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THE EFFECT OF ELLICITORS ON THE SECONDARY METABOLITES PRODUCTION IN VITRO CULTURES-I

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DIPLOMA THESIS

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STATEMENT

I state that I made this diploma thesis by myself under supervision of Assoc.Prof. PharmDr.Lenka Tůmova, CSc.

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CONTENT

ABSTRAKT

Účinné látky vždy pocházely z rostlin. Rostliny však byly schopny vyrobit jen velmi malá množství a to byl důvod, proč se zkouší mnoho alternativních způsobů výroby, jeden z nich je kultivace tkáňových kultur. Tato metoda používá fragment z živé tkáně nebo orgánu, odebíraný z neporušené rostliny, nebo již existující explantátové kultury, za účelem pěstování umělého růstového média. I tento způsob však není schopen produkovat velké množství ve srovnání s extrakcí z terénních rostlin. Elicitace je považována za možný způsob, jak zvýšit produkci sekundárních metabolitů. Tato metoda využívá rostlinám vlastní obranný systém, aby se zvýšila produkce sekundárních metabolitů in vitro. Sloučenina, která se používá k dosažení tohoto jevu se nazývá elicitor. Během naší experimentální práce jsem použil jako spouštěče sloučeninu Ethephon (2-chlorethylfosfonová kyselina) na kalusů a suspenzi kultur Hypericum perforatum, se záměrem sledovat jeho vliv na produkci flavonoidů. Tento experiment byl založen na třech různých koncentracích a šesti různých ochranných časech, přičemž v 6, 12, 24, 72, 168 hodin. Maximální efekt spouštěče byl dosažen s koncentrací C1 (1mg/100ml) po 12 hodinách a o koncentraci C3 (100mg/100ml) po 72 hodinách.

ABSTRACT

Active compounds have been always originated from plants. Plants though, were able to produce only very low amounts of them and that was the reason for trying many alternative ways of production, one of them being plant tissue culture cultivation. This method is any fragment of living tissue or organ taken from an intact plant or an already existing explant culture, with the intention of growing an artificial growth medium. Even this method though, is not able to produce large amounts compared to extraction from field plants. Elicitation is considered a possible way to increase the production of secondary metabolites. This method used the plant's own defense system, in order to increase the production of secondary metabolites *in vitro.* The compound which is used to produce the effect is called elicitor. During our experimental work I used as an elicitor the compound Ethephon (2-Chloroethylphosphonic acid) upon callus and suspension cultures of *Hypericum perforatum,* with intention to observe its effect on flavonoid

production. This experiment was based on three different concentrations and six different withdrawal times, being 6, 12, 24, 72, 168 hours. The maximum effect of elicitor was reached with concentration c_1 (1mg/100ml) after 12 hours and with concentration c_3 (100mg/100ml) after 72 hours.

I INTRODUCTION

1 SECONDARY METABOLITES

Secondary metabolites are chemicals of organic nature which are involved with plant's defense system, rather than other primary functions such as normal growth, development, and reproduction. Secondary metabolites can be classified as: Alkaloids, Glycosides, Terpenoids, Flavonoids, Coumarins, Tannins, Lignans. Most of herbal drugs are thankful to secondary metabolites for their activity and this is what attracted pharmacognosists to further study them. [1]

1.1 FLAVONOIDS

Flavonoids are polyphenolic compounds that are found in nature ubiquitously and divided into categories such as: flavones, flavonols, isoflavones, anthocyanidins, catechins. [2] They are found on flowers, fruits and leaves, being responsible for their color. Flavonoids are also responsible for the protection of the plant against the harming effects of UV radiation. [3]

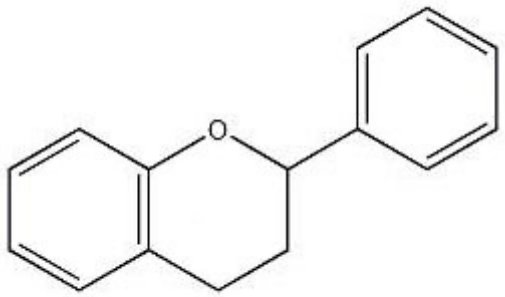
1.1.1 Chemical composition

Flavonoids are being derived from the same biosynthetic source and inevitably they appear a common chemical structure, called 2-phenyl chromate skeleton. Flavonoids are divided into 12 classes. Each of which represents the degree of the central pyran ring being oxidized. This ring can be opened and recyclized again into a ring.

- 2- phenyl chromanes
- Flavans

- Flavan 3-ols. Flavan -3,4-diols
- 2- benzyliden coumaranones
- Chalcones . Dihydrochalcones
- 2- phenyl benzopyriliums
- 2-phenyl chromones [3]

FIGURE 1: REPRESANTATION OF THE 2- PHENYLCHROMAN SKELETON [3]



1.1.2 Occurrence

Flavonoids of the glycoside form are accumulated in the vacuoles being water-soluble. According to the species, they are found on the mesophyl or the epiderm of leaves or sometimes even at both locations. In case of flowers, they are found only in the epidermal cells. On the leaf cuticles, they exist in the form of free aglycones, being totally or partially methylated on the hydroxy group. [3]

1.1.3 Characterization and extraction

Flavonoids are being characterized by methods such as HPLC, TLC, UV spectroscopy,

mass spectrometry, NMR techniques and spectrophotometric methods, as well as HPLC used for quantization.

At high temperatures glycosides are extracted with the use of methanol or ethanol mixed with water. The next phase is solvent evaporation under vacuum and then multi extractions of liquid-liquid type using non-miscible solvents until the aqueous phase is left. [3]

1.1.4 Therapeutic effect

Flavonoids possess the ability to diminish fragility and permeability of capillaries.[3]The most common therapeutic effect of them is their antioxidant activity, which is , *in vitro*, compared with the antioxidant activity of vitamin C and E. A disproportion between reactive oxygen species and antioxidants can lead to damage of the cell due to oxidative stress, further leading to disease such as atherosclerosis, cancer, and neurodegenerative disorders. Flavonoids may provide considerable protection against such diseases among with the help of other antioxidant enzymes and vitamins.[2]Other effects of flavonoids include anti-inflamatory, anti-alergic, anti-microbial and anti-cancer. [4] In the case of anti-cancer effect flavonoids are topoisomerase inhibitors and are able of induction of DNA mutations in the MLL gene, which is strongly associated with acute neonatal leukemia. [5, 6]

2 PLANT TISSUE CULTURE

From ancient times humanity has based upon plants, for acquiring active compounds but it was not long enough to realize that plants themselves were not able to produce large amounts .It was why several alternative methods of production of active compounds were being proposed and developed[7]. Firstly it was in 1934 that it was understood that plant cells were able to be cultivated on synthetic media and were also seemed to be of unlimited growth [8].Greater efforts took place on this field during 1950’s and ever since the plan tissue culture technique is being used in areas such as breeding of plants, studies

concerning production of secondary metabolites and the production of ornamental plants. [9]

Plant tissue culture is considered any fraction of living tissue which is used to assist an artificial growth medium, derived by any already developed explant culture or by a plant itself. [10]Every plant cell is of totipotent type, meaning that they carry all genetic information, not connected to the organ which was derived from. Theoretically a single cell can be multiplied by division and thus develop a tissue of roughly attached cells. This is known as callus. Practically callus culture is formed by taking a small portion of plant tissue from a part of the plant in which cell division is still progressing. That particular part of the plant is known as the explant. [9]Callus culture is subcultured with in intervals of 3 to 6 weeks and is able to be sustained for few years with the possibility of the cells being cultivated in a liquid medium, thus assembling a suspension culture. [8,9] The callus culture has then, the ability to develop and actually form different organs such as stems, leaves, roots or potentially a whole plant, using certain growth substances or appropriate hormones. [9]

2.1 CONDITIONS FOR PLANT CULTIVATION *IN VITRO*

2.1.1 Aseptic conditions

For the tissue to develop properly, it is essential that it should stay away from any kind of infection by certain microorganisms. In order to achieve that, the explant pass through a sterilizing process which is indeed up to a level that is not harmful to the plant cells. Then the plant part is treated with sterile water and finally is transferred to the sterile growth medium under aseptic conditions. [9]

2.1.2 NUTRIENT MEDIA

The most commonly used tissue culture media are Gamborg's B5, Shenck and Hildebrandt and Murashige and Skoog. All nutrient media for plant tissue cultures are developed in order to enable plant tissue to be maintained in a strictly artificial environment. Flasks or petri-dishes are the two common containers of a callus culture

using a semi-solid or liquid medium. Although the container must be preventing infection by organisms, it is also essential that it is able to allow exchanging of gases with the exterior environment. When flask is used, then its mouth should be closed with loose material such as cotton wool in order to prevent contamination of microorganisms and allowing exchange of gases. In the case of petri-dish there is no such necessity. [9]

2.1.2.1 Composition of nutrient media

Nutrient media consist of microelements, macroelements, vitamins and bio-factors, one carbon source, an undefined mixture of natural substances and finally plant growth regulators.

Microelements: Necessary microelements are: boron, manganese, iron, cobalt, molybdenum, iodine, silicon, aluminum, zinc, copper, nickel.

Macroelements: Essential for cultivation of intact plants and with a quantitative content of more than 30mg/L. : calcium, sulfur, phosphorus, magnesium, potassium.

Vitamin and bio-factor: vitamin C, nicotinic acid, group B vitamins, and bio-factors-biotin, myo-inositol.

Carbon source: the source of carbon that is most commonly used is sucrose and in some cases fructose.

Undefined mixture of natural substances: protein hydrolysate or organic extracts derived from coconut milk can be used to induce culture growth.

Plant growth regulators: There are two groups of plant growth regulators commonly used: Cytokinins and Auxins. Cytokinins are responsible for cell division. Zeatin, 6-dimethyl aminopurin, benzylaminopurin are commonly used. Auxins are compounds of organic type, being responsible for the lenthwise development of the stems and having an opposite role upon the roots. The naturally occuring auxine indole-3- acetic acid but a-naphtaleneacetic acid and 2,4- dichlorophenoxyacetic acid are more frequently used. [9,10,11,12]

2.1.3 Environmental factors

Light: Secondary metabolites and be accumulated and intensity of biosynthetic process and can be altered in intact plants.

Aeration : The presence of oxygen is crucial ,since plant cells are classified as aerobic.

Temperature greatly influences the course of the cultivation and also the time required for the number of cells to double. According to the species the range of temperature can be different but 25-30 degrees Celsius is considered to be optimum for in vitro cultivation. [9,11]

3 HYPERICUM PERFORATUM

3.1 CLASSIFICATION

KINGDOM: Plantae

DIVISION: Angiosperms

CLASS: Rosids

ORDER: Malpighiales

FAMILY: Hypericaceae

GENUS: Hypericum

SPECIES: *H. perforatum*

3.2 DESCRIPTION

Hypericum perforatum (also known as St. John's wort) is a stoloniferous perennial herb with extensive creeping rhizomes. It has erect stems which are branched in the upper section and able to reach 1 meter. Its leaves are of yellow-green color having on their

surface translucent dots giving them a perforated image which is responsible for the name ‘perforatum’. Its flowers are yellow with conspicuous black dots, having a length of 2.5 cm, five petaled. The flowers appear in broad cymes. Sepals are pointed with grandular dots. The plant is found indigenously in Europe but has been brought in many temperate areas throughout the world. [13,14,15]

3.3 CHEMICAL COMPOSITION

Hypericum perforatum contains a variety of constituents such as flavonoids, (kaempferol, quercitin, myricetin, isoquercitin, luteolin, amentoflovone, rutin, hyperoside, hyperin), proanthocyanidins (catecin and epicathecin), phloroglucinols (hyperforin, furohyperforin, adhyperforin), naphtodiathrones (hypericin and pseudohypericin, isohypericin, emodin-anthranol,cyclo-pseudohypericin) and essential oils (consisting of mono and sesquiterpens). [13,14, 15]

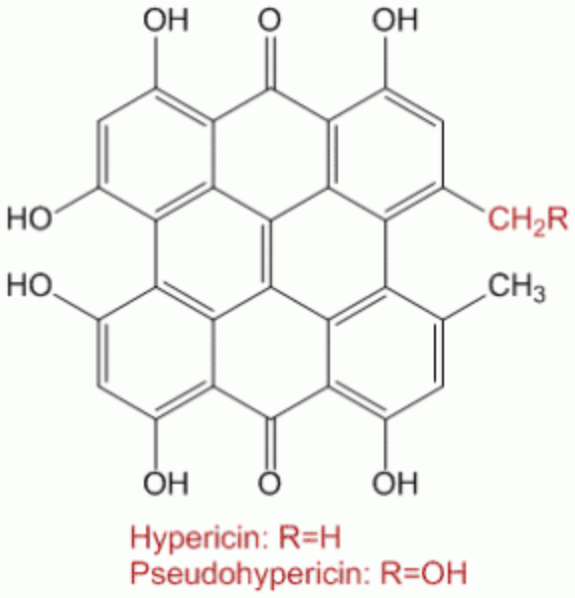


Figure 2 : Structure of hypericin and pseudohypericin - two major constituents of St. John's Wort

3.4 PHARMACOLGICAL ACTIVITY AND USES

The most common use of the drug is its antidepressant activity. The current belief for the mechanism of action is the inhibition of re-uptake of excitatory neurotransmitters (noradrenaline, dopamine, serotonine). Hypericum shows also antiviral and antibacterial activity due to the presence of hypericin and pseudohypericin. It is proposed that, when hypericin is photoactivated, produces a photo- oxidation effect resulting in the destruction of viruses. Hypericum finally has a wound-healing effect due to due to the presence of tannins which possesses an astrigent activity. [13,14 ,15]

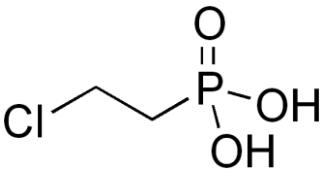
4 ETHEPHON

Ethephon is an organic molecule that acts as ethylene generator and eventually as a plant growth regulator .It is responsible for the regulation of different phases of plant's growth and development with its use depending on time of application, concentration and plant species. Its field of application includes blackberries, apples, cherries, pineapple, tobacco, bromeliads, guava, ornamentals, tomatoes, wheat, cotton, coffee, grapes, cucumber etc. The mechanism of action is based on liberation of ethylene and its absorption by the plant, producing a change in the process of growth. Another use of ethephon is the fastening of ripening of vegetables and fruits. [17,18, 19] Ethephon is classified as general use pesticide (GUP) and any product that contains ethephon has the signal words 'CAUTION', 'DANGER' , 'WARNING'. [16] Various commercial herbicides contain ethephon with names such as Cerone, Flordimex T-extra, Etherel, Arvest, Etheverse, Bromeflor. [16, 17]

4.1 PHYSICAL PROPERTIES

Ethephon's IUPAC name is 2-chloroethylphosphonic acid [18], its CAS number is 16672-87-0, its molecular weight is 144.5 g/mol , it is readily soluble in water, ethanol, methanol, acetone, isopropanol and slightly soluble in non-polar solvents like toluene and benzene, its melting point is 74 degrees Celsius and it appears as colorless solid. [18]

Figure 3: Representation of Ethephon's structure [18]



4.2 TOXICITY

4.2.1 Acute toxicity

Studies were taken place upon rats, in which ethephon was given for a 13 weeks to 20 rats per dose per sex at 0 , 50, 100, 200 mg/kg/ day. Both brain cholinesterase and plasma cholinesterase were observed to have differences, at all doses, with the control dose. In contrast red blood cell cholinesterase did not show differences with the control dose at neither of sexes.[20] Acute oral LD50 for rats was reported as 3,400 mg/kg to 4,229 gm/kg, for solution of ethephon 24% in propylene glycol.[21] Studies were also taken upon dogs, in which ethephon was given for 13 weeks inside the food to 4 dogs per sex per dose level at 0, 5.0 , 25.0 , 187.5 mg/kg/day. The activity of plasma cholinesterase was decreased at all doses in both males and females. The red blood cell activity was decreased in the males at all doses with an exception of 5.0 during 8^{th} week at the 25 and at the 25.0 and 187.5 mg/kg/day in females. The activity of brain cholinesterase was worth mentioning only at 187.5 mg/kg/day. [20]

4.2.2 Chronic toxicity

A 78 week study took place, using Swiss albino mice which were fed with food containing 0, 4.5, 45, 150 mg/kg/day of ethephon. The activity of plasma cholinesterase

was inhibited at the 45 and 150 mg/kg/day in both females and males. For the plasma cholinesterase the lowest effect level (LEL) was, for both sexes, 45 mg/kg/day and the No Observable Effect Level (NOEL) was 4.5 mg/kg/day again for both sexes. The red blood cell cholinesterase was notably decreased in females at the level dose 45 and 150 mg/kg/day [20] and in males the activity was nominally decreased at high and mid doses. The activity of brain cholinesterase did not appear to be different from control doses, at any dose in both sexes. In a study that lasted 2 years, rats were fed with diet containing equal or greater amount of 12.500 mg/kg/day and did not yield any illness effect with an exception of the end of the trial and at the highest dose. [22] For rats the highest dose showing no adverse effect was 375 mg/kg/day for 90 days. [18]

4.2.3 Teratogenic effect

For rats the No Observable Effect Level was reported as 600 mg/kg/day whereas for rabbits the No Observable Effect Level was 50 mg/kg/day. [17, 23]

4.2.4 Carcinogenic Effect

Mice were used for a carcinogenicity study in which 70.6 -72.1 % ethephon was tested. During 78 weeks mice showed no evidence of carcinogenicity at doses of 0, 15.5, 156, 1630 mg/kg/day. [23]

4.3 ENVIRONMENTAL FATE

Ethephon was found to be of low to moderate potential of contaminating ground water, based on soil thin layer chromatography (TLC) tests, due to its low to moderate mobility in soil.[20]It was found that in soil ethephon rapidly degrades to ethylene, phosphoric acid and chloride ions. [18,21] In commodities treated with ethephon, residues of monochloroacetic acid were found, which is a potential degradation product of a impurity found in ethephon.[20] In plants there is rapid degradation of ethephon to ethylene, chloride and phosphate. [18, 20]

4.4 EFFECT ON OTHER PLANTS

Tomato plants were treated with ethephon and showed significantly enhanced ethylene

and lycopenen production and accelerated fruit ripening, suggesting that ethephon induces disease resistance in tomato fruits. [31] A study upon onion and garlic, indicated that ethephon decreased shoot length and biomass, increased values of MDA (lipid peroxidation) and peroxidase activity and decreased production of glucose, fructose and ascorbic acid. [32] Sweet sorghum was treated with ethephon, with the results indicating that ethephon has a n important dwarfing effect on stalk length and significantly reduces lodging rate. It also exhibited smaller biomass and stem yield. [33] Cherry trees that were sprayed with ethephon showed significantly lower anthocyanin content, firmness, antioxidant activity and soluble solids concentration (SSC).[34] Ethephon was applied on pyrethrum (*Chrysanthemum cinerariaefolium)* in order to observe effect on growth and pyrethrins accumulation. Results showed that at concentrations of 50, 100, 250, 500 mg /L the pyrethrins level was significantly increased while at 50, 100 mg/L the fresh and dry flower yield was significantly increased as well.[35] In another study, which was done over three cop seasons, ethephon increased the sucrose, Brix and sorbitol content of 'Scarletspur Delicious' apple juice. Ethephon treatment in spearmint (*Mentha spicata*) decreased stomatal conductance, exchange rate of CO2, chlorophyl content and produced an induction of phenotypic changes, thus modifying essential oil accumulation and plant growth. [37] In cell culture of *Pueraria uberosa* there was an increase in isoflavonoids accumulation after 48 hours of treating with 100μM, and the increase was up to 14 times larger. [38]

5 ELICITATION

5.1 METHODS FOR INCREASING THE PRODUCTION OF SECONDARY METABOLITES

It was always of great interest to scientists the production of active compounds and tissue cultures, due to the potential of producing the desired products on a year-long large-scale. Disappointingly, this amount has proved to be considerably low in comparison with the main production method which is the extraction of plants that

have grown on the fields. [10] Other methods, able to induce production of secondary metabolites include: selection of high-yielding cell lines, optimizing the environmental factors and nutrient media, cultivation of organs and elicitation. [9]

5.2 ELICITATION

Elicitation is the method of increasing the production of secondary metabolites by using the defense system of the plant. [24]

5.3 ELICITORS

Elicitor is recognized any compound that is able to induce accumulation of antimicrobial phytoalexins and to produce any type of defending response. [25]

Elicitors are classified as biotic and abiotic ones. Biotic elicitors are a) molecules that mimic the action of an endogenous signal in order to stimulate the defense system of a plant [25] b) endogenously derived elicitors c) intact organisms like bacteria and viruses d) organic molecules like oligosaccharides, proteins, glycoproteins. [26] The second group of abiotic elicitors consist of a) inhibitors b) salts of heavy metals like CuSo4 and HgCl2 c) detergents [26] d) UV radiation. [10]

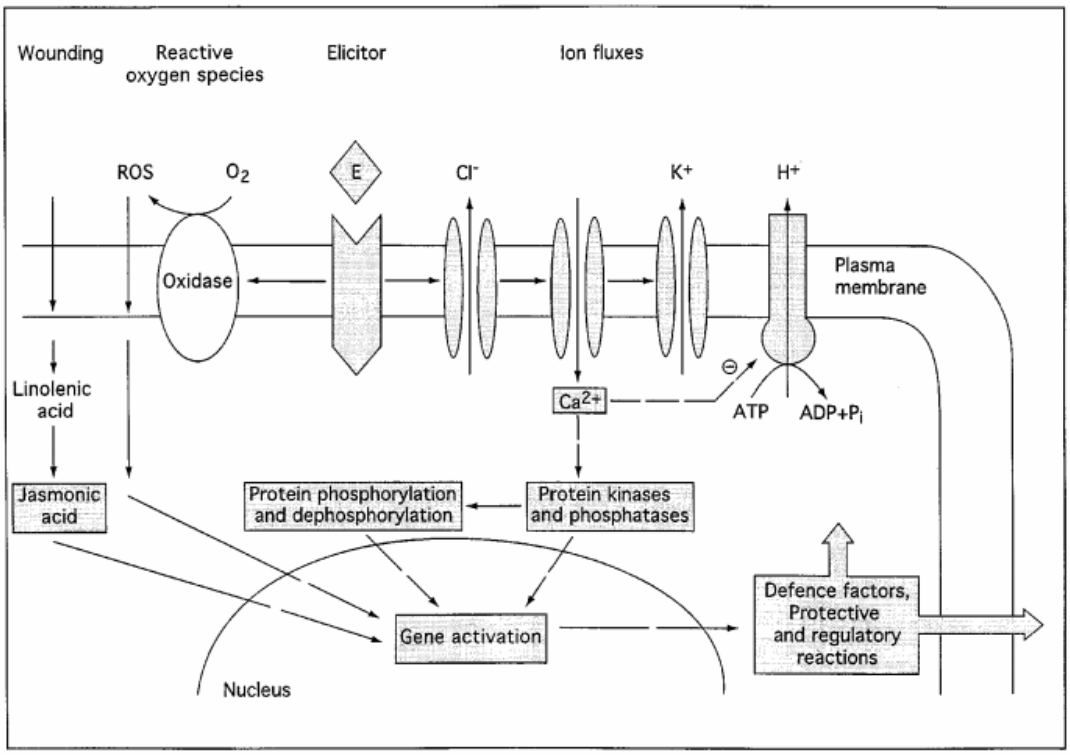
Elicitors can be further classified as endogenous and exogenous. Endogenous elicitors can either be released from the cell wall, like oligogalacturonans or from an infective organism like olygomers of chitin being hydrolyzed by from the cell wall of pathogenic fungi. [27]

5.4 MECHANISMS OF ELICITATION

Activation of appropriate genes is responsible for the majority of defense reactions of the plant. Elicitors do not make use of this approach but through signal transfer compounds .For the signal to travel from the elicitor receptor in the cytoplasm membrane to the DNA, exist different systems: a) production of active oxygen species like superoxide [27] b) the adenosine monophosphate system in which elicitor binding to specific receptor located in membanes causes the GTP to bind to G-protein. The G-protein enables activation of ATP-ase in order to synthesize cAMP

which in turn alters the action of phophatases and proteinkinases. The latter alters the level of intracellular proteins (enzymes) that are phosphorylated and stress metabolites being produced. c) transferring via calcium ions. [28]

Image: representation of activation of defense systems being activated by elicitors. [40]



6 CHROMATOGRAPHY

This method is used to separate, determine and identify the constitution of mixtures that consist of many components. Its characteristic is the use of a mobile phase and a stationary phase. The concept of this method is that the components of an analyte are transferred through the stationary phase by the movement of the mobile phase. The separation takes place according the different migration rates of the components participating in the mobile phase. Two basic types of chromatographic methods are used: Planar chromatography and column chromatography. In the case of column

chromatography there is further classification based on the type of the mobile phases, which can be gas or supercritical fluid and liquid. [29]

6.1 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography is the type of elution chromatography that is used in the widest range, for determing and separating materials of inorganic, organic and biological nature. According to the type of the stationary phase exist few types of high performance liquid chromatography. These are: adsorption or liquid-solid chromatography, ion-exchange, partition or liquid-liquid chromatography, affinity chromatography, size-exclusion chromatography. [29]

II THE AIM OF THE WORK

The aim of the diploma thesis is to evaluate the effects of **ethephon** (as abiotic elicitor) on the secondary metabolites production in callus and suspension cultures. To find whether this abiotic elicitor is able to increase production of secondary metabolites in cultures *in vitro*.

III MATERIALS

1 BIOLOGICAL MATERIAL

In this experiment it was used callus culture developed from germinated seeds of *Hypericum perforatum* in the 90^{th}- 96^{th} passages.

2 LABORATORY APPARATUS AND MACHINES

The following list contains the laboratory apparatus and machines that were used in this experiment (List 1)

MACHINE PRODUCER

MACHINE	LOCATION
Analytic scale A 200S	**Sartorius, Germany**
Autoclave PS 20 A	**Chirana , Czech Republic**
Autosampler Jacco AS-2055 Plus	**Jasco, Japan**
Column LiChristopher RP-18 250x4 (5 µm)	**Merck, Germany**
Column thermostat Jetstream 2 Plus	**Jetstream, Japan**
Compartment with laminar flow Fatran LF	**Pokrok, Slovakia**
Diode detector Jasco MD-2015 Plus	**Jasco, Japan**
Mycrofilter (0,45 µm)	**Tessek , Czech Republic**
Pump Jasco PU-2089 Plus	**Jasco, Japan**
Warm-aerial sterilizer Chirana SVS9/1	**Chirana , Czech Republic**
Water bath type 1042 GFL	**Germany**

3 CHEMICALS

The following list contains the chemicals that were used for this experiment (List 2)

CHEMICAL	PRODUCER
Ajatin Profarma-Produkt	Czech Republic
Ammonium nitrate, p.a	Penta, Czech Republic
Anhydrous sodium sulphate	Lachema, Czech Republic
Calcium chloride p.a.Z	Penta, Czech Republic
Casein hydrolysate	Imuna, Slovakia
Cobalt chloride p.a.	Lachema, Czech Republic
Copper (II) sulphate p.a.	Lachema, Czech Republic
Disodium edentate	Lachema, Czech Republic
Distillated water	Department of Analytical Chemistry, Faf UK HK Czech Republic
Ethanol 96%	Lachema, Czech Republic
Ethephon (2-chloroethylphosphonic acid)	
Ethyl acetate p.a.	Lachema, Czech Republic
Ferrous sulphate	Lachema, Czech Republic
Glacial acetic acid	Penta, Czech Republic
Glycine	Aldrich, USA
Hydrochloric acid	Penta, Czech Republic
Magnesium sulphate p.a.	Lachema, Czech Republic
Methanol HPLC grade	Merck, Germany

Methanol p.a	Penta Czech Republic
Monopotassium phosphate p.a	Lachema, Czech Republic
Myo-Inositol	Fluka, Switzerland
Nicotinic acid	Lachema, Czech Republic
o-phosphorus acid	Lachema, Czech Republic
Potassium iodide p.a.	Lachema, Czech Republic
Pyridoxine	Koch-Light Laboratories, Great Britain
Sodium molybdate	Lachema, Czech Republic
Sucrose	Lachema, Czech Republic
Thiamine	Koch-Light Laboratories, Great Britain
Zinc sulphate	Lachema, Czech Republic
α-naphtyleneacetic acid	Sigma, USA

IV METHODS

1 *IN VITRO* CULTURES CULTIVATION

1.1 NUTRIENT MEDIUM PREPARATION

For this experiment as growth medium was used Murashige and Skoog [39], its composition being explained in the table

COMPOUND	CONCENTRATION
MACROELEMENTS	
Ammonium nitrate (NH_4NO_3)	**1,650 mg/l**
Calcium chloride ($CaCl_2 \cdot 2H_2O$)	**440 mg/l**
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	**370 mg/l**
Potassium phosphate (KH_2PO_4)	**170 mg/l**
Potassium nitrate (KNO_3)	**1,900 mg/l**
Microelements	
Boric acid (H_3BO_3)	**6.2 mg/l**
Cobalt chloride ($CoCl_2 \cdot 6H_2O$)	**0.025 mg/l**

Cupric sulphate ($CuSO_4 \cdot 5H_2O$)	**0.025 mg/l**
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	**27.8 mg/l**
Manganese sulphate ($MnSO_4 \cdot 4H_2O$)	**22.3 mg/l**
Potassium iodide (KI)	**0.83 mg/l**
Sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$)	**0.25 mg/l**
Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	**8.6 mg/l**
$Na_2EDTA \cdot 2H_2O$	**37.2 mg/l**
Vitamins and organics	
i-Inositol	**100 mg/l**
Niacin	**0.5 mg/l**
Pyridoxine · HCl	**0.5 mg/l**
Thiamine · HCl	**0.1 mg/l**
IAA (indole-3-acetic acid)	**1–30 mg/l**

Glycine (recrystallized)	**2.0 mg/l**
Edamine S	**1.0 g/l**
Sucrose	**30 g/l**
Agar	**10 g/l**

Every compound was weighted with the use of an analytical scale and compounds that were in a low amount we used pippeting, for their measurement after preparing their water solutions. Dissolving of every component was carried out to 1000 ml with distilled water in volumetric flask. An ethanol solution of α-napthaleneacetic acid (α-NAA), in the amount of 1mg per 1 L of growth medium), was added as a growth regulator.

1.2 CALLUS CULTURE CULTIVATION

In order to prepare the callus culture I used Erlenmeyer flasks which were first cleaned by hot water with a detergent, and then they were rinsed with potable water and in the final stage dried at 200^0C using warm-aerial sterilizer. Every flask was stuffed with a 'bridge' of filter paper onto which 30 ml of nutrient medium was poured. Then the flasks were sterilized in autoclave at 120^0C for 15minutes after first being closed with aluminum foil. After being saturated with MS growth medium, the callus cultures were transferred into Erlenmeyer flasks. All handling with callus cultures took place in a laminar flow chamber which was illuminated by UV lamp and cleaned using 96% ethanol. The flasks and the foil used to cover them were sterilized with iodine solution of Ajatin and both scalpels and pincers were sterilized with warm aerial sterilizer at 200^0C for 2 hours. The controlled conditions for

growing of callus cultures were: Temperature: 25^0C, Hours in the light: 16, Hours in the dark: 8.

1.2.3. SUSPENSION CULTURE CULTIVATION

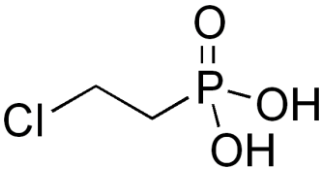
The suspension culture was then derived from the callus culture mechanically by shaking in Murashige and Skoog nutrient medium supplemented with α-naphtylacetic acid (NAA) as a growth regulator at a concentration of 5.4×10^{-5} mol/L. *S.* and was cultivated at the same temperature and photoperiod conditions as *H. perforatum* callus cultures.

The subject of my study was also to determine the hyperoside releasing into nutrient medium of callus and suspension cultures.

2 ELICITATION METHOD

2.1 ELICITORS PREPARATION

For our experimental work, as an elicitor was used Ethephon (2-chloroethylphosphonic acid), its structure being represented in figure:



2-chloroethylphosphonic acid

Three different concentrations were used, designated as c_1 (1mg/100ml), c_2 (10mg/100ml), c_3 (100mg/100ml). In order to prepare c_3 we weighted 0.100g of ethephon and then diluted them in 100 ml of ethanol 96%. For the concentration c_2, 10 ml of the previous solution (c_3) was used and diluted to 100 ml of ethanol 96%. In the end, for the concentration c_1, 10 ml of the second solution (c_2) was used and diluted to 100 ml of

ethanol 96%.

2.2 ELICITATION

I proceeded with elicitation during 21st day after last subcultivation took place. Throughout the application of elicitor, sterile pipettes were used. 32 flasks were utilized with 8 of them serving as control samples containing 1 ml of ethanol. The other 24 flasks were filled, by pipetting, with 1 ml of certain concentration of elicitor. 6 lines of 4 flasks each were designated 6, 12, 24, 48, 72, 168, corresponding to the time of withdrawal of each line. Calluses were then dried upon filter paper at room temperature and pulverized before they got weighted.

3 CONTENT OF FLAVOINOID DETERMINATION IN HYPERICUM PERFORATUM CULTURE *IN VITRO*

3.1 PRINCIPLE

The principle of this determination is chromatography, using High Performance Liquid Chromatography (HPLC).

3.2 ANALYSIS

In a water bath under a reflux condenser the callus tissues were extracted (0.1 g) with the use of 10 ml of methanol 80% V/V for 10 minutes. The product of the extraction was filtered and then, using vacuum distillation, concentrated to 10 ml. From these, 1.7 ml was inserted to vials using microfilters (0.45 μm) and then was used for HPLC analysis.

HPLC analyses were done on:

- chromatograph Jasco (pump PU-2089,

- detector MD-2015
- autosampler AS-2055
- column thermostat Jetstream II plus).
- Column MERCK LiChrospher RP-18 250x4 (5µm) fitted with guard column.

Mobile phase: from the start to 10 min- isocratic elution, then gradient elution. Flow rate 1,0 ml/min.

Eluent A= 8% acetonitrile with 0,15% phosphoric acid, eluent B= 100 % acetonitrile,

- time 0 (100% eluent A)
- time 10 min (100% A)
- time 30 min (89,1%A, 10,9% B)
- time 55 min (43,5%A, 56,5% B)

Injected volume 100 µl.

Detection of spectra in wavelengh 200-650nm. Main spectrometric detection at 254 nm.

Peak areas were integrated, spectra were compared with standards and concentrations of individual compounds were calculated with Jasco software ChromPass.

Used standards: chrysin, quercetin, apigenin, rutin, vitexin, hyperoside, quercitrin, hesperidin, myricetin, baicalin, baicalein, chlorogenic acid and caffeic acid

This method was used for identifying the flavonoids in the samples. In the case that some flavonoid was detected, precise method for determination was used. There was hyperoside in some samples, so this method for hyperoside determination was used:

Simultaneous determination of hyperoside and quercitrin:

HPLC chromatograph Jasco (pump PU-2089, detector MD-2015, autosampler AS-2055, column thermostat Jetstream II plus).

Column MERCK LiChrospher RP-18 250x4 (5µm) fitted with guard column.

Mobile phase: gradient elution, flow rate 1,5 ml/min.

Eluent A= 8% acetonitril with 0,15% phosphoric acid, eluent B= 100 % acetonitril,

- time 0 (90% of A, 10% B)
- time 10 min (80% A, 20%B)
- time 23 min (75% A, 25% B)

Injected volume 20 µl.

Detection of spectra in wavelengh 200-650 nm. Main spectrometric detection at 254 nm.

Peak areas were integrated, compared with standards and concentrations of individual compounds were calculated with Jasco software ChromPass.

4 STATISTICAL ANALYSIS

For statistical analysis of the effect of elicitor, an independent two sample t-test was utilized, in which the t-statistic to detect the difference between the means was calculated according the following equation:

$$t = \frac{|x_1 - x_2|}{\sqrt{n_1 s_1^2 + n_2 s_2^2}} * \sqrt{\frac{n_1 n_2 (n_1 + n_2 - 2)}{n_1 + n_2}}$$

(equation no.1)

Where t= tested criteria, x1=mean of control sample and x2=mean of experimental sample, s1=standard deviation of control sample and s2=standard deviation of experimental sample, n1=number of participants in control sample and n2=number of participants in experimental sample. The overall number of freedom is the result of the equation v= n1 + n2 – 2 (equation4). The statistical importance of the value, is the probability that the absolute value of t could large or lager by any chance, under the null hypothesis of equal means. The number of participants is n1=n2=3 and the number of degrees of freedom is v=4, due to the performance of 3 parallel determinations. The critical value t (t(v)p) is 2,78 for 4 degrees of freedom and for confidence range of 95%. If the calculated value t is higher than the critical value, then the results can be considered as statistically significant. For withdrawn samples after 6, 12, 24, 48 we used the t value of control sample after 24 hours and for withdrawn samples of 168 hours, the t value of control sample after 168 hours.

V RESULTS AND DISCUSSION

(Table 1) Content of hyperoside (%) in callus cultures of *Hypericum perforatum* after elicitor treatment

CONCENTRATION (MG/100ML)	TIME OF EXPOSURE(HOURS)	CONTENT OF HYPEROSIDE (%)
1 (c_1)	6	0
	12	0
	24	0
	24K	0
	48	0
	72	0
	168	0
	168K	0
10 (c_2)	6	0
	12	0
	24	0
	24K	0
	48	0
	72	0
	168	0
	168K	0
100 (c_3)	6	0
	12	0
	24	0
	24K	0

	48	0
	72	0
	168	0
	168K	0

K –control (without ethephon treatment)

Elicitation with ethephon on callus cultures of *Hypericum perforatum* proved to be have no effect on production of hyperoside (see Table 1). None of the three tested concentrations at any exposure time had an effect on increasing hyperoside content.

(Table 2) Content of hyperoside (%) in suspension cultures of *Hypericum perforatum* after elicitor treatment

CONCENTRATION (MG/100ML)	TIME OF EXPOSURE(HOURS)	CONTENT OF HYPEROSIDE (%)
1 (c_1)	6	0
	12	0
	24	0
	24K	0
	48	0
	72	0
	168	0
	168K	0
10 (c_2)	6	0
	12	0
	24	0

	24K	0
	48	0
	72	0
	168	0
	168K	0
100 (c_3)	6	0
	12	0
	24	0
	24K	0
	48	0
	72	0
	168	0
	168K	0

K –control (without ethephon treatment)

Results showed that elicitation with ethephon on suspension cultures of *Hypericum perforatum*, had no effect on production of hyperoside, either (see Table 2). Concentrations c_1, c_2, c_3 were ineffective in increasing the presence of hyperoside, at any exposure time.

(Table 3) Content of hyperoside (%) in nutrient medium of callus cultures of *Hypericum perforatum* after elicitor treatment

CONCENTRATION (MG/100ML)	TIME OF EXPOSURE(HOURS)	CONTENT OF HYPEROSIDE (%)
1 (c_1)	6	0
	12	0
	24	0
	24K	0
	48	0
	72	0
	168	0
	168K	0
10 (c_2)	6	0
	12	0
	24	0
	24K	0
	48	0
	72	0
	168	0
	168K	0
100 (c_3)	6	0
	12	0
	24	0
	24K	0

	48	0
	72	0
	168	0
	168K	0

K –control (without ethephon treatment)

Results indicated that there was no releasing of hyperoside in the medium of callus culture of *Hypericum perforatum* (see Table 3). None of the three different concentrations (c_1, c_2, c_3) produced an increase in hyperoside content, at any exposure time.

(Table 4) Content of hyperoside (%) in nutrient medium of suspension cultures of *Hypericum perforatum* after elicitor treatment

CONCENTRATION (MG/100ML)	TIME OF EXPOSURE(HOURS)	CONTENT OF HYPEROSIDE (%)
1 (c_1)	6	0
	12	1,1
	24	0
	24K	0
	48	0
	72	0
	168	0
	168K	0
10 (c_2)	6	0
	12	0

	24	0
	24K	0,1
	48	0,3
	72	0,2
	168	0,2
	168K	0
100 (c_3)	6	0
	12	0
	24	0
	24K	0,1
	48	0,1
	72	1,1
	168	0,3
	168K	0

K –control (without ethephon treatment)

Medium of suspension cultures of *Hypericum perforatum* were the only which showed that there is increase in hyperoside production. At the concentration of c_1 (1mg/100ml) and after 12 hours, there was increase in content of hyperoside which was considered as highly statistically significant. After 24 hours there was observed no effect of ethephon on hyperoside releasing into medium. At concentration of c_2 (10 mg/100ml) and after 48 hours there was a 3-fold increase in hyperoside releasing. At the same concentration and after 72 and 168 hoursthere was a 2-fold increase of hyperoside releasing into medium. At the highest concentration c_3 (100mg/100ml) there was an increase after 72 hours and it was considered as very statistically significant. After 168 hours there was a 3-fold increase in hyperoside releasing into medium in comparison with control (168K). (see Table 4)

As a summary, I must say that there was no increase in the production of flavonoids in callus cultures, suspension cultures and medium of callus cultures. The only results were shown at the medium of suspension culture. In another study, where *N*-(3-iodo-4-methylphenyl) pyrazine-2-carboxamide was used as elicitor on callus culture of *Silybum marianum*, the most important effect was observed at concentration of 10mg/100ml and after 6 hours. [31] There can be several reasons for the low production. One of them can be considered, the fact that we used very old cultures (90-96th passages). Another can be considered the use of ethanol, in both control samples and also as a solvent in elicitors. Results can be influenced more by errors with small values, thus making both statistical analysis and determination of content less reliable. The use of a non suitable elicitor can be taken under consideration, whereas the medium of growth can not be considered reason for low production, since the same medium has been used for years in many other experiments.

VI CONCLUSIONS

Elicitation effect with ethephon (2-chloroethylphosphonic acid) on hyperoside production in callus cultures, suspension cultures and its releasing into medium of callus cultures was ineffective, since in none of them was an increase in production of flavonoids. In the case of nutrient medium of suspension culture the highest hyperoside releasing, was observed with concentration c_1 (1mg/100ml) after 12 hours and with concentration c_3 (100mg/100ml) after 72 hours of elicitor application. These two results could be the object for further investigations. I highlight the need for next experiments with younger callus and suspension cultures, because the ones used for this experimental work were very old.

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