DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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TROPANE ALKALOID PRODUCTION AND RIBOFLAVINE EXCRETION IN THE FIELD AND TISSUE CULTURES OF HENBANE (*HYOSCYAMUS NIGER* L.)

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Dissertation is accepted for the commencement of the degree of Doctor of Pharmacy on January 18th, 2006 by the Council of the Faculty of Medicine, University of Tartu, Tartu, Estonia

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Commencement: May 3rd, 2006

Publication of this dissertation is granted by the Faculty of Medicine, University of Tartu

ISSN 1024–395X
ISBN 9949–11–276–1 (trükis)
ISBN 9949–11–277–X (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr 128

To Aarne

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I–V):

I Pudersell, K., Vardja, R., Vardja T., Raal, A. Arak, E. Plant nutritional elements and tropane alkaloid production in the roots of henbane (*Hyoscyamus niger*). Pharmaceutical Biology 2003, 41, 4: 226–230.

II Vardja, T., Vardja, R., Pudersell, K., Tohver, A. Riboflavin excretion from the excised roots of *Hyoscyamus niger*. Pharmaceutical Biology 2004, 42, 4–5: 353–359.

III 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, 1, 14–24.

IV Pudersell, K., Kiho, K., Vardja, R., Vardja, T., Raal, A., Arak, E. Söötme koostise mõju koerapöörirohu (*Hyoscyamus niger* L.) juurekultuuridele. Eesti Rohuteadlane, 1998, 3, 3–8.

V Pudersell, K., Vardja, R., Vardja, T., Arak, E. Raal, A: Influence of iron and putrescine on the scopolamine production and riboflavine excretion in the root and hairy root cultures of henbane (*Hyoscyamus niger* L.). Proceedings of the 1st BBB Conference on Pharmaceutical Sciences, Siófok, Sept. 26–28, 2005.

ABBREVIATIONS

AAW	n-amyl alcohol/acetic acid/water
BA	benzyladenine
BAW	n-butanol/acetic acid/water
C_{10}	$C_{10}H_{20}$
2,4-D	2,4-dichlorophenoxyacetic acid
DMRT	Duncan' s Multiple Range Test
ELISA	Enzyme-Linked Immunoabsorbance Assay
GUS	β-glucuronidase
HPLC	High Perfomance Liquid Chromatography
IAA	indolylacetic acid
IBA	indolylbutyric acid
IEC	Ion Exchange Chromatography
Knop-M	Knop-M is modified Knop medium [Knop, 1865], containing in addition to the macronutrients of Knop medium also the micro-nutrients, vitamins and organic additives of MS medium.
LS	Linsmeyer-Skoog
MS	Murashige-Skoog
NAA	naphthaleneacetic acid
ND	not detected
NMR	Nuclear Magnetic Resonance
NPTII	kanamycine resistance
NT	Nagata-Takebe
PAW	pyridine-amyl alcohol-water
PC	Paper Chromatography
RIA	Radioimmunoassay
SH	Shenk-Hildebrandt
spp.	species
ЧДА	pure for analysis (in Russian)
T	Tritium (^3H)
T-DNA	transformation-DNA
TLC	Thin-Layer Chromatography
WI	water saturated with isoamyl alcohol
x-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide
YMB	Yeast-Mannitol

1. INTRODUCTION

Plants are a rich source for numerous medicinal substances [Wagner *et al.*, 1989]. Despite the fact that the number of synthetic medicinal substances on the drug market has grown significantly during the last decade, 25% of prescription medicines still contain one or more herb-derived substances, as their main constituent. In order to simplify processing and enhance production yield, many medicinal substances are currently being produced synthetically. However, in the case of certain medicinal substances, as with the majority of alkaloids, chemical synthesis has been found to be prohibitevly expensive and natural material is therefore the only economical source for such compounds [Oksman-Caldentey *et al*., 1996].

The alkaloids are the most numerous group of active herbal substances, there are known to be over 10000 alkaloids [Leete, 1990]. One of these groups is called tropane alkaloids.

Tropane alkaloids have significant medicinal importance as they are compounds with a variety of pharmacological effects. Atropine sulfate and scopolamine hydrobromide are being used in medicine. As tropane alkaloids belong to a group of natural medicinal substances, requiring production from natural sources due to their complex chemical structures [Oksman-Caldentey, Hiltunen, 1996] they are mainly produced by extraction from cultivated plant material. Atropine, a racemic mixture of DL-isomers of hyoscyamine, is formed from L-hyoscyamine during extraction and further isolation.

Tropane alkaloids are present in different species of the *Solanaceae* families *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia* [Murav'eva, 1991]. Within the family of *Hyoscyamus,* the plant species *Hyoscyamus muticus* and *Hyoscyamus albus* have been the most closely studied [Oksman-Caldentey, 1987; Oksman-Caldentey *et al*., 1987; Sauerwein *et al*., 1993]. *Hyoscyamus niger* (henbane), the only species from *Hyoscyamus* present as natural flora in Estonia has been relatively less studied.

Plant material may be collected from wild plants, however the simultaneous breeding of plants in a field culture enables direct, control and improve their growth more effectively. When breeding field cultures with the intention of studying hyoscyamine and scopolamine yield, it is very important to estimate the mass of different vegetative organs. For example the roots are the site of tropane alkaloid biosynthesis [Hashimoto *et al.*, 1991; Kanegae *et al.*, 1994; Suzuki *et al*., 1999; Nakajima *et al.*, 1999], whereas the leaves are used as a herb and the stems affect the transport of the tropane alkaloids.

There are several reasons for considering plant tissue cultures as an alternative to field cultures; they allow for a shortened growth period, eliminate the need for herbicides and pesticides as well as maintaining constant growth conditions and product quality [Oksman-Caldentey, 1986].

Hairy root cultures are very important in addition to common tissue cultures. The infection of plant cells for example, with *Agrobacterium rhizogenes* results in hairy root formation at the infection site. These hairy roots, produced with *A. rhizogenes* are characterized by rapid growth [Hamill *et al.*, 1986; Jung, Tepfer, 1987], genetic stability [Aird *et al.*, 1988] and long-term stability of *Ri*-transformation [Joao, Brown, 1994]. The hairy roots produced are able to generate more active substances than an infected plant organ [Kamada *et al.*, 1986; Christen *et al.*, 1989; Oksman-Caldentey *et al.*, 1989; Moyano *et al*., 2002; 2003; Bonhomme *et al*., 2000; Cusido *et al*., 1999; Zhang *et al*., 2004; Yoshimatsu *et al*., 2004].

Different aspects of biosynthesis and production of tropane alkaloids have been studied thouroughly [Hartmann *et al.*, 1986; Hashimoto *et al.*, 1987; 1991; Oksman-Caldentey, 1987; Oksman-Caldentey *et al.*, 1987; 1996; Leete, 1990; Walton *et al.*, 1990; Robins *et al.*, 1991; Kutchan, 1995; Rabot *et al.*, 1995; Pinol *et al.*, 1999; Jung *et al.*, 2001; Khanom *et al*., 2001; Medina-Bolivar, Flores, 1995; Rothe *et al*., 2001; Kang *et al*., 2004; Zhang *et al.*, 2004; Haekkinen *et al.*, 2005; Richter *et al.*, 2005; Mino *et al.*, 2005; Stenzel *et al.*, 2006].

The production of tropane alkaloids in the tissue cultures depends to a large degree on media composition [Dixon, 1985]. The effect of different factors, such as a different source of nitrogen, plant growth regulators and different growth conditions on the production of tropane alkaloids has been studied using tissue cultures [Oksman-Caldentey *et al.*, 1987; 1994; Biondi *et al.*, 1993; Rhodes *et al.*, 1989; Robins *et al.*, 1991; Tone *et al.*, 1997]. Different authors have studied the effect of the plant nutritional element calcium on tropane alkaloid production (Curtis *et al.*, 1995; Hilton *et al.*, 1995]. Pinol *et al.* (1999) have studied the effect of different ion concentrations of calcium in media on tropane alkaloid accumulation in hairy root cultures of *Datura stramonium* L. and found that a decrease in calcium concentration did not affect root growth, but did diminish peroxidase activity, which probably plays a role in the breakdown of products of secondary metabolism. At lower calcium concentrations the hairy root clones produced less hyoscyamine. Hilton *et al.* (1994) studied growth as well as sucrose and mineral ion consumption of hairy root cultures of *Datura stramonium* L., *Datura candida* & *aurea* L., *Datura wrightii* L., *Hyoscyamus muticus* L. and *Atropa belladonna* L. On the basis of the results it became clear that the cultures consumed all the ammonium and phosphate ions and some of the magnesium ions [Hilton *et al.*, 1994].

Among plant growth regulators indolylbutyric acid (IBA) is necessary for the growth of most root cultures [Robins *et al.*, 1995] by inducing cell division. The effect of putrescine, a hyoscyamine precursor [Leete, 1990], like the plant growth regulator itself has also been studied in different tissue cultures [Robins *et al.*, 1991; Bais *et al.*, 1999; Biondi *et al.*, 1990; Rugini *et al.*, 1988; 1993; Ballester *et al.*, 1999].

The changing of the media from root cultures of *H. niger* to yellow during a growth cycle in the dark was observed during experiments. The yellow colour

disappeared when cultures were grown under lights, but was maintained in cultures grown in the dark even after enzyme inactivation by heating. This enabled us to conclude that the compound of yellow luminecence, excreted into the media by the root cultures of *H. niger*, might be a flavin pigment.

The excretion of the riboflavine by the roots of intact tobacco plants was initially observed in 1958 [Pound, Welkie, 1958]. Later the same phenomenon was observed for several dicotyledonous plants, sugar beet, pepper and lettuce, during iron deficency. The addition of iron into liquid growth medium caused a decrease in the excretion of riboflavine [Welkie, Miller 1989]. A change of the iron content of the media cause a change in growth conditions and riboflavine excretion, but the same was not observed for the growth of the roots [Welkie, 1995]. The increase in the content of manganese of the medium also caused an increase in riboflavine excretion. It has been suggested that excessive manganese in the medium causes an iron deficiency [Shimizu *et al.*, 1998]. A salt stress did not change the excretion of riboflavine from the roots of the intact plant [Welkie, Miller, 1992].

2. REWIEW OF LITERATURE

2.1. Henbane

2.1.1. Plant description

Henbane (*Hyoscyamus niger* L., *Solanaceae*) is an annual, biennial or perennial herbal plant [Bown, 1995].

The native location of the plant is southern Europe, from where it has spread to many countries. In Estonia the henbane is present along side cattle-runs, roads, rubbish and fences [Tammeorg *et al*., 1984].

The radial leaves are being situated as a rosette around the neck of the root. These are elongated and egg-shaped, being over 30 cm long, pointed and stalky with toothed edges. They have a greenish-gray colour and are covered with sticky hairs. [A Modern Herbal, 2005]. The plant has unpleasant odour and is covered with long, sticky hairy glands and shorter, two-, three- or four-cell simple hairs. The root is pulpy, furrowed slantwise, branched and has a thickened neck at the root. The stems are 20–100 cm high, erect and dull-edged. Leaves are elongated and egg-shaped, pinnately split, pulpy, greyish-green with the lower leaves stalked and the upper leaves in half amplexicaul. Flowers are sitting, situating in swirls at the top of the stems. The crown is bell-shaped, dirty yellow, with violet reticulated veins and a reddish-violet throat. The fruit is a bulged capsule. Seeds are greyish-brown, reniform, flat [ENSV Floora, Kd.4, 1969].

The leaves of henbane are used as a herb (*folium Hyoscyami*) [Gosudarstvennaja farmakopeja SSSR, 1990; Tammeorg *et al.*, 1984; Muravj'eva, 1991; Atlas lekarstvennyh rastenii, 1962].

2.1.2. Chemical composition

All vegetative organs of the plant contain tropane alkaloids. The roots contain 0.15–0.18%, leaves 0.10%, stems about 0.02% and seeds about 0.10% of alkaloids [British Herbal Comp., 1992]. The annual form of *H. niger* contains far less active substances than the biennial form [A Modern Herbal, 2005]. The main alkaloids are hyoscyamine and scopolamine and in addition a small amount of atropamine (apoatropine) and cuscohygrine. The glycosides have also been found within the plant along with flavonolglycosides, such as quercitrine, spireoside, hyperoside and rutine, [Sokolov, 1990] and also the flavonoid aglycones quercetine and kempferole [Duke, 2005]. The plant also contains in small amounts some volatile amines; choline, methylpyrroline and pyridine. Esculetine and other coumarins, atroscine, chlorogenic acid, gamma-aminobutyric acid, proteins, resin, scopetole, scopine, scopoline, tetramet-

hylputrescine, several mucilagines, as well calcium, magnesium, potassium, sodium, iron, manganese, copper, zinc and arsenic have all been isolated from the plant [Duke, 1992]. Seeds contain 34% fat oil [British Herbal Comp., 1992], and some free fatty acids (palmitic, oleinic, stearinic and myristinic acids) [Duke, 1992].

2.1.3. Medicinal importance

The alkaloids, hyoscyamine and scopolamine are compounds with a typical parasymphatetic effect. The medicinally used atropine sulfate, a mixture of DL-isomers of hyoscyamine (optically inactive), also belongs in this group. The biological activity of L-hyoscyamine is 2–2.5 times higher than that of atropine. In small therapeutical doses the substances in the first instance have a peripheral postganglial cholinergic blocking effect. At increasing doses a central effect on the higher nervous system is present. These alkaloids decrease the secretion of tears, saliva, gastric and intestinal glands and have the spasmotic effect on most smooth muscles. Their effect on the heart is a positive chrono-, dromo- and inotropic one. In the eyes the substances can cause mydriase and paralysis of accommodation muscles. Scopolamine has a better central efficacy. In comparison with other similar herbs (*Atropa spp.*, *Datura spp*. etc.) the henbane contains porportionally more scopolamine in its mixture of alkaloids, and because of which the herb is characterized by a sedative effect on the central nervous system [Martindale, 1996; Allikmets *et al.*, 1982].

Atropine is used as an antidote for intoxications with cholinomimetic substances. Atropine and m-cholinomimetics have a one-way antagonism with atropine readily superseding the cholinomimetics at their receptors.

Scopolamine has the same indications as atropine. Central, sedative, anti-parkinsonistic effects and effects against seasickness have led to scopolamine being used more [Allikmets *et al*., 1982].

The herb of henbane is used only per doctor's prescription as a sedative in parkinsonism and as a pain-killer. The oil extract of henbane (*oleum Hyoscyami*) is used topically for neurologic and rheumatic pains by rubbing into the skin [Blinova *et al.*, 1996; British Herbal Comp., 1992; Tammeorg *et al.*, 1984; Allikmets *et al.*, 1982].

2.2. Tropane alkaloids

2.2.1. Characterization and main structures of tropane alkaloids

There are over 150 known compounds which contain bicyclic heterocycle 8-azabicyclo(3.2.1)octane. The tropane alkaloids hyoscyamine, scopolamine and atropine also belong in this group. The structure of tropane alkaloids is based on the tropane ring, and is a methyl derivate of 8-azabicyclo(3.2.1)octane. Tropane alkaloids are esters of the aminoalcohols, scopine and tropine with tropic acid. Hyoscyamine is the ester of tropine and tropic acid. This is an optically active compound, occurring in plants as an L-isomer, but changes to the optically inactive racemate atropine during its isolation from plants. The other compound, scopolamine, is the ester of scopine and tropic acid, which is also optically active L-isomer. The optically inactive racemate of scopolamine is called atroscine. The appropriate formulas are represented in figure 1 [Leete, 1990].

2.2.2. Biosynthesis of tropane alkaloids

The amino acids, ornithine, arginine, lysine, asparagine acid, tyrosine and tryptophane occur as precursors of alkaloids in plants. The main reactions in the biosynthesis of alkaloids are carboxylation, oxydative desamination and trans-amination [Blinova *et al*., 1996].

2.2.2.1. Biosynthesis of 1-methyl-Δ^1-pyrrolinium salt

Biosynthesis of the 1-methyl-Δ^1-pyrrolinium salt is the intermediate stage in tropane alkaloid biosynthesis, with ornithine and arginine known precursors at this stage. Acetic acid is the precursor of these amino acids in plants [Leete, 1990]. The experiments, performed on the basis of hairy root cultures of *H. albus,* demonstrated that ^{13}C-acetate inserts into the structure of tropane alkaloids in the C-3 position [Sauerwein *et al*., 1993]. The acetic acid gives α-ketoglutaric acid through the Krebs cycle. This gives glutamic acid, in the reduction of which the glutamic aldehyde is formed. The last gives ornithine, directly or over several intermediates [Leete, 1990]. At the same time studies performed on the basis of *Erytroxylum coca* and *Datura innoxia* have shown that 1-methylpyrrolidin-2-acetic acid does not act as a precursor in the biosynthesis of tropane alkaloids [Huang *et al*., 1996].

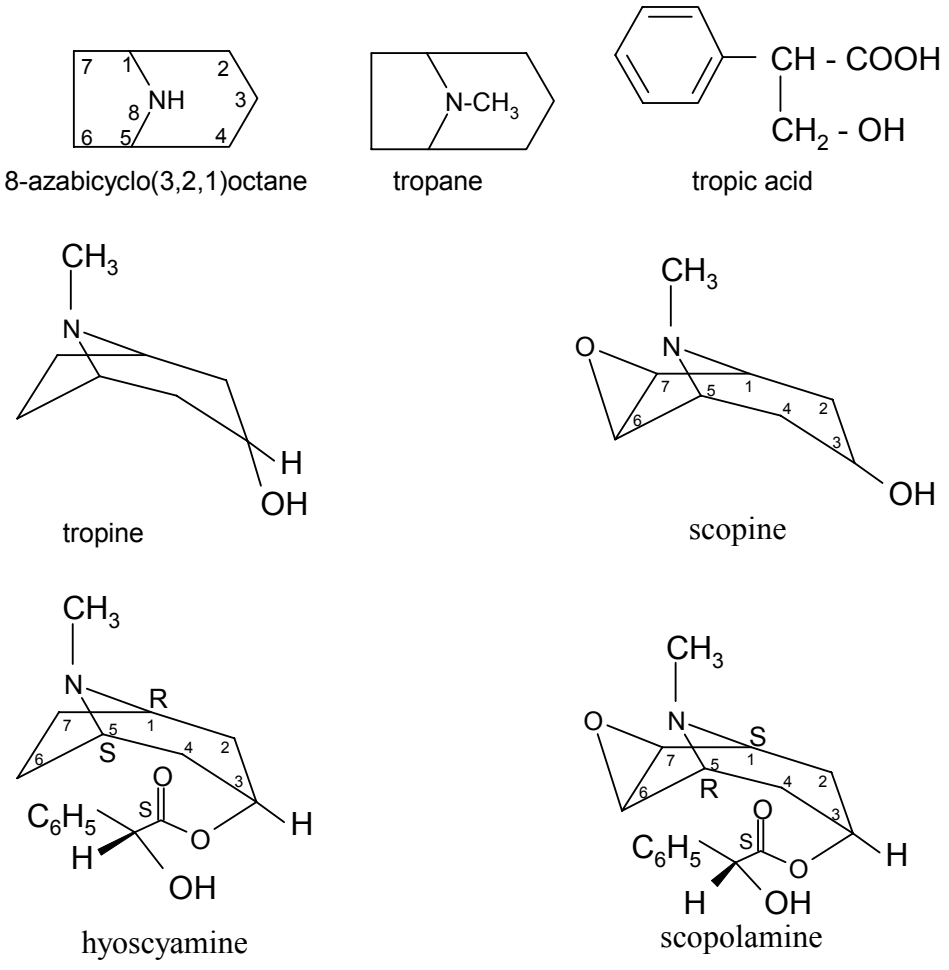


Figure 1. Main structures of tropane alkaloids.

The next important intermediate in the biosynthesis of tropane alkaloids is putrescine, which is the product of primary metabolism [Robins, 1993]. Ornithine or arginine may act as precursors of this compound in plants. Putrescine acts as a precursor in the formation of polyamines spermine and spermidine, regulating the cell functions and hydroxycinnamoyl putrescine [Smith, 1990]. "Binded putrescine" is a possible intermediate in the formation of putrescine *via* ornithine. Ornithine decarboxylase (coenzyme pyridoxal phosphate) occurs as the key enzyme in the formation of "binded putrescine". The N-metylation of putrescine is followed by decarboxylation (enzyme putrescine N-methyltransferase), which may also occur on the basis of "binded putrescine" [Leete, 1990].

Arginine is another possible precursor of putrescine; in this case the putrescine is formed by agmatine and N-carbomoylputrescine (key enzyme arginine decarboxylase) [Leete, 1990].

It was found that N-methylputrescine may be formed directly from ornithine by δ-N-methylornithine as well. The other possible precursor for δ-N-methylornithine is α-N-methylornithine. The aforementioned routes are more characteristic for biosynthesis of nicotine and other similar alkaloids and have little importance in the biosynthesis of tropane alkaloids [Leete, 1990; Walton *et al*., 1990].

The oxidative desamination of N-methylputrescine (enzyme N-methylputrescine oxydase) is thereafter an important stage in the formation of 1-methyl-Δ^1-pyrroliniumsalt. As a result of this reaction N-methylaminobutanale is formed. The iminiumsalt is formed through the cyclization of N-methylaminobutanale. The intermediate in this process is 2-hydroxy-1-methylpyrrolidine. In addition the minor constituents hygrine, cuscohygrine, 1-methyl-2-pyrrolidinone, 1-methylpyrrolidine and 1-methyl-Δ^2-pyrroline are formed as demonstrated by data obtained from different *Solanaceae* plants [Leete, 1990].

2.2.2.2. Formation of tropane ring

It has been found that not only C-3, but the C-2 and C-4 carbons in the tropane ring are derived from acetic acid [Leete, 1990]. This fact was interpreted as follows. As a result of condensation of two molecules of acetylcoenzyme A the acetoacetylcoenzyme A is formed. This β-ketothioester is condensed in the position C-2 with 1-methyl-Δ^1-pyrroliniumsalt. The ester is then formed and during the hydrolysis of which a hygrine-1'-carboxyl acid is formed. Following the decarboxylation of this β-ketoacid the hygrine is formed. It is suggested, that the tropane ring is formed from hygrine over 5-acetonyl-1-methyl-Δ^1-pyrroliniumsalt. The final cyclization in the result of the *Mannich* reaction results in tropinone (figure 2) [Leete, 1990].

2.2.2.3.Tropinone reduction

The noted stage is presented on the figure 3. Two enzymes – tropinone reductases I and II are taking part in the reduction of tropinone. Tropinone reductase I is giving tropine, which is taking part in the formation of hyoscyamine; reaction with tropinone reductase II is giving unwanted intermediate pseudo- or ψ-tropine [Dräger *et al.*, 1988].

2.2.2.4. Tropine esterification

Tropine or hydroxytropane may form esters with several natural acids, like formic, acetic, propanic, butanic and benzoic acids, also with m-hydroxybenzoic acid, phenyllactic acid, tiglic acid and naturally with tropic acid [Leete, 1990].

Phenylalanine is the precursor of tropic acid in plants. During the formation of tropic acid the following stages may be separated [Leete, 1990]:

phenylalanine → phenylpyruvic acid → phenyllactic acid → tropic acid

The stereochemical transformation of the atoms both in L- and D-phenylalanine during the formation of tropic acid is shown in figure 4 [Leete, 1990].

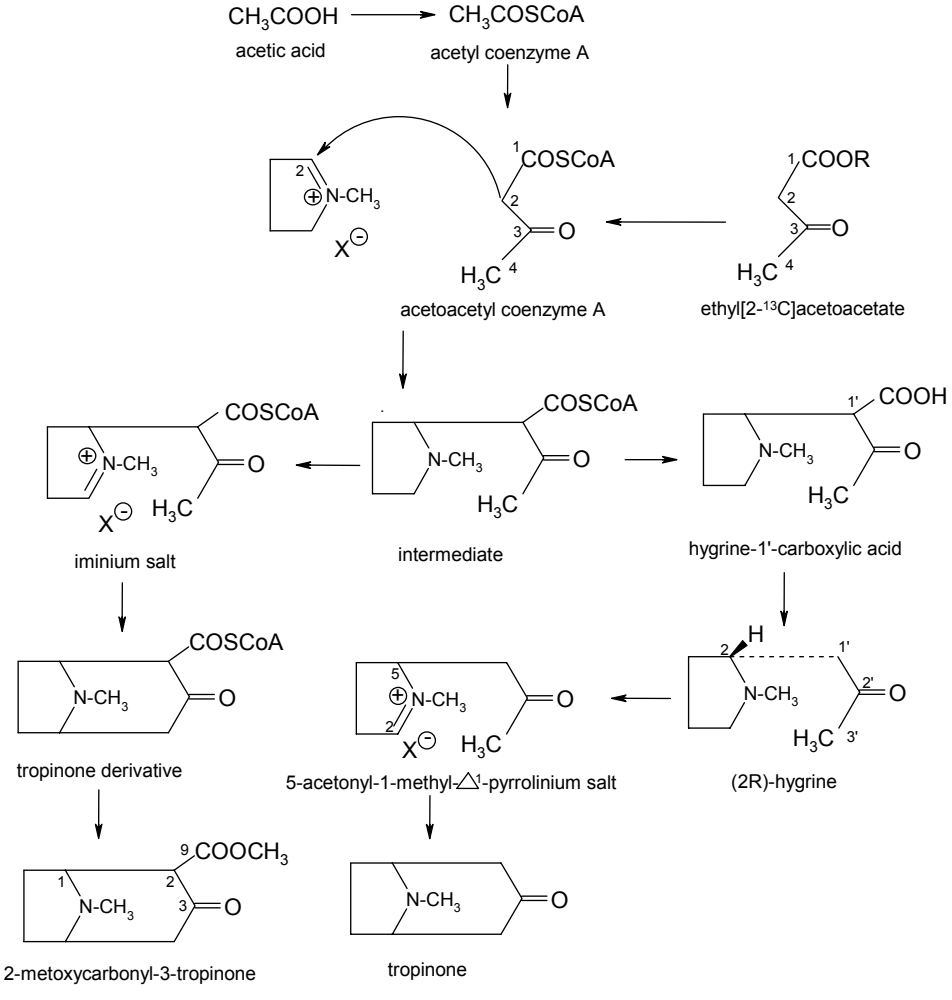


Figure 2. Formation of tropane rings

The precursor of minor aromatic amino acids is also usually phenylalanine, except for isoleucine, which is the precursor of tiglic acid. Tiglic acid is the precursor in the formation of tigloyl coenzym A, which along with acetyl coenzym A takes part in the esterification of pseudotropine [Rabot, 1995].

Hyoscyamine is formed through the esterification of tropine with tropic acid [Leete, 1995].

2.2.2.5. Formation of scopolamine

Previously it had been proposed that two possibilities occur in the formation of scopolamine: from the hyoscyamine over 6β-hydroxyhyoscyamine or over 6β-hydroxyhyoscyamine and 6,7-dehydrohyoscyamine (figure 5) [Hashimoto *et al.*, 1987].

The results of these studies have shown that the formation of scopolamine still does not involve dehydratation and that scopolamine is formed as hyoscyamine-6,7-epoxide by the dehydrogenation of 6β-hydroxyhyoscyamine with the aid of hyoscyamine 6β-hydroxylase (figure 5) [Hashimoto *et al*., 1987].

2.2.2.6. The enzymatic regulation and localization of biosynthesis of tropane alkaloids

The following enzymes, which participate in the biosynthesis of tropane alkaloids have been determined and isolated: ornithine decarboxylase, arginine decarboxylase, putrescine N-methyltransferase, N-methylputrescine oxydase, tropinone reductases I and II and hyoscyamine 6β-hydroxylase [Robins, 1993; Nakajima *et al.*, 1994; 1999; Suzuki *et al.*, 1999; Richter *et al.*, 2005; Ghosh, 2000].

The biosynthesis of both hyoscyamine and scopolamine is localized in the roots of the plant [Robins, 1993], whereas hyoscyamine 6β-hydroxylase, the enzyme, regulating the biosynthesis of scopolamine, is localized in the pericycle of the young roots only [Hashimoto *et al.* 1991].

The intensity of the biosynthesis of scopolamine is limited by the presence of hyoscyamine and the formation of 6β-hydroxyhyoscyamine is a key stage in the production of scopolamine. Similar key stages and processes have also been found in the production of hyoscyamine. The results of these studies only allow us to suppose that the formation of hyoscyamine through the esterification of tropine with tropic acid may be one such key action [Robins, 1993].

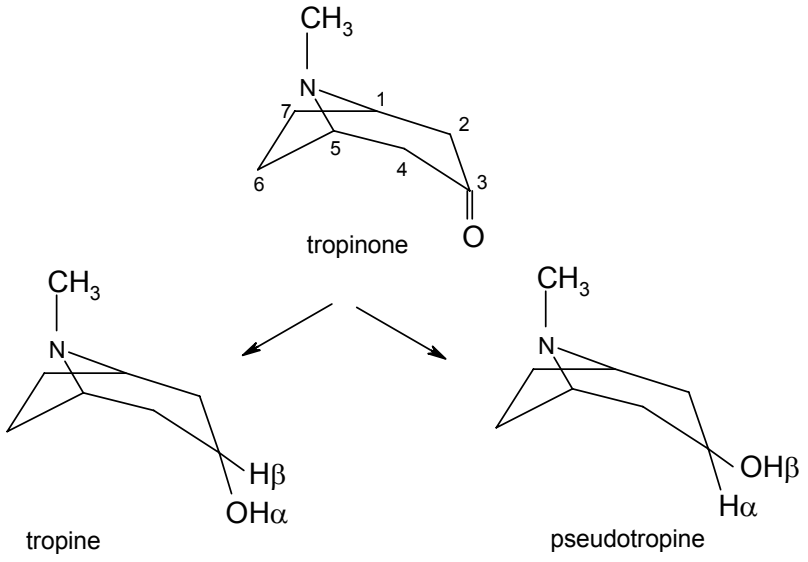


Figure 3. Tropinone reduction.

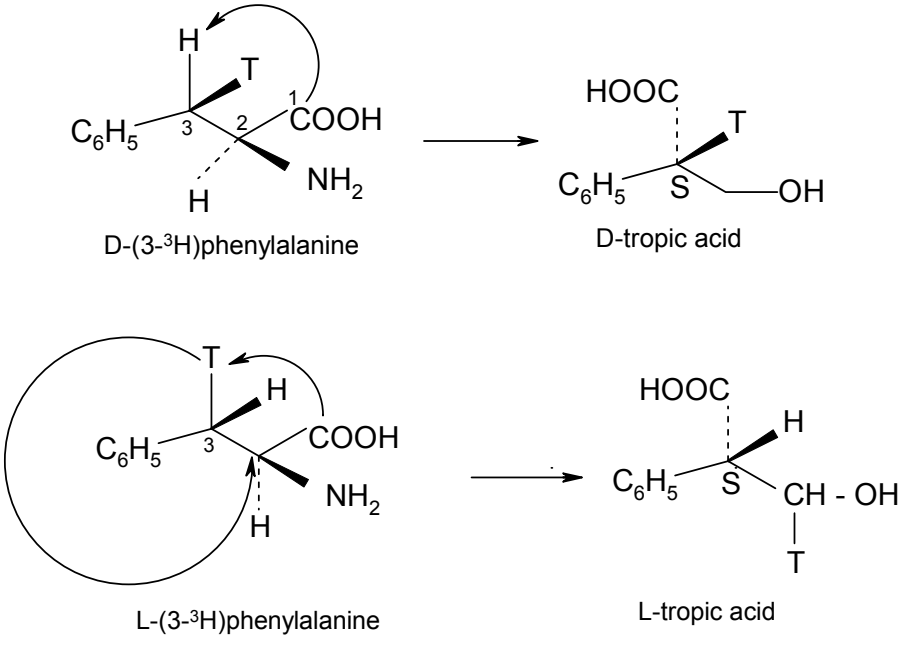


Figure 4. Stereochemical processes in the formation of tropic acid.

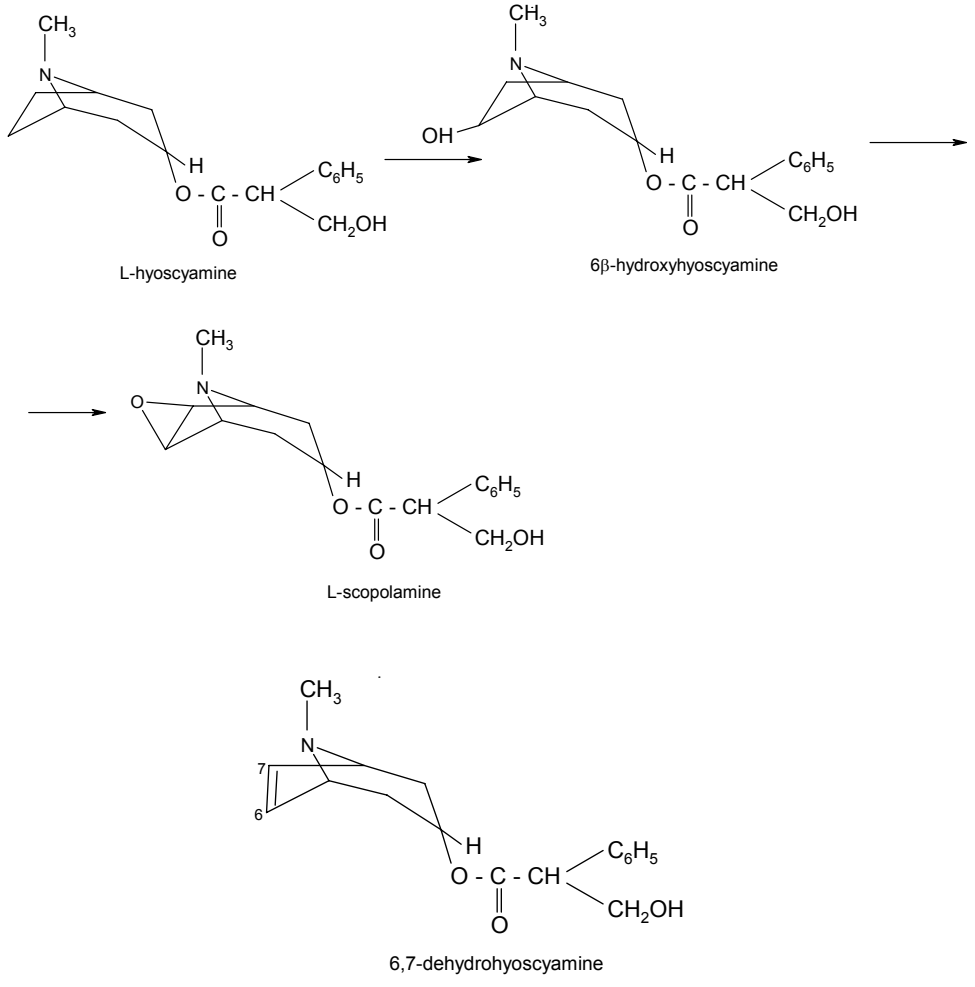


Figure 5. Biosynthesis of scopolamine.

2.2.2. Isolation and analysis of tropane alkaloids

In the qualitative determination of tropane alkaloids several sedimentation and colour reactives have been used (reactives of *Dragendorff*, *Mayer*, *Marquis* etc.), in addition to thin-layer chromatography, spectral analysis and other analytical methods (Ladygina *et al.*, 1983].

In the quantitative analysis of tropane alkaloids several analytical methods have been applied, including gravimetry, titrimetry, colorimetry, thin-layer chromatography, gas chromatography, high perfomance liquid chromatography,

immunological and biological analytical methods [Oksman-Caldentey, 1987; Walters, 1978].

When titrimetric analysis of tropane alkaloids is used it has been found necessary to clean the extract with the repeated exchange of the solvent after extraction of alkaloids with ether in the presence of concentrated ammonia [Ladygina *et al.*, 1983].

Using several physicochemical methods of analysis (different kinds of chromatography) it has been found that after the extraction of the tropane alkaloids with acidic [Witte *et al*., 1986; Hartmann *et al*., 1986] or alkaline [Witte *et al*., 1986; Ylinen *et al.*, 1986] solvent it is neccessary to clean the extract by exchange with the solvent [Witte *et al*., 1986] or by the column chromatography [Witte *et al*., 1986; Hartmann *et al.*, 1986; Ylinen *et al.*, 1986].

Immunological methods of analysis (ELISA, RIA) have appeared to be so selective, that there is no need for the preliminary cleaning of the analysed extract. Tropane alkaloids have been extracted using methanol prior to immunological analysis [Oksman-Caldentey, 1987].

2.3. Plant nutritional elements

2.3.1. Definition and characterization of plant nutritional elements

Plant nutritional elements are the chemical elements necessary for plant growth and development and for which none can be subsituted with any other element due to their specific functions. According to the previous definition the following nutritional elements have been previously identified: carbon, hydrogen, oxygen, nitrogen, phosphorus, sulphur, potassium, calcium, magnesium, iron, manganese, copper, zinc, molybdenum and boron [Kärblane, 1996].

At the moment and in addition to carbon, hydrogen and oxygen a further 17 chemical elements are primarly required in plant development [Loneragan, 1997].

Plant nutritional elements are necessary for the normal functioning of the protoplasma of plant cells, formation of the cell structures and cell development, also for the regulation of the metabolic processes in the cells. Many nutritional elements are also connected with catalytic processes in plants, contributing to enzymes as cofactors or acting as the inhibitors of the enzymes. This last role is carried out by micronutrients as well as some macronutrients (e. g. calcium, magnesium and potassium) have an importance in enzyme regulation also [Kinzel, 1982].

2.3.2. Physiological and biochemical importance of plant nutritional elements

Since this study has concentrated on the plant nutritional elements calcium, potassium, magnesium and iron, thus only these elements are discussed in the following review of literature.

2.3.2.1. Calcium

The content of calcium in the plants is in the range of 0.2–0.3% [Kuldkepp, 1994]. Calcium is a heavily recycled nutrient, forming immobilised salts with organic acids in leaves (e. g. with oxalic acid). In particular older leaves contain a lot of calcium [Kinzel, 1982]. Calcium is also present in plant seeds. For example, the seeds of *H. niger* contain 7.42 ppm of calcium [Duke, 1992].

Calcium is transported from the roots in the ionic form or in a complex compound into the leaves (Kinzel, 1982].

From the point of view of uptake calcium is an antagonist for potassium, sodium, ammonium, magnesium, iron, manganese, several heavy metals and especially for hydrogen. Due to this fact the excess of calcium in the earth somewhat decreases the harmful effect of toxic concentrations of some of the previously mentioned ions on the development of the plants. For example the studying the effect of calcium on the activity of malate dehydrogenase, have shown that calcium may effect the activity of the enzyme through it's effect on the quaternary structure of the enzyme [Kinzel, 1982]. Also it has been found experimentally using tissue culture of *H. muticus*, immobilized by the calcium alginate gel that calcium caused an increase in the production of sesquiterpenes, induced by *Rhizoctonia solani* [Curtis *et al.*, 1995]. The effects of calcium on the production of tropane alkaloid hyoscyamine have also been studied using hairy root cultures of *Datura stramonium* and has been shown that the ability of hairy root clones to produce hyoscyamine decreased with the decreased content of the calcium in the medium [Pinol *et al*., 1999].

2.3.2.2. Potassium

Plants contain on average 0.4–1.6%, of potassium, with more of this present in young, actively growing parts. Potassium is basicly present as chlorides, hydrocarbonates and -phosphates and also in salts of pyruvic, oxalic and citric acids [Kuldkepp, 1994].

The physiological and biochemical role of potassium in plants is very diverse. The following functions for potassium have been proposed:

1. As the one-valent ion, potassium penetrates readily into cells, increasing the permeability of cell membranes for other nutrients.
2. Potassium favours the hydratation of protoplasma and the movement of its particles thereby decreasing the viscosity of protoplasma.
3. Potassium favours the synthesis of saccharides, also intensifying the synthesis of lignine and and subsequent lignification of the cell walls [Kinzel, 1982].
4. Potassium also participates in the regulation of redox processes in plants. Experimentally it has been found that potassium intensifies the oxydative processes in the roots of rice (*Orysa sativa* L.), activating several reducers [Chen *et al*., 1997].
5. Potassium is an antagonist for calcium and magnesium.
6. Potassium activates many enzymes, participating in the photosynthetic processes [Miidla, 1984; Kärblane, 1996].

2.3.2.3. Magnesium

Plants contain 0.02–0.5% of magnesium per dry weight, depending on the plant species, organ, tissues and nutritional level [Kuldkepp, 1994].

In plants magnesium is present as salts (magnesium oxalate, phytine) and in the chlorophyll molecule where it has a specific and important role as a free molecule or in chelates (10–15% of the magnesium in the plant is contained in chlorophyll) [Kärblane, 1996].

The main functions of magnesium in plants are listed below [Kärblane, 1996]:

1. Magnesium binds pyrrole within the molecule of chlorophyll.
2. Mg^{2+}-ion is found in magnesium pectates with the pectins in the cell walls, being important in the formation and maintenance of the structure of cell walls.
3. Magnesium activates the processes of phosphorylation in photosynthesis, glycolysis, citrate cycle and redoxprocesses. The Mg^{2+}-ion activates phosphokinases and phosphotransferases in the transfer reactions of phosphoryl groups, enolases and carboxylases in glycolysis and dehydrogenases in citrate cycle. On the basis of cell suspension cultures of the common grape (*Vitis vinifera*) it has been shown that the Mg^{2+}-ion increases the activity of S-adenosylmethionine : cyanidine-3-glycoside 3'-O-methyltransferase [Bailly *et al*., 1997].

The cations of magnesium have the ability to be recycled within the plant [Kärblane, 1996].

The Mg^{2+}-ion is an antagonist for Ca^{2+}- and K^{+}-ions [Kinzel, 1982].

The metabolism of magnesium in the plant is in large part connected with the metabolism of nitrogen. Experiments have shown that in the addition to

ammonium nitrate, young plantlets of the spruce (*Picea abies* [L.] Karst.) have shown characteristics of magnesium deficiency, probably caused by the ruining of the N/Mg balance [Kölling *et al.*, 1997].

2.3.2.4. Iron

Iron is a heavy metal and its content in plants is very small [Kärblane, 1996], about 0.02% [Miidla, 1984]. Iron contributes to the composition of some important enzymes, taking part in the formation of chlorophyll and oxidative processes in the plants. Most of the iron in the plants (80–90%) is bound with organic substances and due to this has a reduced mobility. Calcium, as the neutralizer of the medium, depresses the uptake of the iron [Miidla, 1984]. In plants iron is present as chelates and at low levels as ions. The physiological function of the iron is based on its ability to form chelates and cause oxidation [Kärblane, 1996]. Iron is present in several prostetic groups with porphyrine. There is more known the compound of protoporphyrine IX with two-valent iron, named haem. The complex of three-valent iron and protoporphyrine is known as ferriprotoporphyrine or haemine. The ferroproteide haemoglobine, similar to blood haemoglobin, having hem in its prostetic group, is found in the root tubercles of *Papiliconaceous* plants. Iron is present in the composition of cytochromes, cytochrome oxidase, peroxidase and catalase. Iron is localized in the ferredoxine of chloroplasts and takes part in the changing of its oxidation step in the enzyme reactions in the synthesis of chlorophyll, especially in photophosphorylation. Mitochondrial and nuclear structures are relatively rich in iron [Miidla, 1984]. Iron is also important in the regulating of the redoxprocesses in the plants [Kärblane, 1996].

Plants uptake iron both in cations (Fe^{2+}) and chelates (e. g. Fe^{3+}-chelates).

2.3.3. Isolation and analysis of plant nutritional elements

For the isolation of plant nutritional elements from plant material the incineration of the plant material, and also, if necessary, the treating of the ash with hydrochloric acid or other chemical substances, have been applied. Several methods have been applied for the determination of plant nutritional elements in plant material, mainly X-ray diffractometry [Arak *et al*., 1996], chromatography [Hilton *et al*., 1995], spectrophotometry, spectral analysis, polarography and isotope analysis as well as potentiometry and several other chemical methods (gravimetry, complexonometry) [Reimets *et al.*, 1970].

2.4. Plant tissue cultures

The first tissue culture, based on the herb (*Catharanthus roseus* L.) was obtained in 1945. In 1947 it was shown that the tissue cultures of henbane (*Hyoscyamus niger* L.) are able to produce tropane alkaloids – hyoscyamine and scopolamine. In 1957 the same data were obtained using tissue cultures of *Atropa belladonna* L. From the point of view of manufacturing of medicinal substances, a greater interest against tissue cultures has arisen since the 1950s, with the publication of first articles discussing the possibility for the application of tissue cultures in the manufacturing of medicinal substances such as plant secondary metabolites (Volossovič, 1970]. On the basis of tissue cultures several active substances (from the alkaloids, e. g. hyoscyamine, scopolamine, solasodine, nicotine etc.) have been studied in several plant species (e. g. *Atropa* spp., *Hyoscyamus* spp., *Duboisia* spp., *Scopolia* spp., *Nicotiana* spp., *Dioscorea deltoidea* L., *Dioscorea* spp. etc.) [Volossovič, 1970; Oksman-Caldentey, Hiltunen, 1996].

Experiments with suspension cultures of *Ammi visnaga* L., *Digitalis* spp., *Datura* spp., *Melilotus officinalis* L. and *Catharanthus roseus* L. have shown that the ability of the plant cells to synthesize characteristic alkaloids, steroids, coumarins and furocoumarins is maintained in the tissue culture. In some cases, e. g. in the tissue culture of *Datura metel* L. the production of alkaloid hyoscyamine was even higher, when compared with the intact plant.

Usually plant tissue culture produces the same active substances as the plant used for the explant. In some cases, e. g. in *Digitalis lanata* L. and *Digitalis purpurea* L. the tissue culture was able to also produce substances not synthesized and or present in the parent plant [Volossovič, 1970].

2.4.1. Common procedures and media in the maintenance of plant tissue cultures

When working with tissue cultures it is necessary to sterilize all the explants and ensure sterile and constant growth conditions (constant temperature, lighting and shaking regimen along with the composition of the medium) [Gamborg *et al.*, 1975, Dixon, 1985, Oksman-Caldentey, 1987, Life technologies™ Catalogue, 1994].

Several media are used for the *in vitro* maintenance of plant tissue cultures, including root cultures. There are also other media including MS [Dixon, 1985; Gamborg, 1975] and B5 [Gamborg, 1975] media. In the growing of plant tissue cultures also for example SH [Dixon, 1985] and NT media are used [Oksman-Caldentey, 1987; Oksman-Caldentey *et al.*, 1987]. The main composition of these media are relatively similar, containing mineral salts and organic substances, which may be divided into 6 main groups: inorganic macronutrients,

inorganic micronutrients, iron salts, organic compounds and plant growth regulators [Dixon, 1985].

The inorganic salts, K^+- and NO_3^--ions, are necessary at concentrations of 20–25 mM. The presence of NH_4^+-ions in the medium is not absolutely necessary. Ammonium may cause the perishing of the cell in the concentration over 8 mM. The suitable concentration of PO_4^{3-}-, SO_4^{2-}- and Mg^{2+}-ions is 1–3 mM.

Recommended micronutrients are I, B, Mn, Zn, Mo, Cu, Co and Fe. The latter is recommended to be used as a chelate, binded with Na_2EDTA.

With regards to the organic compounds, then sucrose and glucose are regarded to be the most suitable, at a concentration of 2–4% [Dixon, 1985].

2.4.1.1. Vitamins

Of the vitamins only thiamine is absolutely necessary. Recently it has been found that pyridoxine and nicotinic acid as well myoinositole also improve cell growth. Riboflavine is also a significant constituent of some media [Vardja, 2001].

The main structure of riboflavine or vitamin B_2 is the core flavine. In addition its structure also contains two methyl- and reduced ribose groups [Talvik, 1996].

Riboflavine is a relatively thermostabile, fluorescent substance, soluble in water. Under the influence of light it readily gives off free radicals [Zilmer *et al.*, 2001].

Riboflavine is synthesized in all tissues of plants taking part in the composition of respiratory enzymes in plants. Enzymes, containing a derivative of riboflavine as their coenzyme, are termed flavoproteins [Miidla, 1984].

The excretion of riboflavine from intact plants of tobacco has been observed since 1958 [Pound, Welkie, 1958]. The same phenomenon was observed in several dicotyledonous plants (sugar beet, lettuce, pepper) due to iron deficiency. The excretion of riboflavine decreased upon addition of iron into the media. [Welkie, Miller, 1989]. The excretion of riboflavine increased also in response to the addition of manganese into the medium, which probably caused the iron deficiency [Shimizu *et al.*, 1998].

2.4.1.2. Plant growth regulators

Auxines (2,4-D, NAA, IAA, IBA) and cytokinines (chinetine, BA) have been used more often with the purpose to improve the plant growth [Vardja, 2001]. On the basis of some data the putrescine might be the plant growth regulator as well [Bais *et al.*, 1999; Robins *et al.*, 1991].

IBA is a synthetic auxin, containing butyric acid in the lateral chain of its indole structure. IBA decomposes due to high temperature and light. At 121 ºC 80% of IBA decomposes [Vardja, 2001]. Robins *et al.* (1995) have described the favouring effect of IBA on the cell division and depression of the production of tropane alkaloids in the root cultures of *H. niger*, maintained in the LS medium [Robins *et al*., 1995].

Putrescine is 1,4-butanediamine [Talvik, 1996] and one of intermediates in the biosynthesis of tropane alkaloids and at the same time one of the products of primary metabolism in the plants. [Robins, 1993]. The precursors of putrescine in plants may be both ornithine and arginine. On the basis of data from the literature putrescine has been added to rooting *in vitro* media of some woody plants, with the purpose of getting of better results [Biondi *et al.*, 1990; Ballester *et al.*, 1999; Rugini *et al.*, 1988; 1993]. Putrescine, added to the medium of hairy root cultures of chicory, stimulated the synthesis of inhibitors of polyamine synthesis and at the same time improved the growth of the roots [Bais *et al.*, 1999]. Putrescine and agmatine depressed the accumulation of tropane alkaloids in the hairy root cultures of *Datura stramonium* [Robins *et al.*, 1991]

Other organic additives, for example protein hydrolysate, yeast extract, malt extract and coconut milk have also been used [Gamborg, 1975].

pH of the media is regulated to 5.5–5.8, using 0,2 N solution of KOH or HCl [Gamborg, 1975].

From the substances in lower concentrations the preparation of concentrates (microelements, vitamins, plant growth regulators) is recommended [Dixon, 1985; Gamborg, 1975].

In the table 1 the compositions of some widely used media have been presented.

2.4.2. The problems of productivity of plant tissue cultures

There are several reasons, why only sanguinarine, rosmarinic acid, berberine, saponines of ginseng and scopolamine are still being produced industrially [Oksman-Caldentey, Hiltunen, 1996]. First of all, the biosynthesis of active substances in the plant cells has a reciprocal relationship with cell growth. The level of the synthesis of secondary metabolites is minimal during stages of intensive cell growth [Oksman-Caldentey, 1987].

Secondly, the productivity of tissue culture is usually lower, than in the plants used as explants. For example, if the content of scopolamine in the leaves of different species of *Hyoscyamus* family varied between 0.107–0.529% from dry weight, the content of scopolamine in the plant cell cultures of the same leaves was in the range of 0.00018–0.012% of dry weight. The productivity of callus and suspension cultures decreases upon consecutive multiplication,

decreasing, for example about 1000 times during the quadruple multiplication in the callus cultures of *Hyoscyamus muticus* [Oksman-Caldentey, 1987].

Usually the biosynthesis of plant secondary metabolites is localized into certain vegetative or generative organs, due to this it is not possible to achieve their production on the basis of plant tissue cultures, derived from the other plant organs. For example, in *Atropa belladonna* and *Rauwolfia serpentina* all the biosynthesis of alkaloids is localized in the roots [Volossovitš, 1970]. Some active substances (aethereal oils, some alkaloids) need specific differentiated glands or receptacles for their production and accumulation. The excretion of the active substances into the surrounding medium may also be a problem [Parr, 1989].

Table 1. Composition of MS, B5 and Knop-M media

Compound	Molecular mass	Content in the medium (mg/l)		
		MS	B5	Knop-M
Macronutrients				
NH_4NO_3	80.09	1 650	–	–
KNO_3	101.10	1 900	250	250
$Ca(NO_3)_2 \cdot 4H_2O$	236.15	–	–	1 000
$CaCl_2 \cdot 2H_2O$	147.02	440	150	–
$MgSO_4 \cdot 7H_2O$	246.50	370	250	250
KH_2PO_4	136.09	170	–	250
$NaH_2PO_4 \cdot 7H_2O$	137.98	–	150	–
$(NH_4)_2SO_4$	132.14	–	134	–
Micronutrients				
KJ	166.01	0.83	0.75	0.83
H_3BO_3	61.84	6.2	3.0	6.2
$MnSO_4 \cdot 4H_2O$	223.09	22.3	–	22.3
$ZnSO_4 \cdot 7H_2O$	287.55	8.6	2.0	8.6
$Na_2MoO_4 \cdot H_2O$	241.95	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	249.68	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$	237.93	0.025	0.025	0.025
$FeSO_4 \cdot 7H_2O$	278.00	27.8	27.8	27.8
Na_2EDTA	372.20	37.3	37.3	37.3
Vitamins and organic additives				
Myoinosite	180.16	100	100	100
Nicotinic acid	123.11	0.5	1.0	0.5
Pyridoxine	337.28	0.5	1.0	0.5
Thiamine	75.1	0.1	10.0	0.1
Glycine		2.0	–	2.0

2.4.3. Possibilities to improve the productivity of plant tissue cultures

A lot of experiments have been performed with the idea of achieving plant tissue cultures with high and stabile productivity. There are several possibilities.

1. Use of different media compositions. One- or two-stage culture system may be applied in the maintenance of plant tissue cultures. During the two-stage culture system maintenance of the cells occurs in the medium, favouring their growth. Thereafter the composition of the medium is changed with the aim to depress cell growth and favour the intensive biosynthesis of active substances [Bassetti *et al.*, 1997]. In using a one-stage culture system the composition of the medium has to be very well balanced, in order to achieve both intensive growth and biosynthesis of active substances in the plant tissue cultures.
2. Cell immobilization. The growth of the cells may be depressed by their immobilization into the network of polymers (agarose, agar-agar, gelatine, sodium alginate, artificial polymers etc.), directing the cells to synthesize more active substances [Curtis *et al.*, 1995].
3. Use of stress conditions. The effect of stress conditions on the growth and synthesis of active substances has been studied in several plant tissue cultures. For example the growth and scopolamine biosynthesis in suspension cultures of *Hyoscyamus muticus* has been studied under the following stress conditions: different content of sucrose in the medium (0–10 g/l), acidic (pH 2–3) or alkaline (pH 9–10) medium, high temperature (30–37°C), different concentrations of plant growth regulators (auxines, cytokinines) and different light regimens. On the basis of the results it may be concluded that the change in the growth conditions decreases the growth of the cells and favoures the biosynthesis of active substances [Oksman-Caldentey *et al*., 1987].
4. The excretion of the active substances to the surrounding medium. In addition to suitably high level of production (growth plus biosynthesis) of active substances, in some cases it is necessary to get the active substances excreted into the surrounding medium. It is for example, necessary, if the immobilized cell culture is continuously applied for the production of according active substances. One possibility is to apply the substances, increasing the leakage of cell walls. Such substances (e.g. dimethylsulphoxide) are usually toxic to the cells. Several biostimulative substances, like chitosan, some fungus-derived substances, heavy metals etc., have also a positive effect on the plant cells, increasing the production of active substances in addition to the improvement in plant cell wall permeability. [Sim, Chang, 1997].
5. Hairy root cultures. All the bacteria of genus *Agrobacterium* have the common characteristic – to transfer the part of DNA in their plasmid

(T-DNA) to the plant genome. The genus of *Agrobacterium* forms one class of the α-subdivision of *Proteobacteria* with the bacterial families of *Rhizobium*, *Phyllobacterium* and *Rickettsiae*. The family of *Agrobacterium* contains five species: *Agrobacterium rhizogenes*, *A. tumefaciens*, *A. radiobacter*, *A. rubi* and *A. vitis* [Otten *et al.*, 1992].

From the listed *Agrobacterium* species, two – *A. rhizogenes* ja *A. tumefaciens* are essential from the point of view of plant biotechnology. The hairy root cultures, based on the infection with *A. rhizogenes* seem to be very important in the improvement of the growth and biosynthesis of active substances in the world of tissue cultures. After insertion to the plant genome the genetical information of the DNA of *A. rhizogenes* results in the formation of tumour-like quickly multiplying roots. Such root cultures have found to be of much higher genetical and biochemical stability, if compared e.g. with conventional root cultures [Oksman-Caldentey, Hiltunen, 1996].

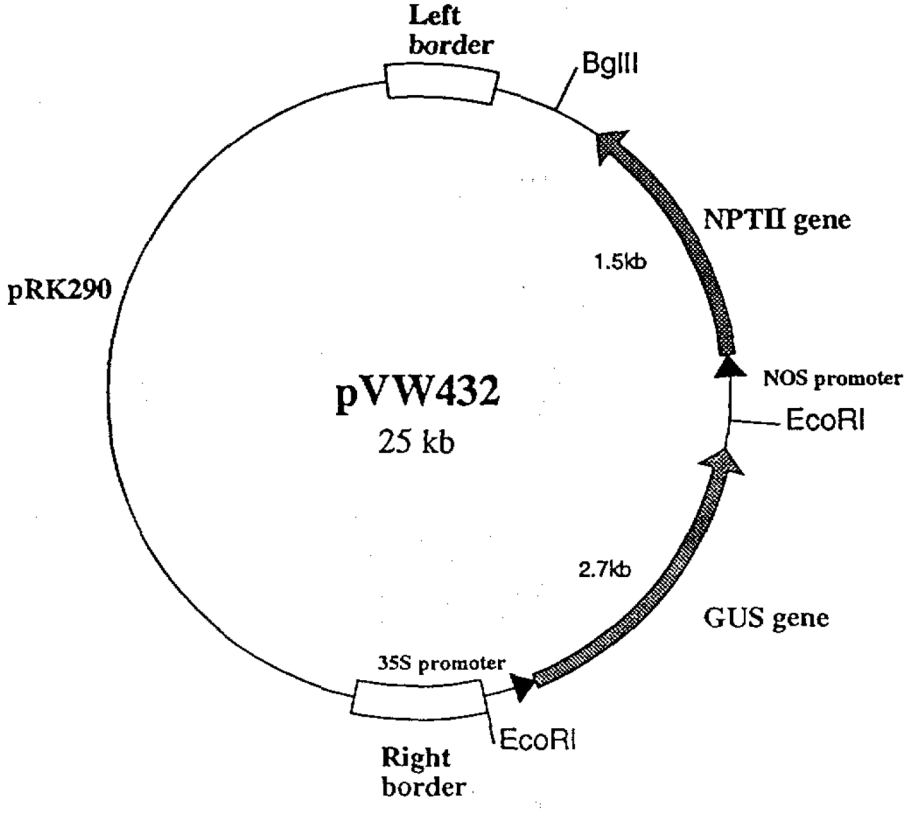


Figure 6. Scheme of the plasmid of pVW432 of *Agrobacterium* spp. [Ainsworth *et al.* 1995].

Figure 6 demonstrates the scheme of the plasmid of *Agrobacterium* spp. During infection the part of the DNA between the right and the left border, T-DNA, is transferred to the plant genome. The left and the right border and remaining part of plasmid are not transferred to the plant genome during infection, simply participating in the insertion of T-DNA into the plant genome. In the plasmid, presented on the figure, the part of natural T-DNA is replaced by two host genes – neomycine phosphotransferase gene and β-glucuronidase gene. The presence of the aforementioned genes enables one to distinguish transgenic tissue cultures and plants later.

The part of T-DNA, characteristic to the *Agrobacterium* may abstractly be halved, the part along with the the right border is T_R-DNA, T_L-DNA is found near to the left border [Oksman-Caldentey, Hiltunen, 1996].

The production of active substances in the hairy root cultures depends on their level of organization and its stability. The organization level of the roots depends on their linear growth, lateral branching and increase of root diameter due to of secondary growth.

Similarly to callus and suspension cultures the hairy root cultures also achieve their maximum of secondary metabolite production in the late stationary growth phase.

The pathogenic effect of *Agrobacterium* spp. on the different plant species is diverse, which needs to be analysed the planning of the according experiment. The virulence of different strains of *Agrobacterium* against the same plant species may also differ [Vanhala *et al.*, 1995]. It is also known that there may occur differences between the growth, accumulation of active substances and morphology of hairy root cultures, obtained by the different strains of *Agrobacterium rhizogenes* [Shanks, Bhadra, 1997]. It has been found that by the systematic selection of clones the stabile hairy root clones of high yield may be obtained. The growth intensity and the level of the production of active substances is different during the different growth stages [Rhodes *et al*., 1989].

Different hairy root clones may need different amounts of basic nutrients, so it may be necessary to optimize the suitable conditions for growth and production of active substances for every individual case separately.

2.5. X-ray diffractometric analysis

X-ray diffractometric analysis is a physicochemical method, based on the use of X-ray diffraction. The frame of the structure of crystalline substances occurs for natural frame of diffraction for X-rays. The X-rays reflect from the crystal only in certain position, where the rays, reflecting from several parallel atomic level, interfere [Azaroff *et al.*, 1968; Klug *et al.*, 1954].

2.5.1. Qualitative analysis

The X-rays reflect from the internal level surfaces of the atoms under the angles, characteristic for single substances, fixed by the according measuring equipment as spikes by the recording equipment, forming the diffractogram [Utsal *et al.*, 1993]. The distances between the atomic levels and the intensities of the reflexes can be found from the diffractogram of the certain substance. For the qualitative analysis the diffractometric analysis is performed at the maximum level of sensitivity, in order to determine all the sample constituents. The distances between atomic levels, calculated on the basis of obtained diffractograms, are compared with the according data from the catalogues [Utsal *et al.*, 1993].

2.5.2. Quantitative analysis

For the performance of quantitative analysis the diffractometric analysis is made at a sensitivity level where all the heights of the spikes of characteristic phases on the diffractogram remain measurable with maximum accuracy. On the basis of different studies and experiences the method of artificial mixtures or internal standard has been recommended. The method is based on the X-ray diffractometric analysis of the 1 : 1 mixture of sample and internal standard, the content of the substance in the sample is calculated on the basis of the relation between the intensities of the most intensive ie. typical reflexes of the studied substance and internal standard [Visser *et al.*, 1964]. In the primary studies the internal standard has been corundium (α-Al_2O_3) [Visser *et al.*, 1964], later TiO_2, ZnO, Cr_2O_3 and CeO_2 have been used as well [Hubbard *et al.*, 1977]. On the basis of the data of later studies quartz (SiO_2) has been suitable internal standard in the quantitative analysis of the several mixtures of the natural minerals [Utsal, 1987].

2.6. Chromatographic analysis

Chromatography is a physicochemical analytical method, based on the separation of the sample components in the result of the repeated distribution of the particles between the mobile and stationary phases.

2.6.1. Qualitative analysis

The constituents of the sample may be identified with the aid of the reference substances or their mixtures or on the basis of the dependence of the retention data of the constituent from its physicochemical characteristics [Gol'bert *et al.*, 1974].

2.6.2.Quantitative analysis

Chromatographic quantitative analysis normally uses the correlation between the peak area and injected amount of the substance. The reproducibility of the results depends to a large extent on the analytical conditions [Bidlingmeyer, 1992].

The pike is characterized by it's area, height and width versus half of the height [Gol'bert *et al.*, 1974].

In chromatographic quantitative analysis the results are found on the basis of internal normalization, absolute calibration or a method of internal standardization. In the latter case a certain amount of known substance (an internal standard) is being added to the sample mixture. With the internal standards a calibration curve is constructed on the basis of the results. Instead of absolute peak areas and concentrations their relationships to the appropriate values of internal standard are used in the calculations. The choice of the substance for internal standard is important to provide sufficient difference from the retention times of the studied substances and the sharpness of the peak. The peak should also be well measurable in the chromatographic conditions used [Gol'bert *et al.*, 1974].

2.6.3. HPLC

HPLC is one method of liquid chromatography, where the liquid mobile phase is directed through the compact stationary phase with the aid of high pressure [Snyder *et al*., 1988; Lim, 1987; Bidlingmeyer, 1992].

2.6.4. IEC

IEC is one method of HPLC, which is based on the resolved exchange of ions of the studied sample and the stationary polymeric resin, ion exhanger or ionite [IUPAC Compendium of Chemical Terminology, 1997]. The components of the mixture are separated due to of the ability of ion exchanger to bind the ions differently (difference in the diffusion coefficients). Ion exhangers may be

divided to cationites and anionites on the basis of their acidic-alkaline properties.

Cationites are acidic ion exchangers, containing for example the carboxyl- or sulphonylgroups, which protons may be exchanged with cations. Anionites contain the groups of alkaline properties, like the aminogroups [BL43 Biochemistry Techniques, 2005].

2.7. Determination of tropane alkaloids

Different authors have examined several methodologies for the HPLC determination of tropane alkaloids, from tablets [Walters, 1978], combined pharmaceutical preparations [Pennington *et al.*, 1982], biological fluids [Buch *et al.*, 1994; Nakanishi *et al.*, 1992], as from plant material [Roos *et al.*, 1986; Chao *et al.*, 1991; Stalcup *et al.*, 1991; Vitale *et al.*, 1995; Dräger, 2002; Zanolari *et al.*, 2003; Kletter *et al.*, 2004].

On the basis of the data from the literature the mostly used stationary phase was octadecylsilyl compound (C_{18}) [Walters, 1978; Pennington *et al.*, 1982; Buch *et al.*, 1994; Roos *et al.*, 1986; Chao *et al.*, 1991]. Acetonitrile or methanole were used for the organic component of mobile phase [Walters, 1978; Chao *et al.*, 1991; Auriola *et al.*, 1991; Nakanishi *et al.*, 1992; Pennington *et al.*, 1982; Roos *et al.*, 1986]. The other components of mobile phase were acidic buffer solutions, including solutions of octanesulphonic acid (pH 3.5) [Walters, 1978], tetramethylammoniumphosphate (pH 2.0) [Pennington *et al.*, 1982], acetic acid [Roos *et al.*, 1986] and sodium phosphate buffer (pH 2.5) [Nakanishi *et al.*, 1992]. At the same time the alkaline ammonium acetate buffer solution has been used as well [Auriola *et al.*, 1991].

The asymmetry of the spikes of analysed components has been noted [Roos *et al.*, 1986]. This may be caused by the free silanol groups on the stationary phase. For the blocking of free silanol groups on the stationary phase, 0.0033% triethylamine is added to the mobile phase.

The UV-detection has been used in the part of the noted cases. Detection has been made on the wavelengths of 254 nm [Pennington *et al.*, 1982], 230 nm [Walters, 1978], 220 nm [Pennington *et al.*, 1982] and 210 nm [Nakanishi *et al.*, 1992]. Using of the wavelength of 230 nm resulted in the sensitivity of <0.5 μg of alkaloid per 50 μl injected solution [Walters, 1978], 220 nm enabled to determine until 0.02 μg of alkaloid per 20 μl injected solution [Pennington *et al.*, 1982]. On the wavelength of 210 nm 0.25–0.34 mg of atropine in the gastrointestinal drugs was determined [Nakanishi *et al.* 1992]. During the last years the detections by ^{1}H- and ^{13}C-NMR-spectroscopy [Kletter *et al.*, 2004], mass-spectrometry [Auriola, 1991; Vitale *et al.*, 1995] and nuclear magnetic resonance spectroscopy [Zanolari *et al.*, 2003] have been preferred. Use of an

ion pair containig mobile phase resulted in the sensitivity for atropine 200 ng/ml [Buch *et al.* 1994], in determination in blood serum and albumin solutions.

Several compounds of alkaline nature have been used as internal standards in the quantitative determination, like procaine [Buch *et al.*, 1994], lidocaine hydrochloride [Walters, 1978] and theophylline [Pennington *et al.*, 1982].

2.8. Determination of nutritional elements

The X-ray diffractometric analysis of plant material cannot be performed without preliminary processing due to the organic matter, which gives a diffusal maxium on the diffractogram and does not allow us to identify the other components. The organic matter may be removed by the preliminary thermal processing of the sample on the sufficiently high temperature [Arak *et al.*, 1996].

The composition of asbest dust in the earth and acricultural crops has been studied on the basis of X-ray diffractometry and polarisation microscopy. It has been found that the dust contains magnesium, calcium, chromium and nickel [Czuba *et al.*, 1992]. The effect of water vapour on the structure of magnesium stearate has been studied by the X-ray diffractometry [Bracconi *et al.*, 2003].

The content of inorganic anions and organic acids in the beverages has been determined by ion exchange chromatography. The same method has been applied for the determination of sulphates, nitrates, chlorides, ammonium, sodium potassium, calcium and magnesium in the different samples. Ion exchange chromatography has also been applied for the estimation of the content of calcium and magnesium in the human blood serum [Thienpont *et al.*, 1994]. The content of calcium and magnesium in the water samples has been studied by the high perfomance liquid chromatography, using graphite-based stationary phase with o-chresolphthalein complexone containing mobile phase [Puall *et al.*, 1997].

3. AIM OF THE STUDY

The aim of the present study was to investigate the effect of plant nutritional elements, including calcium, magnesium, potassium, iron as well as plant growth regulators such as indolylbutyric acid and putrescine, on tropane alkaloid production and riboflavine excretion in *H. niger*.

The following specific tasks were established on the basis of this aim:

1. To study the baseline content of tropane alkaloids such as hyoscyamine and scopolamine as well as plant nutritional elements, including calcium, magnesium and potassium, in the vegetative organs of *H. niger* during different growth phases.
2. To study the production of tropane alkaloids and excretion of riboflavine by root and hairy root cultures of *H. niger*.
3. To study the effect of medium composition and levels of indolylbutyric acid on the production of tropane alkaloids and excretion of riboflavine by root and hairy root cultures of *H. niger*.
4. To study the effect of altering calcium levels in media and observing its effect on the production of tropane alkaloids as well as on the content of calcium, magnesium and potassium in root and hairy root cultures of *H. niger*.
5. To study the effect of altering magnesium levels in media and observing its effect on the production of tropane alkaloids as well as on the content of calcium, magnesium and potassium in root and hairy root cultures of *H. niger*.

4. MATERIALS AND METHODS

4.1. Study material

4.1.1. *In vivo* plant material

Plantlets from the seeds of biennial *H. niger* were cultivated in the experimental base of the Institute of Pharmacy, University of Tartu. The plant material was collected throughout summer in 1996 and 1997 in three different growth stages: young plant, flowering, and ripening stage. Roots, upper and lower leaves and stems were separated immediately after collection and the plant material was dried for 2 to 3 weeks at room temperature. Subsequently, the plant material was ground and sifted through the sieve giving the particle size <1 mm. In addition, loss of drying of samples was determined [European Pharmacopoeia, 2005].

4.1.2. *In vitro* plant material

The seeds of annual *H. niger* (gift from the Biocenter of Viikki, University of Helsinki) were used as the starting material for *in vitro* approaches.

The seeds were sterilized according to procedure by Gamborg [Gamborg, 1975]. Briefly, the seeds were moisturized with distilled water, containing 3 to 4 drops of *Tween 80*. After removing the solution the seeds were placed into the gauze bag containing 9% solution of chloride of lime and sterilized under the laminary flow with constant shaking. Thereafter the seeds were washed three times with sterile distilled water to achieve 95% purity of the seeds.

The seeds were germinated on the solid medium, containing 20% of salts and the vitamins of MS medium, 1% of sucrose and 8% of agar. The temperature in the phytothrone was 22 ± 2°C, day/night cycle 16/8 h and light intensity 20 μM m^{-2} s^{-1}. The percentage of the seeds germinated in 10 days was 90%. Approximately 3 cm long plantlets derived from the seeds in three weeks.

Subsequently, the roots were separated from the plantlets and maintained in liquid MS medium maximum for 28 days.

4.1.3. Bacterial strain

Agropine strain *A. rhizogenes* LBA 9402 (gift from the Norwich laboratory, England), carrying the NPT II and GUS genes was used for transformation. *A. rhizogenes* was maintained on YMB medium (0.2 g/l $MgSO_4$; 0.1 g/l NaCl; 0.5 g/l K_2HPO_4; 10.0 g/l mannitole; 0.4 g/l yeast extract, 50 μg/ml kanamycin, 15 g/l of agar-agar, pH 7.0) at –10°C [Mugnier, 1997]. The bacterium was

subcultured in YMB medium at 25 ± 2°C in the dark on a rotatory shaker (90 rpm) for 24 h before transformation procedure.

4.1.4. Transformation procedure

The leaves from sterile plants were cut into pieces with approximate size of 0.5 x 0.5 cm and co-cultivated for 30 min with fresh *A. rhizogenes* culture diluted 4 times in ½ MS liquid medium making up suspension 10^5 cells/ml. Subsequently, the leaf segments were dried on sterile filter paper, placed onto the MS solid medium (0.7% agar) containing 0.5 g/l of cefotaxime (*Claforan*®, *Roussel*, France) and 100 mg/l of kanamycin (to select kanamycin-resistant primary hairy roots) and incubated in illuminated phytotrone (20 μmol m^{-2} s^{-1}) at 22 ± 2°C. The formation of 2 to 4 roots on the leaf segments was observed in approximately 14 days after the inoculation.

One root was excised from each of the leaf pieces. These roots were called hairy root clones. The root clones were transferred into 300 ml conical flasks, containing 50 ml of MS liquid medium, supplemented with 2% sucrose and 200 mg/l of cefotaxime to purify the roots from the bacteria. The root clones were subcultured 6 times every third week. The purification and multiplication of the root clones was carried out on the shaker at 90 rpm in the dark phytotrone at 25 ± 2°C. The lack of the bacteria was regulated by the maintenance of the clones on the YMB medium.

4.1.5. *GUS*-assay

The presence of GUS activity in the root clones was detected histochemically [Jefferson, 1987] using x-gluc as a substrate.

4.1.6. *In vitro* maintenance of tissue cultures

Sterile root and hairy root material was maintained in respective media (listed below) in the dark phytotrone at 25 ± 2°C on the shaker at 90 rpm up to 28 days; until 10 parallel experiments were carried out. The amount of the explant was 600 ± 50 mg for the root cultures and 100 ± 10 mg for the hairy root cultures per 50 ml of the medium.

Following media were used in the study:

- The root and hairy root cultures of *H. niger* were maintained in the media of following composition:
  - MS medium
  - Knop-M medium

- B5 medium
- Modified MS with IBA 0.5 mg/l
- Modified Knop-M with IBA 0.5 mg/l
- Modified B5 with IBA 0.5 mg/l
- Modified MS (iron-deficient)
- Modified MS with putrescine of 80 mg/l
- Modified MS (iron-deficient with putrescine of 80 mg/l

- In addition, the root cultures of *H. niger* were maintained in the media of following composition:
  - Modified MS medium with supplementary (double) calcium content (440 mg/l $CaCl_2 \cdot 2H_2O$, 1340,6 mg/l of calcium gluconate)
  - Modified MS medium with reduced amount (half) of calcium content (220 mg/l $CaCl_2 \cdot 2H_2O$)
  - Modified MS medium with supplementary (double) magnesium content (740 mg/l $MgSO_4 \cdot 7H_2O$)
  - Modified MS medium with reduced amount (half) of magnesium content (185 mg/l $MgSO_4 \cdot 7H_2O$)
- The hairy root clones 1 and 8 of *H. niger* were maintained in the media of following composition:
  - Modified B5 medium with supplementary (double) calcium content (150 mg/l $CaCl_2 \cdot 2H_2O$ + 457,5 mg/l of calcium gluconate)
  - Modified B5 medium with reduced amount (half) of calcium content (75 mg/l $CaCl_2 \cdot 2H_2O$)
  - Modified B5 medium with supplementary (double) magnesium content (500 mg/l $MgSO_4 \cdot 7H_2O$)
  - Modified B5 medium with reduced amount (half) of magnesium content (125 mg/l $MgSO_4 \cdot 7H_2O$)

4.2. Determination of tropane alkaloids, plant nutritional elements and riboflavine

4.2.1. Isolation of tropane alkaloids

The content of tropane alkaloids was determined by HPLC. The experimental material was dried at room temperature and tropane alkaloids were extracted from the plant material with ethyl aether [Gosudarstvennaja farmakopeja SSSR, 1990] as follows.

Accurately weighed (±0.0001 g) ground plant material, with particle size <1 mm was placed into the 100 ml flask and 4 ml of 25% solution of ammonium hydroxide and 20 ml of ethyl aether were added. The flask was

covered with tinfoil and the mixture was shaken on the rotatory shaker for 1 h. The extract was filtered through the filter paper and the tissue 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 of ethyl aether, which was also added to the extract (filtered). Following the addition of 5 ml of water to the filtrate, the mixture was shaken and stratified, and water layer was discarded. The procedure was performed three times. The ethyl aether layer was placed into the accurately weighed dry bottle. The distribution flask was flushed three times with 5 ml of ethyl aether, which was also added to the extract.

Finally, aether was evaporated on the sand bath under the suction until the sample was dry and the bottle was weighed accurately.

4.2.2. Quantitative determination of tropane alkaloids

Five ml of the mixture of phosphate buffer and 96.6% ethanol [Gosudarstvennaja farmakopeja SSSR, 1990] (1:4) was added to the desiccated extract in the bottle and the bottle was weighed accurately. The phosphate buffer was prepared with dipotassium hydrogen phosphate [ЧДА] and potassium dihydrogen phosphate [ЧДА].

The procaine hydrochloride [Gosudarstvennaja farmakopeja SSSR, 1990] was used as the internal standard according to which the calibration was performed. There were not observed the pikes missing on the chromatogram while using of procaine hydrochloride as internal standard.

For elaboration of chromatographic conditions the hyoscyamine hydrochloride (*Hyoscyamini hydrochloridum*) and scopolamine hydrobromide (*Scopolamini hydrobromidum*) from Sigma were used.

Equipment:
1) column *Tessek*, C_{18}, 3 x 150 mm, with the average particle size of 5 μm;
2) UV-detector *Millichrom*, detection wavelength 210 nm;
3) pump SP 8 700 from *Spectra Physics*;
4) writing equipment ЛКС4-003, working scale 100mV.

Cromatographic conditions:
a) mobile phase containing phosphate buffer solution (pH 6.0) : 96.6% ethanol (1:4), 0.0033% of triethylamine [Reahim, ЧДА] (for blocking of free silanol groups of the packing of the column) was used;
b) flow rate of the mobile phase 0.25 ml/min;
c) mean pressure 14 MPa;
d) rolling rate of paper of the writing equipment 18 cm/h;
e) injection volume 10 μl.

The content of tropane alkaloids was calculated, using procaine hydrochloride as internal standard, in mg/g dry weight and loss of drying of plant material was also determined.

The sensitivity of the methodology for both tropane alkaloids was ≥ 0.02 mg/g dry weight.

4.2.3. X-ray diffractometric determination of plant nutritional elements

Accurately weighed ground plant material (10–15 g) with the particle size <1 mm was placed into the aluminium dish of constant mass. The dish was placed into the oven and the oven was covered by the metal plate. The thermopair sensor was placed into the dish and the material was heated up. The rise of temperature was observed and the maximum temperature was registered (450 ± 25°C). The sample was heated for 3 h, mixing it in 15 min intervals. When the ash was uniformly grey, the sample was cooled and weighed accurately.

The sample was ground to dust in the agate mortar and packed into the moisture-consistent packaging. The ash was ground once more before the X-ray diffractometric analysis and was regarded thoroughly fine, when it was staying on the sample base, kept in the vertical position. The sample base was an aluminium plate carrying the ground ash in it's grave of 1 mm. The prepared sample was analysed by X-ray diffractometry. The preparation of the sample enabled to perform both qualitative and quantitative analysis.

The following equipment was used in the X-ray diffractometric analyses:

1) X-ray diffractometer *DRON-3M*;
2) X-ray tube *BSV-27* (copper anode);
3) high tension source *IRIS*;
4) goniometric mechanism *GUR-8*, for the measuring of diffraction angles in the range of -100° to +167°;
5) scintillation counter *BDS-6*.

The diffractometer had measuring error of the angle Θ ±0.1° and ±0.05° with working rate 2°/min and 1°/min, respectively.

The relative error for X-ray diffractometric determination of plant nutritional elements was 2% ($p<0.05$). The chemical constituents of the ash were detectable by X-ray diffractometry, when their content in the ash was >1%.

4.2.4. Determination of plant nutritional elements by ion exchange chromatography

The accurately weighed sample (0.2–0.4 g) was placed into the crucible of known mass. The temperature was gradually brought up (to avoid burning of the plant material) and the sample was heated in the muffle oven at 300 °C for 30 min, whichafter the temperature was brought up to 600 °C and the sample was heated for 2 ½ h. Subsequently, the sample was cooled down and weighed accurately.
The ash was dissolved in 8.3% hydrochloric acid [Gosudarstvennaja farmakopeja SSSR, 1990] and the solution was diluted with deionized water (9 or 99 ml).

Equipment:
1) column *Alltech Universal Cation*, 100 x 4.6 mm, mean particle size of 7 μm;
2) conductivity detector *IJD-1*;
3) pump model 510 *Millipore*® from *Waters*;
4) oven *Oven IO-1* from *Inkrom*;
5) computer program *Kromex-32*;
6) water cleaning equipment *Analyst HP*.

Chromatographic conditions:
1) mobile phase 2.5 mM oxalic acid;
2) mobile phase flow rate 1 ml/min;
3) mean pressure 10 MPa;
4) injected volume 50 μl.

The system was calibrated for determination of calcium, magnesium and potassium, using respective salts.

4.2.5. Loss of drying

Loss of drying was determined in two samples up to 1 g, according to the requirements of European Pharmacopoeia [European Pharmacopoeia, 2005]

4.2.6. Determination of riboflavine in the media

4.2.6.1. Isolation of flavins

The growth medium (40 ml) was filtered through the filter paper (*Filtrak*®, VEB Spezialpapierfabrik, Niederschlag, Germany) and passed through a C_{10}-column (5 x 2.3 mm). Yellow substance was retained at the top of the column during application and washing with water. The column was washed with 40 ml of water to remove growth medium components and other substances. Yellow pigments were eluted with ethanol of various concentration in the effluent volume, which depended on the ethanol concentration. The effluent obtained with 30% ethanol was routinely used for PC, TLC and spectrophotometric measurements.

4.2.6.2. Chromatography

Paper chromatography was performed on *Whatman* chromatography paper *1 Chr*. [Whatman International Ltd, Maldstone, England]. The mobile phases used were upper phase of BAW 4:1:5 v/v, PAW 1:1:2, and WI. TLC was carried out on *Silufol*® plates (150 mm x 150 mm) [Kavalier, Czechoslovakia] with mobile phases such as BAW and the upper phase of AAW 3:1:3; v/v.

Photolysis of riboflavine and other yellow substances was carried out on a silica plate or chromatography paper under UV or high pressure mercury lamp with a UV filter. The spot of riboflavine solution from yellow fraction of ethanol effluent from C_{10} column was chromatographed in one direction. The chromatogram was dried, illuminated in UV-light for 5–30 minutes and thereafter chromatographed once more with the same solvent in the perpendicular direction. The products of photolysis were developed after second run on the paper or silica plate.

4.2.6.3. Spectrophotometry

The concentration of riboflavine was determined using a fluorimeter *Analiz* [Geologorazvedka, Saint Petersburg, Russia] modified by Dr. A. Tohver. The maximum of the exitation wavelength was 436 nm and emitted light was passed through a 500 nm cut-off filter. The concentration was calculated using standard curve established with authentic riboflavine [Chemapol, Prague, Czechoslovakia]. Spectra were recorded using a *Specord UV-VIS* spectrophotometer [Carl Zeiss, Jena, Germany]. All laboratory procedures were carried out in the dark or with dim red light.

4.3. Statistical analysis

The data were subjected to analysis of variance. The means were compared using DMRT.

The methods, chosen for the analyses are well-known and widely used in the work of experimental establishments, contributed to this thesis.

5. RESULTS

5.1. Tropane alkaloid, calcium, magnesium and potassium content and biomass differences in the vegetative organs of *H. niger* in different growth stages

As shown in Table 2 the percentage of the roots in the biomass of *H. niger* was the highest in young plants (30.1%) and decreased approximately twice by the ripening stage. The content of hyoscyamine was the highest in the roots of young plants of *H. niger* (0.38 mg/g) and decreased below detection limit in the ripening stage. In opposite to hyoscyamine the content of scopolamine gained the maximum level (0.15 mg/g) in the ripening stage. Similarly to scopolamine, the content of calcium decreased in the roots of the flowering *H. niger* compared to that in young plants but gained maximum value (15.72 mg/g) in the ripening stage. In addition, the content of magnesium was 1.83 mg/g in the roots of young plants of *H. niger* but was not detectable in flowering and ripening stages. However, the content of potassium increased gradually during the growth cycle up to 34.40 mg/g in the ripening stage.

Table 2. Hyoscyamine, scopolamine, calcium, magnesium and potassium content and biomass differences in the roots of *H. niger* in different growth stages

Biomass and constituents of roots	Young plant	Flowering plant	Ripening plant
Dry biomass (% from whole)	30.1 ± 4.5^{a}	18.9 ± 0.6^{b}	15.3 ± 1.2^{c}
Hyoscyamine content (mg/g)	0.38 ± 0.01^{a}	0.36 ± 0.01^{a}	ND
Scopolamine content (mg/g)	0.09 ± 0.02^{a}	0.06 ± 0.001^{a}	0.15 ± 0.003^{b}
Calcium content (mg/g)	13.12 ± 0.28^{a}	10.32 ± 0.20^{b}	15.72 ± 0.32^{a}
Magnesium content (mg/g)	1.83 ± 0.05^{a}	ND	ND
Potassium content (mg/g)	27.81 ± 0.55^{a}	30.14 ± 0.62^{a}	34.40 ± 0.70^{a}

Values are means of 4 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

The differences in biomass and constituents of lower stems of *H. niger* throughout the growth stages are shown in Table 3. The percentage of the lower stems in the biomass of *H. niger* was the lowest in young plants (8.1%), increasing up to 4 times by the ripening stage. The content of hyoscyamine was the highest in the young plant stage (0.51 mg/g) and decreased below detection limit in the ripening stage. Compared to hyoscyamine the content of scopolamine was over 25 times lower in the lower stems of young plants of *H. niger* but increased 36 times by the ripening stage. In addition, the content of calcium and potassium decreased during the growth cycle in the lower stems of

H. niger approximately 2.4 times opposite to the content of magnesium which increased approximately 1.3 times.

In the Table 4 are shown the differences in biomass and constituents for upper stems of *H. niger* throughout the growth stages. The percentage of the upper stems in the biomass of *H. niger* was the highest in young plants (8.1%) following decrease approximately 3.5 times by the ripening stage. The content of hyoscyamine was the highest in young plants (0.51 mg/g) and decreased approximately 5 times by the flowering stage and remaining the same in the ripening stage. When compared to the content of hyoscyamine the amount of scopolamine was over 25 times lower (0.02 mg/g), in the young plant stage of *H. niger* but in opposite to the content of hyoscyamine it was gradually increasing throughout the growth stages, gaining 0.08 mg/g by the ripening stage.

Table 3. Hyoscyamine, scopolamine, calcium, magnesium and potassium content and biomass differences in the lower stems of *H. niger* in different growth stages

Biomass and constituents of lower stems	Young plant	Flowering plant	Ripening plant
Dry biomass (% from whole)	8.1 ± 0.4^a	25.2 ± 4.3^b	34.5 ± 4.1^b
Hyoscyamine content (mg/g)	0.51 ± 0.01^a	0.10 ± 0.002^b	ND
Scopolamine content (mg/g)	0.02 ± 0.003^a	0.09 ± 0.002^b	0.72 ± 0.01^c
Calcium content (mg/g)	10.16 ± 0.20^a	8.42 ± 0.18^a	4.32 ± 0.10^b
Magnesium content (mg/g)	0.94 ± 0.02^a	1.08 ± 0.05^b	1.22 ± 0.02^b
Potassium content (mg/g)	28.90 ± 0.59^a	15.27 ± 0.31^b	10.82 ± 0.21^c

Values are means of 4 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

In addition, the content of calcium measured in the upper stems of *H. niger* decreased during the growth cycle. However, the contents of magnesium and potassium in upper stems were highest in flowering plants (1.48 mg/g and 31.43 mg/g, respectively) and the lowest in the stems of young plants and in the ripening stage of *H. niger*, respectively.

Table 4. Hyoscyamine, scopolamine, calcium, magnesium and potassium content and biomass differences in the upper stems of *H. niger* in different growth stages

Biomass and constituents of upper stems	Young plant	Flowering plant	Ripening plant
Dry biomass (% from whole)	8.1 ± 0.4^{a}	22.7 ± 5.5^{b}	28.9 ± 3.3^{b}
Hyoscyamine content (mg/g)	0.51 ± 0.01^{a}	0.10 ± 0.002^{b}	0.11 ± 0.006^{b}
Scopolamine content (mg/g)	0.02 ± 0.003^{a}	0.06 ± 0.003^{b}	0.08 ± 0.002^{c}
Calcium content (mg/g)	10.16 ± 0.20^{a}	8.38 ± 0.16^{a}	8.08 ± 0.16^{a}
Magnesium content (mg/g)	0.94 ± 0.02^{a}	1.48 ± 0.03^{b}	1.24 ± 0.03^{b}
Potassium content (mg/g)	28.90 ± 0.59^{a}	31.43 ± 0.41^{a}	15.44 ± 0.31^{b}

Values are means of 4 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

The differences in biomass and constituents of lower leaves of *H. niger* throughout the growth stages are shown in Table 5. The percentage of the lower leaves in the biomass of *H. niger* was the highest in young plants (61.4%), decreasing about 3.5 times by the ripening stage. The content of hyoscyamine was the highest (0.27 mg/g) in the lower leaves in the ripening stage and the lowest (0.02 mg/g) in the flowering stage. In opposite to latter the content of scopolamine was the highest in the lower leaves of *H. niger* in the flowering stage (0.63 mg/g). The content of scopolamine and calcium in the lower leaves of *H. niger* increased gradually during the growth cycle reaching the amount of 1.13 mg/g and 49.74 mg/g, respectively by the ripening stage. The content of magnesium was not detectable in the leaves of young plants of *H. niger*, but was increasing until 11.52 mg/g by the ripening stage. The content of potassium was the highest in the lower leaves of the flowering plants of *H. niger* (17.69 mg/g), decreasing for the ripening stage until 8.76 mg/g.

The differences in biomass and constituents of upper leaves of *H. niger* throughout the growth stages are shown in Table 6. The percentage of the upper leaves in the biomass of *H. niger* was the highest in young plants (61.4%), but was decreasing approximately 4.5 times by the ripening stage, similarly to the lower leaves. The content of hyoscyamine in the upper leaves of *H. niger* was the highest in young plants (0.24 mg/g) and decreased gradually during the growth cycle being the lowest in the ripening stage (0.06 mg/g).

Table 5. Hyoscyamine, scopolamine, calcium, magnesium and potassium content and biomass differences in the lower leaves of *H. niger* in different growth stages

Biomass and constituents of lower leaves	Young plant	Flowering plant	Ripening plant
Dry biomass (% from whole)	61.4 ± 7.6^{a}	18.7 ± 3.1^{b}	17.6 ± 2.5^{b}
Hyoscyamine content (mg/g)	0.24 ± 0.006^{a}	0.02 ± 0.001^{b}	0.27 ± 0.005^{a}
Scopolamine content (mg/g)	0.12 ± 0.002^{a}	0.63 ± 0.02^{b}	1.13 ± 0.02^{c}
Calcium content (mg/g)	18.00 ± 0.36^{a}	46.42 ± 0.94^{b}	49.74 ± 1.00^{b}
Magnesium content (mg/g)	ND	6.54 ± 0.27^{a}	11.52 ± 0.23^{b}
Potassium content (mg/g)	16.58 ± 0.35^{a}	17.69 ± 0.35^{a}	8.76 ± 0.18^{b}

Values are means of 4 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

The content of scopolamine was the highest in the flowering and the lowest in the young plant stage in the upper leaves of *H. niger*. The content of calcium increased during the growth cycle in the upper leaves of *H. niger*, being the highest in the ripening stage (58.72 mg/g). Similarly to lower leaves the content of magnesium was not detectable in the upper leaves of young plants of *H. niger*, but was increasing to the highest level in the ripening stage (9.49 mg/g). The content of potassium was the highest in the upper leaves of the flowering *H. niger* (36.09 mg/g), decreasing for the ripening stage until 15.39 mg/g.

Table 6. Hyoscyamine, scopolamine, calcium, magnesium and potassium content and biomass differences in the upper leaves of *H. niger* in different growth stages

Biomass and constituents of upper leaves	Young plant	Flowering plant	Ripening plant
Dry biomass (% from whole)	61.4 ± 7.6^{a}	14.6 ± 2.4^{b}	13.4 ± 3.4^{b}
Hyoscyamine content (mg/g)	0.24 ± 0.006^{a}	0.14 ± 0.002^{b}	0.06 ± 0.002^{c}
Scopolamine content (mg/g)	0.12 ± 0.002^{a}	0.28 ± 0.006^{b}	0.17 ± 0.003^{a}
Calcium content (mg/g)	18.00 ± 0.36^{a}	18.60 ± 0.38^{a}	58.72 ± 1.18^{b}
Magnesium content (mg/g)	ND	4.61 ± 0.10^{a}	9.49 ± 0.19^{b}
Potassium content (mg/g)	16.58 ± 0.35^{a}	36.09 ± 0.72^{b}	15.39 ± 0.31^{a}

Values are means of 4 replicates ± standard error; means within rows having different letters are significantly different accoriding to DMRT at $P > 0.05$.

5.2. Tropane alkaloid and riboflavine production and morphological differences in the root and hairy root cultures of *H. niger*

As shown on the Figure 7 significant morphological differences were found in the studied hairy root clones. Clone 1 was morphologically similar to the wild type roots as the roots were not so much branched which is characteristic to the hairy roots, but were still thicker when compared to the wild type roots. In addition, the roots of hairy root clone 6 were very thick, with very short lateral branches. The remaining clones were severely branched, supplied with shorter (clone 2) or longer (clone 9) lateral branches of different tightness.

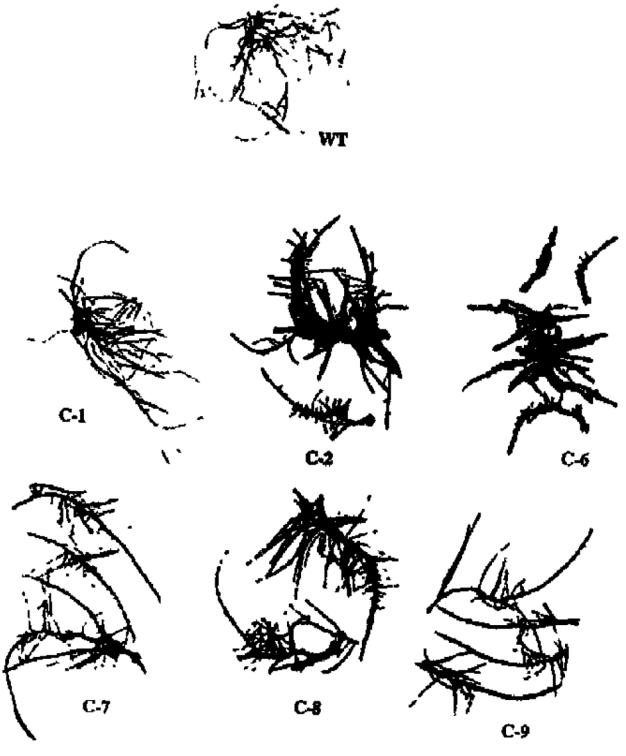


Figure 7. Part of a bundle (contact copies) of wild type (WT) and hairy root clones (C).

As shown in Table 7, the activity of the GUS-gene which is referring to the transgenicity, was determined in the hairy root clones 1, 2, 6, 8 and 9. Although the exterior characteristics of the root clone 7 referred to possible transgenicity the activity of the GUS-gene was not found in the clone 7, similarly to wild type roots. Moreover, the growth of the wild type roots was the worst, 0.1470 g/flask followed by the clone 1 with 1.3 times bigger growth. The remaining hairy root

clones were giving approximately 2.4 times better growth (0.3290–0.3830 g/flask), being the highest in the hairy root clone 2. The content of hyoscyamine in the wild type roots (2.12 mg/g) was approximately twice as high as hyoscyamine content in the clone 1 but over 6 times higher, when compared to the remaining hairy root clones.

Table 7. Growth, tropane alkaloid and riboflavin content in the root and hairy root clones of *H. niger*

Root clone	GUS activity	Growth (g/flask)	Hyoscya-mine content (mg/g)	Scopola-mine content (mg/g)	Riboflavine content in the medium (mg/l)
Wild type	–	0.1470 ± 0.0120^{d}	2.12 ± 0.22^{a}	5.86 ± 0.24^{c}	3.12 ± 0.18^{a}
Hairy root					
Clone 1	+	0.1890 ± 0.0180^{c}	1.14 ± 0.02^{b}	88.87 ± 1.77^{d}	2.18 ± 0.11^{b}
Clone 2	+	0.3830 ± 0.0230^{a}	ND	ND	0.07 ± 0.01^{c}
Clone 6	+	0.3410 ± 0.0190^{ab}	0.351 ± 0.007^{c}	8.55 ± 0.17^{b}	0.09 ± 0.01^{c}
Clone 7	–	0.3460 ± 0.0280^{ab}	0.373 ± 0.007^{c}	2.03 ± 0.41^{d}	0.06 ± 0.02^{c}
Clone 8	+	0.3660 ± 0.0220^{ab}	0.285 ± 0.006^{d}	1.04 ± 0.21^{d}	0.08 ± 0.01^{c}
Clone 9	+	0.3290 ± 0.0160^{b}	ND	ND	0.02 ± 0.02^{d}

Values are means of 10 replicates ± standard error; means within columns having different letters are significantly different according to DMRT at $P > 0.05$.

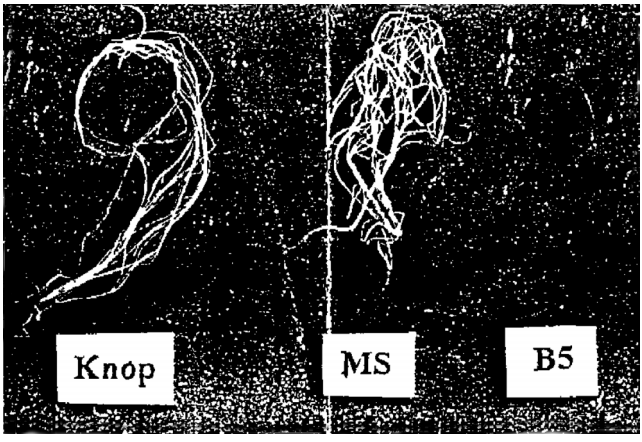
Nevertheless, the content of scopolamine was average in the wild type roots (5.86 mg/g) but over 15 times higher in the hairy root clone 1 and between 1.04–8.55 mg/g in the remaining root clones. Furthermore, hyoscyamine and scopolamine were not detected in the hairy root clones 2 and 9. In addition, the content of yellowish pigment – riboflavine was the highest in the medium of the wild type roots (3.12 mg/l) which was also observed by visual means as the colour of the medium turned strongly yellow during the growth cycle. The content of riboflavine was 1.4 times lower in the medium of the hairy root clone 1, but over 50 times lower in the media of the remaining clones, when compared to the wild type roots.

As shown in the Figures 8^{a} and 8^{b} no significant morphological differences were found in the root and hairy root cultures, maintained in the MS, Knop-M and B5 media. Due to the addition of plant growth regulator IBA (0.5 mg/l) the special aggregates, containing the callus, surrounded by the root capsule were derived both in root and hairy root cultures of *H. niger*.

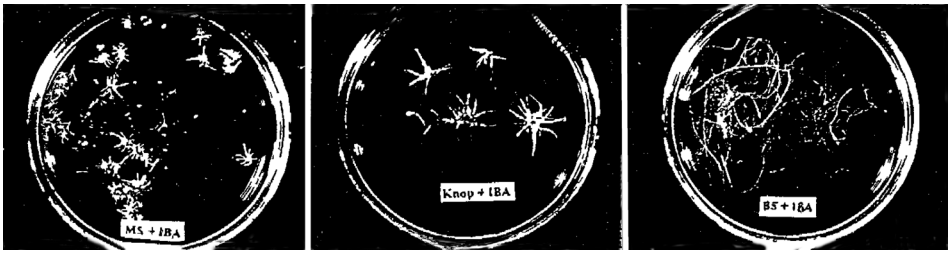
In addition, the growth of the root cultures of *H. niger* maintained in the media of different composition was 0.1320–0.1470 g/flask (Table 8), being the highest in the B5 medium and the lowest in the Knop-M medium. In the maintenance of the cultures at the presence of IBA improved the growth of the

cultures considerably (0.3003–0.4071 g/flask) being the highest in the B5 medium and the lowest in the MS medium. The growth of the hairy root clone 1 of *H. niger* maintained in the media of different composition was 0.1450–0.1880 g/flask being the highest in the B5 medium and the lowest in the Knop-M medium, similarly to the wild type roots. In the maintenance of the hairy root clone 1 of *H. niger* in the MS, Knop-M and B5 media with IBA the growth of the cultures was in the range of 0.1884–0.3257 g/flask being the highest in the MS medium and the lowest in the Knop-M medium. However, the growth of hairy root clone 8 of *H. niger* was 0.2850–0.3600 g/flask being the highest in the B5 medium and the lowest in the Knop-M medium, similarly to the data of hairy root clone 1. The growth of hairy root clone 8 of *H. niger* maintained in the media of different composition at the presence of IBA was 0.1331–0.2811 g/flask being the highest in the B5 medium and the lowest in the Knop-M medium.

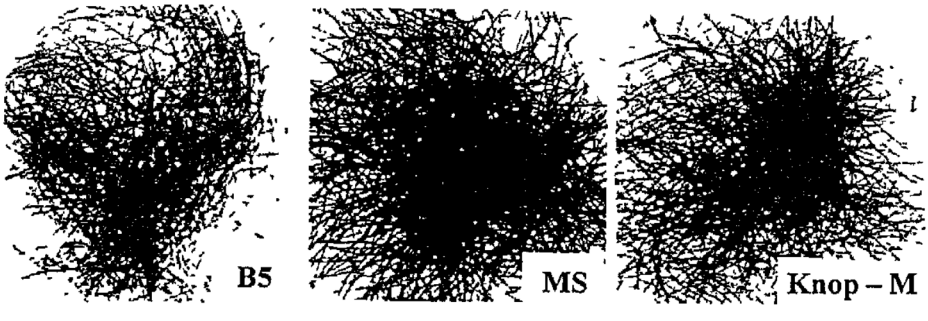
The content of tropane alkaloid hyoscyamine in the root culture of *H. niger* maintained in the MS and Knop-M media was 2.32 and 2.31 mg/g, respectively (Table 8). In the presence of IBA the particular data were 0.46 and 1.39 mg/g, respectively. The content of scopolamine in the maintenance of the root cultures of *H. niger* in the MS and Knop-M media was 5.87 and 0.93 mg/g and in the presence of IBA 0.19 and 0.03 mg/g, respectively. There is no data about the content of tropane alkaloids in the maintenance of the root cultures of *H. niger* in the B5 medium. The content of tropane alkaloid hyoscyamine in the hairy root clone 1 of *H. niger* maintained in the media of different composition was 2.18–4.47 mg/g being the highest in the Knop-M medium and the lowest in the MS medium. The content of hyoscyamine in the hairy root clone 1 of *H. niger* maintained in the media of different composition in the presence of IBA was 0.25 and 1.27 mg/g in the Knop-M and B5 media, respectively and was not detectable in the MS medium. The content of scopolamine in the same material was 7.89–65.60 mg/g being the highest in the Knop-M medium and the lowest in the MS medium. In the presence of IBA the particular data were 6.32–27.02 mg/g being the highest in the Knop-M and the lowest in the B5 medium. The content of hyoscyamine in the hairy root clone 8, maintained in the MS, Knop-M and B5 media was 0–1.16 mg/g being the highest in the B5 medium and was not detectable in the MS medium. The content of scopolamine of the same clone was 3.92–6.12 mg/g in the media of different composition being the highest in the B5 medium and the lowest in the Knop-M medium.



Wild type roots of *H. niger* maintained in Knop-M, MS and B5 media

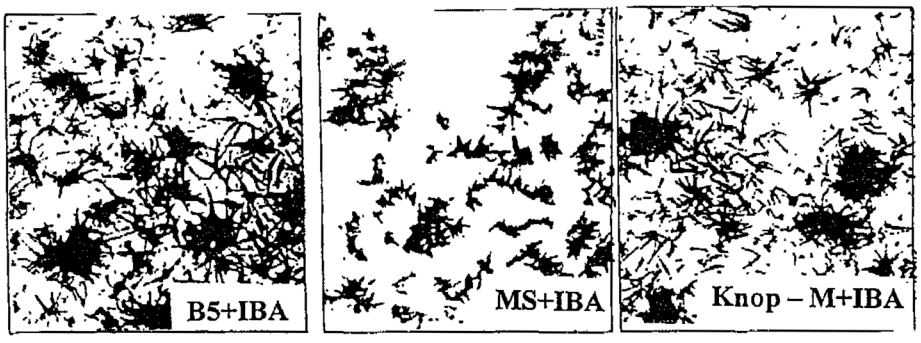


Wild type roots of *H. niger* maintained in modified Knop-M, MS and B5 media (with IBA)



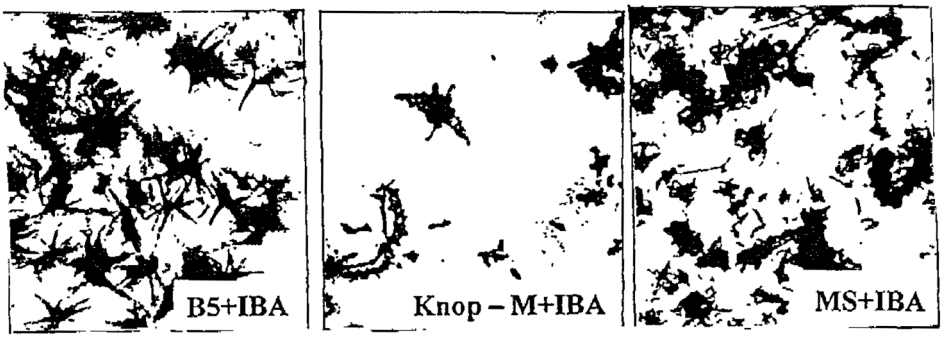
Hairy root clone 1 of *H. niger* maintained in MS, Knop-M and B5 media

Figure 8[a]. Morphological differences in the root and hairy root cultures of *H. niger* maintained in the media of different composition



Hairy root clone 1 of *H. niger* maintained in modified MS, Knop-M and B5 media (with IBA)

Hairy root clone 8 of *H. niger* maintained in MS, Knop-M and B5 media



Hairy root clone 8 of *H. niger* maintained in modified MS, Knop-M and B5 media (with IBA)

Figure 8[b]. Morphological differences in the hairy root cultures of *H. niger* maintained in the media of different composition

The wilde type root and hairy roots clone 8 of *H. niger* were maintained in the defined media (Table 9). The content of tropane alkaloids was determined in the plant material and the content of riboflavine was estimated in the media at the end of the growth cycle. There were no morphological differences in the cultures maintained in the media of different composition.

The growth of the root cultures of *H. niger* maintained in the media of different composition was 0.0722–0.1550 g/flask being the highest in the putrescine containing MS medium and the lowest in the iron-deficient MS medium (Table 9).

The content of hyoscyamine was not detectable in the root cultures of *H. niger* maintained in the media of different composition. The content of scopolamine was 3.42–29.97 mg/g in the root cultures of *H. niger* maintained in the media of different composition being the highest in the standard MS medium and the lowest in the iron-deficient MS medium.

The content of the riboflavine was 1.22–4.83 mg/l in the media of root cultures of *H. niger* at the end of the growth cycle being the highest in the iron-deficient MS medium in the presence of putrescine and the lowest in the normal MS medium at the putrescine content of 80 mg/l.

The growth of the hairy root clone 8 of *H. niger* maintained in the media of different composition was 3270–0.1841 g/flask being the highest in the MS medium with putrescine and the lowest in the iron-deficient MS medium containing 80 mg/l of putrescine.

The content of hyoscyamine in the hairy root clone 8 of *H. niger* maintained in the media of different composition was not detectable. The content of scopolamine in the hairy root clone 8 of *H. niger* maintained in the media of different composition 2.56–37.23 mg/g being the highest in the normal MS medium and the lowest in the iron-deficient MS medium containing 80 mg/l of putrecine.

In addition, the content of riboflavine was 0.11–0.88 mg/l in the media of hairy root clone 8 of *H. niger* at the end of the growth cycle being the highest in the iron-deficient MS medium and the lowest in the normal MS medium at the putrescine content of 80 mg/l.

Table 8. The growth and the content of tropane alkaloids in the root and hairy root cultures of *H. niger* maintained in the media of different composition

Root clone and the medium	Growth (g/flask)	Alkaloids in roots (mg/g)	
		Hyoscyamine	Scopolamine
Wild type			
MS	0.1430 ± 0.0130^{e}	2.32 ± 0.22^{c}	5.87 ± 0.42^{d}
Knop-M	0.1320 ± 0.0080^{f}	2.31 ± 0.19^{c}	0.93 ± 0.08^{f}
B5	0.1770 ± 0.0090^{c}	ND	ND
MS + IBA	0.3003 ± 0.0100^{g}	0.46 ± 0.22^{f}	0.19 ± 0.05^{g}
Knop-M + IBA	0.3300 ± 0.0090^{g}	1.39 ± 0.29^{g}	0.03 ± 0.004^{h}
B5 + IBA	0.4071 ± 0.0120^{h}	ND	ND
Clone 1			
MS	0.1600 ± 0.0070^{d}	2.18 ± 0.11^{c}	7.89 ± 0.39^{c}
Knop-M	0.1450 ± 0.0100^{e}	4.47 ± 0.22^{a}	65.60 ± 3.28^{a}
B5	0.1880 ± 0.0120^{c}	3.75 ± 0.19^{b}	28.48 ± 1.42^{b}
MS + IBA	0.3257 ± 0.0116^{g}	ND	7.79 ± 0.39^{c}
Knop-M + IBA	0.1884 ± 0.0147^{c}	0.25 ± 0.01^{f}	27.02 ± 1.35^{b}
B5 + IBA	0.2714 ± 0.0191^{b}	1.27 ± 0.06^{g}	6.32 ± 0.32^{d}
Clone 8			
MS	0.2980 ± 0.0160^{b}	ND	4.73 ± 0.24^{e}
Knop-M	0.2850 ± 0.0190^{b}	0.47 ± 0.02^{e}	6.12 ± 0.31^{d}
B5	0.3600 ± 0.0140^{a}	1.16 ± 0.06^{d}	3.92 ± 0.21^{e}
MS + IBA	0.2283 ± 0.1495^{g}	0.25 ± 0.01^{f}	3.88 ± 0.19^{e}
Knop-M + IBA	0.1331 ± 0.0048^{f}	1.50 ± 0.08^{g}	3.00 ± 0.15^{e}
B5 + IBA	0.2811 ± 0.0276^{b}	3.13 ± 0.16^{b}	3.34 ± 0.17^{e}

Values are means of 10 replicates ± standard error; means within columns having different letters are significantly different according to DMRT at $P > 0.05$.

Table 9. Growth, tropane alkaloid content and riboflavine excretion in the wild type and hairy root clone 8 of *H. niger* in the media with different iron and putrescine content

Root clone and the medium	Growth (g/flask)	Alkaloid content (mg/g)		Riboflavine content in the medium (mg/l)
		Hyoscyamine	Scopolamine	
Wild type				
I	0.1266 ± 0.1050^{a}	ND	29.97 ± 0.60^{a}	3.89 ± 0.18^{a}
II	0.8160 ± 0.0043^{c}	ND	3.94 ± 0.08^{b}	4.83 ± 0.14^{d}
III	0.1550 ± 0.0053^{a}	ND	11.21 ± 0.22^{c}	1.22 ± 0.04^{c}
IV	0.0722 ± 0.0023^{b}	ND	3.42 ± 0.07^{b}	3.45 ± 0.02^{a}
Clone 8				
I	0.1226 ± 0.0035^{a}	ND	37.23 ± 0.75^{d}	0.13 ± 0.02^{b}
II	0.0327 ± 0.0080^{b}	ND	2.56 ± 0.05^{e}	0.13 ± 0.02^{b}
III	0.1841 ± 0.0029^{a}	ND	7.88 ± 0.16^{c}	0.11 ± 0.01^{b}
IV	0.0510 ± 0.0049^{b}	ND	7.99 ± 0.16^{c}	0.99 ± 0.05^{c}

Explanations: I – MS, 100,17 μM Fe^{3+} (standard); II – Fe^{3+} -deficient MS + 80 mg/l of putrescine; III – MS, 100,17 μM Fe^{3+} + 80 mg/l of putrescine; IV – Fe^{3+}-deficient MS
Values are means of 10 replicates ± standard error; means within columns having different letters are significantly different according to DMRT at $P > 0.05$.

5.3. Tropane alkaloid production and the content of calcium, magnesium and potassium in the root cultures (Wild type) of *H. niger* maintained in the media with different calcium and magnesium content

The growth of the root cultures of *H. niger* maintained in the media with different content of calcium was 0.1148–0.1390 g/flask (Table 10) and was the highest in the medium with double calcium content and the lowest in the medium with standard calcium content.

The content of hyoscyamine was of 2.2–7.8 mg/g in the root cultures of *H. niger* maintained in the media of different calcium content and was the highest in the medium with lower calcium content and the lowest in the medium with double calcium content. In contrast, the content of scopolamine was the highest (24.8 mg/g) in the medium with double calcium content and the lowest (1.5 mg/g) in the medium with lower calcium content in the root cultures of *H. niger*.

The content of calcium and potassium was 2.65–5.02 mg/g and 17.09–22.39 mg/g, respectively, in the root cultures of *H. niger* maintained in the media with different calcium content and was the highest in the medium with double calcium content and the lowest in the medium with lower calcium content. In addition, the content of magnesium was 1.32–1.36 mg/g in the root

cultures of *H. niger* maintained in the media with different calcium content and was the highest in the medium with lower calcium content and the lowest in the medium with double calcium content.

The growth of the root cultures of *H. niger* maintained in the media of different content of magnesium was 0.8980–0.1361 g/flask (Table 11) and was the highest in the medium with double magnesium content and the lowest in the medium with lower magnesium content.

The content of hyoscyamine was not detectable in the root cultures of *H. niger* maintained in the media with different magnesium content. Furthemore, the content of scopolamine was 6.19–25.64 mg/g in the root cultures of *H. niger* maintained in the media with different magnesium content and was the highest in the medium with standard magnesium content and the lowest in the medium with double magnesium content.

Table 10. Growth and the content of hyoscyamine, scopolamine, calcium, magnesium and potassium in the root cultures of *H. niger* maintained in the media with different calcium content

Growth and the content of root cultures	Calcium content in the media (mg/l)		
	59.9	119.7	239.4
Growth (g/flask)	0.1326 ± 0.0463^{a}	0.1148 ± 0.0297^{a}	0.1390 ± 0.0341^{a}
Hyoscyamine content (mg/g)	7.8 ± 1.6^{a}	5.7 ± 0.1^{a}	2.2 ± 0.3^{b}
Scopolamine content (mg/g)	1.5 ± 0.2^{a}	17.7 ± 3.6^{b}	24.8 ± 0.6^{c}
Calcium content (mg/g)	2.65 ± 0.07^{a}	3.56 ± 0.09^{b}	5.02 ± 0.12^{c}
Magnesium content (mg/g)	1.36 ± 0.04^{a}	1.35 ± 0.04^{a}	1.32 ± 0.04^{a}
Potassium content (mg/g)	17.09 ± 0.53^{a}	18.25 ± 0.56^{a}	22.39 ± 0.69^{b}

Values are means of 10 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

The content of calcium was 1.14–2.06 mg/g in the root cultures of *H. niger* maintained in the media with different magnesium content and was the highest in the medium with lower magnesium content and the lowest in the medium with double magnesium content. In contrast, the content of magnesium and potassium was the highest (2.58 mg/g and 21.78 mg/g, respectively) in the medium with double magnesium content and the lowest (1.56 mg/g and 21.43 mg/g, respectively) in the medium of lower magnesium content in the root cultures of *H. niger*.

Table 11. Growth and the content of hyoscyamine, scopolamine, calcium, magnesium and potassium in the root cultures of *H. niger* maintained in the media with different magnesium content

Growth and the content of the root cultures	Magnesium content in the media (mg/l)		
	18.05	36.10	72.2
Growth (g/flask)	0.0898 ± 0.0188^{a}	0.1309 ± 0.0104^{b}	0.1361 ± 0.0098^{b}
Hyoscyamine content (mg/g)	ND	ND	ND
Scopolamine content (mg/g)	9.74 ± 0.12^{a}	25.64 ± 0.51^{b}	6.19 ± 0.12^{a}
Calcium content (mg/g)	2.06 ± 0.05^{a}	1.49 ± 0.04^{b}	1.14 ± 0.03^{c}
Magnesium content (mg/g)	1.56 ± 0.06^{a}	1.89 ± 0.08^{a}	2.58 ± 0.10^{b}
Potassium content (mg/g)	21.43 ± 0.39^{a}	21.61 ± 0.04^{a}	21.78 ± 0.40^{a}

Values are means of 10 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

5.4. Tropane alkaloid production and the content of calcium, magnesium and potassium in the hairy root cultures of *H. niger* maintained in the media with different calcium and magnesium content

As shown in Table 12 the growth of the hairy root clone 1 of *H. niger* maintained in the media with different content of calcium was 0.0590–0.0790 g/flask and was the highest in the medium with standard calcium content and the lowest in the medium with double calcium content.

The content of hyoscyamine was 4.49–6.58 mg/g in the hairy root clone 1 of *H. niger* maintained in the media with different calcium content and was the highest in the medium with double calcium content and the lowest in the medium with standard calcium content. The content of scopolamine was 6.47–47.68 mg/g in the hairy root clone 1 of *H. niger* maintained in the media with different calcium content and was the highest in the medium with lower calcium content and the lowest in the medium with standard calcium content.

Moreover, the content of calcium was 2.83–3.50 mg/g in the hairy root clone 1 of *H. niger* maintained in the media with different calcium content and was the highest in the medium with double calcium content and the lowest in the medium with lower calcium content. In contrast, the content of magnesium was the highest (0.66 mg/g) in the medium with lower calcium content and the lowest (0.51 mg/g) in the medium with double calcium content in the hairy root clone 1 of *H. niger*. Furthermore, the content of the potassium was 3.60–18.87 mg/g in the hairy root clone 1 of *H. niger* maintained in the media with different calcium content and was the highest in the medium with lower calcium content and the lowest in the medium with standard calcium content.

Table 12. Growth and the content of hyoscyamine, scopolamine, calcium, magnesium and potassium in the hairy root clone 1 of *H. niger* maintained in the media with different calcium content

Growth and the content of the hairy root clone 1	Calcium content in the media (mg/l)		
	20.4	40.8	81.6
Growth (g/flask)	0.0700 ± 0.0040^{a}	0.0730 ± 0.0040^{a}	0.0590 ± 0.0030^{a}
Hyoscyamine content (mg/g)	6.35 ± 1.27^{a}	4.49 ± 0.90^{b}	6.58 ± 1.32^{a}
Scopolamine content (mg/g)	47.68 ± 9.54^{a}	6.47 ± 1.30^{b}	26.66 ± 5.34^{c}
Calcium content (mg/g)	2.83 ± 0.07^{a}	2.98 ± 0.08^{a}	3.50 ± 0.09^{b}
Magnesium content (mg/g)	0.66 ± 0.02^{a}	0.55 ± 0.02^{b}	0.51 ± 0.02^{b}
Potassium content (mg/g)	18.87 ± 0.58^{a}	13.60 ± 0.42^{b}	18.26 ± 0.56^{a}

Values are means of 10 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

As shown in Table 13 the growth of the hairy root clone 1 of *H. niger* maintained in the media with different magnesium content was 0.0570–0.0870 g/flask and was the highest in the medium with double magnesium content and the lowest in the medium with lower and standard magnesium content.

The content of hyoscyamine was not detectable in the hairy root clone 1 of *H. niger* maintained in the media with different magnesium content but the content of scopolamine was 7.49–20.89 mg/g in the hairy root clone 1 of *H. niger* maintained in the media with different magnesium content and was the highest in the medium with double magnesium content and the lowest in the medium with normal magnesium content.

The content of calcium was 1.83–2.40 mg/g in the hairy root clone 1 of *H. niger* maintained in the media with different magnesium content and was the highest in the medium with lower magnesium content and the lowest in the medium with double magnesium content. In contrast, the content of the magnesium was the highest (1.35 mg/g) in the medium with double magnesium content and the lowest (0.089 mg/g) in the medium with lower magnesium content in the hairy root clone 1 of *H. niger*. In addition, the content of potassium was 35.04–37.51 mg/g in the hairy root clone 1 of *H. niger* maintained in the media with different magnesium content and was the highest in the medium with double magnesium content and the lowest in the medium with standard magnesium content.

Table 13. Growth and the content of hyoscyamine, scopolamine, calcium, magnesium and potassium in the hairy root clone 1 of *H. niger* maintained in the media with different magnesium content

Growth and the content of the hairy root clone 1	Magnesium content in the media (mg/l)		
	12.17	24.34	48,68
Growth (g/flask)	0.0570 ± 0.0030^{a}	0.0570 ± 0.0030^{a}	0.0870 ± 0.0050^{b}
Hyoscyamine content (mg/g)	ND	ND	ND
Scopolamine content (mg/g)	15.60 ± 0.31^{a}	7.49 ± 0.15^{b}	20.89 ± 0.42^{c}
Calcium content (mg/g)	2.40 ± 0.06^{a}	2.20 ± 0.06^{a}	1.83 ± 0.05^{a}
Magnesium content (mg/g)	0.089 ± 0.04^{a}	0.95 ± 0.04^{b}	1.35 ± 0.05^{b}
Potassium content (mg/g)	35.52 ± 0.65^{a}	35.04 ± 0.64^{a}	37.51 ± 0.69^{a}

Values are means of 10 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

The growth of the hairy root clone 8 of *H. niger* maintained in the media with different calcium content was 0.1000–0.1310 g/flask (Table 14) and was the highest in the medium with lower calcium content and the lowest in the medium with double calcium content.

The content of hyoscyamine was 5.27–6.47 mg/g in the hairy root clone 8 of *H. niger* maintained in the media with different calcium content and was the highest in the medium with lower calcium content and the lowest in the medium with standard calcium content. The content of scopolamine was 38.16–72.56 mg/g in the hairy root clone 8 of *H. niger* maintained in the media with different calcium content and was the highest in the medium with standard calcium content and the lowest in the medium with double calcium content.

The content of calcium was 1.74–3.21 mg/g in the hairy root clone 8 of *H. niger* maintained in the media with different calcium content and was the highest in the medium with double calcium content and the lowest in the medium with lower calcium content. In contrast, the content of the magnesium was the highest (0.92 mg/g) in the medium with lower calcium content and the lowest (0.69 mg/g) in the medium with double calcium content in the hairy root clone 8 of *H. niger*. Furthemore, the content of potassium was 17.47–23.05 mg/g in the hairy root clone 8 of *H. niger* maintained in the media with different calcium content and was the highest in the medium with lower calcium content and the lowest in the medium with standard calcium content.

Table 14. Growth and the content of hyoscyamine, scopolamine, calcium, magnesium and potassium in the hairy root clone 8 of *H. niger* maintained in the media with different calcium content

Growth and the content of the hairy root clone 8	Calcium content in the media (mg/l)		
	20.4	40.8	81.6
Growth (g/flask)	0.1310 ± 0.0070^{a}	0.1050 ± 0.0060^{b}	0.1000 ± 0.0050^{b}
Hyoscyamine content (mg/g)	6.47 ± 1.30^{a}	5.27 ± 1.05^{a}	5.69 ± 1.14^{a}
Scopolamine content (mg/g)	50.44 ± 10.09^{a}	72.56 ± 14.52^{a}	38.16 ± 7.63^{b}
Calcium content (mg/g)	1.74 ± 0.04^{a}	1.91 ± 0.05^{a}	3.21 ± 0.08^{b}
Magnesium content (mg/g)	0.92 ± 0.03^{a}	0.77 ± 0.02^{b}	0.69 ± 0.02^{b}
Potassium content (mg/g)	23.05 ± 0.71^{a}	17.47 ± 0.54^{a}	19.83 ± 0.61^{a}

Values are means of 10 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

The growth of the hairy root clone 8 of *H. niger* maintained in the media with different magnesium content was 0.1310–0.1530 g/flask (Table 15) and was the highest in the medium with standard magnesium content and the lowest in the medium with lower magnesium content.

The content of hyoscyamine was not detectable in the hairy root clone 8 of *H. niger* maintained in the media with different magnesium content. Nevertheless, the content of scopolamine was of 9.10–25.71 mg/g in the hairy root clone 8 of *H. niger* maintained in the media with different magnesium content and was the highest in the medium with lower magnesium content and the lowest in the medium with double magnesium content.

The content of calcium was 2.83–3.50 mg/g in the hairy root clone 8 of *H. niger* maintained in the media with different magnesium content and was the highest in the medium with lower magnesium content and the lowest in the medium with double magnesium content. In contrast, the content of the magnesium was the highest (2.36 mg/g) in the medium with double magnesium content and the lowest (1.19 mg/g) in the medium with lower magnesium content in the hairy root clone 8 of *H. niger*. In addition, the content of the potassium was 44.83–48.18 mg/g in the hairy root clone 8 of *H. niger* maintained in the media with different magnesium content and was the highest in the medium with lower magnesium content and the lowest in the medium with normal magnesium content.

Table 15. Growth and the content of hyoscyamine, scopolamine, calcium, magnesium and potassium in the hairy root clone 8 of *H. niger* maintained in the media with different magnesium content

Growth and the content of th hairy root clone 8	Magnesium content in the media (mg/l)		
	12.17	24.34	48.68
Growth (g/flask)	0.1310 ± 0.0070^{a}	0.1530 ± 0.0080^{a}	0.1450 ± 0.0080^{a}
Hyoscyamine content (mg/g)	ND	ND	ND
Scopolamine content (mg/g)	25.71 ± 0.51^{a}	15.12 ± 0.30^{b}	9.10 ± 0.18^{c}
Calcium content (mg/g)	3.50 ± 0.09^{a}	2.98 ± 0.08^{a}	2.83 ± 0.07^{a}
Magnesium content (mg/g)	1.19 ± 0.05^{a}	1.67 ± 0.07^{b}	2.36 ± 0.10^{c}
Potassium content (mg/g)	48.18 ± 0.88^{a}	44.83 ± 0.82^{b}	44.84 ± 0.82^{b}

Values are means of 10 replicates ± standard error; means within rows having different letters are significantly different according to DMRT at $P > 0.05$.

6. DISCUSSION

Field cultures offer a possibility to study the production of tropane alkaloids. Thus we examined the use of field cultures of *H. niger*, collecting the plants at young, flowering and ripening stages. From the collected plants different vegetative organs (roots, lower and upper stems and leaves) were separated, in which the content of tropane alkaloids hyoscyamine and scopolamine were determined by high perfomance liquid chromatography. In addition, the content of plant nutritional elements calcium, magnesium and potassium was determined by X-ray diffractometry.

On the basis of these results it was concluded that the content of hyoscyamine decreased and the content of scopolamine increased in the roots of *H. niger* as the plant ripened. The results were expected, as hyoscyamine is a precursor in the biosynthesis of scopolamine via 6β-hydroxylation [Hashimoto *et al.*, 1991]. The content of the calcium varied in the roots of *H. niger* during the growth cycle in a manner similar to scopolamine. The content of magnesium and potassium decreased in the roots of *H. niger* during the growth cycle. The content of hyoscyamine decreased in the lower stems of *H. niger* until it ripened, accordingly scopolamine content increased almost 36 times until it ripened and 8 times from the flowering to the ripening stage. During the growth cycle, the content of the calcium and potassium decreased, whereas the magnesium content increased in the lower stems of *H. niger*. The content of hyoscyamine decreased in the upper stems of *H. niger* until it ripened while the scopolamine content somewhat increased. The content of calcium increased in the upper stems of *H. niger* during the growth cycle, the according magnesium and potassium contents increased for the flowering stage and decreased for the ripening stage. The content of hyoscyamine decreased in the lower leaves of *H. niger* for the flowering stage, increasing for the ripening stage at the same time; the content of the scopolamine increased significantly in the lower leaves for the ripening stage. The content of calcium and magnesium increased in the lower leaves of *H. niger* during the growth cycle. The content of potassium increased a little bit for the flowering stage, decreasing for the ripening stage. The content of hyoscyamine decreased in the upper leaves of *H. niger* at the ripening stage. The content of scopolamine increased significantly in the upper leaves for the flowering stage and subsequently decreasing at the ripening stage. Similar to the lower leaves, the content of calcium and magnesium also increased in the upper leaves of *H. niger* at the ripening stage. The content of potassium increased in the leaves for the flowering stage and decreased for the ripening stage.

The results, obtained on the basis of field cultures of *H. niger* demonstrated that more hyoscyamine is being produced in the whole plant when compared with scopolamine, whereas the production of hyoscyamine is prevalent in *H. niger* during its first growth stages, the production of scopolamine dominates

at the end of the growth cycle. As the alkaloid scopolamine is much more valuable when compared with hyoscyamine [Oksman-Caldentey *et al.*, 1996], the application of field cultures for the purpose of the production of tropane alkaloids is not suitable. The results allow us to assume that calcium and magnesium play an important role in the production of tropane alkaloids in the roots of *H. niger*. The complex of three nutritional elements studied (calcium, magnesium and potassium) interact to affect the transport of each other and potentially also the transport processes of both alkaloids between plant organs. This may be significant since according to data from the literature [Hashimoto *et al*., 1991; Kanegae *et al.*, 1994; Suzuki *et al*., 1999; Nakajima *et al.*, 1999] the biosynthesis of the alkaloids proceeds in the roots of the plants whereas the leaves are being used as the herb and source for active substances [Murav'jeva, 1995]. Moreover, the root material of *H. niger* may be suitable for further studies on the production of tropane alkaloids.

Tissue culture methodology offers a number of benefits and is used extensively in the experimental study of the production of tropane alkaloids, allowing the use of one plant organ for a study (e. g. the roots).

Hairy root cultures, based on infection with *A. rhizogenes* are one of the most important achievements in the culture of plant material and enhancing the synthesis of active substances. Hairy root cultures have shown better genetic and biochemical stability when compared with the wild type roots [Oksman-Caldentey *et al*., 1996; Sevon, Oksman-Caldentey, 2002].

In the present study, six hairy root clones with the aid of *A. rhizogenes* supplemented to the root cultures of *H. niger* were derived from clone 1 which had the most morphological similarity with wild type roots. The production of scopolamine in this clone was almost 20 times higher, when compared with the root culture of *H. niger*; At the same time the production of scopolamine in the other hairy root clones was significantly lower, being the lowest in the clone 8. The production of hyoscyamine in all hairy root clones of *H. niger* was below that of its production in the wild type roots of *H. niger*, being the lowest in the hairy root clone 8. Due to the differences in the morphology and productivity of the cultures the hairy root clones 1 and 8 were chosen for the further experiments.

The growth and productivity of the plant tissue cultures depends significantly on the composition of the medium, due to this different results may be obtained in using of different media compositions. The root and hairy root cultures of *H. niger* were maintained in MS, Knop-M and B5 media. MS medium was found to be optimal for the further studies of the production of tropane alkaloids in the root cultures of *H. niger*. Based on the growth data of the *H. niger* hairy root clones 1 and 8, B5 medium was found to be optimal for their maintenance in further experiments.

Plant growth regulators (IBA, kinetine, α-naphthalene acetic acid etc.) may be used in the composition of medium, the suitable content of which varies for different plant species. Robins *et al.* (1995) have applied IBA in the root

cultures of *H. niger*, maintained in LS medium and noticed its inhibiting effect on the production of tropane alkaloids, although morphological changes in the cultures were not described in this study. In the present study the formation of calluses, surrounded by the root capsules was observed in both root and hairy root cultures of *H. niger*. Similarly to the results of previous studies the addition of IBA into medium inhibits the production of tropane alkaloids in root and hairy root cultures of *H. niger* with the exception of hyoscyamine production which increased slightly in the hairy root clone 8 of *H. niger*.

In contrast to the root cultures of other plants (e. g. *Armoracia rusticana*, *Solanum tuberosum*, *Prunus avium* etc.) growth medium of the root cultures of *H. niger* turned to a green-yellow colour if maintained in dark, under constant growth conditions. The colour disappeared under light, but was not maintained in the dark following inactivation of the enzymes by heating. This result suggested that the compound of yellow luminescence, excreted by the root cultures of *H. niger*, might have a flavine structure. During further studies it was found that the compound, excreted into the medium, was riboflavine. There is no data from earlier studies, regarding the excretion of riboflavine into the medium in the maintenance of root cultures of *H. niger*.

The excretion of the riboflavine was found in several dicotyledonous plants (sugar beet, pepper, lettuce) with iron deficiency. The addition of iron into the liquid growth medium caused a decrease in the excretion of riboflavine [Welkie, Miller 1989]. With the changes of iron content of the media and growth conditions subsequent changes in riboflavine excretion were observed. However, the same was not observed, regarding the growth of the roots [Welkie, 1995].

Arising from the present study the root and hairy root cultures (clone 8) of *H. niger* were maintained in different media compositions, modifying the content of iron and putrescine (precursor of hyoscyamine) in the MS medium and studying the effect of different media compositions on the production of scopolamine and riboflavine in *H. niger*.

On the basis of these results it became clear that the production of tropane alkaloid scopolamine in the root and hairy root cultures of *H. niger* was highest in the conventional MS medium. With the addition of putrescine to the MS medium at a concentration of 80 mg/l the production of scopolamine decreased significantly, 2.2 and 3.2 times accordingly, when compared with data from conventional MS medium. The production of scopolamine decreased even more in iron deficient medium, which was not improved by the addition of 80 mg/l putrescine into the medium (the production was more than 10 times lower, when compared with the data from the MS medium). The production of hyoscyamine was not observed in this experiment.

Robins *et al.* (1991) found also that the additive of putrescine or agmatine in the media did not enhance the accumulation of tropane alkaloids, but decreased [Robins *et al.*, 1991].

The excretion of yellow flavine pigment riboflavine was observed at higher levels in the root cultures of *H. niger*, with the most intensive change occurring in the iron-deficient MS medium, containing 80 mg/l of putrescine. The addition of iron into the medium decreased the excretion of riboflavine about 4 times in the root cultures of *H. niger*. The excretion of riboflavine in the hairy root clone 8 was significantly lower when compared with previous results. The excretion of riboflavine in the hairy root clone 8 was at its highest they were maintained in iron-deficient MS-medium, when other media were used the excretion of riboflavine was about 8 to 9 times lower when compared with the previous results.

Similarly to earlier studies it can be concluded that iron deficiency leads to an increase in the excretion of riboflavine in the root cultures. It could be proposed that iron plays a role in the inhibition of the synthesis of riboflavine in the plants. The ability to synthesize riboflavine decreased significantly in the hairy root cultures of *H. niger* when compared with the relevant root cultures.

On the basis of the results obtained from the investigation of the field cultures of *H. niger* it became clear that calcium may play a role in the production of tropane alkaloids in *H. niger*. At the same time it was found that the uptake of calcium may be connected with the assimilation of magnesium as well.

On the basis of these results it became clear that increasing the content of the calcium in the medium increased the production of scopolamine (*circa* 17 times) and decreased the production of hyoscyamine (*circa* 3 times) in the root cultures of *H. niger*. The changes in the content of magnesium in the medium caused an approximate four-time decrease in the production of scopolamine in the root cultures of *H. niger*. The changing of the calcium content in the medium resulted in an increase of tropane alkaloid production in the hairy root clone 1 of *H. niger*, whereas the better productivity of the culture (7 times) was observed with decreasing calcium content of the medium. The changes in the content of magnesium in the medium caused an increase in the production of scopolamine in the hairy root clone 1 of *H. niger*, whereas better productivity (1.9 times) was observed in the increasing of the calcium content of the medium. The change in the calcium content of the medium increased the production of hyoscyamine in the hairy root clone 8 of *H. niger*. Larger changes were observed with a decrease in the content of calcium in the medium. The production of scopolamine inversely decreased in hairy root clone 8 of *H. niger* with the changing of the calcium content of the medium. With the increase of the magnesium content in the medium, the production of scopolamine decreased about 2.5 times in the hairy root clone 8 of *H. niger*. Changes in the content of the calcium in the medium affected the content of calcium, magnesium and potassium in the root and hairy root cultures of *H. niger*. With a decreasing magnesium content in the medium there was an increase in the calcium content of all cultures studied.

The bigger and more significant changes are as follows. With a decreasing calcium content in the medium the content of the calcium in the root cultures of *H. niger* decreased about 1.3 times, at the same time the content of the calcium in the culture increased about 1.4 times with an increasing calcium content in the medium. With an increasing magnesium content of the medium a decrease in the calcium content of the root cultures of *H. niger* of about 1.4 times was observed. If the content of the calcium in the medium was decreased twice, the content of potassium in the hairy root clones 1 and 8 of *H. niger* increased (1.4 and 1.3 times, accordingly), the same happened with the magnesium content in the same cultures (1.2 times in both clones). With an increasing magnesium content of the medium the content of the magnesium both in root and hairy root cultures of *H. niger* increased 1.4 times. With a decreasing magnesium content in the medium, the content of magnesium in the cultures decreased, 1.4 times in hairy root clone 8, 1.1 times in clone 1 and 1.2 times in the root culture.

Collectively these results suggest that in addition to calcium, magnesium may also play a significant role in the production of tropane alkaloids. It is not possible to exclude the significance of potassium as well. The results confirm once more the fact that plant nutritional elements calcium, magnesium and potassium effect the uptake of each other in *H. niger*. The results obtained effect as the basis in the further derivation and enhancing of the methods for the processing of tropane alkaloids.

7. CONCLUSIONS

The main results of the thesis express the effect of the calcium, magnesium and potassium on the assimilation of each other in the *H. niger* and on the tropane alkaloid production in the tissue cultures of *H. niger*.

More exact results are as following:

1. The production of hyoscyamine is dominant in *H. niger* during the first growth stages, the production of scopolamine dominates in the final growth stages.
2. The produced active substances are transported into the higher plant organs, preferentially into the leaves during the life cycle of *H. niger*.
3. The plant nutritional elements calcium, magnesium and potassium effect the uptake of each other in *H. niger*.
4. The addition of plant growth regulator IBA into the composition of the medium results in a change in the morphology and decrease in the production of tropane alkaloids in the root and hairy root cultures of *H. niger*.
5. The production of scopolamine decreases with the addition of putrescine into the medium of the root and hairy root cultures of *H. niger*.
6. The iron deficiency results in the decrease in the scopolamine production and increase in riboflavine excretion in the root and hairy root cultures of *H. niger*.
7. Changes in the calcium content of MS and B5 media have varying effects on the production of tropane alkaloids in the root and hairy root cultures of *H. niger*.
8. Changes in the magnesium content of MS and B5 media have varying effects on the production of tropane alkaloids in the root and hairy root cultures of *H. niger*.
9. An increase in the calcium content of the medium results in an increase in the content of calcium and potassium and a decrease in the content of magnesium both in the root and hairy root cultures of *H. niger*.
10. An increase in the magnesium content of the medium results in an increase of the content of magnesium and potassium and the decrease in the content of calcium both in the root and hairy root cultures of *H. niger*.

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SUMMARY IN ESTONIAN

Tropaanalkaloidide ja riboflaviini produktsioon koera-pöörirohu (*Hyoscyamus niger* L.) põllu- ja koekultuurides

Arvukaima rühma taimseid toimeaineid moodustavad alkaloidid, mida tuntakse üle 10 000 [Leete, 1990]. Nendest ühe rühma moodustavad tropaanalkaloidid. Tropaanalkaloidid omavad märkimisväärset meditsiinilist tähtsust kui mitmesuguste farmakoloogiliste toimetega ained. Meditsiinis kasutatakse neist alkaloididest atropiinsulfaati ja skopolamiinvesinikbromiidi. Et tropaanalkaloidide keemiline süntees pole nende keeruka struktuuri tõttu majanduslikult otstarbekas [Oksman-Caldentey, Hiltunen, 1996], siis on nende peamiseks saamismeetodiks ekstraheerimine kasvatatud taimsest materjalist, kusjuures atropiin (hüostsüamiini DL-isomeeride ratseemiline segu) tekib ekstraheerimise ja edasise töötlemise käigus L-hüostsüamiinist.

Tropaanalkaloide sisaldavad maavitsaliste (*Solanaceae*) sugukonna perekondade *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* ja *Scopolia* erinevad liigid [Murav'jova, 1991]. Perekonna *Hyoscyamus* liikidest on erinevates uurimustes enam tähelepanu pööratud liikidele *Hyoscyamus muticus* L. ja *Hyoscyamus albus* L. [Oksman-Caldentey, 1987; Oksman-Caldentey *et al*., 1987; Sauerwein *et al*., 1993]. Suhteliselt vähem on uurimustesse haaratud liiki *Hyoscyamus niger* L. (koerapöörirohi), mis ainsana perekonna *Hyoscyamus* liikidest esineb Eestis ka looduslikult.

Taimset materjali võib koguda looduslikelt isenditelt või toota koekultuurina. Põllukultuuridega võrreldes pakuvad koekultuurid tunduvalt enam võimalusi, tagades kasvutsükli lühenemise (kuu piiresse), võimaldades vältida taimekaitsevahendite kasutamist, kindlustades püsivate kasvutingimuste rakendamise ning jätkuvalt kvaliteetse tootega [Oksman-Caldentey, 1986].

Tavapäraste koekultuuride kõrval omavad suurt tähtsust karvjuurekultuurid. Taimerakkude nakatamine nt. *Agrobacterium rhizogenes*' e abil põhjustab karvjuurte moodustumise nakatamiskohal. *A. rhizogenes*' e baasil saadud karvjuurekultuure iseloomustab kiire kasv [Hamill *et al.*, 1986; Jung, Tepfer, 1987], geneetiline stabiilsus [Aird *et al.*, 1988] ja Ri-transformatsiooni pikaajaline püsivus [Joao, Brown, 1994]. Saadud karvjuured on võimelised produtseerima rohkem toimeaineid nakatatud taimega võrreldes [Kamada *et al.*, 1986; Christen *et al.*, 1989; Oksman-Caldentey *et al.*, 1989; Moyano *et al*., 2002; 2003; Bonhomme *et al*., 2000; Cusido *et al*., 1999; Zhang *et al*., 2004; Yoshimatsu *et al*., 2004].

Tropaanalkaloidide biosünteesi ja produktsiooni aspekte on jätkuvalt uuritud [Hartmann *et al.*, 1986; Hashimoto *et al.*, 1987; 1991; Oksman-Caldentey, 1987; Oksman-Caldentey *et al.*, 1987; 1996; Leete, 1990; Walton *et al.*, 1990; Robins *et al.*, 1991; Kutchan, 1995; Rabot *et al.*, 1995; Pinol *et al.*, 1999; Jung

et al., 2001; Khanom *et al*., 2001; Medina-Bolivar, Flores, 1995; Rothe *et al*., 2001; Kang *et al*., 2004; Richter *et al.*, 2005].

Toimeainete produktsioon koekultuurides oleneb suurel määral kasutatava söötme koostisest [Dixon, 1985]. Taimetoiteelement kaltsiumi mõju tropaanalkaloidide produktsioonile on uurinud mitmed autorid (Curtis *et al.*, 1995; Hilton *et al.*, 1995]. Pinol jt. (1999) on uurinud ioonse kaltsiumi erineva söötmekontsentratsiooni mõju tropaanalkaloidide akumulatsioonile *Datura stramonium*' i L. karvjuurekultuurides ja teinud kindlaks, et kaltsiumi kontsentratsiooni vähendamine ei mõjutanud juurte kasvu, küll aga vähendas peroksidaasi aktiivsust, mis tõenäoliselt osaleb sekundaarse ainevahetuse produktide lagundamises. Madalama kaltsiumikontsentratsiooni vähenes ka karvjuurekloonide võime produtseerida hüostsüamiini. Hilton jt. (1994) on uurinud on uurinud *Datura stramonium*' i L., *Datura candida* & *aurea* L., *Datura wrightii* L., *Hyoscyamus muticus*' e L. ning *Atropa belladonna* L. karvjuurekultuuride kasvu ning suhkru ja mineraalsete ioonide omastamist. Töö tulemusena selgus, et täielikult kasutati kultuuride poolt ära söötmes esinev ammoonium- ja fosfaatiooni varu ning osaliselt ka magneesiumiooni tagavara [Hilton *et al.*, 1994].

Söötmetele lisatavatest taimekasvuregulaatoritest on indolüülvõihape vajalik enamike juurekultuuride kasvuks [Robins *et al.*, 1995], indutseerides rakkude jagunemist. Putrestsiini, kui hüostsüamiini biosünteesi eellase [Leete, 1990] mõju taimekasvuregulaatorina on uuritud samuti mitmetes koekultuurides [Robins *et al.*, 1991; Bais *et al.*, 1999; Biondi *et al.*, 1990; Rugini *et al.*, 1988; 1993; Ballester *et al.*, 1999].

Uudse efektina täheldati eelkatsete käigus *H. niger*' i juurekultuuride söötmete värvumist kollaseks kasvutsükli vältel pimedas. Kasvatamisel valguse käes söötme kollane värvus kadus, kuid mitte kasvatamisel pimedas pärast ensüümide inaktiveerimist kuumutamisel. Nimetatud tähelepanekud viisid järelduseni, et *H. niger*' i juurekultuuridest söötmesse vabanevaks kollaselt luminestseeruvaks ühendiks on flaviinpigment.

Söötme rauasisalduse ja kultuuri kasvutingimuste muutmisel on täheldatud muutusi riboflaviini eritumises erinevate taimede juurtest, kuid need ei olnud üheselt kantavad juurte massile [Welkie, 1995]. Spinati koekultuurides suurenes söötme riboflaviinisisaldus ka selle mangaanisisalduse suurendamisel. Arvatakse, et mangaani liig söötmes põhjustab rauavaegust [Shimizu *et al.*, 1998]. Söötme erinev soolade sisaldus ei põhjustanud muutusi riboflaviini eritumises intaktse salatitaime juurtest [Welkie, Miller, 1992].

Uurimuse eesmärk

Eespooltoodust lähtuvalt seati töö eesmärgiks uurida taimetoiteelementide kaltsiumi, magneesiumi, kaaliumi ja raua ning taimekasvuregulaatorite indolüülvõihappe ja putrestsiini mõju tropaanalkaloidide ja riboflaviini produktsioonile *H. niger*' is.

Eesmärgist tulenevalt püstitati järgmised ülesanded:

1. Uurida tropaanalkaloidide hüostsüamiini ja skopolamiini ning taimetoiteelementide kaltsiumi, magneesiumi ja kaaliumi sisaldust *H. niger'* i vegetatiivsetes organites erinevate kasvufaaside lõikes.
2. Hinnata tropaanalkaloidide ja riboflaviini produktsiooni *H. niger*' i juurekultuurides ja taime erinevates karvjuurekloonides.
3. Uurida erinevate söötmekombinatsioonide ja indolüülvõihappe mõju tropaanalkaloidide ning riboflaviini produktsioonile *H. niger*' i juure- ja karvjuurekultuurides.
4. Uurida söötme erineva kaltsiumisisalduse mõju tropaanalkaloidide produkstioonile ja kaltsiumi-, magneesiumi- ning kaaliumisisaldusele *H. niger*' i juure- ja karvjuurekultuurides.
5. Uurida söötme erineva magneesiumisisalduse mõju tropaanalkaloidide produktsioonile ja kaltsiumi-, magneesiumi- ning kaaliumisisaldusele *H. niger*' i juure- ja karvjuurekultuurides.

Uurimismaterjal ja meetodid

In vivo urimismaterjalina kasutati Tartu Ülikooli farmaatsia instituudi katsebaasis kultiveeritava *H. niger*' i kaheaastast varieteeti.

In vitro taimse materjali saamiseks kasutati *H. niger*' i 1-aastase varieteedi seemneid, mis pärinesid Helsingi Ülikooli farmaatsiaosakonnast. Seemned steriliseeriti ja idandati tahkel söötmel, mis sisaldas 20% MS-söötme sooli ning vitamiine, 1% sahharoosi ning 8% agarit. 21 päeva möödumisel eraldati seemikutelt steriilsetes tingimustes juured, mida kasvatati katseks vajaliku materjali saamiseks 28 päeva jooksul vedelas MS-söötmes.

Karvjuurte saamiseks kasutati *Agrobacterium rhizogenes*' e agropiintüve 9402 (plasmiid pRi 1855) (*Norwichi* laboratoorium).

- *H. niger*' i juure- ja karvjuurekultuure kasvatati alljärgneva koostisega söötmetes:
  1) MS sööde
  2) Knop-M sööde
  3) B5 sööde
  4) Modifitseeritud MS sööde (IBA 0,5 mg/l)
  5) Modifitseeritud Knop-M sööde (IBA 0,5 mg/l)

6) Modifitseeritud B5 sööde (IBA 0,5 mg/l)
7) Modifitseeritud MS sööde (ilma rauata)
8) Modifitseeritud MS sööde (putrestsiin 80 mg/l)
9) Modifitseeritud MS sööde (ilma rauata + putrestsiin 80 mg/l)

- *H. niger*' i juurekultuure kasvatati lisaks järgmise koostisega söötmetes:
  1) Modifitseeritud MS sööde (440 mg/l $CaCl_2 \cdot 2H_2O$ + 1340,6 mg/l kaltsiumglükonaati)
  2) Modifitseeritud MS sööde (220 mg/l $CaCl_2 \cdot 2H_2O$)
  3) Modifitseeritud MS sööde (740 mg/l $MgSO_4 \cdot 7H_2O$)
  4) Modifitseeritud MS sööde (185 mg/l $MgSO_4 \cdot 7H_2O$)

- *H. niger*' i karvjuurekloone 1 ja 8 kasvatati järgmise koostisega söötmetes:
  1) Modifitseeritud B5 sööde (150 mg/l $CaCl_2 \cdot 2H_2O$ + 457,5 mg/l kaltsiumglükonaati)
  2) Modifitseeritud B5 sööde (75 mg/l $CaCl_2 \cdot 2H_2O$)
  3) Modifitseeritud B5 sööde (500 mg/l $MgSO_4 \cdot 7H_2O$)
  4) Modifitseeritud B5 sööde (125 mg/l $MgSO_4 \cdot 7H_2O$)

Antud töö raames määrati tropaanalkaloidide sisaldus taimses materjalis kõrgrõhuvedelikkromatograafiliselt.

Taimetoiteelementide sisalduse määramiseks taimses materjalis rakendati röntgendifraktomeetriat ja ioonvahetuskromatograafiat.

Riboflaviin isoleeriti söötmetest erinevate kromatograafiliste meetodite abil ja määrati seejärel fluorimeetriliselt.

Tulemuste saamiseks rakendatud analüüsimeetodite valik põhines nende üldisel tunnustatusel ja kasutatavusel konkreetsetes uurimisasutustes, kus toimus tööde läbiviimine.

Uurimuse peamised tulemused

Tropaanalkaloidide produktsiooni uurimiseks rajati *H. niger*' i põllukultuurid avamaal, kogudes materjali noore taime, õitsemis- ja viljumisstaadiumis 1996. ja 1997. aastal. Kogutud taimedel eraldati erinevad organid (juured, alumised ja ülemised varred ning lehed), milles määrati kõrgrõhuvedelikukromatograafiliselt tropaanalkaloidide hüostsüamiini ja skopolamiini sisaldus ning röntgendifraktomeetriliselt taimetoiteelementide kaltsiumi, magneesiumi ja kaaliumi sisaldus.

H. niger' i põllukultuuridelt kogutud materjali analüüsil saadud tulemused näitavad, et intaktses taimes produtseeritakse üldiselt kogu taime lõikes enam hüostsüamiini, võrreldes skopolamiiniga, kusjuures hüostsüamiini produktsioon prevaleerub *H. niger*' is selle kasvutsükli algul, taime kasvutsükli lõpus on ülekaalus aga skopolamiini produktsioon. Et alkaloid skopolamiin on hüostsüa-

miiniga võrreldes tunduvalt väärtuslikum [Oksman-Caldentey *et al.*, 1996], siis pole põllukultuuride rakendamine tropaanalkaloidide tootmise eesmärgil otstarbekas. Saadud tulemustest nähtub, et kaltsiumil ja magneesiumil on täita oluline roll tropaanalkaloidide produktsioonis *H. niger*' i juurtes; uuritud kolme toiteelemendi – kaltsiumi, magneesiumi ja kaaliumi kompleks mõjub üksteise ja tõenäoliselt ka mõlema alkaloidi transpordile eri taimeorganite vahel. Nimetatud fakt võib osutuda väga oluliseks. Vaatamata sellele, et vastavalt kirjandusandmetele [Hashimoto *et al*., 1991; Kanegae *et al.*, 1994; Suzuki *et al*., 1999; Nakajima *et al.*, 1999] toimub alkaloid skopolamiini biosüntees taime juurtes, kasutatakse droogi ja toimeainete allikana siiski taime lehti [Murav'jova, 1995]. Samas oli lähtuvalt eespool toodust mõistlik kasutada tropaanalkaloidide produktsiooni edasisel uurimisel *H. niger*' is taime juurematerjali.

Selleks pakub häid võimalusi koekultuurimetoodika, mis ühtlasi annab tehniliselt hea võimaluse haarata uurimusse vaid üks taimeorgan tervikuna (nt. taime juured).

Taimebiotehnoloogias omavad suurt tähtsust ka *A. rhizogenese* nakkusel põhinevad karvjuurekultuurid, mis on oluliseks saavutuseks taimse materjali paljundamisel ja toimeainete sünteesi intensiivistamisel.

Käesolevas töös saadi lisaks *H. niger*' i juurekultuuridele *A. rhizogenes*'e baasil veel ka kuus karvjuureklooni, millest morfoloogiliselt oli harilikule juurele kõige lähedasem kloon 1. Tropaanalkaloid skopolamiini produktsioon nimetatud kloonis oli ligi 20 korda kõrgem, võrreldes *H. niger*' i juurekultuuriga; samas jäi skopolamiini produktsioon *H. niger*' i ülejäänud karvjuurekloonides tunduvalt madalamale tasemele, jäädes madalaimaks kloonis 8. Hüostsüamiini produktsioon jäi kõigis *H. niger*' i karvjuurekloonides madalamale selle produktsioonist *H. niger*' i juurekultuurides, jäädes madalaimaks samuti karvjuurekloonis 8. Lähtuvalt erinevustest kultuuride produktiivsuses ja morfoloogias rakendati edasistes katsetes karvjuurekloone 1 ja 8.

Taimsete koekultuuride kasv ja produktsioon sõltub oluliselt kasutatava söötme koostisest, mistõttu on erinevate söötmekombinatsioonidega võimalik saada väga erinevaid tulemusi. *H. niger'* i juure- ja karvjuurekultuure kasvatati enamkasutatavates, MS-, Knop-M- ja B5-söötmetes. Lähtuvalt tropaanalkaloidide produktsioonist osutus edasisteks katseteks *H. niger*' i juurekultuuride kasvatamisel sobivaimaks MS-sööde. Lähtuvalt *H. niger*' i karvjuurekloonide 1 ja 8 kasvuandmetest erineva koostisega söötmetes osutus edasisteks katseteks nende kasvatamisel sobivaimaks B5-sööde.

Söötmete koostises võib kasutada ka taimekasvuregulaatoreid (IBA, kinetiin, α-naftaleenäädikhape jne.), mille sisaldus on erinevate taimeliikide jaoks erinev. Robins jt. (1995) on rakendanud IBA-d *H. niger*' i juurekultuurides LS-söötmes ja täheldanud selle tropaanalkaloidide produktsiooni pärssivat toimet, samas viitamata saadud kultuuride morfoloogilistele muutustele. Käesolevas töös täheldati IBA lisandi toimel söötmes juurekapsliga ümbritsetud kalluste moodustumist nii *H. niger*' i juure- kui ka karvjuurekultuurides. IBA lisamine

söötme koostisse mõjus pärssivalt tropaanalkaloidide produktsioonile *H. niger*' i juure- ja karvjuurekultuurides, erandina suurenes IBA lisamisel söötmesse mõnevõrra vaid hüostsüamiini produktsioon *H. niger*' i karvjuurekloonis 8.

Erinevalt muude taimede (nt. *Armoracia rusticana*, *Solanum tuberosum*, *Prunus avium* jt.) juurekultuuridest täheldati *H. niger*' i juurekultuuride puhul söötme värvumist kollaseks. Edasisel uurimisel osutus söötmesse eralduvaks aineks riboflaviin. Varasemad uurimused riboflaviini eraldumisest söötmesse koera-pöörirohu harilike juurekultuuride kasvatamisel puuduvad.

H. niger`i juure- ja karvjuurekultuure (kloon 8) kasvatati erinevates söötmekombinatsioonides, varieerides MS-söötme raua- ja putrestsiinisisaldusega (hüostsüamiini eelühend), uurides söötme erinevate koostiste mõju skopolamiini ja riboflaviini produktsioonile *H. niger*' is. Töö tulemusena selgus, et tropaanalkaloididest skopolamiini produktsioon *H. niger*' i juure- ja karvjuurekultuurides oli suurim tavapärase koostisega MS söötmes, ilma putrestsiini lisandita. Putrestsiini lisamisel MS-söötmesse kontsentratsioonis 80 mg/l vähenes skopolamiini produktsioon tunduvalt kasvatatud juure- kui ka karvjuurekultuurides, vastavalt 2,2 ja 3,2 korda tavapärase MS-söötmega võrreldes. Veelgi enam vähenes skopolamiini produktsioon raua defitsiidi korral söötmes, mida ei suurendanud ka putrestsiini lisamine söötmesse kontsentratsioonis 80 mg/l (produktsioon tavapärase MS-söötmega võrreldes enam kui 10 korda madalam). Hüostsüamiini produktsiooni antud katse tulemuste põhjal ei täheldatud. Sarnaseid tulemusi on saanud ka Robins jt. (1991), leides, et *D. stramonium*' i karvjuurekultuuride söötmetesse lisatud putrestsiin ja agmatiin ei soodustanud tropaanalkaloidide akumulatsiooni kudedesse, seda pigem pidurdades [Robins *et al.*, 1991].

Riboflaviini produktsiooni täheldati enam *H. niger*' i juurekultuurides, kusjuures see oli suurim MS-söötmes rauadefitsiidi ja putrestsiini sisalduse juures 80 mg/l. Raua lisamine söötmesse vähendas riboflaviini produktsiooni *H. niger*' i juurekultuurides ligemale 4 korda. Riboflaviini produktsioon *H. niger*' i karvjuurekloonis 8 oli tunduvalt vähem väljendunud, jäädes eespool toodud tulemustega võrreldes kümneid kordi madalamale. Riboflaviini produktsioon karvjuurekloonis 8 oli suurim kasvatamisel MS-söötmes raua defitsiidi juures, ülejäänud söötmekombinatsioonides jäi riboflaviini produktsioon eespool tooduga võrreldes *ca* 8–9 korda madalamaks.

Uuriti söötme kaltsiumi- ja magneesiumisisalduse mõju tropaanalkaloidide produktsioonile ning kaltsiumi-, magneesiumi- ja kaaliumisisaldusele *H. niger*' i juure- ja karvjuurekultuurides.

Töö tulemusena selgus, et söötme kaltsiumisisalduse suurendamisel suurenes skopolamiini ja vähenes hüostsüamiini produktsioon *H. niger*' i juurekultuurides. Muutused söötme magneesiumisisalduses tingisid skopolamiini produktsiooni ligikaudu neljakordse vähenemise *H. niger*' i juurekultuurides. Söötme kaltsiumisisalduse muutmine põhjustas tropaanalkaloidide produktsiooni suurenemise *H. niger*' i karvjuurekloonis 1, kusjuures kultuuri enamat

produktiivsust täheldati söötme kaltsiumisisalduse vähendamisel. Muutused söötme magneesiumisisalduses tingisid skopolamiini produktsiooni suurenemise *H. niger*' i karvjuurekloonis 1, kusjuures suuremat produktiivsust võis märgata söötme kaltsiumisisalduse suurendamisel. Söötme kaltsiumisisalduse muutmisel suurenes hüostsüamiini produktsioon *H. niger*' i karvjuurekloonis 8, kusjuures enam söötme kaltsiumisisalduse vähendamisel. Skopolamiini produktsioon *H. niger*' i karvjuurekloonis 8 vastupidiselt vähenes söötme kaltsiumisisalduse muutmisel, kusjuures enam söötme kaltsiumisisalduse suurendamisel. Söötme magneesiumisisalduse suurenemisel vähenes skopolamiini produktsioon *H. niger*' i karvjuurekloonis 8 umbes 2,5 korda.

Söötmete erinev kaltsiumisisaldus mõjutas *H. niger*' i juure- ja karvjuurekultuuride kaltsiumi-, magneesiumi- ja kaaliumisisaldust. Ehkki saadud erinevused polnud just väga suured, väärib märkimist nende läbiv ühesuunalisus: söötme kaltsiumisisalduse vähenemisel vähenes kõigil juhtudel ka kultuuri kaltsiumisisaldus; samas aga suurenes kultuuri kaltsiumisisaldus söötme kaltsiumisisalduse tõstmisel. Söötmete magneesiumisisalduse vähendamisel suurenes kõigi jälgitud kultuuride kaltsiumisisaldus, mis omakorda söötme magneesiumisisalduse suurendamisel jällegi vähenes.

Uurimuse tulemuste kõrvutamisel saab väita, et lisaks kaltsiumile on tropaanalkaloidide produktsioonis oluline roll täita ka magneesiumil. Samuti ei saa välistada ka kaaliumi olulisust. Tulemused kinnitavad, et taimetoiteelemendid kaltsium, magneesium ja kaalium mõjutavad *H. niger*' is üksteise omastamist.

Saadud tulemused võivad olla rakendatavad tropaanalkaloidide tootmisprotsesside edasiarendamise ja täpsustamise eesmärgil.

Järeldused

Töö tulemusena selgus, et taimetoiteelemendid kaltsium, magneesium ja kaalium mõjutavad üksteise omastamist koera-pöörirohus, mõjutades samas erinevalt taime koekultuurides toimuvat tropaanalkaloidide sünteesi.

Lisaks selgus töö tulemusena järgmist:

1. *H. niger*' is prevaleerub taime arengu esimestes faasides hüostsüamiini produktsioon, arengu lõppfaasis aga skopolamiini produktsioon.
2. *H. niger*' i elutsükli jooksul toimub produtseeritud toimeainete pidev transport juurtest kõrgemal asetsevatesse taimeosadesse, eelistatult lehtedesse.
3. Taimetoiteelemendid kaltsium, magneesium ja kaalium mõjutavad *H. niger*' is üksteise omastamist.
4. Taimekasvuregulaatori IBA lisamisel söötme koostisse muutub *H. niger*' i juure- ja karvjuurekultuuride morfoloogia ning väheneb tropaanalkaloidide produktsioon.

5. Skopolamiini produktsioon *H. niger*' i juure- ja karvjuurekultuurides väheneb taimekasvuregulaatori putrestsiini lisamisel söötmesse.
6. Rauadefitsiidi korral väheneb skopolamiini produktsioon ja suureneb riboflaviini eritumine söötmesse *H. niger*' i juure- ja karvjuurekultuurides.
7. Muutused MS-ja B5-söötmete kaltsiumisisalduses ei mõjuta ühesuunaliselt tropaanalkaloidide produktsiooni *H. niger'* i juure- ja karvjuurekultuurides.
8. Muutused MS-ja B5-söötmete magneesiumisisalduses ei mõjuta ühesuunaliselt tropaanalkaloidide produktsiooni *H. niger'* i juure- ja karvjuurekultuurides.
9. Söötmete kaltsiumisisalduse tõstmisel suureneb nii *H. niger*' i juure- kui ka karvjuurekultuuride kaltsiumi- ja kaaliumisisaldus ning väheneb magneesiumisisaldus.
10. Söötme magneesiumisisalduse tõstmisel väheneb *H. niger*' i juure- ja karvjuurekultuuride kaltsiumisisaldus ning suureneb magneesiumi- ja kaaliumisisaldus.

ACKNOWLEDGEMENTS

This work was carried out at the Department of Pharmacy, Medical Faculty, University of Tartu and partly at the Institute of Experimental Biology, Estonian Agricultural University in 1995–2003. The study was supported by the ESF Grant No. 3031 (The study of the tropane alkaloid production in the field and tissue cultures of the medicinal plants, 06.01.1997–31.12.1999) and ESF *Ph. D.* Scholarship DARFA 0180896 S99 (Study of the production of tropane alkaloid in the tissue cultures of *Solanaceae* plants. 1998–2002).

I want to extend my warmest thanks to my direct supervisor during all my studies Dr. Elmar Arak (Division of Pharmacognosy and Pharmaceutical Organization), who suggested the topic of this study and introduced me to the field of plant tissue cultures. His wide experience, valuable advice and encouragement have greatly contributed to making this work come true.

I wish to express my deepest gratitude to Dr. Ain Raal, Head of the Division of Pharmacognosy and Pharmaceutical Organization, for his continuous interest and encouragement at all stages of this investigation.

I owe my thanks to Dr. Evi Padu and Dr. Ursel Soomets for their valuable critical comments on the manuscript and to my opponent, Dr. Kirsi-Marja Oksman Caldentey.

I am much indebted to my co-workers at the Institute of Experimental Biology of the Estonian Agricultural University, Mrs. Rael Vardja and Mr. Tõnis Vardja for the pleasant collaboration and valuable discussions in the field of plant tissue cultures.

Many thanks to Dr. Viljar Pihl, Dr. Jaan Pentšuk and Dr. Ivo Leito for their technical assistance and valuable advices in the field of HPLC and IEC.

I thank a lot Dr. Kalju Utsal for his advices and technical help in the field of X-ray diffractometry.

My best and warmest thanks to all the students, co-operating to the thesis during their *B. Sc.* studies: Ave Raitma, Marge Raidpere, Katri Kiho, Hergo Prei, Signe Raudleht, Janne Rosenthal, Hille Paat, Indrek Raidlo, Kuuno Vaher, Kairi Rooma and Piret Siitoja.

I express my sincere thanks to all my colleagues and staff at the Division of Pharmacy and Pharmaceutical Organization, who have made the time of investigations enjoyable and inspiring to me.

I should like to thank Dr. Sean Hynes and Dr. Heidi Annuk for the revision of the language of the manuscript and Mrs. Kerly Kustavus for her technical assistance.

Finally, my warmest thanks are due to my family for their continuous support and understanding, to my husband Urmas for his patience and encouragement during this work and to our little son Aarne, giving the meaning to my life.

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The subject of the research has been the production of tropane alkaloids in the field and tissue cultures of henbane (*Hyoscyamus niger* L.) and the estimation of the role of plant nutritional elements in the production of tropane alkaloids. Eleven scientific publications and 8 presentations at international scientific conferences, in addition 9 presentations in the local scientific conferences.

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Teadustegevus

Põhilisteks uurimisvaldkondadeks on olnud tropaanalkaloidide produktsiooni uurimine koera-pöörirohu (*Hyoscyamus niger* L.) põllu ja koekultuurides ning taimetoiteelementide rolli hindamine tropaanalkaloidide produktsioonis. 11 teaduslikku artiklit ja 8 ettekannet rahvusvahelistel konverentsidel, lisaks 9 ettekannet siseriikliku tähtsusega konverentsidel.