

## Plant protoplasts in stress conditions – pioneer studies of Professor Edward Pojnar and their continuations

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This presentation is to commemorate Professor Edward Pojnar (1919-2011), Head of the Department of Botany (1974-1990) of the current University of Agriculture in Krakow, who initiated research on isolated plant protoplasts in Poland. He had learned the procedure of protoplast isolation by an enzymatic method in Professor E.C. Cocking's Laboratory of Plant Physiology at the University of Nottingham. In 1969, the postdoctoral thesis of Edward Pojnar was published as the first paper on protoplast isolation and culture in Poland.

Present paper provides a survey on Professor Pojnar's research, as well as on further studies inspired by his pioneer achievements. Among others pioneer studies, for example in mutation breeding he exploited fast neutrons as mutagen in cultured protoplasts. Fast neutrons can be used in plant breeding as a mutagen that acts at the level of a single-cell explant, and protoplasts are convenient objects for rapid testing of a mutagen action at the micro level. In our laboratory we continue research using protoplasts as material in studies on plant susceptibility and resistance to viral pathogens. The conducted studies revealed that a wild line of tomato was highly tolerant to TSWV infection. Therefore protoplasts were used as donor material to introduce anti-TSWV genes into tomato cultivars by means of somatic hybridization. Simultaneously, studies on legume protoplasts were also conducted in the Department, and a few years later on monocot protoplasts as well. These both groups

of plants are regarded as difficult in *in vitro* culture, due to their susceptibility to stress factors, such as protoplast isolation itself and culture in artificial conditions. For developing novel, interesting ornamental forms, protoplast isolation and culture were optimized for tulip, hyacinth, narcissus and *Cymbidium* orchids.

With the view of enhancing the resistance of vegetable asparagus to *Fusarium* isolation and culture, protocols were developed for ornamental asparagus naturally resistant to those pathogens. Attempts have also been made to improve culture conditions for regeneration of legume plants, such as white clover, yellow lupin and grasspea. Recent research exploiting protoplast technology, focused on the structural changes involved in regeneration, are also discussed. Originally, the isolated protoplasts were considered to be a new protoplast-plant system for genetic manipulation. Nowadays, due to public opposition (especially in Europe), they are regarded rather as a convenient material for somatic hybridization, cybridization, protoclonal variability, or proteomics and metabolomics. In most studies exploiting plant protoplasts is essential for developing efficient methods for the isolation of viable protoplasts of high morphogenetic potential, and the regeneration of complete plants. Therefore our team from the Department of Botany and Plant Physiology in Cracow continue research to understand and bypass the existing barriers in protoplast regeneration.

## Plant cell response to stresses

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Plant cells cultured *in vitro* are influenced by stresses that usually do not occur *in planta*. Among them there are: different atmosphere content in vials, non-physiologically high hormone concentrations, vitamin, trophic and mineral compound concentration, opposite gradient of hormones, different light spectrum and constant temperature. Defence responses of plants proceed on several levels: subcellular, cellular, and systemic. Using *in vitro* culture many plant defence responses may be investigated, however in callus and cell suspensions or protoplast culture only reactions proceeding on subcellular and cellular level may be studied. In *in vitro* culture conditions the followings are usually studied: the influence of heavy metal ions, osmotic stress (drought and salinity), temperature (cold or heat), light stress, biotic stress (bacteria or fungus metabolites, toxins), influence of chemical compounds (herbicides), and oxidative stress, recognized as secondary stress accompanying another stresses.

Signals of various stresses may have differentiated character. They can be the chemical molecules (hormones, herbicides, ions, toxins) or they can act as physical signals, for example temperature or light.

The molecules are recognized by specific protein receptors bound with cell membranes or located in cytoplasm. One domain of the receptor plays a role of kinase activating effector protein. Temperature is received by cell membranes or cytoplasmic colloids, while light by phytochromes. All signals initiate signal transduction leading to transcription of defence genes. Some plant

responses to several stresses are specific. Cold treatment initiates the synthesis of antifreeze proteins which protect the cell against forming ice crystals. High temperature mainly destabilizes cell membranes and binds with them proteins involved in photosystems in chloroplasts and the electron chain in mitochondria. High temperature increases dramatically the amount of heat shock proteins. Salinity stimulates higher water accumulation in vacuoles to dilute salt concentration. Moreover, the cells accumulate osmolytes to decrease the osmotic potential in vacuoles. Heavy metals are very toxic especially to the enzymes. They are trapped by specific phytochelatins, small peptides with active sulfhydryl group, and are transported to the vacuole. The above mentioned responses to stresses relate only to the changes proceeding within the cell, while many other response processes are induced at the systemic level. Defence mechanisms also involve many changes that are non-specific and are observed as a response to various factors. Plant defence responses proceeding at the cellular level include mainly synthesis of reactive oxygen species, activation of antioxidants, synthesis of phenylpropanoids (phytoalexins, flavonoids for example anthocyanins, lignin, tannins), synthesis of pathogenesis-related proteins, dehydrins, hormones (abscisic, jasmonic, salicylic acid, ethylene), sugars, fatty acids, phytosterols and polyamines. In the first phase of action many stresses affect mainly cell membranes and processes that are closely associated with their structure and properties.

## Stress as a factor of evolution

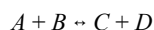
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The authors formulate a thesis that stress may also be considered in isolation from living organisms. They base on the definition of stress which describes it as each factor that disturbs the state of homeostasis. This definition also refers to molecular systems, not only to living organisms.

The equilibrium of any chemical reaction is described by the law of mass action, done by the equation:



where the letters *A*, *B*, *C*, and *D* represent reactive chemical compounds. [*A*], [*B*], [*C*] and [*D*] represent the molar concentrations of these compounds. When these reagents are mixed in any proportions of their amounts they start to react, but finally their concentrations stabilize and the final values of [*A*], [*B*] etc. fulfill the following equation, known as the law mass action:

$$\frac{[C] \times [D]}{[A] \times [B]} = K$$

where *K* is the reaction constant, independent of the initial concentrations of the substance, but characteristic for the reaction and hence called the equilibrium constant of the reaction. If the system being in equilibrium is interrupted of the state, the reaction is activated by which the disturbing factor is nullified and the system returns to the equilibrium. A steady-state for the reaction is analogous to the state of homeostasis for the system composed of organism and its environment, and the change in the concentration of the reacting substances is analogous to the stress that acts on the organism. The throwing off both equilibrium (for chemical reaction) or stress (for the organism) forces a "reaction" of the molecules or of the body, leading to the restoration of homeostasis. This means that one can talk about stress in relation to the era of molecular, cellular, and later stages of the evolution, and is also possible to consider the role of stress in the evolution of the Universe – before the creation of the Earth itself. In the next part of the lecture an examples will be presented which support the important role of stress in the evolution.

One of the main conditions of assuming any object as a living object, is the existence of homeostasis and, stress, as opposite to the former state. It can be assumed that the system does not evolve when it lasts in a state of homeostasis, while the variability of various systems and their ability to evolve are revealed under stress. This gives the stress a much more important function than was assumed earlier.

Next the conditions, appeared as necessary to the life come into being, will be listed. A particular attention is paid to the diversity of elements that build the Earth, which indicates that the formation of the Planet was preceded by the evolution of at least two generations of stars and accompanying stresses on a cosmic scale. A molecular stage of evolution on the Earth might have started in the geothermal vents, as the places of very strong gradients (temperature, concentrations of minerals leached out of the magma and pressure gradients) that drove this evolution.

Stress may also be considered as promoter of later than molecular one stages of the evolution of life. Attention will be drawn to the role of the cell membrane in maintaining gradients of concentration of various cells components and the reasons for which the main of cell membrane components are phospholipids. Also discussed is part of various stresses and gradients in the process of enzymatic catalysis, the role of low temperature stress in the regulation of flowering of plants and finally the stress occurring under in vitro conditions that induce the most surprising responses of cells and tissues, such as organo- and embryonic and gametogenesis, somatic fusion of protoplast etc.

The stress thus appears to be the main stimulator of the evolution. This evolution may refer to the entire Universe from the first moments after the Big Bang, through the evolution of galaxies and individual stars with planets, including our Sun, and ending with the evolution leading to the emergence of life and civilization.

## Plant-beneficial bacteria in *in vitro* cultures

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It is hard to imagine a plant that does not coexist with bacteria, but this coexistence can be of a very different character – from pathogenesis to a far-reaching protection against biotic and abiotic stresses and interference in the processes of growth and development. Bacteria co-existing with plants are often described as their second genome – the microbiom. Both the bacteria colonizing the surface of the above- and underground plants (epiphytes), and inhabiting the interior of plants – vascular bundles, and the spaces between-cells and cell interiors (endophytes) are important for plant life. The impact of bacteria consists in the inactivation of harmful compounds produced by plants, such as methanol, facilitation of the absorption of elements from the soil, assimilation of atmospheric nitrogen, production of protective substances, such as elicitors and osmoprotectants, induction of resistance, providing growth regulators, and the effect on the metabolism of plant cells using signaling elements. Today many strains of bacteria are known to be beneficial to plants. They are already used as bioactive preparations in the growth of plants or are just being developed for practical use. The positive effect of such bacteria is the greater, the less favorable are the growing conditions.

Also in plant tissue cultures, bacteria are often or usually present. Their detection is possible if they can be cultured on any microbiological medium or if their amounts are large enough to be detected by molecular methods.

Bacteria associated with plant tissue cultures are also important, although to a lesser extent; because plant explants are generally grown in nearly optimal conditions. However, the idea of bacterization of the plant tissue cultures was first expressed by Digat et al. (1987) and then by others (Nowak, 1998). *Burkholderia phytofirmans* PsJN (earlier known as *Pseudomonas* PsJN) is the most studied beneficial bacterium for the plant tissue culture (Sessitsch et al., 2005). It is able to colonize easily, in the endophytic manner a range of various plants. It was reported as stimulating the growth of the root system, es-

pecially in the zone of root hairs, stiffing stems due to lignin deposits around vascular system, increasing the dry matter content, the contents of phenolics, chlorophyll and cytokinins. Their endogenous presence increases the resistance to the low levels of bacterial and fungal pathogens, and improves water management. Different strains of *Azospirillum brasilense*, *Azotobacter chroococcum*, *Azorhizobium caulinodans*, *Pseudomonas fluorescens*, *P. aureofaciens* and *P. putida*, *Halomonas desiderata* as well as a number of Metylobacteria, Bacilli and Curtobacteria were also described as useful. Cultures of bacteria that infect plant tissue cultures are largely responsible for the lack of reproducibility in physiological experiments based on the *in vitro* culture technique and reproducibility of protocols between commercial laboratories. Bacteria producing compounds acting as growth regulators may be capable of inducing regeneration of adventitious shoots or somatic embryos in the recalcitrant genotypes (Murthy et al., 1999; Kalyaeva et al., 2003). However, it is assumed that the most useful application of bacteria to plant tissue cultures is the increase of resistance to stress, which microshoots can undergo when moving to the *in vivo* conditions (Thomas J., 2010; Vettori et al., 2010). Beneficial bacteria contaminating plant tissue cultures during the acclimatization can protect microplants against pathogenic fungi i.e. *Verticillium dahliae* (Sharma and Nowak, 1998) or *Botrytis cinerea* or abiotic stresses (Sziderics et al., 2007).

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## When plant cells can survive ultra-low temperatures

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Research on the effect of ultra-low temperatures on plant organisms started in the early 20th century, but it was not until the 1950s and 70s when the modern approach was initiated in northern countries of our hemisphere, such as Canada, the USA, the UK, Japan, Russia, and France. Fifty-six years have passed after the first successes by Sakai (1956) in cryopreservation of willow shoots in liquid nitrogen, which had opened the possibility for long-term storage of plants. Depending on plant species, various organs, tissues or cells: seeds, pollen, shoot apices, dormant buds, excised embryonic axes, zygotic or somatic embryos, callus, and cell cultures are cryopreserved, i.e. stored in ultralow temperatures. Cold acclimation is a complex, physiological adaptive response induced to protect cells against lethal freezing injury. According to the two-factor hypothesis of freezing injury formulated by Mazur (2004), intracellular dehydration and ice formation are the primary deleterious events.

The water status in combination with physiological factors is the most influential determinant of survival of plants or their organs at low temperature. It is possible to cryopreserve plants safely by the process of vitrification, i.e. solidification of liquids without crystallization. This is associated with the glassy state, which is amorphous, lacking organized structure but has the mechanical and physical properties of the solid state. The key to the development of cryoprotective vitrification strategies is to increase cell viscosity to the point at which the ice formation is inhibited and water becomes vitrified on the exposure to cryogenic temperature.

Critical for the development of cryopreservation is the control and/or avoidance of intracellular ice nucleation. Biological antifreeze proteins (AFPs) are naturally occurring compounds found in many plants and insects. Low-molecular AFPs inhibit ice crystal growth by binding ice nuclei, which decrease the freezing point of the solution, thereby allowing survival at subzero temperatures. Such AFPs are found in apoplasts of frost-hardy conifers: in needles of *Abies*, *Picea*, *Tsuga* and also in embryonic axes of seeds of the families Aceraceae and Fagaceae. In our experiment the activity of cold-labile enzyme, lactate dehydrogenase (LDH) was higher after freezing in liquid nitrogen in the presence of protein fraction from these tissues. The cryoprotective activity of AFPs from the above plants was 6- or 10 fold more effective than that of compatible solutes, such as sucrose (a commonly used cryoprotectant) and BSA. Thus AFPs, but also sucrose, and other cryoprotectants, help to enable survival of plant cells in ultra-low temperatures. The most common colligative (penetrating) cryoprotectants used in plant cryopreservation are glycerol, dimethyl sulphoxide (DMSO), methanol and the glycols with smaller molecular weight.

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## Methods of protecting ferns in cryogenic conditions

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Among many different methods of *ex situ* plant biodiversity conservation, the methods that employ liquid nitrogen (LN) play a very important role. Their main advantage is high effectiveness in reducing the life processes that occur in cells, so that the “aging” of the plant material is almost completely inhibited. Other benefits are the relatively low cost of a long-term storage and the possibility of collecting diverse plant material, not only the seeds or spores, but also pieces of tissues such as buds, meristems, embryos or gametophytes. Freezing of that kind of plant material in liquid nitrogen must be certainly preceded by choosing an appropriate cryoprotection method. So far, the achievements in the field of cryopreservation in combination with *in vitro* propagation of cultures have been successful for the preservation of spores and gametophytes viability of several *Pteropsida* species, which may now be easily stored for long time. The aims of the studies undertaken in the Botanical Garden – CBDC in Powsin were: 1) development of cryo-treatment conditions for cryopreservation of *Pteropsida* spores and/or gametophytes and 2) the long-term cryostorage of spores and gametophytes of selected fern species in a manner allowing to maintain their high viability.

Our studies on spore cryopreservation included a four-year-long monitoring of the viability of fern chlorophyll and non-chlorophyll spores stored at LN temperature ( $-196^{\circ}\text{C}$ ) and at  $5^{\circ}\text{C}$ . We found that the spores which were not additionally treated by drying, but immediately cryostored after their harvest, maintained 100% of viability.

The research resulted in the development of an efficient cryopreservation method for gametophytes of seven woody fern species and two herbaceous ones. These species differ from one another by low temperature and the desiccation stress tolerance (Mikuła et al., 2011). The application of a two-week long preculture and an encapsulation-dehydration technique allowed us to achieve a high gametophyte survival (from 70% to 100%) after freezing in LN.

In the next stage, the studies on gametophyte cryopreservation were carried out on fern species having high (*Asplenium adiantum-nigrum*, *A. adulterinum*, *A. cuneifolium*,) and extremely low (*Ceratopteris thalictroides*) tolerance to abiotic stresses in their natural habitat. As a result of these experiments, it was shown that among all *Pteropsida* species studied so far, these four newly introduced species mentioned above were characterized by viability that was extremely different from others. Gametophytes of the three *Asplenium* species demonstrated a survival of 100%, even without a previous preculture, while gametophytes of water fern *C. thalictroides* survived cryopreservation only in 42%. A modification of the cryotreatment conditions allowed us to improve the survival rate of *C. thalictroides* gametophytes by more than 50%. This was obtained by a reduction of the size of alginate capsules, in which the plant material was enclosed, and by the exclusion of light from the cryogenic procedures. A natural high tolerance of *Asplenium* gametophytes to abiotic stresses enabled us to eliminate the two-week-long preculture without affecting their viability in the post thawing culture. In the case of water fern gametophytes, sensitive to dehydration stress, an additional modification of the encapsulation-dehydration technique was required to protect them against LN effects.

The presented studies broaden the general knowledge of cryopreservation of fern gametophytes and spores. They also open up new possibilities for collecting *Pteropsida* gametophytes in liquid nitrogen. We hope that in the near future our experience gained so far will result in a special program of a biodiversity cryoprotection of endangered fern species.

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## Vitrification method for cryopreservation of embryogenic tissues of spruce trees (*Picea* spp.)

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Embryogenic tissues of coniferous species are usually stored in liquid nitrogen by using the slow-freezing method, since this method was first successfully applied in cryopreservation of *Picea abies* embryogenic cultures. Recently it has been found that a rapid-freezing method based on the vitrification phenomenon is an alternative to the above cryopreservation method of spruce tissue cultures. The greatest advantage of this method is that it is simple and requires no toxic cryoprotectants, such as DMSO (which can contribute to metabolic changes in cells, including the genetic material).

The aim of this study was to investigate if this method is effective in cryopreservation of *Picea abies* (Norway spruce) and *P. omorika* (Serbian spruce) embryogenic tissues (ETs), and if the plant material stored with this method is genetically stable.

One- and two-year-old embryogenic tissues of *Picea abies* and *P. omorika*, respectively, were successfully cryopreserved after sucrose preculture and air desiccation over silica gel (so called pre-growth-dehydration method). ETs were precultured for 7 days on a medium with increasing concentrations of sucrose (0.25 M sucrose for 24 h, 0.5 M for 24 h, 0.75 M for 2 days, and 1.00 M for 3 days) and next air-dried, to a water content of 20%, placed in cryovials, and rapidly immersed in liquid nitrogen for 24 h. Part of ET of *P. abies* was precultured in the presence of sucrose and 10  $\mu$ M ABA. Next the tissues were thawed in a water bath at 42°C and transferred to the media with decreasing concentrations of sucrose (1.00-0.25 M, for 1.5 h at each concentration), and finally placed on a proliferation medium.

The survival rate of the tissues within a few weeks after thawing was more than 50% and 90% for *P. abies* and *P. omorika*, respectively. ET growth was slow in

the early weeks after thawing, but over time it gradually intensified. Proliferated ETs were friable and white, like before cryostorage. After staining of the thawed tissues with acetocarmine, only meristematic cell groups of the embryogenic region of proembryos, which differentiated into bipolar structures (with suspensors) during the post-thaw regrowth, were observed. These structures gave rise to somatic embryos (SEs) at the precotyledonary stages 2-3 weeks after placing on the medium supplemented with 20  $\mu$ M ABA and 1  $\mu$ M IBA. After 5 weeks, cotyledonary SEs had a normal structure. They were also capable of germination. A genetic analysis of 5 microsatellite regions (SpAGC1, SpAGC2, SpAGG3, SpAC1F7, SpAC1H8) in the DNA of cryopreserved *P. abies* ET and SEs obtained from restored ET after freezing in liquid nitrogen suggests genetic stability of the stored tissue after using this cryopreservation method. Both ET as SEs had normal diploid genotypes. Nine different alleles were detected among the 5 microsatellite loci tested. Except locus SpAGC2, all SSRs loci were heterozygotic. No mutations were observed in any of the loci. In cells of the analysed plant material from *in vitro* culture, subjected to preculture and stored in liquid nitrogen, only loci and genotypes identical to ET achieved from one zygotic embryo were detected.

Our results show that the presented method may be used for effective cryopreservation of embryogenic cultures of both tested *Picea* species, although its performance depends on the genotype of the stored ET line.

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## How does isolation stress influence (re)organization of cell structures in protoplasts of *in vitro* recalcitrant plants?

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Disturbances in morphological plasticity limit successful *in vitro* propagation of recalcitrant species. Protoplast culture is a particularly severe culture system, since isolated protoplasts have to cope not only with developmental reprogramming, but also serious infringement of cell integrity. Although an enzymatic digestion of cell wall components is essential to protoplast production, it also induces a stress reaction, from which protoplasts have to recover in an artificial environment. Cell wall regeneration is the first visible activity taken up by isolated protoplasts and the success of this process determines further behavior of protoplasts in culture. Studies on cell wall biosynthesis were usually conducted on protoplasts easily dividing in culture, e.g. *Solanaceae* representatives (Wang et al., 1991). In contrast, little is known about cell wall regeneration in protoplast culture of recalcitrant plants, such as numerous grain legumes and monocots. Here we present the results of our study on cell-wall regeneration, conducted in protoplast cultures of four recalcitrant plant species: yellow lupin (*Lupinus luteus* L.) and grasspea (*Lathyrus sativus* L.) (both belonging to grain legumes), and ornamental monocots: hyacinth (*Hyacinthus orientalis* L.) and asparagus (*Asparagus densiflorus* "Sprengeri" (Kunth) Jessop). The rate of cell wall resynthesis was investigated together with the arrangement of cellulosic fibres on a surface of protoplast-derived cells using Calcofluor White staining. Additionally, cytochemical detection of arabinogalactan proteins in cell walls was performed using  $\beta$ -glucosyl Yariv reagent. Quick cell wall renewal occurred in cultures of grasspea and hyacinth, where the percentage of protoplast-derived cells accounted for 45-50% after 24h. On the contrary, in asparagus and lupin cultures the

presence of a cell wall in a half of population was not observed until 5 and 7 days, respectively. Grasspea, hyacinth and asparagus cells budded intensively. Moreover, in lupin, asparagus and hyacinth, the cellulosic material of the cell wall was disorganized and unevenly distributed. Arabinogalactan proteins (AGPs) were mainly detected in spherical cells with condensed cytoplasm. No AGPs were observed in the budding cells. We concluded that an incorrect cell wall structure/composition, together with a deregulated cell cycle might contribute to protoplast recalcitrance in the examined species. Abnormalities could be a result of an isolation process itself and defective stress-recovering mechanisms. Subsequently, these could arrest regeneration competences and direct cells at apoptosis pathway.

A supplementary study on cellular rearrangements during protoplast culture was also conducted. We compared cell wall regeneration between two genotypes of yellow lupin and grasspea. Mesophyll protoplast-derived cells were stained with Calcofluor White and aniline blue to detect cell wall  $\beta$ -glucans, and with  $\beta$ -glucosyl Yariv reagent to detect arabinogalactan proteins. Also, attempts were made to provide reliable data on cytoskeleton (re)arrangements during the early stages of protoplast culture. Immunocytochemical detection of tubulin and actin was performed three times during a 10-day long culture. The results obtained in the supplementary study will be presented during the Conference.

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## Response of the ascorbate–glutathione cycle to salinity stress in cucumber (*Cucumis sativus* L.) cells in culture

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Saline environments can induce a great number of responses in plants ranging from the readjustment of transport and metabolic processes to growth inhibition. In high salt environments plants are stressed in two ways: by the increase in the osmotic potential in the rooting medium and by the toxic effect of a high concentration of Na<sup>+</sup> and Cl<sup>-</sup>. Changes in the osmotic potential might cause disturbances in the water balance in plants reducing turgid and photosynthesis. On the other hand, high Na<sup>+</sup> and Cl<sup>-</sup> levels reduce absorption of essential nutrients and, additionally, can be toxic by competing with K<sup>+</sup> in biochemical processes.

The ascorbate-glutathione cycle is an important defense mechanism against active oxygen species. The cycle was found in the chloroplasts, mitochondria, peroxisomes and cytosol. The main enzyme of the cycle is ascorbate peroxidase (APX), which cooperates with ascorbate. This enzyme reduces H<sub>2</sub>O<sub>2</sub> to water and is coupled with monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR).

In recent years, the examination of salt tolerance and the production of osmolytes in tissue culture has gained attention. In particular, the role of proline in salt tolerance of agriculturally important crop plants such as alfalfa, wheat, soybean, rice, beans, and cucumber has been examined. Many varieties identified as salt tolerant have produced higher levels of proline (compared to salt sensitive varieties) when exposed to salt.

The main purpose of experiment was to determine the pro- and antyoxidative processes in cytosol and mitochondria in an *in vitro* scheme and to check how the acclimation influenced the parameter activity. The question was: which of all the examined parameters is connected with the defense against salinity stress?

Changes in the Halliwell-Asada pathway enzyme activities, as well as TBARS and proline content in cucum-

ber cell suspension acclimated and non-acclimated were examined 0.5, 1 and 3h after a single application of 20 mM (mild stress) and 50 mM NaCl (severe stress).

The mild stress caused an increase in APX activity in cytosol compartment from the beginning of the experiment while the severe stress induced enhancement of this enzyme activity from the 3 h of experiment. The maximum increase in APX activity was observed after lower NaCl solution in acclimated cells in cytosol compartment. Moreover, in mitochondria there was an increase in APX activity after both NaCl solutions until 3 h. The severe stress caused a decrease in MDHAR activity up to 0.5 h. The maximum decrease was noted on the 3 h in the non-acclimated cell suspension.

The results suggest that the APX and GSH-Px (glutathione peroxidase) play the main role in the acclimation process. The constitutive activities of these enzymes were higher than in non-acclimated cell suspensions likewise the concentration of the proline, which points indicates better adaptation to salinity stress. Moreover the higher concentration of thiobarbituric acid reactive substances (TBARS) as a marker of lipid peroxidation enabled faster mobilization of the antioxidant system.

The salinity stress caused a dysfunction in the regeneration or synthesis of ascorbate and glutathione, which resulted in a decrease in the concentration of the reduced forms of these antioxidants, which, in turn, lowered the redox ratios.

The obtained data show that two phases may be distinguished: nonspecific (until to 0.5h) and specific (after 1h) connected with detoxification and regeneration and/or synthesis of nonenzymatic antioxidants. APX, GSH-Px and GST seem to be the earliest reacting enzymes to salinity stress and DHAR, GST (glutathione S-transferase), GR are connected with second phase.

# The molecular and physiological analysis of suppressor mutant of *abh1* revealed a new face of already known players of ABA signaling in *Arabidopsis thaliana*

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Although the importance of abscisic acid (ABA) in plant development and responses to abiotic and biotic stresses is well recognized, the molecular basis of signaling pathway remains to be elucidated. Mutants in particular genes related to ABA are widely used as a tool for getting insight into mechanisms of ABA signal transduction and ABA-dependent stress response. We used a genetic approach of suppressor screening in order to decipher the interaction between known players of ABA signaling. *ABH1* (*CBP80*) encodes a large subunit of CBC (CAP BINDING COMPLEX) and *abh1* mutant is drought tolerant and hypersensitive to ABA during seed germination (Hugouvieux et al., 2002). It is known that ABH1 (CBP80) together with a small subunit of CBC – CBP20 takes part in biogenesis of miR159 which is a negative regulator of *MYB33* and *MYB101*. The decreased level of miR159 in *abh1* mutant leads to the accumulation of *MYB33* and *MYB101* transcripts and consequently results in (the) inhibition of the germination process in the presence of low concentration of ABA (Kim et al., 2008). In order to evaluate the role of ABH1 (CBP80) or find new components interacting with ABH1 (CBP80) during seed germination in ABA presence, a suppressor mutants of *abh1* was generated after chemical mutagenesis. The mutant named *soa1* (*suppressor of abh1 hypersensitivity to ABA 1*) displays an ABA insensitive phenotype. The genetic analysis showed that *soa1* is dominant in relation to *abh1* and segregates as a single locus. Based on physiological response to a wide spectrum of physiological assays during different stages of development, we used a candidate-genes approach to identify a suppressor gene.

The molecular analysis revealed that mutation causing the phenotype of *soa1* occurred in *ABI4* (*ABA insensitive 4*) gene. The C577T mutation identified in *ABI4* is an already known allele – *abi4-101*. The co-segregation of C577T mutation in *ABI4* with suppressor phenotype was confirmed. Additionally, we investigated the close neighborhood of *ABI4* on chromosome 2. and sequenced ABA-related genes in order to exclude the possibility of the influence of another gene on the *soa1* phenotype. Our analysis clearly showed that the suppressor gene is *ABI4*. We also crossed the original *abi4-101* mutant with *abh1* and obtained the same result of genetic relationship between both loci – the phenotype caused by *abi4-101* mutation was dominant over *abh1*. All physiological tests, such as response to different doses of ABA, NaCl, mannitol and glucose, as well as morphological observations, like stomata number showed that a phenotype of *soa1* was similar to *abi4-101* mutant. qRT-PCR analysis of *MIR159* and miR159 targets – *MYB33* and *MYB101* in wild-type, *abh1*, *soa1* and *abi4-101* together with bioinformatics approach allowed us to establish a working model of interaction between *ABI4* and *ABH1*.

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## Cryopreservation of embryonic axes of recalcitrant and orthodox tree seeds (*Acer* spp.)

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The cryopreservation of embryonic axes (EAs) of *orthodox* seeds of Norway maple (*Acer platanoides*) and *recalcitrant* seeds of sycamore (*A. pseudoplatanus*) was conducted after air dehydration. The *orthodox* seeds tolerate desiccation to a low moisture content (MC) and can be stored at subzero temperatures, while *recalcitrant* seeds are sensitive to desiccation and exposure to low temperatures. The aim of the present study was to determine the possible injuries of EAs during the cryopreservation process: desiccation, pre-cooling to  $-40^{\circ}\text{C}$ , immersion in liquid nitrogen ( $\text{LN}_2$ ) and thawing cycle. EAs of *A. platanoides* tolerated desiccation to 5% MC, while EAs of *A. pseudoplatanus* were more sensitive and their viability decreased after drying below 10% MC. Results of differential thermal analysis (DTA) showed that non-freezable water in EAs of Norway maple occurs below 27% MC, while in sycamore below 36% MC. Successful cryopreservation was achieved when EAs of Norway maple were dried to 15-10% MC. The highest recovery after cryopreservation of EAs of sycamore was achieved after desiccation to 20-15% MC. Membrane injury during desiccation and cryopreservation was tested using electrolyte leakage test and analyses of malondialdehyde (MDA) and free fatty acid (FFA) contents, the products of lipid peroxidation and de-esterification. Lipids are the major class of biomolecules targeted by reactive oxygen species (ROS) in membranes. Lipid peroxidation is of concern because it affects membrane

integrity and alters its functions, leading to cell death. The increase in electrolyte leakage from cells as a result of dehydration was observed only in the case of *A. pseudoplatanus* EAs.

The dehydration and freezing of EAs were accompanied by a gradual increase in the production of ROS: superoxide ( $\text{O}_2^{\cdot-}$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in both species. On average, the increase in  $\text{H}_2\text{O}_2$  content in desiccated axes of *A. platanoides* was 28.4% higher than in *A. pseudoplatanus*. However, the increase in  $\text{O}_2^{\cdot-}$  content in EAs of *A. pseudoplatanus* was 49% higher than in *A. platanoides*. The marked accumulation of ROS coincided with the decrease in activities of antioxidant enzymes, such as guaiacol peroxidase (POX) and superoxide dismutase (SOD) in EAs of Norway maple and POX in sycamore. Oxidative stress during cryopreservation caused a decline of the reduced form of glutathione (GSH) in EAs of both species and vitamin E ( $\alpha$ -tocopherol) in *A. pseudoplatanus*. Vitamin E is an important antioxidant that is directly involved in scavenging oxygen free radicals. The obtained results showed that the cryopreservation of EAs of *A. platanoides* and *A. pseudoplatanus* without cryoprotectants could be used for preservation of their germplasm.

### Acknowledgements

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## Cryopreservation of *Gentiana cruciata* (L.): proteomic analysis of dehydration process during sucrose pretreatment helps to understand acquisition of freezing tolerance

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Contemporary plant biotechnology enables us to conserve valuable genetic resources and conduct fundamental research which the aim is to understand the processes at every level of plant organisation. Micropropagation, regeneration, transformation or conservation require storing of plant material. Cryopreservation is a technique which is used frequently for long-term preservation of the experimental material. The increasing of the knowledge of the mechanisms and regulatory processes leading to desiccation tolerance is essential to make a better use of cryo-techniques. The cryopreservation protocol of *G. cruciata* encapsulated cell suspension is based on the acquisition of dehydration tolerance during incubation at successive levels of concentrations of sucrose, being gradually increased. Achieving a high level of dehydration of the material allows cells to survive freezing and storage at ultra low temperatures. The tolerance to dehydration and freezing, which is induced at the stages of pre-treatment, is a requirement for maintaining unchanged high viability of plant material (Mikuła et al., 2011). It is a result of a complex cascade of molecular events leading to the acquisition of tolerance. Conducting the proteomic analysis allows us to observe the response of the cell manifested at the post-translational level (Lopez, 2007). It also enables us to optimize the methodology and assure of safety procedures as well as place plant material for a long-term storage at ultra low temperatures.

The main objective of the present study was to detect and identify *G. cruciata* desiccation responsive proteins, and to observe how the sequence of sucrose treatment induces changes in the protein expression patterns.

In the Wang's protein isolation technique that we slightly modified, a very rich 2D PAGE profiles with a larger number of protein spots were obtained and compared one to each other by Image Master 2D Platinum 7.0 software. The proteins that were differentially expressed were taken forward for MS identification. The results showed that under drought, the stress expression of various proteins was changed, a group of protein spots were up-regulated and another one was down-regulated. The data of an MS analysis confirmed the participation of various processes in the response to the test conditions. Both groups of proteins were involved in various metabolic processes, such as carbohydrate metabolism, glycolysis, induction of somatic embryogenesis. These processes are directly related to the adaptation to stressful conditions as well as the basic physiology of the cell. This proves that the tested conditions affect natural physiological processes that occur in the material, which consequently leads to its ability to survive freezing. It should also be underlined that the protein database for *Gentiana cruciata* is only residual so we were forced to use very broad databases. However, some proteins still might have been left unidentified due to the lack of appropriate database.

The presented proteomic analysis of multi-stage osmotic dehydration makes it possible to observe changes induced by prolonged and gradually intensified dehydration stress. These types of observations have not yet been described.

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## The effect of collection time of wild rose meristems on their survival and regeneration after cryopreservation

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Cryopreservation is a perfect method for a long-term conservation of plant genetic resources, using a very low temperature (liquid nitrogen,  $-196^{\circ}\text{C}$ ). The method has been recognized as a practical and efficient tool for a long-term storage of germplasm. Roses are one of the most important flowers worldwide. The intensive breeding programs for roses require access to extensive genetic resources.

In the present study, explants for cryopreservation were obtained by isolation of meristems from axillary buds of dog roses (*Rosa canina*) collected from natural habitats in the SW part of Cracow in the Pychowice District. One-year-old shoots of roses were collected at three times: during dormancy: in October and at the end of January/beginning of February, and during the vegetation season, in May. Buds with a fragment of bark (measuring about 10-12 mm) were excised from shoots, disinfected with 70% ethanol for 30 s. Subsequently, meristems (0.1-0.2 mm) were isolated from the buds with a fragment of a shoot. Explants were prepared in preservation solutions, first one of MS medium (100%) containing 2 M glycerol + 0.4 M sucrose for 20 minutes and second one: PVS2 (30% glycerol, 15% ethylene glycol, 15% DMSO, 0.4 M sucrose, 100% MS) for 10, 20 and 30 minutes. Next, the explants in an 8- $\mu\text{l}$  droplet of PVS2 were placed onto aluminum foil strips, then transferred to cryotubes and placed in cryogenic dewars in liquid nitrogen. The tissues were re-warmed in a liquid medium containing 1.2 M sucrose for 20 min at a temperature of  $23^{\circ}\text{C}$ . The re-warming solution contained Domestos (1.5%). The explants were washed with sterile water and used for initiation of *in vitro* cultures. Meristems were placed on the medium containing 50% of mineral components of MS medium, 0.3 M sucrose and

0.5% agar Difco Bacto. Twenty four hours later the explants were transferred onto a regeneration medium for roses enriched with  $1.5\ \mu\text{M GA}_3$ ,  $1\ \mu\text{M BA}$ ,  $0.087\ \text{M}$  sucrose, 0.7% agar, pH 5.7. For the first week explants were cultured in the dark and then the light intensity PPFd was increased to  $30\ \mu\text{M m}^{-2}\ \text{s}^{-1}$ . The regenerating lateral shoots of roses were transferred to fresh media every 5<sup>th</sup> week. The survival rate of the meristems after cryopreservation (%) was determined on the 7-14<sup>th</sup> day after re-warming while the regeneration rate (%) was assessed in the 8<sup>th</sup> week. In addition, dry weight of the explants to be cryopreserved was evaluated.

The studies demonstrated that the highest survival rate of *Rosa canina* meristems after cryopreservation, with retainment of the regenerative capacity of shoots, was achieved after a 20-minute pretreatment of meristems with PVS2 solution.

The highest survival rate and regeneration rate were noted for meristems excised from winter one-year-old shoots at the end of January/beginning of February. The survival rate of these meristems reached 97.9% while the respective value for the meristems collected in autumn was 82.4%. The lowest survival rate (6.7%) was observed for explants isolated from rose buds during the vegetation season, in May.

The regeneration rate was lower than the survival rate by several tens of percent. The highest regeneration rate (57.5%) was noted for meristems excised from winter buds, in January/February. The studies showed the best survival after cryopreservation with the highest regenerative capacity for explants characterized by the lowest water content compared to explants collected at other dates (about 0.92 g of water/1g of dry weight).

## Genetic stability of *Asplenium cuneifolium* gametophytes and sporophytes after cryopreservation

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Cryopreservation is a technology of high and still growing importance, but its practical application for the preservation of plant materials is useful only if it does not lead to the genetic changes in the plant of interest. As it was reviewed by Harding (2001) and Mikuła (2010), a majority of the papers published so far have reported on the insignificant or no influence of cryopreservation on the plant genome. However, these results were usually obtained with the help of only one or two types of molecular markers (mostly RFLP, RAPD, AFLP, SSR or ISSR). None of the plant species belonging to *Pteridopsida* has ever been a subject of research concerning its molecular analysis in post-thaw culture before.

The aim of presented studies was to assess the genetic stability of fern *Asplenium cuneifolium* Viv. gametophytes and sporophytes after cryopreservation by encapsulation-dehydration technique. Three different molecular marker systems (RAPD, ISSR, and AFLP) were applied simultaneously.

In the experiments *A. cuneifolium* gametophytes obtained from spores and multiplied *in vitro* were used as an initial plant material. These gametophytes were encapsulated in sodium alginate beads and then exposed to three-day-long osmotic dehydration consisting in incubation of beads in a liquid 1/2 MS medium with an increasing sucrose concentration (from 0.5 to 1.0 M). After that, the beads were air desiccated for 5 h, put into cryovials and immersed directly in liquid nitrogen. Thawing was carried out 3 days later, and the encapsulated plant material was placed on agar-solidified 1/2 MS medium supplemented with 2% sucrose for secondary gametophyte proliferation. To induce sporophyte development, gametophytes were cultured on modified 1/4 MS with 2% sucrose, but without any vitamins and ammonium ions.

Fifteen gametophytes and fifteen sporophytes obtained in post-thawing culture were employed for total genomic DNA isolation. The same number of non-cryopreserved *A. cuneifolium* individuals of both generations was used as a control. DNA was extracted using DNeasy Plant Mini Kit (Qiagen) and its quantity and quality were evaluated with a spectrophotometer and by gel electrophoresis. For both RAPD and ISSR analysis 12 primers were examined, respectively. PCR optimization was performed in T-Gradient thermocycler (Biometra). Products of amplifications were electrophoresed on a 1.5% agarose gel containing ethidium bromide, and examined under UV light. AFLP analysis was carried out according to Vos et al. (1995) with minor modifications. 6 pairs of EcoRI/MseI primer combinations were tested in a selective PCR. The amplified products were separated on a 7% denaturing polyacrylamide gel and silver stained. All DNA profiles were scored manually.

The encapsulation-dehydration method ensured viability of 100% of *A. cuneifolium* gametophytes. A complete recovery of the gametophyte culture took 4 weeks, whereas sporophytes were obtained after 4 months. The optimum PCR conditions allowed to obtain about 140 RAPD bands and over 150 ISSR ones per each DNA sample. A comparison of the electrophoretic separation images of DNA from the cryopreserved and non-cryopreserved plant material enabled checking whether there were any differences between them. The influence of cryopreservation on *A. cuneifolium* genome stability will be discussed.

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## Cryopreservation of riboflavin over-producing cucumber cell lines

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Modern *in vitro* culture techniques have an increasing impact on the development of plant genetic research, breeding and biotechnology. Long-term maintenance of cultures on proliferation media carries the risk of losing their unique biological characteristics. Repeated cell line subculturing and *prolonged* storage at room temperature increase the probability of contamination and somaclonal variation, as well as generate labor, material costs, and potential loss due to apparatus failure. Cryopreservation has attracted attention as a method to preserve plant cell cultures and to safely maintain their genetic integrity (Grout, 1995). The development of plant and animal stem cell biology has led to the production of a new category of germplasm for cryostorage – cell lines characterised by stemness potential (i.e. possessing self-renewal capacity and the potential to multi-type differentiation). The culture of totipotent stem cells (TSCs) and trichoblast derived stem cells (T-dSCs) are the most advanced forms of cucumber suspensions developed in the Department of Plant Genetic, Breeding and Biotechnology (Burza, unpublished; Burza and Bartosik, 2007). A key element of the mechanism controlling the proliferative growth phase of both of these cultures is riboflavin, synthesized and accumulated in vacuoles in the oxidized form, with the ability to emit green autofluorescence (GAF) as a result of excitation with blue light.

The purpose of the current study was to elaborate a cryopreservation method for cultured cucumber SCs. This technology could allow the initiation of biotechnological processes at a laboratory shake flask or a bioreactor scale, at any time, based on thousands of valuable frozen cell lines.

The experiments were conducted on a highly inbred line B obtained from cucumber cultivar Borszczagowski. Suspension cultures were carried out in the dark on modified 1/2 MS media in 100 ml Erlenmeyer flasks. After a period of stabilization they were passaged every

14 days. Cryopreservation was performed using encapsulation-dehydration method (Mikuła et al., 2011). The tissue was closed in alginate beads and subjected to several stages of pre-incubation in media of increased osmotic potential induced by the supplementation with mannitol. The utilisation of mannitol instead of sucrose that is commonly used in such procedures, was necessary due to the specific conditions of the maintenance of TSC and T-dSCs. In the next step capsules with pre-dehydrated cell aggregates were subjected to air desiccation. Then they were frozen in the liquid nitrogen vapor (–196 °C). In order to facilitate the optimization of cryopreservation method, the storage time in liquid nitrogen was calculated to be a minimum of one hour. Two days after thawing the 2,3,5-triphenyltetrazoliumchloride (TTC) reduction (survival) test, and the establishment of suspension cultures in shake flasks were performed. The level of GAF emission by cell aggregates two weeks after thawing, as well as the rate of proliferation were considered as the most reliable parameters for the evaluation of the procedure effectiveness. The results showed no correlation with the outcomes of the TTC test. In conclusion, the riboflavin over-producing cucumber cell lines were fully able to tolerate the developed procedure of cryopreservation, which proves their exceptional tolerance to freezing, osmotic stress and air dehydration.

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## The influence of abiotic stresses on pectin metabolism in flax seedlings

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Flax (*Linum usitatissimum* L.) is a common crop, highly valued as a source of fibre, oil and linseed used in the food, chemical, cosmetics, pharmaceutical and paper industries. The biggest crop losses worldwide are caused by *Fusarium* infection, but also its cultivation is restricted by environmental stress factors.

Pectin is a structurally complex family of polysaccharides and plays a crucial role in plant growth, development, the defense for the pathogen infection and in many other processes. Pectin generally consists of four pectin domains: homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan, with their galacturonic acids (GalUA) residues constituting up to 70% of the overall structure. Based on polysaccharides connections, pectins are divided into three groups: the water soluble fraction (WSF), CDTA soluble fraction (CSF) and Na<sub>2</sub>CO<sub>3</sub> soluble fraction (NSF). The WSF pectin is loosely associated with the cell wall, whereas the CSF and NSF fractions are enriched with ionically and covalently bound pectin respectively.

The aim of this study was to research how different abiotic stresses influence pectin metabolism in flax. Of particular interest for us were hormones (ABA, NAA, GA3), salt, temperature, light and sugar stresses. Screening of a subtractive cDNA library constructed from flax seedlings exposed for 2 days to *F. oxysporum* revealed two sets of gene sequences connected with cell wall sugar polymers (UDP-D-glucuronate 4-epimerase and formate dehydrogenase), which are involved in flax defense response. This was confirmed, together with the

involvement of other key pectin metabolism genes (pectin methylesterase, glutathione-dependent formaldehyde dehydrogenase and s-formylaldehyde hydrolase), in our previous analysis (Wojtasik et al., 2011). UDP-D-glucuronate-4-epimerase catalyses the interconversion of UDP-GalUA to UDP-GlcUA delivering precursors for pectin biosynthesis. Pectin methylesterase is responsible for demethylesterification of pectin making possible pectin degradation by other enzymes like polygalacturonases and rhamnogalacturonases. Methanol, a side effect of pectin demethylesterification, is converted by glutathione-dependent formaldehyde dehydrogenase and s-formylaldehyde hydrolase into formate which can be transformed to CO<sub>2</sub> by formate dehydrogenase.

In order to ascertain the level of expression of these genes, 9-days old flax seedlings exposed to 2-days stress factors were subjected to real-time PCR analysis. We have determined that the biggest changes were detected for three isoforms of pectin methylesterase and formate dehydrogenase genes and they were depended on stress factors and genes. The content of uronic acid in total pectin and three fractions of pectin: WSF, CDTA and Na<sub>2</sub>CO<sub>3</sub> were determined by the spectrophotometry method and obtained data correlated with a gene expression.

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## Regeneration from androgenetic embryos of carrot on media with increased zinc content

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Among the main pollutants of the natural environment are heavy metals, including zinc. Zinc is a micro-nutrient; in small amounts it is essential for proper growth and development of plants. It is a very important component of many enzymes, takes part in redox reactions and is involved in electron transport and other metabolic processes. Zinc excess or deficiency are both harmful to plants. A deficiency of this element in plants manifests itself as the inhibition of growth, chlorosis, necroses, deformation and discoloration of the leaves, and premature ageing. Plants with an excess of zinc are characterized by slow growth rates, reduction in root biomass, browning of root tips, and also deformities. Searching for plants resistant or tolerant to environmental hazards is very necessary and highly topical.

The aim of the experiment was to investigate the effect of increased concentrations of zinc in the form of  $ZnSO_4 \times 7H_2O$  in a culture medium on plant regeneration of carrot from its androgenetic embryos. The  $B_5$  medium (Gamborg et al. 1968) was used as the control with the concentration of  $ZnSO_4 \times 7H_2O$  (2 mg/l) recommended by the authors. Concentrations 10x (Zn-1), 100x (Zn-2) and 1000x (Zn-3) higher than in the control combination were used. The experimental material consisted of embryos derived from anther culture described elsewhere (Górecka et al. 2005).

Three passages were carried out: after 4, 9 and 15 weeks. The resulting plant material was classified into different categories, such as plants, rosettes, secondary embryos and incipient rosettes, while distinguishing among them normal and deformed structures, which were counted and weighed.

There were both beneficial and adverse effects of the increased concentrations of zinc on the regeneration of carrot plants from androgenetic embryos. The results were consistent with references earlier published (Echávarri et al. 2008 and Kothari-Chajer et al. 2008). There have been reports on the positive effects of higher concentrations of zinc on the regeneration of various plant species as well as on the negative effects of increased zinc concentrations on this process. In our experiment, the concentration of 1000x higher than in the control medium was lethal to the androgenetic carrot plants. All the embryos died within the first 4 weeks of culture. During the first passage, more secondary embryos were obtained on the media with 20 mg and 200 mg/l  $ZnSO_4 \times 7H_2O$  than in the control medium. After 15 weeks of culture, the concentration of  $ZnSO_4 \times 7H_2O$  that was 10 times higher than in the control medium caused the highest multiplication of plants. A negative effect of the increased concentrations of zinc in the regeneration medium was the occurrence of deformations, especially of the leaves.

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## Signalling pathways in *Solanum* genotypes induced by biotic stress

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Plants possess integrated signalling networks that mediate the responses to environmental stresses. They transduce extracellular stimuli into intracellular plant responses. Phospholipase A<sub>2</sub> (PLA<sub>2</sub>), a key enzyme in lipid metabolism, mitogen-activated protein kinase (MAPK) cascades and calcium-dependent protein kinases (CDPK) are a crucial components of signalling pathway for plants to defend against numerous potential pathogens. Products of PLA<sub>2</sub> activity, fatty acids and lysophospholipids, can influence plant protein kinase activities, may function as second messengers in signal transduction and serve as substrates for oxylipin biosynthesis. Moreover, in plants, there are some evidence for the involvement of PLA<sub>2</sub>s, MAPK and CDPK activities in the generation of reactive oxygen species (ROS) by NADPH oxidase in response to elicitation.

Changes in PLA<sub>2</sub>, MAPK and CDPK activities were investigated in leaves of *Solanum tuberosum* cv Bzura, *Solanum tuberosum* clone H-8105 and *Solanum nigrum* var. *gigantea* in response to elicitor (culture filtrate, CF) from the *Phytophthora infestans*, the pathogenic oomycete that causes late blight, the most destructive potato disease. These *Solanum* genotypes exhibit field resistance, susceptibility and non-host resistance to *Phytophthora infestans*, respectively.

We found that PLA<sub>2</sub>, MAPK and CDPK activities increased in response to CF treatment but varied with respect to intensity and timing. The highest increase in enzymes activities was noted in *S. nigrum* var. *gigantea*, non-host completely resistant to *P. infestans*, and the lowest in the susceptible H-8105. It means that these

enzymatic activities were positively correlated with the level of plant resistance in response to CF treatment in *Solanum* genotypes.

To elucidate the contribution of specific forms of PLA<sub>2</sub> to plant defence mechanism reasonably selective PLA<sub>2</sub> inhibitors, haloenol lactone suicide substrate (HELSS) and p-bromophenacyl bromide (BPB), which discriminate between Ca<sup>+2</sup>-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) and Ca<sup>+2</sup>-dependent secretory PLA<sub>2</sub> (sPLA<sub>2</sub>), were used. The *in vivo* and *in vitro* effects of the inhibitors on PLA<sub>2</sub> activity were assayed. Moreover, to ascertain a possible relationship between ROS production and PLA<sub>2</sub> activity, we have compared ROS production in response to CF in leaves preincubated with the inhibitors of PLA<sub>2</sub>.

Differences among the genotypes in the effects of each inhibitor on CF-induced PLA<sub>2</sub> activity and on ROS production may reflect the diversity of PLA<sub>2</sub> isoforms in plants. Contrary to BPB, the inhibitory effect of HELSS was observable mainly on CF-induced PLA<sub>2</sub> activity, which suggests that iPLA<sub>2</sub> participates in signal transduction in defence reactions. Various effects of the two inhibitors on PLA<sub>2</sub> activity and ROS production suggest different contribution of sPLA<sub>2</sub> and iPLA<sub>2</sub> to modulation of defence reactions in the interaction between *Solanum* genotypes and *Phytophthora infestans*.

The obtained results widen the knowledge about signalling pathways occurring in *Solanum* genotypes after treatment with elicitor from *P. infestans*.

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## The effect of *in vitro* nitrogen and potassium deficiency on the growth and development of different tomato genotypes

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Tomato is one of the most common crops in the world because of its flavour and high nutritional value. The popularity of tomato prompts breeders to improve certain characteristics of the plant including enhancing the plant resistance to diseases and tolerance to abiotic stresses. Wild relatives of tomato are a valuable source of genes in both conventional and biotechnology breeding programs. Unlike the cultivated tomato cultivars, they are characterized by high genetic variability. *In vitro* cultures allow examination of many plants in a relatively small area. Moreover, the amounts of nutrients in the medium as well as the experimental conditions are strictly controlled and consequently the selection of tolerant plants is more efficient. They can serve as useful tool for testing the tolerance of the plant material for potassium and nitrogen deficiency.

The research material in this study included two species of tomato: *Solanum habrochaites* S. Knapp & D.M Spooner, Peru (LA1777), *S. sitiens* I.M. Johnst., Chile (LA1974) and two *Solanum lycopersicum* L. cultivars: Congolese Tomaat (Congo) and Subarctic Planty (Canada). Seeds of wild species of tomato were obtained from the gene bank of the Tomato Genetics Resource

Center of the University of California at Davis, while seeds of cultivated tomato were obtained from The Centre for Genetic Resources, the Netherlands (CGN) of the Wageningen University. Tomato cultures were initiated from seeds on MS (Murashige and Skoog, 1962) medium. Before culture initiation the seeds were put in sterile distilled water for 12 h, then for 30s in 70% ethanol. Next they were disinfected in 7,5% sodium hypochlorite (NaClO) solution for 15 minutes. After that, tomato cultures were tested for tolerance to the deficiency of nitrogen and potassium. For this purpose, 0.5 cm fragments of stems with the lateral bud were put on MS medium (as a control medium) and five other testing media with decreased nitrogen and potassium concentrations.

A statistical analysis showed significant differences between the four studied genotypes of tomato: in plants high, root length and the number of internodes. Significant differences were also shown between plants of the same species but grown on different media. Wild species of tomato showed to be more tolerant than cultivated plants, and these plants were higher, had longer roots and a greater number of internodes.

## The application of nano silver and gold to micropropagation of chrysanthemum

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For years, silver and gold have been considered substances with antimicrobial properties. For a long period, these metals have been used in a limited scope in *in vitro* cultures, as they are available in the form of gold and silver salts that are toxic to plant tissues due to the low level of purity and interactions with other chemical compounds. The development of nanotechnology allowed the synthesis of nanocolloids of these metals, which, in the synthetic form, have a high degree of purity, stability and durability, as well as antimicrobial and antimucosal properties. Solutions of gold and silver nanocolloids can be used for *in vitro* cultures as explant sterilizing solutions that may be spread over the medium surface or as an additive to the medium. However, in order to introduce plant colloid compounds to mass production of seedlings in *in vitro* cultures, the influence of the discussed compounds on plant growth must be explained to exclude their toxicity to plant tissues.

Therefore, the purpose of this analysis was to determine the influence of gold and silver nanocolloids on the number of medium infections and shoot multiplication, the intensity of callus tissue and somatic seed value for-

mation in chrysanthemum (*Dendratherma grandiflora*) in *in vitro* cultures.

Our research showed that nano colloidal silver with particles of 20 nm is a strongly antimicrobial compound. When added to an MS medium at a 5 mg·dm<sup>-3</sup> concentration, it almost totally eliminated any infections visible on the medium surface, and stimulated the growth of the above-ground part of chrysanthemum – plants multiplied on this type of medium were higher, had larger mass and formed more numerous leaves. However, the root system development was slightly restricted. The positive influence of colloidal silver was observed in a number of somatic seed values and the mass of callus tissue.

Following its addition to MS medium, nano colloidal gold significantly slowed the development of microbiological colonies on the medium surface, but its properties are considerably weaker than those of nano silver. If added to the medium at a concentration of 2 mg·dm<sup>-3</sup>, the substance in *in vitro* cultures had a positive influence on the growth as well as the number of leaves and roots in chrysanthemum.

## The influence of sulfate concentration on thiols metabolism in *Brassica cretica* ssp. *botrytis* in vitro cultures

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Sulfur is an essential macronutrient for the majority of organisms. It is a component of many compounds such as amino acids – cysteine, cystine, methionine, glutathione, enzymes and vitamins. A large content of active sulfur metabolites can be found in plant families like *Brassicaceae* and *Alliaceae*. Plants and microorganisms can assimilate inorganic sulfur as a sulfate. The reduction of this compound to a sulfide leads to a cascade of enzymatic steps and to the synthesis of sulfur-containing amino acids. In contrast, humans and animals lack the capability to reduce sulfate. As a consequence humans and animals rely on their diet for the provision of reduced sulfur in cysteine and methionine. The biological activity of sulfur compounds makes them essential for human diet, so their deficiency may cause many health complications. Thus plants are the most important source of sulfur organic compounds for example, cysteine or lipoic acid. It constitutes an economic interest of sulfur assimilation and sulfur amino acid biosynthesis in higher plants. Additionally the sulfide/disulfide redox system is retained as a detoxification mechanism for reactive oxygen species and signaling mechanism in plant.

The aim of the present studies was to determine the effect of sulfate concentrations on sulfur compounds metabolism in broccoli cultured *in vitro*.

*Brassica cretica* ssp. *botrytis* shoot liquid agitating cultures were maintained on Murashige-Skoog medium

containing 1 mg/l BAP, supplemented with different amounts of sulfate (0; 0.5; 1.5; 3 and 5 mM), under constant artificial light (ca. 4 W/m<sup>2</sup>), at 24 ±2 °C and 2-weeks growing cycles. The levels of the following compounds were measured in methanolic extracts from the cultured broccoli biomass: non-protein sulfhydryl groups (NPSH), glutathione (GSH), cysteine, cystine and sulfane sulfur.

*Brassica cretica* ssp. *botrytis* in vitro cultured plants can assimilate sulfate directly from the medium. The administration of sulfate significantly elevated the analysed compound as NPSH (from 6.65 to 17.16 µmol/g d.w.), glutathione (from 0.31 to 0.45 µmol/g d.w.), cysteine (from 0.51 to 3.59 µmol/g d.w.), and the sulfane sulfur level (from 3.49 to 7.13 µmol/g d.w.). Only the cystine concentration was lowered (from 0.67 to 0.064 µmol/g d.w.). This could be the result of an unbalanced cysteine/cystine status in plants tissues under low medium sulfate concentrations. The thiols redox status disturbance is usually the effect of a stress condition.

In conclusion, sulfate is a good precursor of cysteine, utilized for glutathione biosynthesis, which was confirmed by its capability to the elevate NPSH level and leading to the formation of sulfane sulfur-containing compounds. It could serve as a good experimental model to modulate thiols and other sulfur compounds content in plants. Studies of the ubiquitous functions of sulfur in edible plants provide integration in understanding plant biology from molecular to cellular levels.

## Effect of methyl jasmonate on the synthesis of Amaryllidaceae alkaloids in *in vitro* culture of *Leucojum aestivum* “Gravety Giant”

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*Leucojum aestivum* L. is an important medicinal bulbous plant, belonging to the Amaryllidaceae family, which contains numerous alkaloids (Ptak et al., 2009). Amaryllidaceae alkaloids have important pharmacological properties, such as acetylcholinesterase inhibitory activity, cytotoxicity and antitumoral activity. Galanthamine is the most important alkaloid, considering its use for the treatment of Alzheimer’s disease (Heinrich and Lee Teoh, 2004). The second most important alkaloid, lycorine, shows strong antimitotic activities and it has also been studied for antimalarial and antiviral effects. Galanthamine is produced on a large scale from Bulgarian *Leucojum aestivum* plants and also by chemical synthesis (Berkov et al., 2005). In view of the increasing demand of the pharmaceutical market for this alkaloid, related to population ageing, its supply has become limited. *In vitro* production of secondary plant metabolites could be an alternative source. So far, however, the few reports on *in vitro* culture of *Leucojum aestivum* have shown that the Amaryllidaceae alkaloid accumulation is too low for large-scale production. The use of elicitors to promote secondary metabolism in plant cell and tissue cultures has become a common practice. A biotic elicitor, such as methyl jasmonate (MeJA), is known to induce the production of secondary metabolites.

In our experiment, somatic embryos of *Leucojum aestivum* “Gravety Giant” were cultured in the Rita<sup>®</sup> temporary immersion bioreactor on Murashige and Skoog (1962) medium containing 5 µM of zeatin, 3% sucrose, pH 5.8 for the period of 21 days. The immersion frequency was 5 minutes every 2 hours. The somatic embryos were grown at a temperature of 25°C under white fluorescent light with a 16-h photoperiod (20 µmol m<sup>-2</sup> s<sup>-1</sup>). After 21 days of growth the cultures were treated with MeJA at the concentrations of 0 (control),

5, 50 and 100 µM. Plant materials were harvested 10 h, 24 h, 72 h and 1 week after treatment with the elicitor. The effects of this compound on the growth of somatic embryos, as well as on the amounts of galanthamine and lycorine released to the culture medium and accumulated in the tissues were studied. The LC-MS technique was used for analyses.

The obtained results showed that MeJA did not have a negative effect on the development of somatic embryos into plants. No abnormally developed plants were observed. The plants treated with MeJA for 72 h and those grown on the control medium were characterized by the same fresh weight. However, 1-week exposure to MeJA (irrespective of the concentration used) inhibited plant weight gain as compared to the control. Galanthamine and lycorine were detected in elicited and non-elicited tissues. The highest concentrations of these alkaloids were found at the end of the experiment. Moreover, the effect of MeJA concentration on the levels of galanthamine and lycorine was noted. The addition of MeJA at 50 µM (during 1 week) promoted the accumulation of these alkaloids. Under these *in vitro* conditions the plants contained 0.02% dry weight of galanthamine and 0.1% dry weight of lycorine. However, in the culture media, irrespective of the treatment combination used, only trace amounts of galanthamine and lycorine were found.

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## Factors promoting and inhibiting the hyperhydricity and the shoot formation in *Magnolia* × *soulangiana* “Coates” and “Burgundy” *in vitro*

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Hyperhydricity is a physiological disorder frequently affecting shoots of many herbaceous and woody plant species propagated *in vitro*. Hyperhydric tissues exhibit reduced lignification and decreased levels of cellulose. The affected plants appear glassy and have malformed stems and leaves, reduced shoot formation, a low percentage of rooting and poor survival after transplantation into the soil (Gaspar et al. 1995; Kevers et al. 2004). Hyperhydricity of *Magnolia* × *soulangiana* “Coates” and “Burgundy” was observed when the cultures were established using MS medium containing 1.5 mg l<sup>-1</sup> BAP or 2.5 mg l<sup>-1</sup> *meta*-topolin. It is known that the occurrence of hyperhydricity is affected by multiple factors but the interaction of those factors is still unclear.

The aim of the experiment was to investigate the effect of nitrogen salts (KNO<sub>3</sub>, NH<sub>4</sub>NO<sub>3</sub>) having different ratios in relation to MS medium (100:100, 75:75, 50:50, 100:50, 75:50), carbohydrate type (sucrose, fructose, glucose) and level (20, 30 and 45 g l<sup>-1</sup>), and BAP concentration (0, 0.2, 0.5, 1.0 and 1.5 mg l<sup>-1</sup>) on the hyperhydricity frequency and multiplication rate of *M.* × *soulangiana* “Coates” and “Burgundy” *in vitro*. The cultures were grown at 20°C.

The results showed that at concentrations from 0.2 to 1.5 mg l<sup>-1</sup> BAP significantly stimulated hyperhydricity in both *M.* × *soulangiana* cultivars, when they were grown on MS medium containing 100% nitrogen salts, and sucrose in a concentration of 20 g l<sup>-1</sup>. In “Burgundy”, increased levels of sucrose (30 g l<sup>-1</sup>) in full strength MS medium resulted in up to a fourfold decrease in the number of hyperhydric shoots and a two-fold increase in the multiplication rate (5.0 shoots/explants). At the same time, however, the inhibition of the shoot growth, leaf yellowing and necrosis were

noted. These symptoms increased in the presence of BAP at concentrations above 0.5 mg l<sup>-1</sup>. The symptoms decreased when sucrose was replaced by fructose. High levels of sucrose (45 g l<sup>-1</sup>) and nitrogen (100% in relation to MS), and BAP (1.0-1.5 mg l<sup>-1</sup>) caused the death of the culture. A satisfactory shoot formation and quality of *M.* × *soulangiana* “Burgundy” was obtained on a medium containing 0.5 mg l<sup>-1</sup> BAP and reduced nitrogen salts. The optimal level of nitrogen depended on the carbohydrate type. The best growth and development of “Burgundy” shoots was observed on the medium containing fructose (30 g l<sup>-1</sup>) and 75% strength of both nitrogen salts. In the presence of sucrose, the decreasing of ammonium ions by one half was the most effective. Similarly, in *M.* × *soulangiana* “Coates”, the BAP-stimulated hyperhydricity was overcome by reducing the nitrogen level. The best shoot formation (5.4 shoots/explants) and quality was obtained on the medium with BAP (0.5 mg l<sup>-1</sup>), 75% strength of both nitrogen salts and sucrose or 75% strength of KNO<sub>3</sub>, 50% strength of NH<sub>4</sub>NO<sub>3</sub> and fructose (30 g l<sup>-1</sup>). In both *M.* × *soulangiana* cultivars, the negative influence of glucose was noted.

To sum up, BAP increased hyperhydricity in *M.* × *soulangiana* “Coates” and “Burgundy” in a concentration-dependent manner when C/N was not optimal.

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# Identification and TILLING analysis of barley homologs encoding the negative regulators of abscisic acid signaling

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Drought stress is an environmental condition that seriously affects crop productivity worldwide. In order to decipher the complexity of drought tolerance mechanisms many different approaches have been undertaken. Despite very intensive studies there are still a number of unknown parts of the signaling pathway. The majority of research was carried out with the use of model species *Arabidopsis thaliana*, and the cascade of drought and other stresses response are best elucidated in this species.

The major challenge for plant biologists and crop breeders is understanding the mechanisms of drought tolerance and increasing crops' to drought stress. Using the information from *A. thaliana* it is possible to identify and then functionally analyze genes related to drought in crops such as barley.

The aim of our study was: 1) identification of barley homologs encoding negative regulators of ABA signaling: AtERA1 (Enhanced Response to ABA1) and AtAHG2 (ABA-hypersensitive germination 2); 2) mutational analysis of identified genes using the TILLING approach. AtERA1 gene encodes the  $\beta$  subunit of the farnesyltransferase. This particular enzyme is involved in the post-translational modification of proteins acting as negative regulators of plant sensitivity to abscisic acid (ABA), that is one of the mediators determining a physiological response of plants to water deficit. AtAHG2 encodes a poly(A)-specific ribonuclease. Its expression is upregulated by ABA or stress treatment. Arabidopsis mutants in both genes are hypersensitive to ABA and tolerant to drought (Brady et al., 2003; Nishimura et al., 2005).

*A. thaliana* coding sequences were used as a reference for further bioinformatics analysis. The identifi-

cation of *H. vulgare* EST sequences, homologous to the gene coding sequence was performed using the *Oryza sativa* CDS due to the greater genome similarity of both species. The HvERA1 gene complete mRNA sequence of 1592 bp and corresponding genomic sequence of 5159 bp were isolated. The HvAHG2 gene complete mRNA sequence of 1592 bp and corresponding genomic sequence of 4432 bp were isolated. On the basis of protein sequence obtained by *in silico* translation a highly conserved domain responsible for the reported catalytic properties in both homologs were identified. The TILLING analysis is performed with the use of TILLING population – HoTILLUS consisting of 10 000 M<sub>2</sub> individuals after chemical mutagenesis with the use of sodium azide and MNU. In case of both genes region encoding the conserved domains were analyzed in order to identify mutations that are most likely to result in deleterious effects on the genes' function.

Identification and further analysis of mutants in HvERA1 and HvAHG2 genes would bring important insights into the challenge of increasing drought tolerance of crops.

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## Construction and analysis of cDNA libraries derived from salt-treated leaves of halophyte *Beta maritima*

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Reductions in plant productivity under salt stress conditions are, to a large extent, associated with a decrease in photosynthetic activity. The aim of our studies is to understand the mechanisms of the photosynthetic apparatus protection against elevated salinity in leaves of the halophyte *Beta vulgaris ssp. maritima*. This objective will be achieved by comparing the transcript composition in leaves exposed to toxic levels of salinity, with those incubated under conditions of moderate salinity.

Plant culture conditions and the method of salt stress treatment were optimized during preliminary experiments. Plants were grown under field conditions. Well-developed, young leaves were cut off and placed in plastic containers with their petioles immersed in Hoagland nutrient solution supplemented with various concentrations of NaCl. Given that salt solution was added directly to the transpiration system, this approach allowed to eliminate the defense mechanisms against salinity related to the regulation of Na<sup>+</sup> ion transportation from the root to shoot. Consequently, the exposure of plant's photosynthetic apparatus to salt stress treatment was supposed to be increased. In order to define the impact of salt treatment on metabolism in detached leaves, a number of physiological parameters related to salt stress response were monitored during the incubation in a salt solution. It was found that moderate stress was caused by 150 mM NaCl, whereas 300 mM NaCl provoked a strong stress in the treated leaves. The results of these experiments indicated that 48 hours of incubation in a salt solution was the minimum treatment time after which the physiological consequences of the stressor's action were detectable.

Three full length cDNA libraries were generated from the isolated leaves of *Beta maritima*, treated with

0 mM (control), 150 mM or 300 mM NaCl. The leaf samples for library construction were collected after 48 hour-long incubation in a salt solution. The control leaves and those representing moderate or severe stress served as a material for total RNA isolation. Subsequently, a high quality mRNA was purified from total RNA preparations. mRNA was subjected to reverse transcription and the resulting cDNA was cloned in *E. coli* using the Gateway technology. It is a universal cloning technique based on site-specific recombination, characteristic for bacteriophage lambda. It provides a possibility for easy DNA subcloning using various types of vectors, and allows extensive expression of proteins for functional gene analysis.

Initial assays revealed that the titer of libraries was  $2 \times 10^6$  cfu/ml for the libraries derived from leaves treated with either strong or moderate stress, and  $2 \times 10^5$  cfu/ml for the control library. cDNA libraries were also analyzed to determine the average insert size and percentage of recombinants. All three libraries showed 100% of recombinants. The size of the inserts ranged from 0.25 to 2.4 kb, with the average insert size of 1.05 kb. Based on the results of the preliminary analyzes (the percentage of recombinants, the average insert length) it can be concluded that the constructed cDNA libraries contain inserts of full length and are suitable for further experiments. Future experiments will be aimed on a comparative analysis of the expressed sequence tags (EST) generated from the three libraries. The results may contribute to our understanding of the mechanism underlying the halophyte ability to perform efficient photosynthesis under exposure to salinity.

## Evaluation of tolerance *Osmunda regalis* L. and *Lobelia erinus* L. to cadmium and aluminum ions in *in vitro* culture

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Plants in nature are exposed to various abiotic or biotic stresses. In the case of unfavorable growth conditions, abnormal structures and function of plant organs could be observed. Before the study to determine the tolerance to cadmium and aluminium ions of a protected species *Osmunda regalis* L. (Zarzycki and Szelağ, 1992) and ornamental species *Lobelia erinus* L. preliminary experiment was conducted. The experiment was carried out on hormone free MS medium, taking into account the concentration of macronutrients.

Ferns sterile gametophytes used in the experiment were obtained from spores of *O. regalis*. The spores were washed in a detergent solution and rinsed out with 70% ethanol. After that a proper disinfection treatment, comprising at least 15 minutes of soaking in 15% hydrogen peroxide was done. In the next stage the spores were washed three times for 15 min in sterile distilled water and placed on MS medium.

The experiment was established with well-developed fern gametophytes, which, before being placed in a selective medium had been divided into smaller pieces of 0.3-0.5 cm size.

The seedling of *Lobelia erinus* were obtained by seeds sterilization started by one hour their soaking in sterile distilled water with a few drops of detergent. Next seeds were washed in 70% alcohol. The seed sterilization process involved 15 minutes of commercial bleach Domestos (1:2 with water) treatment and three times water washing.

The experiment used four combinations of MS medium (Murashige and Skoog, 1962) with varying concentrations of macronutrients: MS (control), 1/2, 1/4 and 1/8 the recommended dose. Medium was supplemented to 30 g l<sup>-1</sup> sucrose, 2.0 g l<sup>-1</sup> gerlite and adjusted to pH – 5.8. Culture was carried out in the culture room at 18 °C,

16 h photoperiod. Measurements were made at intervals of two months.

The best results were obtained on 1/2 MS medium. Gametophytes of *O. regalis* were the largest and also the lightest on 1/2 MS medium. On 1/4 MS medium the most compact gametophytes (with the highest fresh weight) and sporophyte of the best developed stage were observed. The lowest survival rate of explants in a two-month period and further culture was found on MS media (25%) and 1/4 MS (60%) and highest (100%) on 1/2 MS. *L. erinus* explants also grew best on 1/2 MS medium.

In main experiment concerning determination of the tolerance to cadmium and aluminium ions 1/2 MS medium as a control for both species was used. Medium was supplemented with 10, 25, 100, 200 (mg l<sup>-1</sup>): aluminium or cadmium at pH = 4.0 of the medium. The material used in the experiment included gametophytes *O. regalis* and seedlings *L. erinus*.

Influence of aluminum toxicity was observed in *O. regalis* gametophytes at a concentration of 10 mg l<sup>-1</sup>. As a result of experiments on seedlings *L. erinus* a stimulating effect of low concentrations of aluminum (10 mg l<sup>-1</sup>) on the growth of shoots and the root system was found. At presence of high aluminum (100 mg l<sup>-1</sup>, 200 mg l<sup>-1</sup>) concentration destructive effects were noted – the seedlings become dwarf and fragile. What's more chlorosis of leaves as well as anatomical changes in the roots were observed. *Lobelia erinus*, a plant popular in horticulture, proved more resistant to aluminum than *Osmunda regalis*, which is a protected species.

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## Effect of sodium chloride on the mitotic activity of root tip cells of onion (*Allium cepa* L.) cultured *in vitro*

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Nowadays soil salinity and water stress are the very serious challenges for agriculture. Many crop plants species are sensitive even to relatively low salinity ( $EC < 2$ ), which restricts water availability by reduction of its potential in the soil solution. Salt stress inhibits growth and causes reduction in shoot and root length (Bekheht et al., 2006; Zeng and Shannon 2000). In consequence, salt stress leads to the yield reduction and economic losses.

Plant salinity tolerance mechanisms are not fully understood, which makes development of effective methods of its alleviation difficult. Onion is considered susceptible to water stress and soil salinity because of the shallow root system. Tolerance of onions for the saline conditions is relatively high at germination, however very low during seedling growth and increases again at about the three- to five-leaf stage (Shannon and Grieve, 1999). The study aimed at the estimation of the effect of different levels of sodium chloride on the type and frequency of cytological changes in the onion root tip.

Seeds of three *Allium cepa* accessions were used. Seeds were surface-disinfected and placed in a 9 cm Petri dish with basal MS (Murashige and Skoog 1962) medium without growth regulators for germination. Five days-old seedlings were transferred to the selection media containing respectively 100, 200, 300 mM of NaCl. The effect of NaCl was observed in the extended period of 0, 7, 14 and 20 days. Roots of onion were fixed in 1:3 acetic acid/ alcohol, hydrolysis of the cell wall was done in 1 N HCl. After that the roots were stained with Schiff's reagent (Sigma) and cells in the root tip squash were examined with the help of a light microscope.

The roots are the first organs exposed to higher salt concentrations in the soil. The observation of the onion root system under saline conditions showed a decrease in the number and length of the roots caused by the influence of higher salt concentrations on the mitotic activity. Microscopic observations have shown that increasing concentrations of sodium chloride caused a variety of mitotic abnormalities. Chromatin fragmentation, lag-gard chromosomes and micronuclei were observed. The highest concentration of NaCl stops completely the cell divisions causing the death of a cell. Our results showed that salt stress conditions play an important role in the meristematic activity in the response of root growth. Little is known about the mechanisms that cause salt stress to affect the cell cycle regulation. The mitotic index is nevertheless an important parameter of detection of its cytotoxic effects. Meristematic cells are considered to be salt-sensitive. Tracking the root system elongation together with the detection of the rate of mitotic abnormalities of the *in vitro* cultured plants could be used as a fast and reliable method for selection or discrimination between salt tolerant and salt susceptible genotypes.

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## Nitrogen stress inhibits cell death of *Nicotiana tabacum* BY-2 cells

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Programmed cell death (PCD) is a conserved suicide program, which is induced to ensure that organisms enter appropriate developmental pattern and growth responses to external challenges. To sustain growth and survival, plant cells need a constant supply of precursors in order to produce macromolecules. A lack or limited access to nutrients leads to the metabolic dysfunction of cells, limiting their growth and proliferation, and consequently may lead to cell death.

Nitrogen is, at least quantitatively, the most important nutrient for plants. Therefore plants have developed many different ways to capture this element, for example they are able to exude proteases by roots and use organic nitrogen as a nitrogen source. It is well known that nitrogen deficiency negatively affects plant growth and development and induces wide reprogramming of primary and secondary metabolism and can also activate the programmed cell death machinery.

Although, to date no homologous of caspases and Bcl-2 family members have been identified, there is some evidence for the existence of evolutionarily conserved PCD in both plants and animals. It has been demonstrated that Bax-like protein may exist in cells, mammalian Bax can initiate plant cell death, Bax-induced cell death is similar to a hypersensitive response and lamina-like protein is cleaved by caspase-6 like proteases.

The aim of our study was to compare the responses of tobacco BY-2 cells cultured on the following media: Linsmaier and Skoog medium (1965) which was treated as a control – (LS), LS with addition of 0.1% of casein – (LS+C), LS without inorganic nitrogen  $\text{NH}_4^-$  and  $\text{NO}_3^-$

– (LS-N) and LS without inorganic nitrogen but with 0.1% of casein supplementation – (LS-N+C).

Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cell cultures at the stationary growth phase were passaged into the complete LS medium or into one of those described above. The parameters of cell culture growth and protein profiles, protein levels and activity of proteolytic enzymes in the cell homogenates in every day of culture growth cycle were analyzed. Additionally, the Western blot technique was used to analyze proapoptotic Bax-like protein expression under nitrogen starvation conditions.

The obtained results have revealed that 1) the addition of protein (casein) to the culture medium increases growth and proliferation of BY-2 cells, 2) the rate of cell growth was lower on the inorganic nitrogen deprived medium (LS-N) than on the same medium supplemented with casein (LS-N+C) and this effect was accompanied by increase in proteolytic activity in the medium, which suggests that cells can use protein as a nitrogen source, 3) cells after nitrogen starvation had a high level of viability in comparison to the control. This result was accompanied by drastically dropped Bax protein expression level in the cells cultured under nitrogen free conditions. Expression of Bax-like protein after UV light was higher than in the control cells. This suggests, that in nitrogen starved cells proapoptotic protein is blocked.

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## Molecular cytogenetics in the analysis of the genotoxic effect induced by abiotic stress in plant genome

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It is important for the prevention of DNA changes caused by abiotic agents to understand the biological consequences of DNA damage and their molecular action. Numerous genotoxicity assay systems have been developed to detect and measure DNA damage induced by different abiotic stresses.

The changes in chromosomal morphology are usually detected with classical cytogenetic techniques. However, the traditional methods of chromosome staining can fail in the analysis of small changes in the chromosome structure. The development of molecular biology has made progress in the methods of detection and estimation of the genotoxicity of different agents. A more detailed analysis of genotoxicity, both at the chromosomal and DNA level, is possible using fluorescent *in situ* hybridization (FISH) which allows the detection and a more detailed analysis of the involvement of a specific chromosome or its fragment in their formation. The changes at the DNA level are detectable using a comet assay. It is used for analyzing the genomic DNA damage and repair by measuring the level of single-, double-strand DNA breaks and alkali-sensitive sites. Fluorescence *in situ* hybridization (FISH) applied on microgels with nuclei (comet – FISH) was also introduced a few years ago, but still relatively few papers have been published on its application in plant cells. Various probes have been applied in FISH with comets however centromere, telomere, ribosomal DNA repeats are the most widely used. With the comet-FISH the distributions of specific genomic regions between the head and the tail can be rapidly examined, and the sensitivity of these regions to the breakage can be estimated after exposure to the damaging agents.

In this study we analyzed the involvement of the rRNA genes and telomeric/centromeric DNA in

micronuclei and comets formation in plant cells after chemical and physical mutagenic treatment. The distribution of these sequences was analyzed in model plant species: *Crepis capillaris* and *Hordeum vulgare*. The application of the postincubation after mutagenic treatment allows to analyze the effectivity of repair processes. An application of rDNA as probes allowed it to be stated that 5S rDNA – bearing chromosomes are involved in micronuclei formation more often than NOR chromosomes. Also this work allowed to compare the origin of physically- and chemically-induced micronuclei in barley cells. FISH confirmed its usefulness in the characterization of micronuclei content, as well as in understanding and comparing the mechanisms of the actions of mutagens applied in plant genotoxicity. The results of comet – FISH showed DNA fragmentation within rDNA sequences. Based on the analysis of the frequencies of the comets with specific distribution of rDNA signals we confirmed that 25S rDNA sequences are not so often involved in comet formation as 5S rDNA sequences. The involvement of 25S rDNA sequences in the nucleolus formation and differences in chromatin structure between the two loci may explain the different susceptibility of 25S and 5S rDNA regions to migrate into the tail. Even though the availability of plant DNA probes is not wide, micronuclei test and comet assay combined with FISH improve the effectiveness of the genotoxicity assessment in plants.

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## Identification and phenotyping of barley mutants with changes in genes encoding transcription factors associated with drought tolerance

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Abiotic stresses such as drought, extreme temperatures and high salinity are the main factors which influence the growth and yield of plants. Several families of transcription factors (TF) have been shown to be involved in the regulation of drought response in plants. We used the TILLING (Targeting Induced Local Lesions in Genomes) strategy to generate and identify mutations in the genes encoding selected transcription factors. We developed a large barley TILLING platform (HorTILLUS) of two rowed malting cultivar "Sebastian", which consists of more than 10.000 M<sub>2</sub> plants. The chemical mutagenesis using combined treatment of NaN<sub>3</sub> and MNU was employed for the creation of HorTILLUS population. We focused on a barley basic domain/Leu zipper (bZIP) transcription factor – *HvABI5* (ABA insensitive 5; acc. num.: HQ456390), two members of Apetala 2/ethylene-responsive factor (AP2/ERF) family: *HvDREB1* (dehydration-responsive element binding protein 1; acc. num.: DQ012941) and *HvDRF1* (dehydration-responsive factor 1; acc. num.: AY223807), and a member of NAC (NAM, ATAF, and CUC) transcription factor family – *HvSNAC1* (stress-responsive NAC 1; acc. num.: JF796130.1).

We identified a homologous gene of rice *SNAC1* in barley based on EST (Expressed Sequence Tag) sequence TC254458 and full-length sequence cDNA FLbaf19c03. We also identified the genomic sequence of *HvABI5* gene.

The obtained sequences of both barley genes were published in GeneBank database.

For all analyzed genes the bioinformatic analysis using CODDLE (Codons Optimized to Discover Deleterious Lesions) program was performed. The regions of the genes with the highest probability to identify changes in the DNA sequence with an impact on the protein function during TILLING screenings were chosen. We were able to identify missense alleles in all studied genes. The overall mutation frequency was one mutation per 337 kb and ranged from 5 to 23 mutations per gene. The screen revealed a total of 60 independent mutations, including 57 (95%) located in exons. Thirty eight (67%) of the exon-located mutations induced a change of amino acid (AA) sequence. The detected mutations were in the homo (50%) or heterozygous (50%) stage.

The reaction of homozygous mutants to drought was evaluated in a greenhouse experiment. A relative water content (RWC) was calculated to check changes in the water status between the selected mutants and the parent variety "Sebastian". The chlorophyll fluorescence parameters were measured with a fluorometer (Hansatech Instruments Ltd) and the maximum photochemical efficiency ( $F_v/F_m$ ) was estimated. Additionally, for mutants carrying changes in *HvABI5* gene response to different ABA (abscisic acid) concentrations was evaluated at the seed germination stage.

## Adaptation and survival of androgenetic carrot plants obtained on culture media with increased copper concentrations and in a substrate with elevated Cu content

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The increase in the emissions of environmental pollutants, including heavy metals, is the cause of many negative effects on plants and the soil, and thus on human health. Plants are generally characterized by resistance to high concentrations of heavy metals. Higher concentrations of these elements can be toxic to plants. Our team in the Institute of Horticulture have conducted trials to obtain plants resistant to higher concentrations of copper. To select such plants, plentiful and diverse plant material is needed. Through androgenesis, using the techniques of anther cultures and isolated microspore cultures, and the valuable materials selected by breeders, numerous androgenetic embryos were obtained, from which those resistant to the stress factor of increased copper content *in vitro*, and then in the soil, were selected.

The regenerated, rooted plants *in vitro*, both from the control medium containing  $0.1 \mu\text{M}\cdot\text{l}^{-1}$   $\text{CuSO}_4 \times 5\text{H}_2\text{O}$  and from the media with increased amounts of copper 1, 10,  $100 \mu\text{M}\cdot\text{l}^{-1}$  were transplanted into 50 ml micropalletes with a substrate composed of 1 part peat, 3 parts sand. 1 l of the substrate was supplemented with 1 g of macro-Scott and 0.4 ml of micro-Pionier. The adapted plants were transplanted from the micropalletes into pots with a diameter of 8 cm from the control media to the control substrate, and from the culture media with increased copper content into the substrate with increased concentration of copper sulfate.

The composition of the control substrate was the same as in the micropalletes. To each liter of the substrate with a higher  $\text{CuSO}_4 \times 5\text{H}_2\text{O}$  content addition was made of 552 mg of this compound and 1 g of macro-Scott. At the stage of 4-5 true leaves, carrot plants from the regeneration media, i.e. the control and those with

increased concentrations of copper sulfate, were transplanted into pots with a diameter of 16 cm and a height of 13 cm. In 1 liter of soil there was 396 mg  $\text{CuSO}_4 \times 5\text{H}_2\text{O}$  for plants with the increased amount of this compound. The control plants were planted in the soil. All the plants replanted into pots with a larger diameter were pitted in a field at the same level as the experimental plot.

On the medium labelled Cu-3, with the highest concentration of copper sulfate, i.e. 1000 times higher than in the control medium, no plants were obtained. Only 38.5% of the adapted plants were obtained from the culture medium with a 10 times higher concentration of  $\text{CuSO}_4 \times 5\text{H}_2\text{O}$ . Better results of adaptation, 55.2%, were obtained among the plants from the medium containing a 100 times higher concentration of copper (Cu-2). The control was found to have the highest percentage of adapted carrot plants *in vitro*. It accounted for 68.8%. All of the control plants survived the replanting into larger pots.

Plants from the medium with a 10 times higher concentration of copper (Cu-1) survived the transfer to substrates with increased  $\text{CuSO}_4 \times 5\text{H}_2\text{O}$  content in 85.7% of cases, and those from the medium with a 100 times higher copper content in 92.3% of cases. The highest survival rate of carrot plants in the field was obtained on a substrate with 396 mg of  $\text{CuSO}_4 \times 5\text{H}_2\text{O}$  after they had been regenerated on the medium with a 100 times higher level of copper sulfate (Cu-2). A lower adaptation rate was observed in field conditions on the same substrate when the plants had come from the culture medium with a 10 times higher Cu concentration than in the control medium.

## Growth of *Nicotiana tabacum* BY-2 cell culture under conditions of nitrogen-sucrose and phosphorus-sucrose starvation

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The availability of food determines the survival of all organisms. Availability of nutrients is especially important in the case of *in vitro* cultures where the composition of the medium determines the possibilities of cell growth. Sucrose is an important source for energy generation and structural constituent of cells. It also regulates gene expression and acts as important regulator of various processes associated with growth and development. Nitrogen (N) and phosphorus (P) belong to a group of essential macronutrients required by plant cells. They are the elements which form part of many biologically important substances and their availability in the environment is essential for growth, development and survival of plants. The lack of, or limited access to nutrients leads to metabolic dysfunction of cells, limiting their growth and proliferation and consequently may lead to cell death. Under conditions of starvation many intracellular signaling pathways switch on, which can lead to cell death or may activate pro-survival mechanisms including autophagy.

The aim of our study was to compare the response of tobacco BY-2 cells lacking a source of sucrose, nitrogen or phosphorus in the medium with the reaction of cells simultaneously nitrogen-sucrose or phosphorus-sucrose starved.

Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cell cultures at the stationary growth phase were passaged into the complete Linsmaier and Skoog

(LS) medium (control) or into that depleted of sucrose (-suc), nitrogen (-N), phosphate (-P) or into that lacking both sucrose and nitrogen (-suc/-N) or sucrose and phosphorus (-suc/-P).

The parameters of cell culture growth and protein profiles, protein levels and the activity of proteolytic enzymes in the cell homogenates in the following days of culture growth cycle were analyzed.

Our results have revealed that: i) double-starvation reduced the increase in the cell number in the culture only slightly more than the single-starvation; ii) both the absence of sucrose, nitrogen or phosphorus and also sucrose and nitrogen or sucrose and phosphorus in the medium inhibited the culture growth but did not cause cell death; iii) in comparison with the control the activities of proteolytic enzymes in the starved cells were several times higher, which was accompanied by a significant decrease in the overall level of proteins in the cells; iv) although in all starved cells protein levels were very low new kinds of proteins were found only in the cells on the -suc, -suc/-N and -suc/-P media.

In conclusion, the responses of tobacco BY-2 cells in the absence of carbon, nitrogen or phosphorus sources in the medium only slightly differ compared to the reactions of cells in the case of combined stress. On the other hand, however, under conditions of dual nutrient stress the observed reactions seem to be induced by the absence of sucrose.



## The accumulation of phenolic acids in *Ginkgo biloba* *in vitro* cultures under different light conditions

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The *Ginkgo biloba* tree is the only surviving genus and species of the *Ginkgoaceae* family. Phytopharmaceuticals from ginkgo leaves are used in medicine mostly in disturbances of cerebral and peripheral circulation. Phenolic acids are known to occur in many plants, in *Ginkgo biloba*, too. They are important compounds that have pharmacological activity (especially anti-inflammatory, cholagogic, hypolipemic, spasmolytic, fungistatic, bacteriostatic). *Ginkgo biloba* has been well investigated chemically for various classes of constituents. It is reported to contain a number of secondary metabolites including terpenoids, polyphenols, allyl phenols, organic acids, carbohydrates, fatty acids and lipids, amino acids and inorganic salts. Plant cell biotechnology of *Ginkgo biloba* focuses on micropropagation or ginkgolides and bilobalide biosynthesis and accumulation. The other secondary metabolites, such flavonoids, have been rarely investigated in *in vitro* cultures. The content of phenolic acids in *Ginkgo biloba in vitro* cultures has not been investigated yet (Beek, 2000).

The aim of this study was to investigate the impact of light and its quality on the phenolic acids accumulation in biomass of *Ginkgo biloba in vitro* cultures.

*G. biloba* callus cultures were established from female leaves explants collected in the Botanical Garden of the Jagiellonian University in Kraków. Two variants of MS medium (Murashige and Skoog, 1962) supplemented with different combination of growth regulators were tested (I - NAA 4 mg/l, BAP 2 mg/l; II - picloram 4 mg/l, BAP 2 mg/l). Callus cultures were maintained under

different light conditions (blue, red, far red light, UV-A irradiation, white light and darkness) in a laboratory of the Department of Ornamental Plants, University of Agriculture in Krakow. The analyses of methanol extracts of biomass from callus cultures were conducted by HPLC method according to Ellnain-Wojtaszek and Zgórką with our modifications (Ellnain-Wojtaszek and Zgórką, 1999). Aglycons of phenolic acids were detected after hydrolysis of glycosides in 2 M aqueous HCl (Harborne, 1998). The experiments showed the impact of light quality on the culture growth. Red and far red light caused inhibition of culture growth or even biomass degradation. Under blue, white light, darkness and UV-A irradiation no changes in biomass growth were noted. Six phenolic acids (o-coumaric, gallic, p-hydroxybenzoic, protocatechuic, syringic, vanillic acids) and cinnamic acid were detected in methanol extracts of *in vitro* material. The results proved the significance of medium and light quality for the accumulation of phenolic acids.

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## Cold temperature stress in isolated microspore culture of rye (*Secale cereale* L.)

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Rye (*Secale cereale* ssp. *cereale*) is a recalcitrant species, the haploids of which are not easily obtained with a conventional method of anther culture. The technique of isolated microspore is more complicated and has been occasionally applied for only a limited number of monocot species, cultivars and strains. The aim of the conducted experiment was to assess the frequency of mechanically damaged microspores after isolation with the help of the mortar. The effect of temperature (4 °C) pretreatment of spikes on microspore viability and the course of culture was also taken in consideration.

The analyses of an isolated microspore in the culture were conducted on 30 genotypes of rye originated from two breeding programs and various provenances.

The application of temperature or osmotic stress is required for the sporophytic development of rye microspores *in vitro* culture. As it may be found in literature, it is well known that 1) low temperature treatment causes death of anthers and increases the number of non-viable microspores, 2) at a temperature of approx. 6 °C the number of microspores retained in the phase of the first mitotic division increases and 3) low temperature causes a non-specific shock and is the trigger of sporophytic microspore development. In order to obtain an analogous result in isolated microspore cultures of cereals, particularly barley and wheat, mannitol is used additionally in various concentrations, enhancing the efficiency of androgenesis and plant regeneration.

For the 30 investigated populations of *Secale cereale* low temperature (4 °C) was adopted as the stress factor. After reaching a specific development phase spikes with microspores in the unicellular stage were excised and exposed to a temperature of 4 °C for a period of 2 up to 42 days in dark and immersed in distilled water.

After isolation, the highest microspore viability response for population 1283C – 91.9% and S1152/10 – 91.8% was observed. The process of mechanical isolation of rye microspores caused their damage within the range from 8.2% to 20.2% of the genotypes originating from Danko and from 8.1% to 16.2% for microspores of PHR genotypes.

An optimal time for the maintenance of high microspore viability was to perform isolation following low temperature stress (4 °C) applied for up to 7 days. A considerable extension of the duration of low temperature (36-42 days) resulted in a reduction of microspore isolation efficiency and a slight reduction of microspore viability.

In the conducted experiment we did not observe any considerably enlarged or dividing microspores. In the course of observations it was found that microspores most frequently accumulated starch and their cytoplasm was shrunk. All of them failed to conduct further mitotic activity.

## Pigments in potato plants growing *in vitro* cultures on media with different contents of humic compounds

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There is much information on the influence of humic acids on the growth of plants *in vivo*. However, only a few works concern the influence of these compounds on plant propagation *in vitro*. Works that concern the latter indicate that the effect of humic compounds may be direct or indirect. The direct effects include the influence on physical and chemical properties of the growing media, the oxidation-reduction potential, the buffering capacity, colloidal properties and the availability of cations. Indirect effects are the influence on respiration, photosynthesis, synthesis of nucleic acids and enzymes, enzyme activity and absorption of mineral salts (Kalembasa and Tengler, 2004).

In the present experiment we investigated the effect of different concentrations of humic compounds (0.01, 0.1 and 1%) added to the medium before autoclaving on the content of chlorophylls and anthocyanins in potato plants cv. Sante grown *in vitro* cultures. The contents of chlorophylls *a* and *b* were determined according to the method developed by Lichtenthaler (1987), and anthocyanins by the modified method according to Mancinelli et al. (1988).

It was found that the highest levels of chlorophyll *a* and *b* in plant were observed after the first 7 days of culture on media containing humus. The highest value was observed in plants grown on the media containing

0.01% humic compounds. It was also observed that the total chlorophyll content (*a* + *b*) was similar to that of chlorophyll *a* and *b* separately and it was the highest after 7 days of culture.

The contents of anthocyanins was a little different than that of chlorophyll. At the beginning of the experiment (after 7 and 14 days) the highest anthocyanins content occurred in the control plants and in the plants growing on the medium with a lower concentration of humus. But after 21 days, a higher content of anthocyanins in plants growing on the media with largest concentration of humus (1%) was observed.

It can be supposed that the humic compounds affecting the stimulation for the content of chlorophylls and significantly affect the process of photosynthesis, and consequently the size of the plants and potato tubers. By increasing the content of anthocyanins, humic acids affect the antioxidant activity in plants and in the products manufactured from them.

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