced higher amounts of tropane alkaloids as a result of elevated biomass production. The highest alkaloid productions were calculated in clone ? 415 following 6 weeks of cultivation in dark (0.63mg hyoscyamine and 0.37% scopolamine / culture) and in clone ? 410 following 3-4 weeks cultivation in light (0.60mg hyoscyamine and 0.77% scopolamine / culture).

Summarizing the results obtained regarding the alkaloid content and production of hairy root clones ? 410, ? 411 and ? 415 we can conclude that clones ? 410 and ? 415 contain hyoscyamine as predominant alkaloid but clone ? 411 contains scopolamine in greatest quantities. It seems that the accidental incorporation of bacterial tDNA resulting genetically unique hairy root clones possessing similar biomass production (e.g. fresh weight, growth value) but significantly different secondary metabolism (e.g. tropane alkaloid production). Although tissues of clone ? 415 produced the highest amount of hyoscyamine and scopolamine in dark, clone ? 410 was selected for further experiments due to its stable and high alkaloid production both in dark and in light.

Investigation of 4 weeks old tissues of hairy root clone ? 410 following HE staining revealed that the structure of hairy roots are similar to the young roots *in vitro D. innoxia* plants. No special differences could be observed between the tissues cultivated in dark or in light. Localisation of alkaloids was studied following the administration of Dragendorff reagent and it was similar as observed in the roots *in vitro* plants.

3.2. EFFECT OF THE COMPOSITION OF CULTURE MEDIUM ON HAIRY ROOT CULTURES

✍ Changing of the sucrose concentration in the medium

Tissues of *D. innoxia* hairy root clone ? 410 were cultivated for 4 weeks in dark in liquid MS medium containing 0-6% of sucrose. High fresh weight was measured in culture media containing 1-2% of

sucrose. However, greater dry weight was detected for the effect of 2-6% sucrose in MS medium. The maximal dry matter content was calculated in medium containing 6% of sucrose which might be the result of the hypertonic conditions causing water deprivation from tissues. The greatest growth value was calculated in MS medium containing 2% of sucrose which corresponds to the sucrose content of basic MS medium.

The optimum sucrose content for both tropane alkaloid (hyoscyamine and scopolamine) content and production of tissues were 2/3%. According to our observations for the effect of sucrose the ration of hyoscyamine, scopolamine or apoatropine did not changed significantly.

✍ **Changing of the $MgSO_4$ concentration in the medium**

Growth and alkaloid production of *D. innoxia* hairy root clone ? 410 – grown for 4 weeks both in dark and in light in liquid MS medium containing 2% of sucrose – was investigated in relation to the Mg^{2+} content of medium. Greatest fresh weight was detected in medium containing 370mg/l $MgSO_4$ (corresponding to the $MgSO_4$ content of basic MS medium) and in medium containing 740mg/l $MgSO_4$ in light. Highest dry matter content – with the lowest fresh weight – was calculated in medium supplemented with 185mg/l $MgSO_4$ both in dark and in light. Maximal growth values were obtained in cultures grown in medium supplemented with 370mg/l $MgSO_4$.

Despite the similarities in the biomass production of tissues grew in dark and in light there were significant differences in optimal $MgSO_4$ concentrations for tropane alkaloid content and production. While the optimal $MgSO_4$ concentration was 370mg/l in dark (0.054% of hyoscyamine and 0.049% of scopolamine), the highest alkaloid content was measured (0.062% of hyoscyamine and 0.060% of scopolamine) and the greatest alkaloid production was calculated in medium containing 740mg/l $MgSO_4$ in light.

According to our results there were no significant differences between the growth (e.g. fresh and dry weight, growth value) of tissues cultivated in dark or in light. However, cultivation in light coupled with elevated $MgSO_4$ concentration was advantageous from the point of view of alkaloid content and production.

3.3. EFFECT OF ABIOTIC STRESS ON CALLUS AND HAIRY ROOT CULTURES

The aim of these experiments was to study the role of endogenous HCHO in transmethylation and oxidation/reduction reactions and its function in primary and secondary metabolism of plant cells so we have investigated the effect of special molecules influencing endogenous HCHO content and metabolism (growth, ageing and tropane alkaloid production) of *in vitro* cultures. In addition to the

control tissues the molecules were applied in different amounts (1ppm, 10ppm, 100ppm and 1000ppm) to solid and liquid media of the plant tissues.

✍ **Effect of dimedone on callus cultures**

Dimedone – which reacts irreversibly with HCHO – was applied in increasing amount to the solid AMS medium of *D. innoxia* callus cultures in order to investigate the endogenous HCHO depriving effect of this molecule on *in vivo* cells and tissues. The tissues were collected after 2, 4 and 6 weeks. Following administration of 1ppm, 10ppm and 100ppm dimedone the fresh weight of tissues reached or slightly exceeded that of the controls. However, for the effect of 1000ppm dimedone significant decrease could be detected. Regarding the formation of fresh weights we could not observe differences in the effect of dimedone on tissues cultivated in dark or in light except the tissues grew on 1000ppm dimedone containing medium. In this case the fresh weight of cultures cultivated in dark almost reached that of the control. Parallel with the growth of tissues dry matter content slightly decreased following the administration of 1ppm, 10ppm and 100ppm dimedone, but 1000ppm – with the exception of 6 weeks old tissues cultivated in dark – resulted a significant increase in its value (20-80%). Administration of 10ppm dimedone in culture medium strongly affected the growth and biomass production of tissues as it was shown by the growth values which are significantly exceeded that of the controls (50-200%) both in dark and in light.

The results of endogenous HCHO determination as dimedone adduct (formaldemethone) showed that the measurable HCHO content of tissues varied between 4.6?g/g and 27.0?g/g. For the effect of dimedone in culture medium – especially in case of 10ppm and 100ppm – the amount of measurable HCHO content decreased. Four weeks old tissues treated with 1000ppm dimedone contained high amount of measurable HCHO exceeding the control level, but the HCHO content of 6 weeks old cultures significantly decreased. The measurable HCHO content of tissues cultivated in light was significantly higher compared to the cultures cultivated in dark.

Following HE staining we observed that the administration of dimedone in culture medium did not influence the structure of tissues, but it strongly affected the ratio of apoptotic cells. The apoptotic index (A_i) determined following TUNEL reaction increased in parallel with the amount of dimedone applied. However, 1000ppm dimedone in culture medium almost ceased DNA fragmentation especially in tissues cultivated in light. Our results showed that – with the exception of 10ppm dimedone in culture medium – elevated A_i could be calculated in tissues grown in dark compared to the cultures cultivated in light.

✍ **Effect of dimedone on hairy root cultures**

Administration of 1ppm, 10ppm and 100ppm dimedone in the liquid MS medium of 4 week-old *D. innoxia* hairy root cultures of clone ? 410 resulted a significant decrease (30-40%) of fresh weight. For the effect of 1000ppm dimedone the fresh weight of tissues dramatically decreased, values reached 15-20% of the controls only. Dry matter content of cultures – with the exception of 100ppm dimedone in medium and cultivation in dark – decreased with the applied dimedone concentration, as well. Application of 1ppm dimedone resulted 60-75% decrease of calculated growth values compared to the control. 10ppm and 100ppm caused further decrease, but for the effect of 1000ppm no growth could be detected. Determination of the pH of culture medium showed that in case of hairy roots cultivated in light – similarly to the tissues cultivated in MS basic nutrient medium – higher pH could be measured both in control and dimedone treated tissues. However, pH values decreased in parallel with increased dimedone concentration in spite of the fact that all media was set to pH 5.7 before application.

Hyoscyamine and apoatropine content of 4 week-old tissues cultivated in dark decreased step by step with increasing dimedone concentration of the culture medium. However, amount of scopolamine significantly increased (150-180%) following the administration of 1ppm and 10ppm dimedone. Hyoscyamine and scopolamine content of tissues cultivated in light slightly increased for the effect of 10ppm and 100ppm dimedone, but these values did not exceeded that of the controls. It is important to remark that 1000ppm dimedone in culture medium practically stopped the synthesis of tropane alkaloids.

Even the administration of 1ppm dimedone caused significant decrease (45-85%) of alkaloid production both in dark and in light. For the effect of 10ppm further decrease and for the effect of 100ppm slight increase could be observed, but hairy roots in medium containing 1000ppm dimedone did not produce alkaloids.

Results of endogenous HCHO determination showed that *D. innoxia* hairy roots contain 300-400? g/g HCHO which is on scale higher than in callus tissues. It might be the result of the more complex metabolism of hairy root tissues or their genetically transformed ability as well.

✍ **Administration of semicarbazide**

Administration of 1ppm semicarbazide – which reacts reversibly with HCHO molecules – into the liquid MS medium of *D. innoxia* hairy root clone ? 410 resulted significant increase of fresh weight (80-100%) following 4 weeks of cultivation. Application of amount of semicarbazide (10ppm, 100ppm and 1000ppm) resulted a sudden decrease of fresh weight. Parallel with these results dry matter content of tissues decreased when 1ppm semicarbazide was added to the medium, but it

increased with lower fresh weights. For the effect of 1000ppm semicarbazide the value of dry matter conetn decreased to the level determined following the administration of 1ppm again. Calculated growth values of 4 week-old hairy roots significantly increased for the effect of 1ppm semicarbazide. However, 10ppm and 100ppm caused sudden decrease and 1000ppm completely stopped the growth of tissues. Following the administration of semicarbazide – according to our previous observations – higher pH values of culture medium could be measured in cultures cultivated in light. For the effect of 1ppm and 10ppm the pH slightly exceeded the control level but 100ppm and 1000ppm resulted a progressive decrease.

The higher amount of semicarbazide we applied the lower tropane alkaloid content could be measured in tissues. A slight increase could be measured slight incresae only in dark, after the administration of 10ppm and 100ppm semicarbazide. 1000ppm in culture medium – according to the application of dimedone – resulted the absence of alkaloids in tissues. None of the alkaloid production values reached that of the control, but progressively decreased till 1000ppm which caused complete inhibition of production.

Endogenous HCHO content of tissues cultivated in light slightly decreased from the value of 345.0? g/g determined in the controls to 286.4? g/g measured in hairy roots grown in medium containing 100ppm semicarbazide. Due to the low growth rate of cultures in 1000ppm semicarbazide containing medium there were no sufficient amount of sample to perform the endogenous HCHO determination.

✍ **Morphological investigation of hairy roots treated with dimedone or semicarbazide**

Following HE staining or the administration of Dragendorff reagent it could be observed that there are no differences in the structure or in the localisation of alkaloids among the control and dimedone or semicarbazide treated hairy roots independent of cultivation in dark or in light. The tissues cultured in 1000ppm dimedone or semicarbazide containing medium did not gave Dragendorff positive reaction reinforcing our data regarding alkaloid content of cultures.

Results of TUNEL reaction verified that neither in control nor in dimedone or semicarbazide treated

✍ **Effect of aminoguanidine**

The hydralazine derivative aminoguanidine was administered in the liquid culture medium of *D. innoxia* hairy root clone ? 410 cultivated in dark in order to study the effects on the growth and tropane alkaloid production of tissues. This highly electrophyl molecule can react with bounded HCHO presented in cells.

Our results showed that the growth values significantly increased following the administration of 1ppm and 10ppm aminoguanidine exceeding the control values. However, higher amounts caused growth inhibition, especially the application of 1000ppm.

The highest hyoscyamine (0.17%), scopolamine (0.13%) and apoatropine (0.02%) content was measured following the administration of 10ppm and 100ppm aminoguanidine. These values were 2 or 3 times higher than in the controls. The highest alkaloid production – according to the alkaloid content – was calculated in tissues treated with 10ppm and 100ppm aminoguanidine which raise the possibility that these are the results of specific effects, just like the change of gene activation through DNA methylation.

✍ **Effect of hydralazine**

Hydralazine was added to the liquid culture medium of *D. innoxia* hairy root clone ? 410 cultivated in dark in order to investigate the effects on the growth and tropane alkaloid production of tissues. Hydralazine – just like aminoguanidine – is a hydrazine derivative and can react with bounded HCHO molecules of the cells.

Effect of hydralazine on the growth of tissues was similar to the effect of aminoguanidine. However, growth values slightly exceeded the control level following the administration of 1ppm hydrazine only. Higher amounts caused strong inhibition of cell proliferation, especially administration of 1000ppm.

10ppm and 100ppm hydralazine – which have negative effect on growth – unambiguously promoted alkaloid production. Highest hyoscyamine (0.024%), scopolamine (0.14%) and apoatropine (0.03%) content was measured in cultures treated with 10ppm hydralazine. These values significantly exceeded the control levels with 135% on the average. Administration of 100ppm hydralazine in culture medium caused around 100% increase of alkaloid content as well. It is important to remark that these treatments with hydralazine – in addition to the increase of alkaloid content – resulted a dramatic increase of alkaloid production. The hyoscyamine content of tissues was 6-7 times higher than the control values. For the effect of 1000ppm hydralazine we could not detect hyoscyamine, scopolamine or apoatropine in measurable quantities.

Summarizing our results it can be concluded that all the created *in vitro* cultures of *D. innoxia* (plant, callus and hairy root cultures) grew well but only the hairy root tissues synthesised tropane alkaloids in significant quantities (0.23% of hyoscyamine and 0.21% of scopolamine) which amount reached that of the *in vivo* plants. However, changes in culturing conditions (e.g. cultivation in light) or in the composition of culture medium (e.g. sucrose and $MgSO_4$ content) provide possibilities for

further improvement of alkaloid production of hairy root clones ?410 and ?415 producing hyoscyamine and clone ? 411 producing scopolamine as main alkaloid.

Investigation of the effect of abiotic stresses (administration of dimedone, semicarbazide, aminoguanidine or hydralazine) showed that the growth, tropane alkaloid production and measurable HCHO level of tissues could be changed significantly. Small amounts of these molecules (1-100ppm) generally resulted significant increase of biomass and tropane alkaloid production raising the possibility of specific effects (e.g. change in gene expression). However, administration of high amounts (1000ppm) resulted dramatic decrease of growth and alkaloid production.

Morphology of *in vitro* cultures investigated were consistent with bibliographical data. According to our observations it can be established that different culturing conditions (light, dark, abiotic stresses) did not influence the basic structure of tissues.

In the cells of *D. innoxia* callus cultures and of the roots of *in vitro* plants we could represent the occurrence of DNA fragmentation by TUNEL reaction indicating apoptotic processes. In the cells of 4 week-old hairy roots (clone ? 410, control or treated with dimedone or semicarbazide) we could not detect DNA fragmentation. It seems that the development of hairy roots might deviate from the previously investigated *in vitro* cultures.

PUBLICATIONS IN THEME OF THE Ph.D. THESIS

Articles:

1, **László, I.,** Szoke, É. and Tyihák, E. (1998) Relationship between abiotic stress and formaldehyde concentration in tissue culture of *Datura innoxia* Mill. Plant Growth Regulation, 25, 195-199.

2, Kiss, A.S., Galbács, Z., **László, I.,** Szoke, É., Galbács, G. (1998) A deutériumtartalom változásának biológiai, biokémiai hatásai. Múzeumi füzetek, az Erdélyi Múzeum-Egyesület Természettudományi és Matematikai Szakosztályának közleményei, 7, 72-76.

3, **László, I.,** Szoke, É., Németh, Zs., Albert, L. (1998) Plant tissue culture as a model for study of diversity in formaldehyde bounding. Acta Biologica Hungarica, 49 (2-4), 247-252.

4, Szende, B., Tyihák, E., Trézl, L., Szoke, É., **László, I.,** Kátay, Gy., Király-Véghely Zs. (1998) Formaldehyde generators and capturers as influencing factors of mitotic and apoptotic processes. Acta Biologica Hungarica, 49 (2-4), 323-330.

5, **László, I.**, Szoke, É., Tyihák, E., Szende, B. (2001) Programmed cell death in *Datura innoxia* Mill. cultures cultivated in light and in dark. Plant Growth Regulation, 33 (3), 231-236.

6, **László I.**, Szoke, É. (2002): Biotechnológiai módszerek alkalmazása terápiás szempontból fontos, növényi eredetu hatóanyagok eloállítására. Képzés egy életen át, II/10, 10-16.

7, **László, I.,** Szoke, É., Tyihák, E., Németh, Zs. and Albert, L. (2003): Identification and measurement of endogenous formaldehyde in *Datura innoxia* Mill. tissues by OPLC, HPLC and MALDI MS techniques. Proceedings of the International Conference on Medicinal and Aromatic Plants, Part II. Acta Horticulturae, **597**, 265-270.

8, Hank, H., **László I.**, Bálványos, I. and Tóth, E. (2003): Effect of magnesium on the growth and alkaloid production of hairy root cultures. Proceedings of the International Conference on Medicinal and Aromatic Plants, Part II. Acta Horticulturae, **597**, 271-274.

Abstract in journal:

1, **László I.,** Szoke É. and Tyihák E. (1998) Effect of Magnesium on the Methylation of Alkaloids in *Datura innoxia* Mill. Tissue Cultures. Magnesium Research, 11, 236-237.

Chapters in books:

1, Kiss, A.S., **László, I.,** Szoke, É., Galbács, Z. and Galbács, G. (1997) The Effect of Deuterium Depleted Medium on Plant Tumors. In: Magnesium: Current Status and New Developments (Theophanides, T. and Anastassopoulou, J. editors) Kluwer Academic Publishers, Dordrecht, Boston, London, 81-84.

2, **László, I.,** Szoke, É., Németh, Zs., Kursinszki, L., Albert, L., and Tyihák, E. (1998) Effect of Magnesium on the Methylation of Alkaloids in *Datura innoxia* Mill. Tissue Cultures. In: Magnesium: Magnesium and Interaction of Magnesium with Trace Elements (Kiss, A.S. editor) Hungarian Chemical Society, Budapest, Hungary, 353-357.

3, Szoke, É., **László, I.,** Liszt, K. (2001) A mákalkaloidok képzodése szövettenyészetekben (a mák alkaloidjainak *in vitro* bioszintézise). In: Magyarország Kultúrflórája, A mák (Papaver somniferum L.). (Sárkány, S., Bernáth, J., Tétényi, P. editors) Akadémiai Kiadó, Budapest, 135-154.

Conferences (lecture, poster):

1, Szoke, É.**,** Lemberkovics, É. and **László, I.** (1995) Bioactive Compounds of Chamomile Hairy Root Cultures. 43rd Ann. Congr. on Med. Plant Res. Abstracts, p. 40. Halle (Germany).

2, Szoke, É.**,** Lemberkovics, É., Troilina, J. and **László, I.** (1995) Essential Oil Formation in Chamomile Hairy Root Cultures. 26th International Symposium on Essential Oils, Abstracts, p. 52. Hamburg (Germany).

3, Kiss, A.S., **László, I.,** Szoke, É., Galbács, Z. and Galbács, G. (1997) The Effect of Deuterium Depleted Medium on Plant Tumors. VIII. International Symposium on Magnesium, Book of Abstracts. p. 26. Heraklion, Crete (Greece).

4, **László, I.** and Szoke, É. (1997) Change of Formaldehyde Level in Tissue Culture of *Datura innoxia* Mill. For Abiotic Stresses. Stress of Life, International Congress of Stress, Abstracts, p. 186. Budapest.

5, **László, I.,** Szoke, É. and Tyihák, E. (1997) The Effect of Abiotis Stresses on Formaldehyde Level in *Datura innoxia* Mill Tissue Culture. Second International Symposium on Natural Drugs, Abstracts p. 144. Maratea (Italy).

6, **László, I.,** Szoke, É. and Tyihák, E. (1997) Change of Formaldehyde Level in Tissue Culture of *Datura innoxia* Mill. For Abiotic Stresses. VI. Semmelweis Science Fair, Abstracts p. 38. Budapest.

7, **László I.,** Szoke É. and Tyihák E. (1998) Tropánvázas alkaloidok vizsgálata genetikailag módosított növényi szövettenyészetekben. Farmakokinetika és Gyógyszermetabolizmus Szimpozium, Abstracts p. 23. Mátraháza.

8, **László I.,** Szoke É. and Tyihák E. (1998) Effect of Magnesium on the Methylation of Alkaloids in *Datura innoxia* Mill. Tissue Cultures. 6th European Magnesium Congress, Book of Abstracts p. 79. Budapest.

9, **László I.,** Szoke É., Tyihák E. (1998) Abiotikus stresszkörülmények hatása *Datura innoxia* Mill. szövettenyészetek formaldehid tartalmára. Pécsi Fitoterápiás Napok, Abstracts p. 30. Pécs.

10, Szende B., Tyihák E., Trézl L., Szoke É., **László I.,** Kátay Gy., Király-Véghely Zs. (1998) Foemaldehyde generators and capturers as influencing factors of mitotic and apoptotic processes. 4th International Conference on the Role of Formaldehyde in Biological Systems, Abstracts p. 18. Budapest.

11, **László I.,** Szoke É., Németh Zs., Albert L. (1998) Plant tissue culture as a model for study of diversity in formaldehyde bounding. 4th International Conference on the Role of Formaldehyde in Biological Systems, Abstracts p. 26. Budapest.

12, **László I.,** Szoke É., Tyihák E., Kursinszki L., Németh Zs., Albert L. (1998) The influence of culturing conditions on *Datura innoxia* Mill. callus and genetically modified tissue cultures. 46th Annual Congress of the Society for Medicinal Plant Research, Abstracts C30 Vienna (Austria).

13, **László, I.,** Szoke, É., Tyihák, E., Kursinszki, L., Németh, Zs., Albert, L. (1998) The influence of culturing conditions on *Datura innoxia* Mill. callus and genetically modified tissue cultures. VII. Semmelweis Science Fair, Abstracts p. 31. Budapest.

14, **László I.,** Szoke É., Tyihák E. and Szende B. (1999) Programozott sejthalál vizsgálata növényi szövettenyészetek sejtjeiben. SOTE PhD Tudományos Napok '99 Abstracts p. 44-45. Budapest.

15, **László, I.,** Szoke, É., Tyihák, E. and Szende, B. (1999) Programmed cell death in plant tissue cultures cultivated in light and in dark. 2000 Years of Natural Products Research, Joint Meeting of: American Society of Pharmacognosy, Association Francaise pour l'Enseignement et la Recherche en Pharmacognosie, Gesellschaft für Arzneipflanzenforschung and Phytochemical Society of Europe, Book of Abstracts P 325 Amsterdam (The Netherlands).

16, **László, I.,** Szoke, É., Tyihák, E., Szende, B. (1999) Investigation of apoptotic processes in *Datura innoxia* Mill. tissue cultures. X. Symposium of Plant Anatomy in Hungary, Programme and Abstracts of Papers pp. 98-99. Debrecen.

17, **László, I.,** Szoke, É., Tyihák, E., Szende, B. (1999) Investigation of apoptotic processes in *Datura innoxia* Mill. tissue cultures. Gyógyszerészek Országos Kongresszusa IX., Congressus Pharmaceuticus Hungaricus XI., Programme and Abstracts p. 66. Siófok.

18, **László, I.**, Szoke, É., Tyihák, E., Szende, B. (2000) Biochemical and morphological investigation of Datura innoxia Mill. tissue cultures. 1st International Colloquium "Health, Environment and Natural Substances", Programme and Abstracts of Papers p. 80. Metz (France).

19, **László, I.**, Szoke, É., Kursinszki, L., Szende, B., Tyihák, E. (2000) Investigation of genetically modified and non-transformed Datura innoxia tissue cultures. The 6th European Congress of Pharmaceutical Sciences, Final Program p. 15. Budapest.

20, **László, I.**, Szoke, É., Szende, B. (2000) Effect of dimedone and semicarbazide on the growth and metabolism of *D. innoxia* tissue cultures. 5th International, Jubilee Conference on Role of Formaldehyde in Biological Systems, Scientific Program and Abstracts p. 7. Sopron.

21, **László, I.**, Kursinszki, L., Szoke, É., Tyihák, E., Szende, B. (2001) *Datura innoxia* Mill. szövettenyészetek biokémiai és morfológiai vizsgálata. Dr. Mozsonyi Sándor Alapítvány emlékülése Díjkiosztása és Tudományos Ülése, Program p.8. Budapest.

22, Bálványos, I., **László, I.,** Vida, K., Hank, H., Kursinszki, L., Tóth, E., Szoke, É. (2001) Effect of magnesium on the genetically modified *Atropa belladonna* cultures. 7th Hungarian Magnesium Symposium, Program and Abstracts pp. 13-14. Siófok.

23, Kiss, A.S., **László, I.,** Stefanovits-Bányi, É., Galbávs, Z., Szoke, É. (2001) Effect of magnesium on the growth of of plant tumors cultivated on culture media with reduced deuterium content. 7th Hungarian Magnesium Symposium, Program and Abstracts pp. 24-25. Siófok.

24, **László, I.,** Szoke, É., Kursinszki, L., Tyihák, E., Szende, B. (2001) Effect of dimedone and semicarbazide on the growth and metabolism of plant tissue cultures. World Conference on Medicinal and Aromatic Plants, Abstracts p. 164. Budapest.

25, Kursinszki, L., **László, I.,** Albert, L., Németh, Zs., Szoke, É. (2001) Determination of tropane alkaloids in plant samples using extrelut column and high-performance liquid chromatography. World Conference on Medicinal and Aromatic Plants, Abstracts p. 170. Budapest.

26, Hank, H., Vida, K., **László, I.,** Bálványos, I., Kursinszki, L., Tóth, E., Szoke, 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 p. 265. Budapest.

27, Hank H., **László I.,** Bálványos I., Kursinszki L., Vida K., Kovács Gy., Tóth E., Szoke É. (2002) Influence of culturing conditions on the primary and secondary metabolism of genetically modified *Atropa belladonna* L. tissues. PhD Tudományos Napok, P 34, Budapest.

28, Hank H., Bálványos I., **László I.,** Vida K., Kursinszki L., Kovács Gy., Tóth E., Szoke 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).

29, Hank H., Bálványos I., **László I.,** Vida K., Kursinszki L., Kovács Gy., Tóth E., Szoke É. (2002) Effect of magnesium on the growth and alkaloid production of hairy roots and reorganised plant cultures. Medical plant research and utilization 2002, Program and Book of Abstracts p. 92. Kecskemét.