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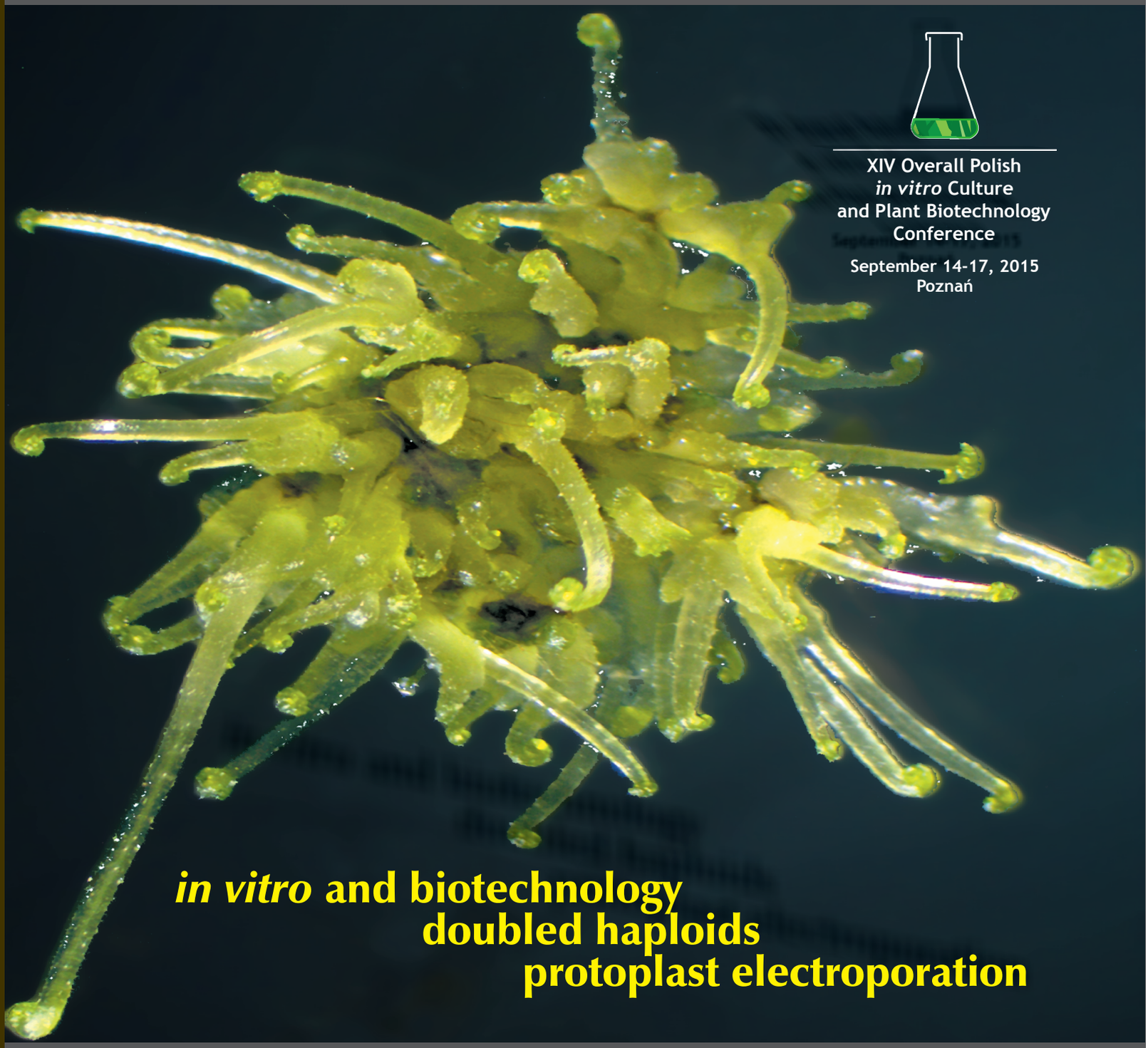

XIV Overall Polish
in vitro Culture
and Plant Biotechnology
Conference

September 14-17, 2015
Poznań

***in vitro* and biotechnology**
doubled haploids
protoplast electroporation

Committee of Biotechnology • Polish Academy of Sciences
Institute of Bioorganic Chemistry • Polish Academy of Sciences

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Front cover: Numerous somatic embryo-derived young sporophytes of *Cyathea delgadii* which developed on stipe explant cultured *in vitro* by Anna Mikuła

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Preface

Allow me to introduce the selected papers, abstracts of lectures, and oral and poster presentations from the XIVth Polish In vitro Culture and Plant Biotechnology Conference, held at the BioCenter of the Poznan University of Life Sciences 14-17th September 2015 in Poznań, entitled: *Structural, physiological and molecular bases of plant differentiation*.

The program of the Conference includes research presented in 115 abstracts in the following sections: plenary lectures – 9, oral presentations – 30 and poster presentations – 76 within the overall framework of four sessions: 1) *Plant differentiation processes in the culture of somatic cells and cells of the generative pathway*, 2) *Physiological and molecular conditions of plant cell response in the processes of morphogenesis*, 3) *Plant cells under stress conditions* and 4) *Exploitation of organ and cell cultures in basic and applied studies*.

The three years since our last meeting (2012) have seen the growth of the subject, with completely new plant species which are the model of our research. Generally, this conference is very similar to the last one, considering the fields of interest of Polish plant biotechnology laboratories. However, it is a shame that the development of plant transformation does not have the opportunity to follow other fields of plant tissue culture and plant biotechnology in our country.

Today is the second time that we have had the opportunity to use the pages of *BioTechnologia* to make our achievements more widely known, not only within our Society but to all readers of the Journal. The selected papers which met Editorial requirements present a broad spectrum of subjects, reflecting a variety of interests. The number of Polish papers presented to an international audience is constantly increasing, showing that we have better opportunities to realize our dreams in plant biotechnology using increasingly more sophisticated tools, including not only the latest technology, but also more expensive chemicals, which is possible thanks to financial support from the Polish Government. What is also noteworthy is that although the competition between ours laboratories is tough, our publications have increasingly been gaining recognition on the international stage.

I do hope that this volume of the Journal will be of interest to young plant tissue "culturists" and plant genome "manipulators" who love to play with plant organs, tissues, cells, organelles and their genomes in the quiet of a laboratory where they may realize their own scientific dreams.

I wish to express my gratitude to all participants for the effort and care with which they have prepared their publications and abstracts. I extend my thanks to the reviewers, who have done a very good job of ensuring the scientific merit of each publication. I would like to thank the staff of the Journal and especially Dr. Paweł M. Stróżycki for his professional work. Last but not least, I wish to thank my closest co-workers Dr. Anna Mikuła and Dr. Karolina Tomiczak, the leading members of both Conference Committees, for their work on the organization of the conference.

Prof. dr hab. Jan J. Rybczyński

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Journal of Biotechnology, Computational Biology and Bionanotechnology REVIEW PAPER

Doubled haploids as a material for biotechnological manipulation and as a modern tool for breeding oilseed rape (*Brassica napus* L.)

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Abstract

Haploids and doubled haploids (DHs) have been produced in *Brassica* ssp. using anther or isolated microspore cultures. Since 1982, when Lichter developed a method of isolated microspores culture, this technique has been constantly improved and modified. Haploids and DH of *Brassica napus* have been extensively used in genetic studies, such as gene mapping, location of quantitative trait locus, marker / trait association studies, and genomics. Furthermore, the oilseed rape haploid induction technique can nowadays be efficiently combined with several other plant biotechnological techniques, enabling several novel breeding achievements, such as mutation breeding, the breeding of hybrid varieties, genetic transformation, and resynthesis of *B. napus*. The development and improvement of *in vitro* androgenesis of oilseed rape allowed DH populations to be obtained on a large scale. Today DH lines are used in the genetic analysis of quantitative traits and to analyze the impact of the environment on the yield and quality of the seeds. Currently, DH technology has become more efficient and hence can be widely applied in winter oilseed rape breeding. Additionally, DH technology is used to shorten the process of breeding new varieties. An open pollinated *B. napus* cv. Monolit (Plant Breeding Strzelce Ltd., Co.) is the first Polish winter oilseed rape variety obtained using DH technology. The breeding cycle of this variety is about four years shorter compared to a classical breeding program. The second Polish cultivar obtained using DH technology, cv. Brendy (Plant Breeding Smolice Ltd., Co.) was developed in a similarly short period. In the breeding of new oilseed rape hybrid varieties, homozygous restorer lines are also exploited. Our basic research used oilseed rape DH technology with a focus on improving the qualitative traits of oilseed rape and is presented here, based on the major researches conducted worldwide as well as on the results obtained at the Plant Breeding and Acclimatization Institute – National Research Institute in Poznań.

Key words: *Brassica napus* L., oilseed rape, doubled haploids, markers-assisted selection, gene mapping, transformation, breeding

Abbreviations

AFLP	– amplified fragment length polymorphism	RFLP	– restriction fragment length polymorphism
CMS *ogura*	– cytoplasmic male sterility *ogura*	*Rfo* gene	– restorer gene of *ogura* cytoplasmic male sterility
DH	– doubled haploid	RIL	– recombinant inbred line
ISSR	– inter-SSR amplification	RS	– resynthesized *Brassica napus*
MDE	– microspore-derived embryo	SCAR	– sequence characterized amplified region
QTL	– quantitative trait locus	SNP	– single nucleotide polymorphism
PCR	– polymerase chain reaction	SRAP	– sequence-related amplified polymorphism
RAPD	– random amplified polymorphic DNA	SSR	– simple sequence repeat

Introduction

Oilseed rape (*Brassica napus* L.) is the third, after palm and soybean, most important source of vegetable oil in the world; it contributes significantly to the economy of many countries. Due to its economic value and high performance in an *in vitro* culture, oilseed rape is considered as one of the most suitable species that can be improved through biotechnology techniques. One of

the biotechnological methods most useful in basic researches and in plant breeding is androgenesis *in vitro* that aims at the development of haploids and doubled haploids (DH). Microspores isolated from the anthers of oilseed rape can be induced to develop into fully functional haploid embryos, instead of mature pollen grains. The ability of these cells to change their development process in response to environmental stimuli is an exceptional example of their totipotency.

The history of this method is almost a hundred years old. Since the first discovery of the spontaneous development of haploid plants in *Datura stramonium* in 1922 (Blakeslee et al., 1922), particularly after the discovery of *in vitro* androgenesis in anther cultures of *Datura* in 1964 (Guha and Maheshwari, 1964, 1966), the potential of haploids arose. Later, Zentkteler developed an *in vitro* production of *Atropa belladonna* L. haploid plants and in 1972 elaborated a method for obtaining embryos and plantlets from microspores in *in vitro* anther culture of *Lycium halimifolium* Mu, L. (Zentkteler, 1971, 1972).

Following those initial discoveries, many studies have been conducted to develop haploids in *Brassica* plants. Using anther or isolated microspore cultures, haploids and DH have been produced in *Brassica* ssp. *Brassica* anther culture was successfully used by Keller et al. (1975) and Thomas and Wenzel (1975). Alternatively, in 1982, Lichter developed an isolated microspore culture system for *B. napus*. These techniques have been gradually developed and constantly improved (Cegielska-Taras et al., 2002; Forster and Thomas, 2005; Palmer et al., 2005; Smýkalová et al., 2006; Ferri and Keller, 2007; rev. Pratap et al., 2009; rev. Touraev et al., 2009; Bohownik et al., 2011; Takahira et al., 2011; Murovec and Bohanec, 2012). Protocols for isolated microspore culture of *B. napus* vary among laboratories, especially for cultivating donor plants, harvesting buds, isolating microspores, inducing microspores for division, converting microspore-derived embryos to plantlets, and doubling of chromosome. Protocols for *B. napus* have been successfully extended to other *Brassica* species too.

Generally, the yield of androgenic plants in the microspore culture of *Brassica* species is higher than in the anther culture. In the method of microspore culture, every microspore is potentially capable of regenerating into a viable embryo, and each plant, therefore, represents the genetic variation that exists in the population of microspores. Plant regeneration occurs either by direct embryogenesis or by secondary embryogenesis followed by organogenesis. A number of factors influence microspore embryogenesis including genotype, donor plant genotype growth conditions, the stage of explant development, pretreatment composition of the culture medium, and environmental conditions during the culture or the diploidization process. A large part of these factors have to be optimized for each microspore donor genotype (Cegielska-Taras, 2002; Babbar et al., 2004; Gu et al., 2004; Friedt and Zarhloul, 2005; Zhang et al., 2006; Gil-Humanes and Barro 2009; Pratap et al., 2009; Ferrie and Caswell, 2011; Ferrie and Möllers, 2011; Islam and Tuteja, 2012).

The key for increased regeneration efficiency during androgenesis largely depends on the control of two main developmental switches: the induction of microspore cell division and its ultimate commitment to the embryogenic pathway and diploidization rate (Maraschin et al., 2005).

Most plants obtained from the microspore culture of *B. napus* are haploid, which, in turn, must be diploidized. The frequency of spontaneous chromosome doubling is about 10-20% and varies among genotypes (Weber et al., 2005; Takahira et al., 2011); therefore, artificial chromosome duplication is required. The use of antimitotic substances immediately after the microspore isolation is the most effective method of artificial chromosome duplication (Zhou et al., 2002) and of obtaining microspore-derived embryos (Mohammadi et al., 2012). DH plants are produced by doubling the chromosome number of a haploid plant, while the conventional homozygous inbred lines are developed by selfing in successive generations. With the DH method, homozygous plants produced in one generation show a homozygosity of 100% compared to the conventional method, which results in an average level of homozygosity of 96.9%, that is, after five to six generations of selfing (Briggs and Knowles, 1967).

One of the principal advantages of haploid techniques is the fixation of segregating genotypes occurring at a lower frequency, in which the recessive gene coding for specific traits is combined in the homozygous condition (Friedt and Zarhloul, 2005).

Microspore culture plays a significant role in both fundamental and practical research. A number of review articles are found on the use of microspore culture and the resulting DH plants (Maluszynski et al., 2003; Forster and Thomas, 2005; Palmer et al., 2005; Friedt and

Snowdon, 2009; Pratap et al., 2009). This technique is simple and used in the production of *B. napus* DH plants; this method is highly preferred compared to classical breeding methods. Therefore, microspore culture is the method of choice in plant genetic research and breeding programs.

DHs of *B. napus*, besides being used for the production of homozygous lines, are also used for selecting lines that possess traits determined by recessive genes. DHs were also used for the basic research of physiology and biochemistry, induction and selection of mutants, improvement of plants through genetic transformation, and broadening the genetic diversity in the process of resynthesis of *B. napus* (Babbar et al., 2004; Palmer et al., 2005; Friedt and Snowdon, 2009; Pratap et al., 2009).

Basic researches on DH technology of oilseed rape and attempts to improve their qualitative and quantitative traits are presented based on the main researches conducted worldwide as well as on the results obtained at the Plant Breeding and Acclimatization Institute NRI in Poznań. The results presented here as well as the publications issued by our Institute were based on the data obtained from experiments performed using doubled haploid lines developed by a technology elaborated by Cegielska-Taras et al. (2002).

Development of molecular markers

Haploids can efficiently be used in basic studies, for example, to identify molecular markers associated with important traits. For the development of molecular markers, segregating populations for the trait of interest are required. Marker-assisted selection (MAS) allows the detection of desired traits in newly developed varieties at early stages of plant development in breeding programs.

Nowadays, new oilseed rape breeding programs that involve modern methods and techniques of biotechnology, plant physiology, molecular genetics and molecular biology have reached an advanced level. It is expected that new varieties will reveal an excellent result in terms of yield, quality characteristics, or disease and stress resistance. Different, sometimes antagonistic, directions of breeding are carried out in parallel and require increasing flexibility from breeders. To maintain competitiveness in this field, it is necessary to react rapidly to the demands of the oilseed market (Matuszczak, 2013).

However, the need for a rapid change stays in opposition to the traditional model of breeding, or the development of new varieties through the selection of certain genotypes spanning several growing seasons. To make the breeding process more efficient and effective and, as a consequence, to significantly accelerate the development of new varieties, molecular markers are used for the selection (Mohan et al., 1997; rev. Snowdon and Friedt, 2004; Mikołajczyk et al., 2008; rev. Matuszczak, 2013).

Because of their genetic homogeneity, DH lines are extensively used to determine single nucleotide polymorphisms (SNPs) that are specific for agronomically important traits and may be used as molecular markers in breeding programs. Several mapping populations were developed and association studies were performed along with positional cloning and genomic and transcriptomic sequencing experiments using *B. napus* DH lines to analyze the crucial genes and regulatory sequences for further application in basic and applied research (Snowdon and Friedt, 2004).

DH lines were developed to detect SNP in low-linolenic (LL) winter oilseed rape genotypes that were obtained by chemical mutagenesis at the Plant Breeding and Acclimatization Institute-NRI Poznan (Spasibionek, 2006). The LL DH line of winter oilseed rape, DH219/05, was selected from a population of DH lines developed by *in vitro* culture of isolated microspores from the LL M681 mutant line of *B. napus* (C18 : 3 ≤ 3%) obtained using ethyl methanesulfonate (EMS) (Spasibionek, 2006). Total DNA was isolated from a wild-type line and the LL mutant DH219/05 line and *FAD3* desaturase alleles from the homeologous A and C genomes of *B. napus* (*BnaA.FAD3* and *BnaC.FAD3*). Obtained DNA preparations were polymerase chain reaction (PCR) amplified, cloned, and sequenced (Mikołajczyk et al., 2010a). As a result, two statistically important point mutations were detected: one with a C to T transition in the mutant *bnaA.fad3* gene leading to a possible Arg to Cys substitution and another with a G to A transition in the 5′ donor splice site of the mutant *bnaC.fad3* gene, disrupting intron sixth splicing. In addition, the total RNA was isolated from the developing seeds of wild-type and mutant DH lines eight weeks after pollination. They were used for reverse transcription reaction to confirm the detected SNPs as well as to analyze the splicing variants for *bnaA.FAD3* and *bnaC.FAD3* (Mikołajczyk et al., 2010a). Functional genetic markers for monitoring *FAD3* alleles in breeding programs were developed, and the detection

of wild-type and mutant *FAD3* alleles comprised 1) independent PCR amplification of short fragments comprising SNPs and 2) the detection of SNPs based on the microsequencing method (SNaPshot) using allele-specific primers (Mikołajczyk et al., 2010a).

This unique SNaPshot assay enables a precise and unambiguous detection of the allelic variability in programs accompanied by MAS, including recombinant and hybrid breeding of the LL forms of winter oilseed rape.

DH populations to construct genetic maps

A DH population is ideal for genetic mapping (Maluszynski, 2003). Genetic mapping is one of the methods by which important crops are studied to identify the molecular markers which are linked to the valuable traits of the plant under study. In particular, when dealing with quantitative traits, influenced by many various genes, the localization of quantitative trait loci on the genetic map becomes a very important step in the genetic studies being performed.

For mapping *B. napus*, DH populations are commonly used, and it seems that their use has been consistently increasing (Lombard and Delourme, 2001; Parkin et al., 2005; Delourme et al., 2006; Kaur et al., 2009). A good illustration of this general tendency is the fact that among all *Brassica* mapping populations currently listed on the website Brassica.info, 70% (16) are DH populations and only 30% (7) are other types of populations (King and Baten, 2015). The most significant achievement of these studies is the construction of integrated maps of *B. napus*. A good example of such integration is the construction of a genetic map using three distinct DH populations and the subset of RFLP, SSR, RAPD, AFLP and SNP markers. The obtained BnaWAIT_01_2010a integrated linkage map contains 5,162 markers representing 2,196 unique loci (Wang et al., 2011). Other studies focused on the comparison of two maps based on DH (190 lines) and recombinant inbred line (94 lines) populations developed from the same cross (two diverse *B. napus* cultivars "Polo" and "Topas" were crossed). The maps were prepared based on SSR, SRAP, ISSR and SCAR markers. Data from the two genetic maps were used to construct a consensus integrated genetic map covering a total genome length of 2464.9 cM (Geng et al., 2012).

There are at least two reasons for the DH populations to be preferred during the process of map construction. Both reasons arise from the fact that in DH lines, all the loci exist in homozygous state. First, the DH population is practically immortal and it can be maintained over many years simply by selfing each line, without any genetic changes in subsequent generations. This makes it a good material for any genetic study or phenotypic analysis in various environments. Second, the DH population has a very simple pattern of segregation (the theoretical proportion of two alleles is 1 : 1 for each locus), which is of great value if the localization of QTLs for some complex traits is considered. When using dominant markers (e.g., RAPD or AFLP), the use of DH population is the only possible choice because dominant markers are unable to detect loci in the heterozygous state that are present in the F_2 population.

Isolated microspore culture method has been successfully used to produce DH plants and to the development of DH population (Matuszczak et al., 2001). A total of 320 DH plants thus obtained were derived from the F1 hybrid obtained from the crossing of parental lines DH-JN-86 (R1) × DH-ER_2-13/1 (R2). The seeds of the first parent had a low erucic acid and high glucosinolate content, whereas the seeds of the second parent had a high erucic acid and low glucosinolate content. The DH-JN-86 line was derived from the Jet Neuf variety, and the DH-ER_2-13/1 line was from our own breeding materials. The subset of 117 DH lines served as a basis for the construction of genetic map. This mapping population, as well as parental lines, were studied using RAPD (Matuszczak and Krzymański, 1999) and AFLP (Matuszczak, 2002) markers. Today, the obtained genetic map continues to consist of 75 markers grouped in 19 linkage groups. Since the parental forms exhibit variations in important agronomic traits such as erucic acid and glucosinolate contents in seeds, our research focused on the finding of QTLs for these traits. Fifty-eight various QTLs have been localized on the map so far (Matuszczak, 2010; Matuszczak et al., 2011).

The study on the yellow-seeded oilseed rape with improved seed meal quality is another example. This trait in *B. napus* is generally associated with a reduced seed coat thickness and consequent lowering of antinutritive crude fiber and phenolic compounds (Friedt and Snowdon, 2009). Yellow seeds do not occur naturally among the genotypes of *B. napus*. In various research groups, there are many forms of yellow oilseed rape lines obtained by transferring this trait from other yellow-

seeded *Brassica* species or obtained as mutations (Cegielska-Taras, 2002; rev. Rahman and McVetty, 2011). In genetic mapping, these yellow-seeded materials are used to identify the major genes that contribute to reduced seed coat and to develop markers related to this trait (Friedt and Snowdon, 2009). The Polish source of yellow-seeded *B. napus* was derived from the crosses of a spontaneous mutant with lighter seeds, found in the breeding material of a double low winter rapeseed, with segregating, spotted-seed-coat spring line, from earlier crosses of *Brassica napus* × *Brassica rapa var. oleifera.* The lines thus obtained were further improved with respect to quality traits and then those that showed a stable expression for yellow seediness were selected (Bartkowiak-Broda et al., 2011). Using androgenesis *in vitro* of isolated microspores of bright color lines, yellow-seeded DH lines were developed. Two yellow-seeded lines DH Z129 and DH Z114 were used for reciprocal crosses with black-seeded DH lines DH H5-105 and DH W40. Hence, for creating genetic maps of *B. napus*, two segregating populations consisting of 200 DH lines were used. All DH lines were analyzed using RAPD, AFLP, and SSR methods. As a result of the research, 889 polymorphic amplification products were obtained, but for the construction of a genetic map, only 349 of potential genetic markers could be used. Field experiments with these DH lines in two environments were carried out for four years to obtain the data of their phenotypic characteristics. These data will be used to create a genetic map of the yellow-seeded oilseed rape.

Mutagenesis and selection *in vitro*

A mutation technique was applied to improve the yield and quality of the crop and to develop resistance to diseases and pests. Many novel genotypes with desired traits have been obtained (Szarejko, 2003). In *B. napus*, microspores and haploid tissues have been used as a material for mutation (Kott et al. 1996; Cegielska-Taras and Szała, 2001; Szarejko, 2003; Li et al., 2005). A mutagenic treatment of a single, potentially totipotent haploid cell (e.g., an isolated microspore), followed by rapid differentiation in the embryo, represents a unique opportunity for screening homozygotic, non-chimerical M1 organisms for resistance to particular agents. Thus, the mutant production cycle can be shortened significantly. Haploid cells (microspores) or haploid tissue and selection can yield a plant population with genetically fixed novel traits. The advantage of using a large-scale microspore selection system over a traditional somatic tissue selection system is evident (Cegielska-Taras and Szala, 2001). Mutation methods combined with *in vitro* selection, especially at the haploid level, to select genotypes with traits controlled by either recessive or dominant genes, improves the collection of desired mutants. In *B. napus*, haploid cells and tissues have been used to select mutants for herbicide resistance, disease resistance, lower glucosinolate content, fatty acid composition, long pods and short stems, and DH lines with improved resistance to *Sclerotinia sclerotiorum* and better agronomic characters (rev. Li et al., 2005).

The methods of early *in vitro* selection of microspore-derived embryo (MDE) genotypes discussed here appear to be promising for further optimization of DH technology in oilseed rape (Ferrie and Möllers, 2011). The number of PCR-based DNA markers for agronomically important traits is increasing and will allow for a more efficient MAS in segregating MDE populations at an early *in vitro* developmental stage (Ferrie and Möllers, 2011).

One such example is the application of DH techniques with a method of early selection for erucic acid in cotyledons of MDEs. In this study, the seeds of the selected DH lines had higher erucic acid, that is, more than 50%. The erucic acid contents (expressed in percent of the total fatty acid content) of the cotyledons of embryos and of the seeds derived from plant regenerated from the remaining parts of the embryos were highly correlated. Results showed that the selected DH lines had high erucic acid content along with high oil content (Cegielska-Taras et al., 1999).

Genetic modification of *B. napus*

Oilseed rape (*B. napus* L.) is particularly susceptible to *Agrobacterium tumefaciens*-mediated transformation allowing a considerable progress in the development of genetically modified varieties (Friedt and Snowdon, 2009).

The limitations of initial regeneration systems and problems with combining regeneration and transformation within the same cells are the major constraints hindering the advancement of plant transgenesis. The essential elements of successful plant transgenesis include the choice of cells and organs for recipients of the introduced gene and an efficient system for the selection of cells containing an integrated foreign gene in the ge-

nome. In order for the genes to be successfully transferred, the cells and tissues have to represent a high regenerative capacity to develop into a complete and normal transgenic organism. DHs obtained by tissue cultures of microspores and MDEs show uniformity, and as a complete homozygous, they can also be efficiently used in genetic transformation (Fukuoka et al., 1998; Nehlin et al., 2000; Takahata et al., 2005; Cegielska-Taras et al., 2008). The gene introduced into a haploid genome, which in this case was an MDE and subsequent chromosome doubling, gives rise to a homozygous transgenic oilseed rape (Cegielska-Taras et al., 2008b; Cegielska-Taras and Pniewski, 2011).

One of the first successful attempts to obtain a transgenic herbicide-resistant winter oilseed rape was the transformation of haploid MDEs using hypervirulent *A. tumefaciens*' EHA105 strain (Cegielska et al., 2008b). In this way, two biotechnological methods of oilseed rape – DH and genetic modification – were combined.

The elaborated MDE transformation method of winter oilseed rape allows to obtain genetically modified plants (Cegielska-Taras et al., 2008b; Cegielska-Taras and Pniewski, 2011). The main advantage of this method is that as a result of chromosome duplication in the transformed haploid, the introduced trait can be evaluated in a single step, as a transgenic homozygote. The production of transgenic homozygous oilseed rape provides a unique material for further studies of the inheritance and functionality of genes introduced through a subsequent generation, that is, for basic research, breeding programs, and utilitarian purposes. An alternative path to obtain oilseed rape with new quality traits is by transferring particular foreign genes to a plant genome.

Many projects have aimed at obtaining transgenic DH lines; one such example is an attempt to obtain DHs that are tolerant to drought stress. The genetic source that expresses resistance to drought in *B. napus* is unknown. Hence, there is a need to explore the mechanisms that increase the tolerance toward drought stress and also genetic manipulations and to widen the range of genetic variability of this species. It is expected that studies that focus on the above points will result in DH lines that are tolerant to water deficiencies.

Drought stress induces diverse changes at the cellular and molecular level and is associated with the production of plant phytohormones, especially abscisic acid (ABA). The key role in the regulation of ABA signaling is attributed to protein phosphatases such as ABI1 and ABI2 (ABA insensitive). Protein phosphatases, which are negative regulators of ABA signaling, interact with various targets to form a regulatory core modulating cellular processes and stress response pathways. Moreover, the concentration of calcium (Ca^{2+}) in the cytoplasm increases substantially in response to some factors of stress. The information about stress (an increase in the calcium concentration) is sensed by numerous proteins, including Ca-dependent protein kinases (CDPKs). In the study described here, the coding regions of the *Arabidopsis thaliana AtABI1* and *AtCDPK6* genes were inserted into the vector pPKGIB, and these constructs were introduced into *B. napus* via MDEs with *Agrobacterium* mediated transformation (Babula-Skowrońska et al., 2015; Olejnik et al., 2011). Three independent *AtABI1*-overexpressing *B. napus* DH lines such as 41, 42, and 45 were investigated with respect to their drought stress response. The authors found that the overexpression of *AtABI1* in *B. napus* negatively influences several important cellular processes such as relative water content (RWC), chlorophyll accumulation, and the expression profile of several ABA- and/or dehydration stress-inducible genes. These data show that the function of ABI1 protein phosphatase is highly conserved within the *Brassicaceae* family. Furthermore, it functions as a regulatory hub protein for dehydration stress responses in plants. Interestingly, there are six *ABI1*-related genes in the *B. napus* genome, which are formed by polyploidization during evolution. To investigate the functional diversification of the duplicated *BnaABI1* gene copies, two evolutionarily distant *BnaABI1* genes such as *BnaA01.ABI1.a* and *BnaC07.ABI1.b* were selected. The expression pattern of these genes showed differences indicating the differential involvement of the duplicated *BnaABI1* genes in the response of *B. napus* to drought. These results shed new light on the diverse role of the *ABI1* gene family in the drought response plasticity in the *Brassica* polyploid (Babula-Skowrońska et al., 2015).

Resynthesis of *B. napus*

The level of genetic diversity in double-low genotypes is relatively low. It is caused above all by intensive selection of genotypes in terms of two features associated: with improving oil, through eliminating erucic acid and with improving the meal, through the reduction of glucosinolates content. Today, oilseed rape breeders are

seeking genetic diversity in their breeding programs. A particularly successful method used to create a novel genetic variety is wide hybridization in a special resynthesis of *B. napus* from ancestral species *B. oleracea* and *B. rapa.* The resynthesized (RS) oilseed rape (*B. napus*) is potentially of great interest for hybrid varieties breeding because its heterosis effects are reported to be higher in crosses of genetically distant materials. A major problem using RS lines of *B. napus* in hybrid varieties breeding is the quality of their seed oil (high level of erucic acid) and seed meal (high glucosinolates content), which does not comply with the double-low quality of oilseed rape. Additional treatments are needed for breeding before introducing RS *B. napus* in practice. Szała et al. (2015a) described RS oilseed rape obtained through crosses between *B. rapa* ssp. *chinensis* var*. chinensis* (pak choy) and *B. oleracea* ssp. *acephala* var. *sabellica* (curly kale) using embryo rescue technique. Several RS oilseed rape lines obtained by this technique were crossed with double-low winter oilseed rape possessing *Rfo* gene for CMS *ogura* hybridization system. Large numbers of androgenic plant populations (semi-RS DH lines) were developed from F1 hybrids using a microspore *in vitro* culture method. The seeds of the obtained semi-RS DH lines were analyzed biochemically with regard to double-low quality (zero erucic acid and glucosinolates content). From the populations of the semi-RS DH, genotypes with double-low quality and *Rfo* gene were selected. Currently, the obtained semi-RS DH lines with *Rfo* gene and double-low quality are being used to produce new components of winter oilseed rape for the development of CMS *ogura* hybrids (Szała et al., 2015a).

Improvement of the quality of oilseed rape oil

At present, much attention is paid to the nutritional value of oilseed rape oil obtained from double-low cultivars.

With regard to nutrition, oilseed rape is a very valuable oil product, and it is not only a source of essential unsaturated fatty acids but also a source of natural antioxidants with other endogenous bioactive substances in seeds such as tocochromanols, sterols, beta-carotene, and phenolic compounds. The quality of *B. napus* oil is also enhanced by the presence of a series of beneficial hydrophobic compounds including carotenoids, tocochromanols, and phytosterols (Cegielska-Taras et al., 2008a, 2011). Carotenoids and tocochromanols are important antioxidants and essential components of human diet providing a source of provitamin A (beta-carotene) and vitamin E (alpha-tocopherol).

A three-year study was performed on the range of the genetic variation among DHs to determine the effect of the genotype and the environment on tocopherol (T) and plastochromanol-8 (PC-8) content in winter oilseed rape (*B. napus* L.). The plant material consisted of 25 DH lines derived from the F1 hybrid obtained from the cross between two DH lines (Z × H), (Z-114 – yellow seeds; H_2-26 – black seeds) (Sieger et al., 2015). Among all the genotypes analyzed, 11 were selected as satisfactory and stable regarding tocopherol or PC-8 contents. From the breeding point of view, high heritability of traits guarantees the effectiveness of the selection and a rapid biological progress. The results of this study confirm the desirability of making changes to tocopherol and PC-8 contents using DH line technology. The lines obtained of the DH population derived from F1 plants of the cross between yellow and black seeds are characterized by different color of seeds, which correlated with their tocopherol homologues content. The high coefficients of heritability for α-T, γ-T, and PC-8 suggest a possibility of an effective selection based on the phenotype, allowing to obtain DH lines with a stable expression of traits. This suggests that α-T, γ-T, and PC-8 contents depend more on the genotype than on the environmental influence. This dependence is confirmed by a statistical analysis (Siger et al., 2015).

Phytosterols are produced by isoprenoid biosynthetic pathway and possess the properties of lowering LDL cholesterol. Thus, it would be highly desired to select high phytosterol content in oilseed rape oil (Amar et al., 2008). Recently, three QTLs for total phytosterol content were detected in a winter oilseed rape DH population, explaining 60% of the genetic variance for this trait.

The study on 44 DH lines of winter oilseed rape showed a large and significant variation of its total phytosterol and fat contents. The large genotypic differences in total phytosterol content indicate that an effective selection of genotypes with higher phytosterol content, in a cultivar development program, would be possible (Cegielska-Taras et al., 2011).

Hybrid breeding of winter oilseed rape

Currently, the breeding programs of rapeseed worldwide as well as in Poland are focused consistently on the

development of hybrid varieties which display an improved yield performance and better stability owing to the heterosis effect. The benefit of heterosis depends on the specific combining ability and genetic distance of parental lines which should be homozygous. Most breeding programs are based on cytoplasmic male sterility (CMS) systems, controlling cross pollination, while homozygous restorer lines are necessary to exploit heterosis effect. Today, DHs are widely used for producing homozygous parental restorer lines for breeding oilseed rape hybrids (Poplawska et al., 2007; Friedt and Snowdon, 2009).

In the CMS *ogura* system used in Poland as well as in many other breeding centers in the world, the restoration of fertility is achieved by a single restorer gene *Rfo*. The restorer has to be incorporated into a gene pool distant from the gene pool of male sterile lines. DH lines can be produced right away from F1 plants or starting from later selfing generations, which allows for some early testing (Frauen, 1994; Möllers and Iqbal, 2009; Paulmann and Frauen, 1997; Popławska et al., 2006).

DH lines possessing the *Rfo* restorer gene were selected after molecular analyses using specific biochemical and genetic markers. The biochemical assay used to detect the *Raphanus sativus* Pgi-2 isozyme locus closely linked to the *Rfo* gene (Delourme and Eber, 1992) was applied (Poplawska et al., 2007) in addition to other molecular markers. At the beginning, the OPC02 RAPD marker (Delourme et al., 1994) was used for monitoring the *Rfo* gene in parallel to the specific SCAR marker – for the CMS *ogura* (Sigareva and Earle, 1997). Then, the *Rfo* RAPD marker was converted to an SCAR one (Mikołajczyk et al., 2008), which was further combined with the "CMS" SCAR marker for developing a multiplex PCR assay (Mikołajczyk et al., 2010b), making the selection of DH lines with the *Rfo* restorer gene more time-, labor-, and cost-effective.

DH technology in winter oilseed rape breeding programs

Generally, because of the high response of *B. napus* genotypes to microspore culture techniques, the use of DH production has become a common practice in commercial breeding programs and has already resulted in numerous licensed varieties.

The most important benefits of using haploid plants in the breeding of new varieties are as follows: 1) homozygous recombinant line can be developed in one generation instead of numerous backcross generations; 2) selection for recessive traits in recombinant lines is more efficient since these are not masked by the effects of dominant alleles; and 3) varieties can be developed in reduced time period, for example, at least from 12 to 8 years.

Microspore culture is a useful tool in plant breeding because homozygous lines exhibiting the desired agronomic traits can be rapidly selected, which creates an opportunity for faster production of commercial cultivars. A breeding program of Canadian spring canola cultivar Quantum lasted five years (Stringam et al., 1995). It resulted in the selection of DH line which is characterized by a high yield and agronomically superior blackleg resistance. The other winter oilseed rape DH line, cultivar Mohican, was developed in the UK (Kučera et al., 2002). Currently, most of the Canadian spring oilseed rape cultivars that are grown are derived using DH technology (Ferrie and Möllers, 2011).

An open-pollinated cultivar Monolit (Plant Breeding Strzelce Ltd., Co.) is the first Polish winter oilseed rape variety obtained using DH technology (Cichy et al., 2005). The breeding cycle of this variety was about four years shorter than a classical breeding program. The second Polish winter oilseed rape variety being the DH line was registered as cv. Brendy (Plant Breeding Smolice Ltd., Co.). It is a DH line which was licensed in 2013. Both cultivars were obtained from isolated microspore culture *in vitro* at the Plant Breeding and Acclimatization Institute NRI in Poznań.

Numerous DH populations of winter oilseed rape obtained from an isolated microspore culture (Cegielska-Taras et al., 2002) have been widely used in various statistical researches, for example, to study the influence of environmental conditions on the stability of important yield components and the transgression effects of advantageous traits (Szała et al., 2015b).

Conclusions

Androgenesis *in vitro* is one of the most widely used culture techniques which has miscellaneous applications. The production of DH plants through a microspore culture is the milestone in the development of research on the genome of rapeseed at different levels and using different technologies. It is also used to determine the effectiveness of breeding programs.

It seems that the positive results of molecular studies on the identification of genes responsible for trigge-

ring the induction of divisions of isolated microspores of *B. napus* in *in vitro* culture may become another step in the improvement of DH technology.

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References

Amar S., Ecker W., Becker H.C., Möllers C. (2008) *QTL for phytosterol and sinapate ester content in Brassica napus L. collocate with the two erucic acid genes*. Theor. Appl. Genet. 116: 1051-1061.

Babbar S.B., Agarwal P.K., Sahay S., Bhojwani S.B. (2004) *Isolated microspore culture of Brassica: An experimental tool for developmental studies and crop improvement*. Indian J. Biotech. 3: 185-202.

Babula-Skowrońska D.; Ludwików A., Cieśla A., Olejnik A., Cegielska-Taras T., Bartkowiak-Broda I., Sadowski J. (2015) *Involvement of genes encoding ABI1 protein phosphatases in the response of Brassica napus L. to drought stress*. Plant Mol. Biol. 88: 445-457.

Bartkowiak-Broda I., Piotrowska A., Hernacki B., Michalski K., Krotka K. (2011) *Genetic and molecular analysis of specific-origin yellow-seeded winter rapeseed (B. napus L. var. oleifera)*. Proceedings of 13th International Rapeseed Congress, June 05-09, 2011, Prague. www.irc2011.org.

Blakeslee J., Belling M.E., Farnham M.E., Bergner A.D. (1922) *A haploid mutant in the Jimson weed, Datura stramonium L*. Science 55: 646-647.

Bohowmik P., Dirpaul J., Polowick P., Ferrie A. (2011) *A high throughput Brassica napus microspore culture system: influence of percoll gradient separation and bud selection on embryogenesis*. Plant Cell Tiss. Organ. Cult. 1066: 359-362.

Briggs F.N., Knowles P.F. (1967) *Introduction to Plant Breeding*. New York Reinhold Pub. Corp.

Cegielska-Taras T. (2002) *The use of microspores cultures for genetic improvement of winter oilseed rape (Brassica napus)*. Monografie i Rozprawy Naukowe IHAR 18: 7-107 (in Polish).

Cegielska-Taras T., Nogala-Kałucka M., Bartkowiak-Broda I., Siger A., Szała L. (2008a) *Tocochromanols and fat content in seeds of winter oilseed rape (Brassica napus L.) doubled haploids*. In: *Advance in Analysis and Technology of Rapeseed Oil, Monograph I*. Ed. Szłyk E. Toruń: Wyd. Nauk. UMK: 23-33.

Cegielska-Taras T., Nogala-Kałucka M., Rudzińska M., Szała L., Siger A., Kaczmarek Z., Adamska E. (2011) *Study of the comparing of sterols and fat content in populations of doubled haploids of winter oilseed rape (Brassica napus L.)*. In: *Advances in Research and Technology of Rapeseed Oil, Monograph III*. Ed. Szłyk E. Toruń: Wyd. Nauk. UMK: 21-32.

Cegielska-Taras T., Pniewski T. (2011) *The use of herbicides in biotech oilseed rape cultivation and in generation of transgenic homozygous plants of winter oilseed rape (Brassica napus L.)*. In: *Herbicide – Mechanisms and Mode of Action*. Ed. Hasaneen M.N.A. InTech 7: 125-137.

Cegielska-Taras T., Pniewski T., Szała L. (2008b) *Transformation of microspore-derived embryos of winter oilseed rape (Brassica napus L.) by using Agrobacterium tumefaciens.* J. Appl. Gen. 49: 343-347.

Cegielska-Taras T., Szała L. (2001) *Induced mutation for the generation of herbicide-resistant doubled haploids of Brassica napus L*. Biol. Bull. Poznań 38: 43-47.

Cegielska-Taras T., Szała L., Nałęczyńska A., Kołodziej K., Ogrodowczyk M. (1999) *In vitro selection for high erucic acid content in microspore-derived embryos of winter oilseed rape (Brassica napus L.)*. J. Appl. Genet. 40: 305-315.

Cegielska-Taras T., Tykarska T., Szała L., Kuraś L., Krzymański J. (2002) *Direct plant development from microspore-derived embryos of winter oilseed rape Brassica napus L. ssp. oleifera (DC.). Metzger*. Euphytica 124: 341-347.

Cichy H., Budzianowski G., Cegielska-Taras T., Szała L. (2005) *Winter oilseed rape cultivar developed by the use of doubled haploids*. Rośl. Oleiste – Oilseed Crops. XXVI: 589-593 (in Polish).

Delourme R.,.Bouchereau A., Hubert N., Renard M., Landry B.S. (1994) *Identification of RAPD markers linked to a fertility restorer gene for the Ogura radish cytoplasmic male sterility of rapeseed (Brassica napus L.)*. Theor. Appl. Genet. 88: 741-748.

Delourme R. Eber F. (1992) *Linkage between an isozyme marker and a restorer gene in radish cytoplasmic male sterility of rapeseed (Brassica napus L.)*. Theor. Appl. Genet. 85: 222-228.

Delourme R., Falentin C., Huteau V., Clouet V., Horvais R., Gandon B., Specel S., Hanneton L., Dheu J.E., Deschamps M., et al. (2006) *Genetic control of oil content in oilseed rape (Brassica napus L.)*. Theor. Appl. Genet. 113: 1331-1345.

Ferrie A.M.R., Caswell K.L. (2011) *Isolated microspore culture technique and recent progress for haploid and doubled haploid plant production*. Plant Cell Tiss. Organ. Cult. 104: 301-309.

Ferrie A.M.R., Keller W.A. (2007) *Optimization of methods for using polyethylene glycol as a non-permeating osmoticum for the induction of microspore embryogenesis in the Brassicaeae*. In Vitro Cell. Dev. Biol. 43: 348-355

Ferrie A.M.R., Möllers C. (2011) *Haploid and doubled haploids in Brassica spp. for genetic and genomic research*. Plant Cell Tiss. Organ. Cult. 104: 375-386.

Forster B.P., Thomas W.T.B. (2005) *Doubled haploids in genetics and plant breeding*. Plant Breed Rev. 25: 57-88.

Frauen M. (1994) *Use of biotechnology in breeding new varieties of winter oilseed rape in Germany*. Bul. GCIRC 10: 16-29.

Fried W., Snowdon R. (2009) *Oilseed rape*. In: *Oil crops*. Ed. Vollmann J., Rajcan I. Dordrecht, Heidelberg, London, New York, Springer 4: 91-126.

Friedt W., Zarhloul M.K. (2005) *Haploids in the Improved of Crucifers*. In: *Biotechnology in Agriculture and Foresty*. Ed. Palmer C.E., Keller W.A., Kasha K.J. Berlin, Heidelberg: Springer-Verlag 56: 191-213.

Fukuoka H., Ogawa T., Matsuoka M., Ohkawa Y., Yano H. (1998) *Direct gene delivery into isolated microspores of rapeseed (Brassica napus L.) and the production of fertile transgenic plants*. Plant Cell Rep. 17: 323-328.

Geng J., Javed N., McVetty P.B.E., Li G., Tahir M. (2012) *An integrated genetic map for Brassica napus derived from double haploid and recombinant inbred populations*. Hered. Genet. 1: 103. doi: 10.4172/2161-1041.1000103.

Gil-Humanes J., Barro F. (2009) *Production of doubled haploids in Brassica*. In: *Advances in Haploid Production in Higher Plants*. Ed. Touraev A. et al. Springer Science + Business Media B.V. 4: 65-73.

Gu H.H., Hagberg P., Zhou W.J. (2004) *Cold pretreatment enhances microspore embryogenesis in oilseed rape (Brassica napus L.)*. Plant Growth Regul. 42: 137-143.

Guha S., Maheshwari S.C. (1964) *In vitro production of embryos from anthers of Datura.* Nature 204: 497.

Guha S., Maheshwari S.C. (1966) *Cell division and differentiation of embryos in the pollen grains of Datura in vitro*. Nature 212: 97-98.

Islam S.M.S., Tuteja N. (2012) *Enhancement of androgenesis by abiotic stress and other pretreatments in major crop species*. Plant Sci. 182: 134-144.

Kaur S., Cogan N.O.I., Ye G., Baillie R.C., Hand M.L., Ling A.E., Mcgearey A.K., Kaur J., Hopkins C.J., Todorovic M., et al. (2009) *Genetic map construction and QTL mapping of resistance to blackleg (Leptosphaeria maculans) disease in Australian canola (Brassica napus L.) cultivars*. Theor. Appl. Genet. 120: 71-83.

Keller W.A., Rajhathy R. Lacapra J. (1975) *In vitro production of plants from pollen in Brassica campestris*. Can. J. Genet. Cytol. 17: 655-666.

King G.J., Baten A. (2015) *Genetic maps*. Published map data. http://www.brassica.info/resource/maps/published-data.php. Accessed 23 April 2015.

Kott L., Wong R., Swanson E., Chen J. (1996) *Mutation and selection for improved oil and meal quality in Brassica napus utilizing microspores culture*. In: *In vitro haploid production in higher plants*, vol 2. Eds. Jain S.M., Sopory S.K., Veilleux R.E. Springer: Berlin-Heidelberg-New York: 151-167.

Kučera V., Vyvadilová M., Klíma M. (2002) *Utilization of doubled haploids in winter oilseed rape (Brassica napus L.)*. Czech J. Genet. Plant Breed. 38: 50-54.

Li S., Wang H., Zhang J., Fitt B.D., Xu Z., Evans N., Liu Y., Yang W., Guo X. (2005) *In vitro mutation and selection of doubled-haploid Brassica napus lines with improved resistance to Sclerotinia sclerotiorum.* Plant Cell Rep. 24: 133-144.

Lichter R. (1982) *Induction of haploid plants from isolated pollen of Brassica napus*. Z. Pflanzenphysiol. 103: 229-237.

Lombard V., Delourme R. (2001) *A consensus linkage map for rapeseed (Brassica napus L.): construction and integration of tree individual maps from DH populations*. Theor. Appl. Genet. 103: 491-507.

Maluszynski (2003) *Doubled haploid production in crop plants: a manual*. Dordrecht: Kluwer.

Maluszynski M., Kasha K.J., Szarejko I. (2003) *Published doubled haploid protocols in plant species*. In: *Doubled Haploid Production in Crop Plants*. Ed. Manual A., Maluszynski M., Kasha K.J., Forster B.P., Szarejko I. Dordrecht: Kluwer Academic Publishers: 309-335.

Maraschin S.F., de Priester W., Spaink H.P., Wang M. (2005) *Androgenic switch: an example of plant embryogenesis from the male gametophyte perspective*. J. Exp. Bot. 56: 1711-1726.

Matuszczak M. (2002) *Zastosowanie metody AFLP do analizy DNA rzepaku ozimego*. Rośliny Oleiste – Oilseed Crops XXIII: 255-265 (in Polish).

Matuszczak M., Cegielska-Taras T., Szała L., Krzymański J. (2001) *Populacja linii podwojonych haploidów rzepaku ozimego – etapy otrzymywania oraz wstępna charakterystyka za pomocą markerów RAPD*. XIV Zjazd Polskiego Towarzystwa Genetycznego: *Genetyka w służbie człowieka*, 11-13. 06. 2001, Poznań, Poland, Streszczenia: 252 (in Polish).

Matuszczak M. (2013) *Molecular markers for study of oilseed rape (Brassica napus L.) II. The review of markers used for breeding programs*. Rośliny Oleiste – Oilseed Crops XXXIV: 151-166 (in Polish).

Matuszczak M., Krzymański J. (1999) *Poszukiwanie markerów RAPD różnicujących linie rzepaku ozimego o różnych cechach chemicznych*. Rośliny Oleiste – Oilseed Crops XX: 395-414 (in Polish).

Matuszczak M., Tokarczuk I., Szała L., Cegielska-Taras T., Krzymański J., Bartkowiak-Broda I. (2011) *The localization of many QTLs on the genetic map of winter oilseed rape (Brassica napus L. var. oleifera)*. 13th International Rapeseed Congress, Prague, Czech Republic. Abstarct Book: 1026-1029.

Matuszczak M. (2010) *Identyfikacja loci cech jakościowych rzepaku ozimego (Brassica napus L. var. oleifera)*. Praca doktorska, Instytut Hodowli i Aklimatyzacji Roślin – Państwowy Instytut Badawczy, Zakład Genetyki i Hodowli Roślin Oleistych, Poznań.

Mikołajczyk K., Dabert M., Nowakowska J., Podkowiński J., Popławska W., Bartkowiak-Broda I. (2008) *Conversion of the RAPD OPC021150 marker of the Rfo restorer gene into a SCAR marker for rapid selection of oilseed rape*. Plant Breed. 127: 647-649.

Mikołajczyk K., Dabert M., Karłowski W., Spasibionek S., Nowakowska J., Cegielska-Taras T., Bartkowiak-Broda I. (2010a) *Allele-specific SNP markers for the New low linolenic mutant of winter oilseed rape*. Plant Breed. 129: 502-507.

Mikołajczyk K., Dobrzycka A., Podkowiński J., Popławska W., Spasibionek S., Bartkowiak-Broda I. (2010b) *A multiplex PCR assay for identification of the ogura male sterile cytoplasm and the Rfo restorer gene among oilseed rape bre-*

eding forms. Rośliny Oleiste – Oilseed Crops XXXI: 201-210.

Mohan M., Nair S., Bhagwat A., Krishna T.G., Yano M., Bhatia C.R., Sasaki T. (1997) *Genome mapping, molecular markers and marker assisted selection in crop plants*. Molec. Breed. 3: 87-103.

Mohammadi P.P., Moieni A., Ebrahimi A., Javidfar F. (2012) *Doubled haploid plants following colchicine treatment of microspore-derived embryos of oilseed rape (Brassica napus L.)*. Plant Cell Tiss. Organ. Cult. 108: 251-256.

Möllers C., Iqbal M.C.M. (2009) *Doubled haploids in breeding winter oilseed rape*. In: *Advances in haploid production in higher plants*. Ed. Touraev A., Forster B.P., Jain S.M. Heidelberg: Springer 13: 161-170.

Murovec J., Bohanec B. (2012) *Haploids and Doubled Haploids in Plant Breeding*. In: *Plant Breeding*. Ed. Abdurakhmonov I.Y. Croatia: InTech: 5: 87-106.

Nehlin L., Möller C., Bergman P., Glimelius K. (2000) *Transient β – gus and gfp gene expression and viability analysis of micro-projectile bombarded microspores of Brassica napus L.* J. Plant Physiol. 156: 175-183.

Olejnik A., Ludwikow A., Misztal L., Rodakowska E., Cegielska-Taras T., Sadowski J., Bartkowiak-Broda I. (2011) *Molecular analysis of DH lines of winter oilseed rape transformed with genes changing in response to drought stress*. 13th International Rapeseed Congress, Prague, Czech Republic. Abstarct Book: 324.

Palmer C. E. Keller W.A., Kasha K.J. (2005) *Haploids in crop improvement II*. In: *Biotechnology in Agriculture and Forestry 56*. Ed. Nagata T., Lörz H., Widholm J.M. Berlin, Heidelberg, New York: Springer-Verlag.

Parkin I.A.P., Gulden S.M., Sharpe A.G., Lukens L., Trick M., Osborn T.C., Lydiate D.J. (2005) *Segmental structure of the Brassica napus genome based on comparative analysis with Arabidopsis thaliana*. Genetics 171: 765-781.

Paulmann W., Frauen M. (1997) *The firstly restorer F1-hybrids of winter oilseed rape*. GCIRC Bull. 13: 24-25.

Poplawska W., Bartkowiak-Broda I., Szała L. (2007) *Genetic and breeding evaluation of doubled haploid lines with restorer gene for CMS ogura system of winter oilseed rape (Brassica napus L.)*. Brassica 9: 29-32.

Popławska W., Szała L., Cegielska-Taras T., Bartkowiwak-Broda I. (2006) *Genetic and breeding evaluation of doubled haploids lines with restorer gene for CMS ogura system of winter oilseed rape (Brassica napus L.)*. In: *Haploidy i linie podwojonych haploidów w genetyce i hodowli roślin*. Ed. Adamski T., Surma M. Instytut Genetyki Roślin PAN w Poznaniu: 151-157 (in Polish).

Pratap A., Gupta S.K., Takahata Y. (2009) *Microsporogenesis and haploidy breeding.* In: *Biology and Breeding Crucifers*. Ed. Gupta S.K. London: CRS Press Taylor & Francis group: 293-307.

Rahman M., McVetty P.B.E. (2011) *A review of Brassica seed color*. Can. J. Plant Sci. 91: 437-446.

Sigareva M.A., Earle E.D. (1997) *Direct transfer of a cold-tolerant Ogura male-sterile cytoplasm into cabbage (Brassica oleracea ssp. capitata) via protoplast fusion*. Theor. Appl. Genet. 94: 231-220.

Siger A., Michalak M., Cegielska-Taras T., Szała L., Lembicz J., Nogala-Kałucka M. (2015) *Genotype and environment effects on tocopherol and plastochromanol-8 contents of winter oilseed rape doubled haploid lines derived from F1 plants of the cross between yellow and black seeds*. Industr. Crops Prod. 65: 134-141.

Smýkalová M., Větrovcová M., Klima M., Macháčková I., Griga M. (2006) *Efficiency of Microspore Culture for Doubled Haploid production in the Breeding Project "Czech Winter Rape"*. Czech J. Genet. Plant Breed. 42: 58-71.

Snowdon R.J., Friedt W. 2004. *Molecular markers in Brassica oilseed breeding: current status and future possibilities*. Plant Breed. 123: 1-8.

Spasibionek S. (2006) *New mutant of winter oilseed rape (Brassica napus L.) with changed fatty composition*. Plant Breed. 125: 259-267.

Stringam G.R., Bansal V.K., Thiagarajah M.R., Tewari J.P. (1995) *Development of agronomically superior blackleg resistant canola cultivar in Brassica napus L., using doubled haploid*. Can. J. Plant. Sci. 81: 107-108.

Szała L., Sosnowska K., Popławska W., Liersch A., Olejnik A., Kozłowska K., Bocianowski J., Cegielska-Taras T. (2015a) *Introgression of resynthesized Brassica napus L. into parental lines of winter oilseed rape F_1 hybrids* (in press).

Szała L., Kaczmarek Z., Adamska E., Cegielska-Taras T. (2015b) *The assessment of winter oilseed rape DH lines using uni- and multivariate methods of quantitative genetics and mathematical methods*. BioTechnologia, in the issue.

Szarejko I. (2003) *Doubled haploid mutant production*. In: *Doubled haploid Production in Crop Plants*. Ed. M. Maluszynski, Kasha K.J., Forster B.P., Szarejko I. Dordrecht, Boston, London: Kluwer Academic Publishers: 351-361.

Takahata Y., Fukuoka H., Wakui K. (2005) *Utilization of microspore-derived embryos*. In: *Haploids in Crop Improvement II*. Ed. Plamer C.E., Keller W.A., Kasha K.J. Berlin, Heidelberg: Springer-Verlag: 153-169.

Takahira J., Cousin A., Nelson M.N., Cowling W.A. (2011) *Improvement in efficiency of microspore culture to produce doubled haploid canola (Brassica napus L.) by flow cytometry*. Plant Cell Tiss. Cult. 104: 51-59.

Thomas E., Wenzel G. (1975) *Embryogenesis from microspores of Brassica napus*. Z. Pflanzenzucht. 74: 77-81.

Touraev A., Forster B. P., Jain S.M. (2009) *Advances in Haploid Production in Higher Plants*. Springer Science + Business Media B.

Wang J., Lydiate D.J., Parkin I.A.P., Falentin C., Delourme R., Carion P.W.C., King G.J. (2011) *Integration of linkage maps for the amphidiploid Brassica napus and comparative mapping with Arabidopsis and Brassica rapa*. BMC Genomics 12: 101.

Weber S., Ünker F., Friedt W. (2005) *Improved doubled haploid production protocol for Brassica napus using microspore colchicine treatment in vitro and ploidy determination by flow cytometry*. Plant Breed. 124: 511-513.

Zenkteler M. (1971) *In vitro production of haploid plants from pollen grains of Atropa belladonna L.* Experientia 27: 1087.

Zenkteler M. (1972) *Development of embryos and seedlins from pollen grains in Lycium halimifolium Mill. in the in vitro culture*. Biol. Plant. 14: 420-422.

Zhang G.Q., Zhang D.Q., Tang G.X., He Y., Zhou W.J. (2006) *Plant development from microspore-derived embryos in oilseed rape as affected by chilling, desiccation and cotyledon excision*. Biol. Plant. 50: 180-186.

Zhou W.J., Tang G.X., Hagberg P. (2002) *Efficient production of doubled haploid plant by immediate colchicines treatment of isolated microspores in winter Brassica napus*. Plant Growth Regul. 37: 185-192.

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Electroporation and morphogenic potential of *Gentiana kurroo* (Royle) embryogenic cell suspension protoplasts

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Abstract

This article presents our further *in vitro* studies into the morphogenic potential of gentian cells, organs, and tissues after modification of their genome. The objective was to study the effect of electroporation and the introduction of foreign genes on the morphogenic potential of *Gentiana kurroo* embryogenic cell suspension protoplasts. Protoplasts were electroporated with DNA plasmids carrying *nptII* and *bar* genes. The stability of cell membranes, the contents of electroporation buffer, the length of electric pulse, the number of pulses and the strength of the electric field were studied. We determined the highest electroporation efficiency by evaluating the highest protoplast survival rate under specific physical conditions. The best results were achieved in the presence of EB1 electroporation buffer where the viability of protoplasts was 70.1%. Protoplast survival at this higher level required culture temperatures near 0 °C, and a 20 μs electric pulse with an electric field of 1.0 kV/cm. After seven days of agarose embedded protoplast culture, a selective agent – kanamycin – was introduced to the medium. The cell transformation effect was improved by a long term culture of callus, regenerated somatic embryos and transformants in the presence of 50 mg/l kanamycin.

Key words: electroporation buffer, electric field, electric pulse, kanamycin resistance protoplast viability, plating efficiency, transformant regeneration

Abbreviations

AS	– adenine sulfate	Kin	– kinetin
BAP	– benzylaminopurine	kV/cm	– kilowatt/centimeter
Dicamba	– 3,6-dichloro-2-methoxybenzoic acid	MS medium	– Murashige and Skoog medium (1962)
DNA	– desoxyribonucleic acid	NAA	– naphtaleneacetic acid
EB1	– electroporation buffer no. 1	*nptII* gen	– gene coding neomycin phosphotransferase
EB2	– electroporation buffer no. 2	PCR	– polymerase chain reaction
GA_3	– gibberelic acid	μs	– millisecond
kan	– kanamycin	*uidA* (*gus*)	– gene coding glucuronidase
		2,4-D	– dichlorophenoxy acetic acid

Introduction

The alternatives to *Agrobacterium* mediated plant cell transformation are biolistic particle bombardment or protoplast chemical and physical treatments. Electroporation consists of the treatment of plant cells with short high voltage electric pulses. The electric pulse shock causes brief permeability of the plasmalemma for high molecular particles, such as DNA (Bates, 1989). The DNA movement is *via* pores formed after electric pulses in the cytoplasmic membrane (Sowers, 1992). The pores are of temporal character and they are related to the increased dipole moment of hydrophilic heads building cell membrane lipids. The dipole heads of phospholipids dislocate in the direction of the electric field, which causes breaks in the continuity of the cell membrane (Kinosita and Tsong, 1977; Neuman et al., 1982, 1996). In addition to various biological parameters connected with the selection of the proper source of protoplasts and their culture conditions, the pivotal requirement for successful cell transformation are the physical conditions of electroporation, specifically the voltage of the electric field, the number of pulses and length of pulse duration (Sauders et al., 1995).

The specific effect of the electric field on tissues cultured *in vitro* was determined by analyzing the growth of isolated protoplasts as well as with protoplast-derived

calli of Colt cherry (*Prunus avium* × *pseudocerasu*). The plant regeneration capacity of electroporated tissues was also investigated. The callus obtained from the protoplasts and subjected to three successive exponential pulses at 250 V or 500 V showed the largest fresh weight increases between subcultures. It also exhibited the highest morphogenic potential, as manifested by increased frequency of plant regeneration calculated based on the number of shoots per callus. These shoots, in turn, expressed (once more) a higher potential for producing a more prolific root system when compared to those derived from non-electropulsed protoplasts of Colt cherry (Ochatt et al., 1988).

There are numerous examples of the use of electroporation in transformation experiments with organized plant structures such as immature embryo or somatic embryos. Embryo and somatic embryos at the torpedo stage of coffee have been electroporated with DNA containing *gus* and *bar* genes, and plant regeneration through secondary somatic embryogenesis has been obtained (Barton et al., 1991). The presence of *gus* and *bar* genes has been confirmed by PCR reaction performed on DNA isolated from regenerated plants (Fernandez-Da Silva and Menendez-Yuffa, 2003). Similar experiments are needed concerning representative species of the Gentianaceae family.

The Gentianaceae family consists of about 1700 species but only 28 are used for plant tissue culture and biotechnology. Among these, *Gentiana kurroo* (2n = 26) (Behera, 2011) is a critically endangered species endemic to the northwestern Himalayas (Khuroo, 2005). This is a small perennial herb with a stout rhizome bearing a decumbent flowering stem. Due to its multiple uses (Behera, 2011), it is a species that is being over exploited in its natural habitat. Hence, it qualifies as an important endangered medicinal plant and is protected by Indian Law (Khuroo, 2005). Its secondary metabolite spectrum is the richest of all gentian species, even comparable to *G. lutea*. These secondary compounds – gentiopicrine, gentiamarin and the alkaloid gentianinare – are used for the treatment of inflammation, pain, fever, and hepatitis (Latif, 2006).

G. kurroo's reproductive biology is connected with its flowers being dichogamous due to protardy. Stigmatic lobes remain adpressed until almost complete anther dehiscence. The stigma becomes receptive to pollen germination about one week after initiation of anther dehiscence. The flowers are cross-pollinated (Raina et al., 2003). The plant is characterized by being mostly wild and not domesticated due to its poor seedling establishment in nature. However, these plants produce abundant seeds. Seed germination under laboratory conditions commenced from six to twenty eight days with a total record of about 70% (Raina et al., 2011, 2003; Tomar et al., 2012). Macroproliferation by rhizomes appears to be an alternative, easy and effective technique for the multiplication and conservation of this herb (Tomar, 2011).

The first papers summarizing the achievements of gentian plant tissue cultures were published in 1988 and in 1991 (Barešová, 1988; Miura, 1991). Since then, considerable progress in the biochemistry and biotechnology of gentians has been achieved. The establishment of a green leaf mesophyll cell cultures and cell suspension protoplast culture procedures have provided a significant basis for progress in gentian somatic cell genetic manipulation (Jomori et al., 1995; Meng et al., 1996; Fiuk and Rybczyński, 2007; Tomiczak and Rybczyński, 2015). Initially, possibilities were presented for establishing plant multiplication of *G. kurroo* by culturing the shoot tips and nodal segments on MS medium (Murashige and Skoog, 1962) supplemented with BAP (benzylaminopurine) and NAA (naphthaleneacetic acid). For the most effective rooting, individual shoots with 3-4 nodes were implanted on semisolid hormone-free MS medium supplemented with 6% sucrose (Sharma, 1993). The MS medium supplemented with 0.5 mg/l IAA and 0.8 mg/l BAP stimulated more than 80% of apical meristems for rich proliferation (Kaushal et al., 2014). Randomly amplified polymorphic DNA (RAPD) and karyotypic analysis showed the lack of variation, and hence the genetic stability of regenerants, and confirmed the efficacy of the protocol for micropropagating plants of *G. kurroo* over a ten year time period (Kaur et al., 2009). Significant progress in vegetative plant cell manipulation occurred when the somatic embryogenesis of primary explants was described for a few gentian species (Mikuła, 1996a, 1996b). Among the studied gentians, *G. kurroo* appeared to be a highly embryogenic species with tremendous morphogenic potential. Both seedling and leaf explants have been used for culture initiation and the establishment of cell suspensions (Fiuk and Rybczyński, 2008). The derived calli appear to be an excellent source of embryogenic cells and their protoplasts. A very high yield of regenerants *via* somatic embryogenesis from proto-

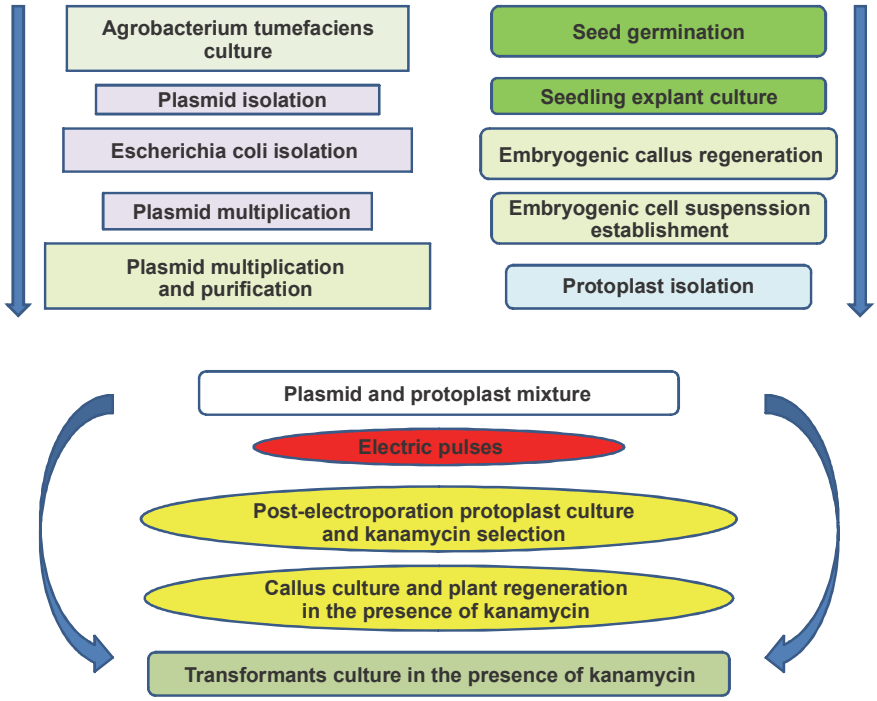

Fig. 1. Diagram of procedures involved in electroporation experiments of *G. kurroo* (Royle) embryogenic cell suspension protoplasts

plast bead culture has confirmed the usefulness of this type of protoplast culture for gentian cell manipulation (Fiuk and Rybczyński, 2007). The cell suspension cultures also produce embryogenic aggregates and somatic embryos which could be used for proteomic studies (Fiuk and Rybczyński, 2007; Niedziela and Rybczyński, 2014). Somatic embryogenesis is a complex process that allows the performance of multidisciplinary studies into the mechanism of embryo induction and its development. There are a huge number of indications that this phenomenon also has great potential for plant propagation (Rybczyński et al., 2007).

This research describes the effect of electroporation and the introduction of foreign genes (*gus* and *ntp*II) on the morphogenic potential of *G. kurroo* embryogenic cell suspension protoplasts (Fig. 1).

Methods

Cell suspension initiation and culture

Seedling explants of *G. kurroo* (cotyledons, hypocotyles and roots) were implanted on an MS medium supplemented with 1.0 mg/l Kin (kinetin) and 0.5 mg/l 2,4-D (2,4-dichlorophenoxy acid) to induce an embryogenic callus culture. A hypocotyl-derived embryogenic callus was used for cell suspension culture establishment on an MS medium supplemented with BAP (benzylaminopurine), NAA (naphthaleneacetic acid), Dicamba (3,6-dichloro-2-methoxybenzoic acid) and AS (adenine sulfate) (Fiuk and Rybczyński, 2007). Cell suspensions were initiated by transferring 1.0 g of embryogenic tissue to 20 ml of liquid medium in 100 ml conical flasks. Once established, the cell suspension cultures were maintained as 80 ml aliquots in large 200 ml conical flasks, with agitation on a rotary shaker at 120 rpm, under diffused light 20 μM $m^{-2}s^{-1}$ at 22 °C. Subcultures of the cell suspension were made every seven days.

For the evaluation of the embryogenic suspension, the production of embryoids was measured by implanting cell suspension fractions (500, 500-300, 300-150 and 150 μm) on MS medium supplemented with 0.5 mg/l GA_3 (gibberellic acid), 1.0 mg/l Kin + 80 mg/l AS.

Protoplast isolation and culture

Protoplasts were isolated from embryogenic cell suspensions which were subcultured every 7 days. One gram of cell suspension was transferred to deep Petri plates and immersed in 20 ml of enzyme mixture. Powdered enzymes – Cellulase RS (YakultHonsha Co., LTD) – 1.5%; Driselase (Fluka AG) – 0.5%; Hemicellulase (Sig-

ma) – 0.25%; Macerozyme R10 (YakultHonsha Co., LTD) – 1.5%; Pectolyase (Seishin Pharmaceutical Co., LTD) – 0.04% – were dissolved in a protoplast washing solution (Frearson et al., 1973). Non-soluble debris were removed by centrifugation. The supernatant was supplemented with 9% of mannitol and 5.0 mM MES (2-[N-Morpholino] ethanesulfonic acid). pH was adjusted to 5.8 (NaOH). The mixture was filter-sterilized using a 0.2 μm membrane filter of Sartorius sterilization system in the cold.

The plant material was incubated in the enzyme mixture for 12 hrs at 28°C in the dark with 30 rpm agitation. The released protoplasts were filtered through a plastic sieve (45 μm) and centrifuged for 10 min at 180 rpm on an MPW-360 centrifuge (MPW Med. Instruments, Warsaw, Poland). The protoplast pellet was resuspended and washed three times with a protoplast washing solution supplemented with 90.0 g/l mannitol. The protoplast yield and densities were determined with a Fuchs-Rosenthal hermocytometer. Protoplasts were embedded in Sea Plaque agarose-gelled PCM medium (MS without NH_4NO_3 + 30.0 g/l glucose + 3.0 g/l Glutamine + 0.5 mg/l 2,4-D + 1.0 mg/l Kin + 9.0% mannitol, pH = 5.8) and cultured in droplets of 100 μl. 10 droplets per 5.0 cm Petri plate were covered by 2.0 ml of a liquid PCM medium.

The effect of kanamycin concentration on protoplast survival

Freshly isolated protoplasts were embedded in agarose PCM and, in the form of 100 μl drops of medium containing protoplasts, these were dropped into a ⌀ 5.0 cm plastic Petri plate. Later, once the droplets (10 per plate) had jellified, Petri plates were filled with 2.0 ml of PCM. After seven days of culture, the medium was exchanged for the PCM supplemented with 25.0, 50.0, 75.0 or 100.0 mg/l kanamycin for transformant selection. The experiment was repeated five times for each antibiotic concentration. The culture period lasted four weeks. The effect of kanamycin concentration on the growth and development of cultured protoplasts was evaluated using a Vanox Olympus light microscope, and this served as the basis for the choice of the antibiotic concentration for further experiments.

Esherichia coli culture and plasmid DNA isolation

Escherihia coli strain HB 101 with plasmid pBI (Jefferson et al., 1987) carrying an *npt II* gene encoding neomycin phosphotransferase under a *nos* promoter and a *gus* gene encoding β-glucuronidase under a CaMV35S promoter was the source of DNA used for electroporation experiments.

E. coli HB 101 cells were maintained on LB medium (Schaad, 1988) containing 5.0 g/l yeast extract, 10.0 g/l tryptophan, and 10.0 g/l NaCl, pH 7.2. Plasmids were isolated by alkaline lysis with some modifications (Sambrook and Russell, 2001). The bacteria cells were treated with 15 μl RNase (10mg/ml) in solution I (50 mM glucose, 25 mM Tris-HCL, pH = 8.0, 10 mM EDTA pH = 8.0). Later, the sample was supplemented with 200 μl solution II (0.2 MNaOH, 1% SDS), shaken gently and incubated in ice for 5 min, and then 150 μl of frozen 7.5 M ammonium acetate was added. The sample was left for 10 min on ice, followed by centrifugation at 14,000 rpm. The DNA was precipitated with 900 μl of 96% EtOH for 2 min. at room temperature and centrifuged at 13,000 rpm for 5 min. The next step of preparation consisted of the washing of the DNA sample with 70% EtOH and centrifugation at 13K rpm for 5 min. Finally, the sample was dried at room temperature and diluted in 30 μl H_20. All centrifugations in this procedure were performed in 1.5 ml Eppendorf tubes and on a SIGMA 2-16PK centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany).

Protoplasts were electroporated using the Generator of Electric Pulses BTX ECM 2001 Electro Cell Manipulator together with Cuvette Safety Stand 630B in BTX Electroporation Cuvettes Plus with 2.0 mm Gap Cuvettes. Protoplasts freshly isolated from the embryogenic cell suspension were suspended in an electroporation buffer at a density of 4×10^5 per ml in the presence of 50 μg/ml plasmid DNA. Two electroporation buffers were tested: EB1 (70 mM KCl, 5 mM $MgCl_2$, 0.1% MES, 9% mannitol, pH = 5.8) and EB2 (4 mM $MgCl_2$, 0.5% MES, 9% mannitol, pH = 5.6). Before electric current treatment, the cuvettes with protoplasts and plasmids in the electroporation buffer were placed in ice for 10 min.

The electroporation parameters tested were: the length of the electric pulse (20, 30, 40 μs and 1.0, 2.0, and 5 ms), the number of pulses (1 or 2), the electric field strength (0.0-1.75 kv/cm), and the temperature (on ice; about 0°C and room temperature; about 20°C) treatment after electroporation. The experiments were repeated five times for each of the above experimental conditions.

After the electric current treatment, protoplasts were incubated at room temperature or on ice for 10 min, followed by protoplasts being washed with liquid

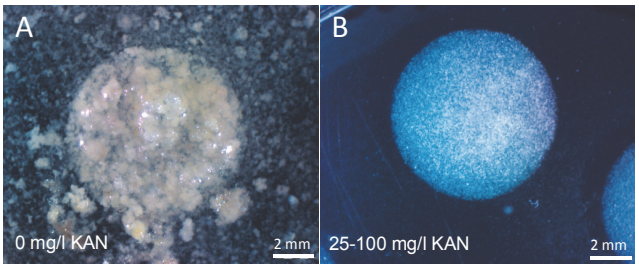

Fig. 2. Sea Plague Agarose embedded control protoplast and kanamycin treated protoplasts of *G. kurroo* after four weeks of the experiment. A) The overgrowth of callus formed by control protoplast culture. Notice spontaneous cell aggregates – shedding from agarose bead into liquid medium. B) Protoplast-derived cell division suppressed culture exposed to 25-100 mg/l kanamycin applied after 7 days of culture

PCM medium and left at room temperature for 24hrs. Then, the liquid medium was substituted by a PCM agarose gelling medium, and cultures were maintained as droplets with a protoplast density of 1×10^5/ml. A total of 2.0 ml of liquid PCM medium was then added to each Petri plate. After one week, the PCM medium was substituted with the same medium supplemented with 50 mg/l kanamycin. Complete osmotic pressure reduction was achieved as follows: mannitol was reduced from 9% to 6% after 3 weeks and then to 3% two weeks later with final reduction of osmotic pressure by week 8.

Agarose droplets with protoplasts already developed into multicellular aggregates were transferred to CSIM medium (MS + 0.5 mg/l 2,4-D + 1.0 mg/l Kinetin) supplemented with the same kanamycin concentration. Mock electroporation was performed as above, except that the electric current was not used. The protoplast morphogenic potential evaluation required a control culture which was carried out as described elsewhere (Fiuk and Rybczyński, 2007).

Protoplast viability and transient gene expression evaluation in the presence of kanamycin

Protoplast viability evaluation was performed twice using 0.01% FDA (Fluorescein diacetate) and UV light λ = 485 on a Vanox (Olympus) microscope: after 24 hrs and after 7 days of culture. After 7 days, the frequency of cell divisions was also scored. The transient gene expression was evaluated by the β-glucuronidase test. The histochemical activity of β-glucuronidase was evaluated again when the calli reached a prominent size and dissection of small pieces from the tissue did not harm subsequent proliferation.

Transformant regeneration from protoplast culture exposed to electroporation

After seven weeks of culture, agarose droplets with prominent calli were transferred to CSIM + Kan medium. The regenerated structures were transferred to a TPM medium (MS + 30.0 g/l sucrose + 8.0 g/l agar, pH = 5.8) supplemented with 50 mg/l kanamycin.

Evaluation of GUS activity

The expression of *gus* in cells, calli and leaf fragments of transformants was assayed using 5-bromo-4-chloro-3-indolylglucuronide (X-Gluc) as a substrate. The tissues were incubated in the reaction mixture including 1.0 mM X-Gluc in DMSO, 20.0 mM phosphate buffer pH = 7.0, 0.25 mM Triton X-100, 3.12 mM methanol. The plant material was covered with a reaction mixture and maintained at 37°C for 24hrs in the dark. After that, the tissues were treated with 70% ethanol (three times) to extract chlorophyll from them in order to visualize a blue pigment (dichloro-dibromoindigo). The blue stain is the effect of GUS activity. Finally, the tissues were fixed with Carnoy's fixative (acetic acid/ethanol: v/v 1:3).

PCR analysis of regenerated plants

In order to confirm the transgenesis, primers for the marker *nptII* gene (selection gene) and for the gene *uidA* (*gus* reporter gene) were used for testing. For the PCR amplification of a 700 bp fragment of the *nptII* gene, the following starters have been used: (5′-GAGGCTATTCGG CTATGACTG-3′) and (5′-ATCGGGAGCGGCGATACCG TA-3′) (Dong and McHughen, 1993). Next, the following thermal profile was adopted: 95°C 15 min., 30× (94°C 30 sec., 56°C 30 sec., 72°C 60 sec.), 72°C 5 min and 4°C. For the *uidA* gene, the following starters were tested: 1 – (5′-TTATCTCTATGAACTGTGCGTCA-3′) and 2 – (5′- TTGGACATACCATCCGTAATAA-3′) with amplification of 679 bp. Primers were designed according to the Primer3 program on the basis of *uidA* gene sequences found in the NCBI database (National Center for Biotechnology Information 2008). After that, the following thermal profile was adopted: 95°C 15 min., 30× (94°C 30 sec., 51°C 1 min., 72°C 2 min), 72°C 10 min and 4°C.

Statistical analysis

A statistical analysis was performed with the application of the Statgraphics Plus4.1 program. The number of samples and their size, and the number of repetitions of experiments are presented.

Results

Electroporation experiments were carried out on freshly isolated protoplasts of *Gentiana kurroo* embryogenic cell suspensions. Electroporated DNA plasmid derived from the HB101 *E. coli* strain carried two gene markers: *nptII* (selection) and *gus* (reporter). Successful electroporation experiments required the determination of the protoplast viability, the content of the electroporation buffer, the length of the electric pulse, the number of pulses and the strength of the electric fields applied. Long-term kanamycin treatment of calli resulted in a selection of regenerants.

The effect of kanamycin on the growth and development of protoplasts in control cultures

To assess the effect of kanamycin on the cell division and growth of protoplast cultures, kanamycin was used at 0.0, 25.0, 50.0, 75.0, and 100.0 mg/l. The use of kanamycin significantly blocked cell divisions of protoplasts and, at 25-75 mg/l, it decreased cell division in protoplast-derived cultures four-fold. The highest level of kanamycin (100.0 mg/l) completely blocked cell division and no further development of the culture was observed. In contrast, those control cultures which expressed a morphogenic potential by forming a callus and regenerating plants when treated with kanamycin did not form a callus and were not able to regenerate plants (Fig. 2).

The effect of the electroporation buffer on protoplast survival

The effect of the buffer composition on protoplast survival was studied after 24 hrs and at 7 days of culture of freshly isolated protoplasts. After one week of culture, protoplast electroporation in the richer EB1 medium resulted in a protoplast viability of 70.1%, while EB2 buffer supported a significantly lower level of survival (55.8%). Protoplast viability was higher at room temperature than on ice, where it dropped to as low as 60%. Conversely, when assessed after 7 days, which led to nutrient protoplast starvation, protoplast survival was higher on ice than at room temperature (Fig. 3).

Effect of electric field on protoplast survival and plating efficiency

The effect of the electric field voltage on protoplast survival was evaluated twice: at 24 hrs and 7 days. A high electric field voltage significantly decreased the protoplast survival, irrespective of the temperature at

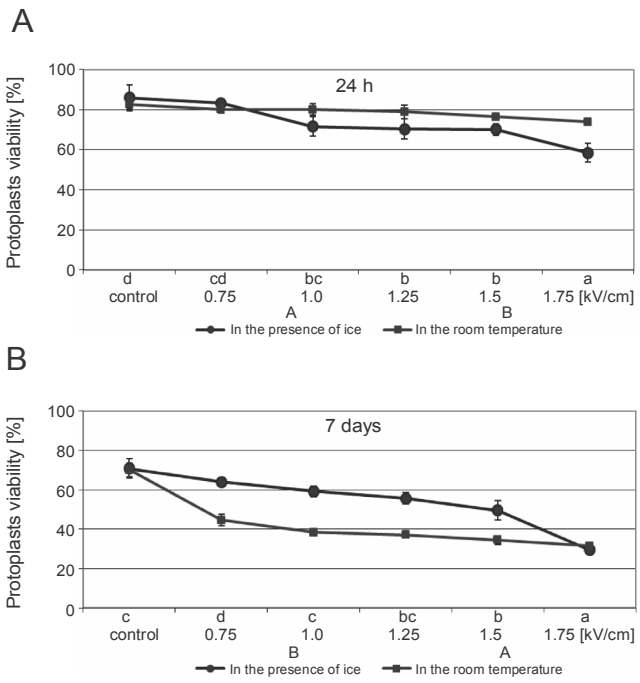

Fig. 3. The effect of temperature (at 0°C and 20°C) and electric field current (1 puls, 20 μs long) on viability of *G. kurroo* protoplasts evaluated A) 24 hrs and B) 7 days after electroporation (values described by the same letters and the size of characters are not significantly different, $p > 0.05$)

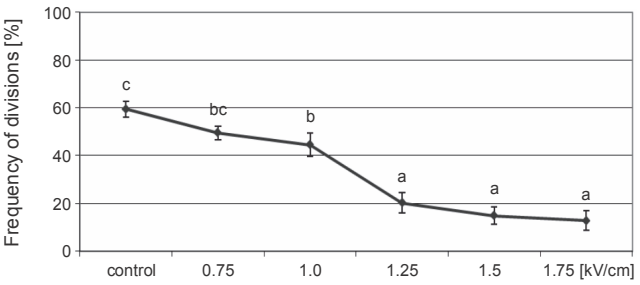

Fig. 4. The effect of electric field on frequency of *G. kurroo* protoplast cell division after one pulse of 20 μs duration. Protoplasts were incubated on ice (data marked by the same letters are not statistically different, $p > 0.05$)

which protoplasts were maintained following electroporation. At the lowest voltage – 0.75 kV/cm – 44.6% of protoplasts survived, while at the highest voltage (1.75 kV/cm) only 31.4% survived. Protoplast incubation on ice produced similar results, with a protoplast survival of 63.8% at 0.75 kV/cm and 29.3% at 1.75 kV/cm after one week of culture (Fig. 4).

Non-electroporated protoplasts showed a plating efficiency of 59.4%. The lowest electric pulse voltage (0.75 kV/cm) reduced the protoplast plating efficiency to

49.5%, while further increases in the electric field strength up to 1 kV/cm resulted in a protoplast division reduction to 44.53%. These data, however, were not significantly different. The elevation of electric field voltage above 1 kV/cm dramatically decreased protoplast division, but results were not significantly different from those for 1.25 or 1.75 kV/cm.

Number of pulses

The number of pulses had a significant effect on the viability and frequency of the cell division of protoplasts. Thus, the percentage of viable protoplasts was nine-fold lower with two successive electric pulses as compared to a single one. With one pulse, the frequency of protoplast division was 44.53%, and this dropped to 6.8% with two pulses.

The effect of short and long time electric pulse treatment on protoplast viability and division

To study the effect of the length of an electric pulse on protoplast viability, five durations of pulses were selected: from 20, 30, 40 μs; and 1.0, 2.0 and 5 ms. The two longest pulses significantly reduced protoplast viability counted after 24 hrs of culture (Fig. 4). After one week of culture, the longest pulse treatments killed all the protoplasts. Protoplasts electroporated for 20, 30 and 40 μs survived at 70, 64 and 60%, respectively, after 24 hrs of culture. After 7 days, the highest level of survival was observed for those samples treated with the shortest electric pulses (20 μs) (Fig. 5).

Long electric pulses decreased the number of dividing protoplasts, and a pulse longer than 40 μs totally blocked the division of electroporated protoplast-derived cells. The highest percentage (44.5%) of protoplast division occurred in the case of 20 μs pulses. Already, a 30 μs long pulse resulted in a drastic decrease to 5.6% in cell division, which was reduced further to 2.9% with 40 μs pulses, although no significant differences were observed between 30 and 40 μs.

Plant regeneration from electroporated protoplasts

Among the experimental conditions for the electroporation of embryogenic cell suspension protoplasts of *G. kurroo* (Fig. 6A and Fig. 6B), only one combination led to callus formation and plant regeneration; namely, one single 20 μs long pulse with a field strength of 1 kV/cm. It is noteworthy, that the control protoplast culture underwent numerous cell divisions and by six

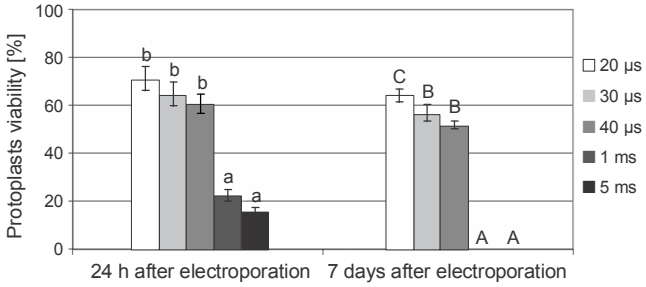

Fig. 5. The effect of the duration of electric pulse on the viability of *G. kurroo* protoplasts after application of one pulse of 1 kV/cm and incubated on ice (data marked with the same letters and size of characters are not significantly different, $p > 0.05$)

weeks the beginning of small callus formation was noticed. Further, the change of culture conditions from liquid to solidified medium resulted in plant regeneration.

Electroporated protoplasts required embedding in agarose droplets supplemented with plant growth medium to start cell division. The initial division of protoplast-derived cells was observed after 5 days of culture (Fig. 6C and Fig. 6D). As expected, supplementing kanamycin to the culture medium after 7 days suppressed cell divisions of non-transformed protoplasts. A microscopic analysis confirmed the mitotic activity of transformed protoplasts which were able to reach a few cell aggregates. However, only an extremely low percentage of protoplasts in kanamycin selection conditions were able to divide and form a callus (Fig. 6E). Two months of protoplast culture in agarose droplets was sufficient for prominent callus formation (Fig. 6F and Fig. 6G). Initially, the transgenic callus consisted of hydrated cells which were white or pale yellow (Fig. 6H). Only the embryogenic regions of the callus were of a deeper yellow color. On the proliferation medium, these regions underwent an intense growth and this resulted in plant regeneration of *G. kurroo*. Indirect somatic embryogenesis from the callus was the only morphogenic pathway observed in these cultures (Fig. 6I and Fig. 6J). Finally, 9 plants were regenerated and later their resistance to kanamycin was confirmed.

Transgenesis confirmation assessment of* gus *gene expression

No expression of *gus* genes on cells and calli or in collected leaf fragments was observed. In contrast to the results obtained for the transformation of cell suspensions (data not published) with *A. tumefaciens*, proto-

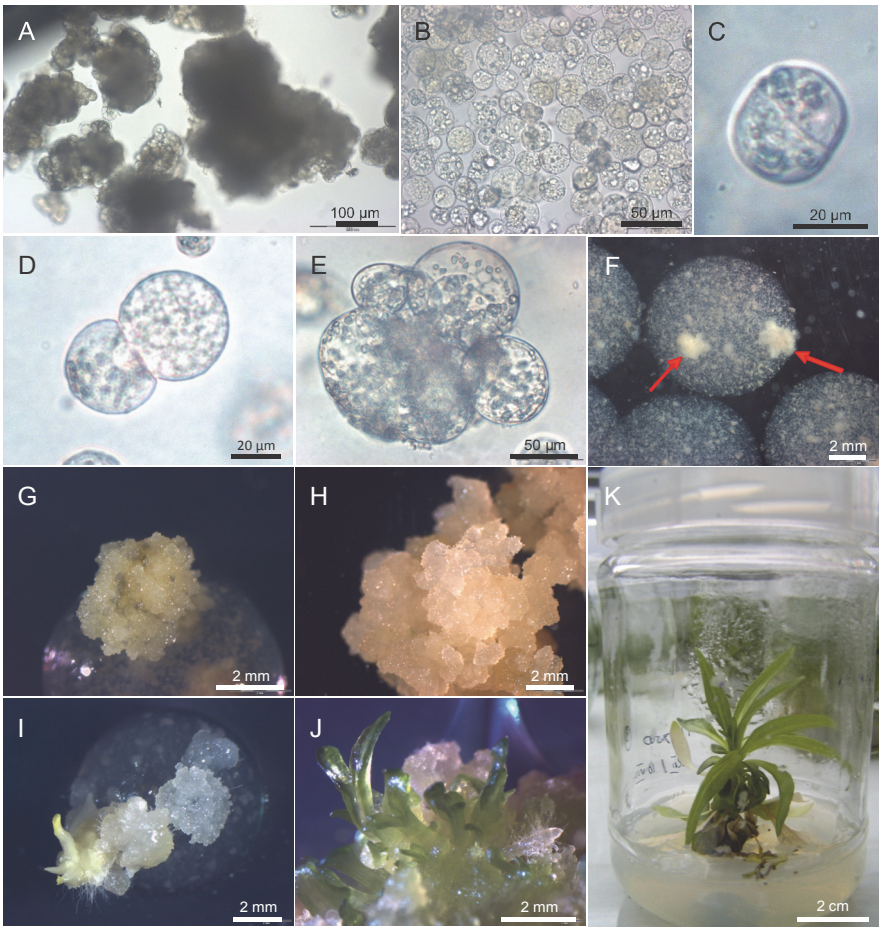

Fig. 6. *G. kurroo* transformant regeneration after embryogenic cell suspension protoplast electroporation: A) General view of plant cell suspension used for protoplast production, B) Freshly isolated protoplasts ready for electroporation experiments, C-D) First cell division of electroporated protoplasts after initial cell wall regeneration on 5th day of culture, E) Few cell aggregates, F) Agarose droplet with developing mini calli, G-H) Overgrowth of transformed callus on the agar proliferation medium, I-J) Somatic embryo formation from callus derived from electroporated protoplasts, K) Transgenic plantlet of *G. kurroo* cultured on 50 mg/l kanamycin selection medium. All cultures were carried out in the presence of 50 mg/l kanamycin

plast transformants did not show any expression of gene coding for β-glucuronidase (blue color).

Discussion

Protoplast transformation opens the possibility of obtaining genetically modified plants from single cells, thus excluding chimerism of tissue and regenerants derived from multicellular explants (Puonti-Kaerlas et al., 1992). The absence of a cell wall makes the protoplast an almost ideal explant for the exploration of electroporation (Tagu et al., 1988; Saunders et al., 1995). However, the most important prerequisite is the development of an efficient methodology of plant regeneration from protoplast cultures. In our experiments, electroporation was carried out on protoplasts derived from embryogenic cell suspension cultures, for which the conditions for protoplast isolation, culture and plant regeneration had previously been described (Fiuk and Rybczyński, 2007). In the discussed paper, protoplasts were placed under the extremely tough stress conditions of an electric field, various buffers and kanamycin treatment. However, this stress was partly compensated by the culture conditions. Our attempts resulted in the expression of genes of the selection agent and the establishment of a callus culture with embryogenic potential.

The chemical composition of the electroporation buffer plays an important role in protoplast survival, as does the electric field. Gupta and coworkers (1988) showed that protoplast viability of conifer plants after electroporation was reduced almost by half. In our experiments,

buffers differed in their composition. These differences resulted in a higher survival (70.1%) in EB1 buffer and lower (55.82%) in EB2 buffer. High protoplast survival is related to the application of the same pH during protoplast isolation and electroporation. In this study, it is necessary to remember that divalent cations ($Ca^{2+}$and Mg^{2+}) play an important role in membrane stabilization, which has been discovered in protoplast fusion with both PEG and electrofusion experiments (Fromm at al., 1985; Niedz et al., 2003). Working with friable structures such as protoplasts, their plasmolemma requires some physical protection in the case of any manipulations, so incubation on ice appeared to act as the agent for biological stabilization. However, the death of the protoplast may be the result of the overheating of a sample which is located between two electrodes, and irreversible membrane damage resulting from the electric field applied. Hence, pre-cooling of the electroporation cuvette lessened the effects of such drastic protoplast treatments. It has been proved that low temperature supports the duration of the pore opening and the efficiency of transformation is higher than with an uncooled sample (Tsong and Kinosita, 1977). This research contradicted the electroporation of citrus protoplasts, where heat shock treatment at 49°C before an electric pulse greatly enhances transient transformation expression (Hidaka and Omura, 1993).

The only condition of electroporation with *nptII* + *uidA* genes that permitted the development of a callus and regenerated plants in the presence of kanamycin selection medium was 1 pulse with a voltage of 1kV/cm and a duration of 20 μs. Other experimental conditions studied resulted in a drastic decrease in the number of dividing protoplasts. A significant decrease in the number of the dividing protoplasts was found when the voltage was higher than 1.0 kV/cm. We did not observe any significant differences between 0.75 and 1.0 kV/cm. It has previously been shown that the voltage used for electroporation will change with the electroporated plant material. For *Stysanthes guianensis* a voltage of 0.25 kV/cm (Quecini et al., 2002) is the best, while for *Citrus sinensis, Daucus carota* (Bower and Birch, 1990; Niedz et al., 2003) it is 0.45 kV/cm, 0.5 kV/cm for *Nicotiana tabacum, Glycine max* (Bates, 1989; Dhir et al. 1991), and 1.0 kV/cm for *Pisum sativum* (Hashimoto et al., 1992). The last result was similar to our results. Most frequently, only one pulse is used; however, sometimes 6 or 10 pulses are required (Bower and Birch, 1990). The pulse duration is experimentally specified and species dependent. For protoplasts of Chinese orange a duration as long as 70-80 ms is the best (Niedz et al., 2003).

In our experiments, only DNA at a concentration of 50 μg/ml was used. A number of papers indicate 100 μg/ml as the most successful concentration of DNA in transient expression. The most effective transformation of *Citrus* protoplasts occurred in the presence of that concentration, while doubling it to 200 μg/ml resulted in a drastic reduction of the phenomenon (Hidaka and Omura, 1993). Not only does the concentration of the plasmid play an important role, but the form (circular or linear) of DNA should also be considered (Quecini et al., 2002). In some plants, linear DNA allowed better results to be obtained than the circular vector (Negrutiu et al., 1987; Shillito et al., 1985).

Because of the inner structure of DNA and the lack of distortions resulting from the structure of a double helix, a circular DNA is significantly less mobile in the electric field (Courey and Wang, 1983; Drew et al., 1988). The size of the linear molecule is significantly smaller because of the lack of its tertiary and quaternary structure, which helps DNA molecules to penetrate the pores of cell membranes (Tanaka, 1988).

The number of plants, only nine, derived from electroporated embryogenic protoplasts is low in comparison to the morphogenic potential of untreated protoplasts (Fiuk and Rybczyński, 2007). However, the system of selection based on kanamycin resistance seems to be very stringent and only the expression of the *nptII* gene ensures the survival of the protoplast and callus in the presence of 50.0 mg/l kanamycin. The lack of a solid cell wall at the initial stages of protoplast cultures that could act as a physical barrier, combined with a relatively early application of kanamycin, could be considered as the factors that inhibit the formation of "escapes".

The experimental system presented here is very labor-intensive and requires knowledge of protoplast culture and plant regeneration techniques. It is nevertheless worthwhile to invest in research into technology for sourcing the whole culture from a single cell.

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Authors' contribution

The authors' contributions to the research are: laboratory experiments – A. Wójcik, writing of the manuscript – J. J. Rybczyński.

References

Amita Tamar, Manhas R.H., Shrivastava R.K. (2012) *Seed germination studies in Gentiana kurroo Rayle – an endangered medicinal herb*. J. Med. Aromat. Plant Species 34: 168-171.

Barton C.R., Adams T.L., Zarovitz M. (1991) *Stable transformation of foreign DNA into Coffea arabica plants*. In: Proceedings of the 14thInternational Conference on Coffee Science. July 14-19th San Francisco, USAASIC Lausanne: 460-464.

Bates G.W. (1989) *Electroporation of protoplasts*. J. Tiss. Cult. Meth. 12: 121-126.

Bower R., Birch R.G. (1990) *Competence for gene transfer by electroporation in a subpopulation of protoplasts from uniform carrot cell suspension cultures*. Plant Cell Rep. 9: 386-389.

Courey A.J., Wang J.C. (1983) *Cruciform formation in a negatively supercoiled DNA. may be kinetically forbidden under physiological conditions.* Cell 33: 817-829.

Dhir S.K., Dhir S., Hepburn A., Widholm J.M. (1991) *Factors affecting transient gene expression in electroporated Glycine max protoplasts.* Plant Cell Rep. 10: 106-110.

Drew H.R., McCall M.J., Calladine C.R. (1988) *Recent studies of DNA in the crystal.* Ann. Rev. Cell. Biol. 4: 1-20.

Fernandez Da Silva R., Menendez-Yuffa A. (2003) *Transient gene expression in secondarysomatic embryo from coffee tissue electroporated with gene bar and gus.* Mol. Plant Pathol. 5: 527-536.

Fiuk A., Rybczynski J.J. (2007) *The effect of several factors on somatic embryogenesis and plant regeneration in protoplast cultures of Gentiana kurroo (Royle).* Plant Cell Tiss. Org. Cult. 91: 263-271.

Fiuk A., Rybczyński J.J. (2008) *Factors influencing efficiency of somatic embryogenesis of Gentiana kurroo (Royle) cell suspension.* Plant Biotech. Rep. 2: 33-39.

Freason E.M., Power J.B., Cocking E.C. (1973) *The isolation, culture and regeneration of petunia leaf protoplasts.* Dev. Biol. 33: 130-137.

Fromm M., Callis J., Taylor L.P., Walbot V. (1987) *Electroporation of DNA and RNA into plant protoplasts*. In: *Methods in enzymology*. Ed. Abelson J.N., Simon M.I. New York, Academic Press: 351-366.

Gupta P.K., Dandekar A.M., Durzan D.J. (1988*) Somatic embryo formation and transient expression of a luciferase gene in Daglas fir and loblolly pine protoplasts.* Plant Sci. 58: 85-92.

Hashimoto T., Yamada T., Tado A., Kawamata S., Tanaka Y., Sriprasertsak P.,Ichinose Y., Kato H., Izutsu S., Shiraishi T., Oku H., Ohtsuki Y. (1992) *Transientexpression in electroporated pea protoplasts: Elicitor responsiveness of a phenylalanineammonia-lyase promoter.* Plant Cell Rep. 11: 183-187.

Jefferson R.A., Kavanagh T.A., Bevan M.V. (1987) *GUS fusions: β-glucoronidase as a sensitive and versatile gene fusion marker in higher plants*. EMBO J. 6: 3901-3907.

Jomori H., Takahata Y., Kaizuma N. (1995) *Plant regeneration from leave-derived calli of Gentians and their protoplast culture*. Acta Horticult. 392: 81-86.

Hidaka T., Omura M. (1993) *Transformation of citrus protoplasts by electroporation*. J. Jap. Soc. Hort. Sci. 62: 371-376.

Kaushal S., Sidana A., Dev K. (2014) *In vitro plant production through apical meristem culture of Gentiana kurroo Royle*. J. Med. Plant Stud. 3: 4-9.

Latif A., Afaq S.H., Khan T.F. (2006) *Antiinflammatory activity of flowertops of Gentiana kurroo Royle extract*. Pharmacol. On line J. 3: 575-580.

Meng Y.-L., Gao Y.-P., Jia J.-F. (1996) *Plant regeneration from protoplasts isolated from callus of Gentiana crassicaulis.* Plant Cell Rep. 16: 88-91.

Murashige T., Skoog F. (1962) *A revised medium for rapid growth and bioassay with tobacco tissue culture.* Physiol. Plant. 15: 473-497.

Negrutiu I., Shillito R., Potrykus I., Baisini M.G., Sala F. (1987) *Hybrid genes in the analysis of transformation conditions.* Plant Mol. Biol. 8: 363-373.

Neumann E., Kakorin S., Tsoneva I. (1996) *Calcium-mediated DNA adsorption toyeast cells and kinetics of cell transformation by electroporation.* Biophys. J. 71: 868-877.

Neumann E., Schäefer-Ridder M., Wang M., Hofschneider P.H. (1982) *Gene transfer into mouse lyoma cells by electroporation in high electric fields.* EMBO J. 1: 841-845.

Niedz R.P., McKendree W.L., Shatters Jr. R.G. (2003) *Electroporation of embryogenic protoplasts of sweet orange (Citrus sinensis (L.) Osbeck) and regeneration of transformed plants.* In Vitro Cell. Dev. Biol. Plant 39: 586-594.

Niedziela A., Rybczyński J.J. (2014) *Primary results of 2-dimensional electrophoresis for protein studies of Gentiana kurroo Royle somatic embryos derived from long-term embryogenic cell suspensions*. Acta Soc. Bot. Pol. 83: 247-250.

Ochatt S.J., Chand P.K., Rech E.L., Davey M.R., Power J.B. (1988) *Electroporation-mediated improvement of plant regeneration from colt cherry (Prunus avium × pseudocerasus) protoplasts.* Plant Sci. 54: 165-169.

Quecini V.M., de Oliveira C.A., Alves A.C., Vieira M.L.C. (2002) *Factors influencing electroporation-mediated gene transfer to Stylosanthes guianensis (Aubl.) protoplasts*. Gen. Mol. Biol. 25: 73-80.

Puonti-Kaerlas J., Ottosson A., Eriksson T. (1992) *Survival and growth of peaprotoplasts after transformation by electroporation.* Plant Cell Tiss. Org. Cult. 30: 141-148.

Raina R., Behera M.C., Chand R., Sharma Y. (2003) *Reproductive biology of Gentiana kurroo Royle*. Curr. Sci. 85: 667-670.

Raina R., Chand R., Sharma Y. (2011) *Conservation strategies of some important medicinal plants.* Int. J. Med. Arom. Plants 1: 342-347.

Sambrook J., Russell D.W. (2001) *Molecular Cloning: A Laboratory Manual*. Vol. 1.

Shillito R.D., Saul M.W., Paszkowski J., Müller M., Potrykus I. (1985) *Highefficiency direct gene transfer to plants.* BioTechnology 3: 1099-1103.

Sowers A.E. (1992) *Mechanisms of electroporation and electrofusion.* In: *Guide to electroporation and electrofusion*. Ed. Chang D.C., Chassy B.M., Saunders J.A., Sowers A.E. San Diego, Academic Press: 119-138.

Tagu D., Bergounioux C., Cretin C., Perennes C., Gadal P. (1988) *Direct gene transfer in Petunia hybrida electroporated protoplasts: evidence for co-transformation with a phosphoenolpyruvate carboxylase cDNA from sorghum leaf*. Protoplasma 146: 101-105

Tanaka T. (1988) *Gels*. Sci. Amer. 244: 124-138.

Tomiczak K., Rybczyński J. J.(2015) *Autotetraploid plant regeneration by indirect somatic embryogenesis from leaf mesophyll protoplasts of diploid Gentiana decumbens* L. In vitro Cell. Dev. Biol. Plant 51: 350-359.

Tsong T.Y., Kinosita K. (1977) *Formation and resealing of pores of controlled sizes inhuman erythrocyte membrane.* Nature 268: 438-440.

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Analysis of genetic components of winter oilseed rape (*Brassica napus* ssp. *oleifera*) regeneration ability under *in vitro* culture

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Abstract

This paper presents the study on the genetic components of regeneration ability of winter oilseed rape (*Brassica napus* ssp. *oleifera*) explants cultured *in vitro.* The general combining ability (GCA) and specific combining ability (SCA) effects and the heritability in the broad and narrow sense were determined for four traits: the efficiency of shoot regeneration from hypocotyls, the number of regenerated shoots per single hypocotyl, the efficiency of shoot regeneration from cotyledons with a petiole, and the number of regenerated shoots per a single cotyledon with a petiole. For the study, explants originating from winter oilseed rape doubled haploid (DH) lines (W15, W69, W70, W78, and W131) and their F1 hybrids were used. Conducted analysis revealed that most of GCA effects were insignificant. None of the DH lines showed positive GCA effect, while lines W15 and W131 exhibited significant negative GCA effects for three of four analyzed traits. There were no significant SCA effects for any of the F1 hybrids. Calculated values of heritability in the broad and narrow sense were very low. The results obtained in the study confirmed that quantitative traits connected with plant regeneration under *in vitro* conditions are characterized by high variability. Although the results have widened knowledge of the genetic determinants of regeneration ability trait, further studies and observations are still required.

Key words: oilseed rape, *in vitro* culture, regeneration ability, GCA, SCA, heritability

Introduction

Winter oilseed rape (*Brassica napus* ssp. *oleifera*) is usually described as a good source of plant material to conduct *in vitro* cultures, but the species reveals a considerable genotypic diversity concerning its regeneration ability. Regeneration is a prerequisite in obtaining valuable plant material under *in vitro* conditions. Genotypes with a high regeneration ratio are a significant tool in biotechnological research, especially in genetic engineering. Despite the progress in research, many important aspects of *in vitro* plant regeneration are poorly known. The present study attempts to investigate the genetic background of the regeneration of rapeseed plants by using a classical genetic analysis. The genetic research and breeding programs depend on the proper diagnosis of the conditions of quantitative trait inheritance. During the selection process, the information about the combining ability of parental components used for crossbreeding is very important. This knowledge is essential for proper selection of suitable parents in identifying promising hybrids. A common method used in a classical genetic analysis is diallel crossing applied to evaluate the combining ability of parents and progeny generations. One possible way to analyze diallel crosses is the method proposed by Griffing (1956a), which divides the total genetic variance for the GCA of parents and the SCA of obtained hybrids. The GCA determines the ability of the tested line of providing an abundant offspring when crossing it with many other lines, while the SCA is characterized by the ability of two different lines of giving an abundant offspring after their mutual crossbreeding (Sprague and Tatum, 1942). The assessment of the general and specific combining abilities allows determining the additive and non-additive gene action as well (Falconer, 1967). Thus, the knowledge of combinatorial abilities helps to understand the nature of the action of genes involved in the expression of quantitative traits and predicts the value of further generations (Machikowa et al., 2011). How

Table 1. DH lines of winter oilseed rape used in the study and F1 hybrids resulting from diallel crosses

DH line	F1 hybrid	DH line	F1 hybrid	DH line	F1 hybrid	DH line	F1 hybrid	DH line	F1 hybrid
W15	W15×W69	W69	W69×W15	W70	W70×W15	W78	W78×W15	W131	W131×W15
	W15×W70		W69×W70		W70×W69		W78×W69		W131×W69
	W15×W78		W69×W78		W70×W78		W78×W70		W131×W70
	W15×W131		W69×W131		W70×W131		W78×W131		W131×W78

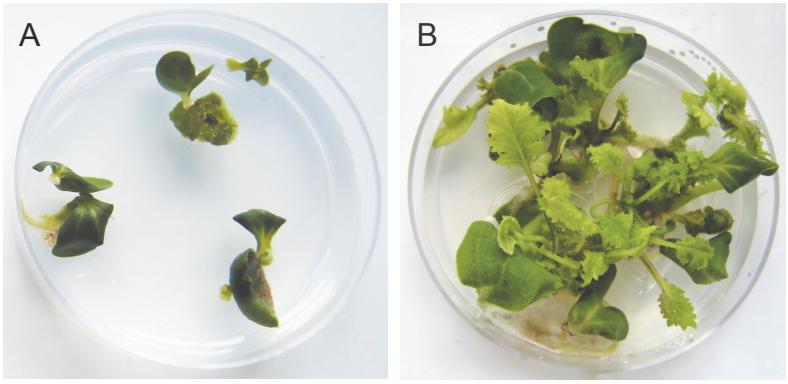

Fig. 1. Hypocotyls and cotyledons of winter oilseed rape doubled haploids cultured *in vitro*:
A) line W15 with low regeneration effectiveness, B) line W78 with high regeneration effectiveness

ever, it is important to know not only the value of general and specific combining abilities, but also how the values obtained are reproducible in subsequent generations, that is, what is their heritability. The broad and narrow sense heritability coefficients are often reported in the literature and are not much applicable for breeding. For the selection process, it is more important to know the heritability in the narrow sense because the additive variance is a measure of similarity between parents and offspring (Falconer, 1974), which allows predicting a response to the selection (Bos and Caligari, 1995).

With regard to the above, the aim of the research was to analyze the effects of GCA and the SCA, and the heritability in the broad and narrow sense on the characteristics that make up the overall picture of the regeneration capability of winter oilseed rape (*Brassica napus* ssp. *oleifera*) explants cultured *in vitro.*

Materials and methods

The plant material included five doubled haploid (DH) lines of winter oilseed rape (*Brassica napus* ssp. *oleifera*), showing different regeneration effectiveness under *in vitro* conditions. In addition, F1 progeny obtained from diallel crosses was also used. DHs included lines with high regeneration effectiveness (W69, W70, and W78) and lines with low regeneration effectiveness (W15 and W131) when cultured *in vitro*. DH lines were obtained in cultures of isolated microspores, in cooperation with the Institute of Plant Breeding and Acclimatization, Department of Oilseed Crops in Poznan. Details concerning DH lines and F1 hybrids are shown in Table 1; explant samples of lines W15 and W78 are depicted in Figure 1.

The effects of the GCA and the SCA and the heritability in a broad and narrow sense were estimated by using the classical two-factor model (Mądry et al., 2010), and the second method of Griffing (1956a), with the assumptions that there is no maternal effect, action of non-allelic genes is independent, multiple alleles do not exist, parents are homozygous, segregation of genes between parents is independent, and the breeding coefficient is equal to 1 (Frankenberger et al., 1981; Griffing, 1956b).

Heritability in the broad sense was defined by the formula:

$$h^2_{bs} = Var(G) / Var(P) \quad (1)$$

where

h^2_{bs} is heritability in the broad sense, Var(G) is the genotypic variance, Var(P) is the total phenotypic variance.

Heritability in the narrow sense was defined by the formula:

$$h^2_{ns} = Var(A) / Var(P) \quad (2)$$

where

h^2_{ns} is the heritability in the narrow sense, Var(A) is the additive variance, Var(P) is the total phenotypic variance.

The significance of the effects of the GCA and the SCA was determined using a Fisher-test with the significance level $\alpha = 0.05$ and $\alpha = 0.01$. GCA effects were estimated for the DH lines in the system "each with each".

The valuation of the genetic components described above was performed for four traits which make up the overall picture of rapeseed explant regeneration capability under *in vitro* conditions. These were as follows: 1) the efficiency of shoot regeneration from hypocotyls, 2) the efficiency of shoot regeneration from cotyledons with a petiole, 3) the number of regenerated shoots per single hypocotyl and the number of regenerated shoots per single cotyledon with a petiole.

All the analyses were performed using the statistical package R (R Core Team, 2012).

Results and discussion

The evaluation of the general combining ability effects revealed that the majority of the GCA effects were not statistically significant. There were no positive GCA effects for any of the DH lines, while significant negative effects of the general combining ability were demonstrated for lines W15 and W131 and three of four analyzed traits. These were as follows: the effectiveness of shoot regeneration from hypocotyls (lines W15 and W131), the number of shoots regenerated per a single hypocotyl (lines W15 and W131), and the number of regenerated shoots per a single cotyledon with a petiole (line W131). The negative values for all DH lines were obtained probably due to the very high variability of the analyzed traits, which also points to the uselessness of these lines as a form of parenting. None of the F1 crossing combinations showed any statistically significant SCA effects for the analyzed traits either. The calculated values of heritability in the broad and narrow sense proved to be very low. The significance of the effects of GCA and SCA for F1 hybrids is shown in Tables 2 and 3, while the values of heritability in the broad and narrow sense are presented in Table 4.

The SCA variance higher than the GCA variance reveals an overwhelming effect of the dominant genes in determining the studied traits. Conversely, a higher GCA variance suggests that the effects of additive genes are involved in determining these traits. If none of the variances is significant, the existence of an epistatic gene effects is indicated (Marinković et al., 2000; Škorić et al., 2000). The results could therefore indicate a little effect of the additive genes in determining the regeneration ability trait. However, a vast majority of statistically insignificant GCA and SCA effects may suggest the existence of effects of other genes. Nevertheless, the calculated GCA effects point that the DH lines showing significant negative effects (W15 and W131) may affect the decrease in value of analyzed traits in the progeny.

A study presented in this paper demonstrates very low heritability values of analyzed traits in the broad and narrow sense. The efficiency of shoot regeneration from hypocotyl heritability in the broad and narrow sense amounted to 0.074 and 0.06, and the efficiency of shoot regeneration from cotyledons with a petiole was 0.035 and 0.008, respectively. The number of regenerated shoots per single hypocotyl was characterized by slightly higher values of heritability, which amounted to 0.11 in a broad sense and 0.085 in the narrow sense. The numbers of regenerated shoots per single cotyledon with a petiole were 0.057 and 0.037 in the broad and narrow sense, respectively. Despite such low values of heritability in both broad and narrow sense, within the F1 hybrids, higher values of the regeneration efficiency were observed, when comparing them to weaker one of their parents. This may prove that the dominant gene effect was higher than the additive effect of genes, and the additive genetic variance could be low in this population when considered separately.

Ono and Takahata (2000) studied the genetic background of winter oilseed rape shoot regeneration using diallel crosses. In those studies, shoot regeneration from cotyledonous petioles was associated with the additive and dominant effect of genes, and the additive effect outweighed the effect of dominance. Heritability in the broad and narrow sense was analyzed, respectively, amounting to 0.973 and 0.819 values.

Akasaka-Kennedy et al. (2005) demonstrated a genetic control of the shoot formation ability for winter oil-

Table 2. The significance of the effects of GCA of three DH lines of winter oilseed rape

DH Line	The efficiency of shoot regeneration from hypocotyls	The efficiency of shoot regeneration from cotyledons with a petiole	The number of regenerated shoots per single hypocotyl	The number of regenerated shoots per single cotyledon with a petiole
W15	−0.177**	−0.772	−0.211**	−1.153
W69	−0.132	−0.644	−0.128	−1.037
W70	−0.138	−0.689	−0.156	−1.003
W78	−0.143*	−0.622	−0.167*	−0.959
W131	−0.199**	−0.744	−0.228**	−1.314*

The significance of the effects on levels of 0.05 –* and 0.01 – **

Table 3. The significance of the effects of SCA for F1 hybrids of winter oilseed rape

F1 hybrid	The efficiency of shoot regeneration from hypocotyls	The efficiency of shoot regeneration from cotyledons with a petiole	The number of regenerated shoots per single hypocotyl	The number of regenerated shoots per single cotyledon with a petiole
W15 × W69	−0.020	0.061	−0.039	0.132
W15 × W70	0.024	−0.061	0.028	−0.146
W15 × W78	−0.026	0.050	−0.028	0.032
W15 × W131	0.086	0.500	0.100	0.771
W69 × W15	0.058	0.000	0.083	0.066
W69 × W70	−0.020	0.033	−0.011	0.027
W69 × W78	0.019	0.100	0.022	0.182
W69 × W131	0.063	0.506	0.039	0.743
W70 × W15	−0.026	0.044	−0.044	0.099
W70 × W69	0.330	−0.200	0.572	−0.662
W70 × W78	0.002	−0.078	0.006	0.016
W70 × W131	0.069	0.639	0.067	0.932
W78 × W15	−0.020	−0.044	−0.033	−0.101
W78 × W69	0.013	0.044	0.006	−0.018
W78 × W70	0.036	0.011	0.050	0.149
W78 × W131	0.074	0.550	0.078	0.910
W131 × W15	−0.031	−0.078	−0.039	−0.123
W131 × W69	−0.042	−0.078	−0.022	−0.040
W131 × W70	0.280	−0.278	0.422	−0.473
W131 × W78	0.019	0.000	0.011	−0.096

Table 4. Heritability in the broad and narrow sense obtained for the four studied traits

Trait	Heritability in the broad sense	Heritability in the narrow sense
The efficiency of shoot regeneration from hypocotyls	0.074	0.060
The efficiency of shoot regeneration from cotyledons with a petiole	0.035	0.008
The number of regenerated shoots per single hypocotyl	0.110	0.085
The number of regenerated shoots per single cotyledon with a petiole	0.057	0.037

seed rape leaf explants, and a strong correlation between the number of shoots formed on a single explant and the efficiency of shoot formation. However, after the comparison of these data with the results of Ono and Takahata (2000), no correlation between the shoot formation from leaves and shoot formation from the cotyledonous petioles was shown. This could either be due to the differences in phytohormones used to conduct the *in vitro* culture in both experiments or the existence of variations in genes controlling the regeneration capacity of leaves and cotyledons.

Sparrow et al. (2004) used a diallel analysis to investigate the genetic control of cabbage (*Brassica oleracea*) shoot regeneration. According to the results, the regeneration of shoots from the cotyledonous petioles of *B. oleracea* was found to be strongly influenced by genes. About 85% of the variance was accounted for genetic variation and the rest was due to environmental factors. In these studies, most of the genetic variation was due to the additive gene action (71%) while a high efficiency of shoot regeneration was observed as a dominant trait over the low ability to regenerate shoots.

Łuczkiewicz et al. (2006) analyzed several quantitative traits associated with the *in vitro* regeneration of *Camelina sativa*. Six different (in terms of analyzed traits) genotypes were crossed using diallel crossing. Cotyledon-hypocotylous explants obtained from F1 hybrid and parental plants were cultured *in vitro,* and the mass of callus and regenerated plant parts and the number of regenerated shoots were evaluated. The evaluation of the GCA and the SCA effects revealed that most of them were not statistically significant. No statistically significant SCA effect on the mass of the callus and regenerated plant parts was proven. The values of heritability were not evaluated at all due to the large error caused by too high quantitative trait variation. The additive effects of genes were not as yet been demonstrated.

Etedali and Khandan (2012) studied the genetic control of callus growth and shoot regeneration of oilseed winter rape mature embryos. Callus growth was recognized using two parameters: the callus diameter measured in millimeters and the fresh weight of callus measured in grams. The GCA effects estimated by researchers were found to be insignificant for all the studied traits, while significant SCA effects were estimated only for the callus concerning traits. In addition, Etedali and Khandar obtained high values of broad sense heritability of studied traits, which amounted to 0.68 for shoot regeneration, 0.89 for callus diameter, and 0.82 for the weight of callus. Conversely, they obtained very low values of heritability in the narrow sense, which were, respectively, 0.03 for shoot regeneration, 0.04 for callus diameter, and 0.03 for the weight of callus. The authors concluded that high values of broad sense heritability of the studied traits indicate that callus growth in oilseed winter rape is genetically controlled; however, low values of narrow sense heritability estimated to be these traits suggest the effect of dominant genes exceeded onto an additive gene action. The researchers found that the genes which control the growth of callus are likely to be different from those controlling the regeneration of shoots.

Our results and of those reported in literature indicate that quantitative traits associated with the regeneration ability under *in vitro* conditions, such as shoot regeneration frequency and the number of shoots per explant, are highly variable, which hinders the genetic analysis of these characteristics. The results of the evaluation of the four traits investigated in this work, which compose the general picture of rapeseed plant regeneration ability, are markedly different from the results of Ono and Takahata (2000) and Sparrow et al. (2004). They do, however, remain in accordance with the results of Łuczkiewicz et al. (2006) and Etedali and Khandan (2012). The differences may have been due to or connected with a sampling error, other genotypes used for testing, or different types of media used in the *in vitro* culture. It is also possible that a certain clonal or epigenetic variation occurred. Although the obtained results undoubtedly contribute to the knowledge of genetic determinants of oilseed rape regeneration ability trait, further studies and observations are still required.

References

Akasaka-Kennedy Y., Yoshida H., Takahata Y. (2005) *Efficient plant regeneration from leaves of rapeseed (Brassica napus L.): the influence of $AgNO_3$ and genotype.* Plant Cell Rep. 24: 649-654.

Bos I., Caligari P. (1995) *Selection methods in plant breeding.* London: Chapman & Hall.

Etedali F., Khandan A. (2012) *Determination of hybrid vigor and inheritance for Regeneration in rapeseed (Brassica napus).* Int. J. Agron. Plant Prod. 3(4): 145-153.

Falconer D.S. (1974) *Inheritance of quantitative traits.* Warszawa: PWN.

Falconer S.P. (1967) *Introduction to quantitative genetics.* New York: The Ronald Press Company.

Frankenberger A., Hasegawa P.M., Tigchelaar E.C. (1981) *Diallel analysis of shoot forming capacity among selected tomato genotypes.* Z. Pflanzenphysiol. 102: 233-242.

Griffing J.B. (1956a) *Concept of general and specific combining ability in relation to diallel crossing system.* Aust. J. Biol. Sci. 9: 463-492.

Griffing J.B. (1956b) *A generalized treatment of the use of diallel crosses in quantitative inheritance.* Heredity 10: 31-50.

Łuczkiewicz T., Nawracała J., Strybe M., Satkiewicz K. (2006) *Genetic analysis of several quantitative traits linked with regeneration of false flax Camelina sativa L. in in vitro tissue cultures.* Biul. Inst. Hod. Aklimat. Rośl. 242: 261-266.

Machikowa T., Saetang C., Funpeng K. (2011) *General and Specific Combining Ability for Quantitative Characters in Sunflower.* J. Agricult. Sci. 3(1): 91-95.

Mądry W., Mańkowski D.R., Kaczmarek Z., Krajewski P., Studnicki M. (2010) *Classical two-factor experience.* In: *Statistical methods based on linear models, applications for experimentation, genetics and plant breeding.* Monogr. i rozpr. nauk. IHAR 34: 37-50.

Marinković R., Škorić D., Dozet B., Jovanović D. (2000) *Line × tester analysis of combining ability traits in sunflower (H. annuus L.).* In: Proc. of the 15th Int. Sunflower Conf. Toulouse, France: 30-35.

Ono Y., Takahata Y. (2000) *Genetic analysis of shoot regeneration from cotyledonary explants in Brassica napus.* Theor. Appl. Genet. 100: 895-898.

R Core Team (2012) *R: A language and environment for statistical computing.* Vienna, Austria: R Found. Statist. Comput.

Škorić D., Jocić S., Molnar I. (2000) *General (GCA) and Specific (SCA) combining abilities in sunflower.* In: Proc. of the 15th Int. Sunflower Conf. Toulouse, France: 23-29.

Sparrow P.A.C., Townsend T.M., Morgan C.L., Dale P.J., Arthur A.E., Irwin J.A. (2004) *Genetic analysis of in vitro shoot regeneration from cotyledonary petioles of Brassica oleracea.* Theor. Appl. Genet. 108: 1249-1255.

Sprague G.F., Tatum L.A. (1942) *General vs specific combining ability in single crosses of corn.* J. Amer. Soc. Agron. 34: 923-932.

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In vitro cultures of animal and human cells

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Tissue culture was developed at the beginning of the twentieth century (1907) as a method for studying the behaviour of human and animal cells free of systemic variations that might arise *in vivo* both during normal homeostasis and under the stress. The technique was established first with disaggregated fragments of tissue, and growth was restricted to the radial migration of cells from the tissue fragment, with occasional mitoses in the outgrowth. As culture of cells from and within such primary explants of tissue dominated the field for more than 50 years, it is not surprising that the name "tissue culture" has remained in use as a generic term despite the fact that most of the expansion in the field in the second half of the twentieth century was made possible by the use of dispersed cell cultures. Disaggregation of explanted cells and subsequent plating out of the dispersed cells was first demonstrated by Rous (Rous and Jones, 1916). In 1950s trypsin became more generally used for subculture, following procedures described by Dulbecco to obtain passaged monolayer cultures for viral plaque assays (Dulbecco, 1952), and the generation of a single cell suspension by trypsinization.

Tissue culture enter its second century since its inception. For the first time it is possible for genetic manipulation of commonly and easily cultured cells, such as skin fibroblasts, to allow their conversion into pluripotent stem cells (iPS), capable of differentiating into a range of different cell types. The field opens up to a whole new scenario: instead of the need for complex selective culture technique, the culture procedures may be used to initiate biochemical regulation and may be used to convert phenotype.

Tissue culturing nowadays is essential and irreplaceable in modern biotechnology as a method supplementing and enriching our knowledge. Without specialized laboratories for tissue culture with equipment for they analysis and chance of cytogenetic and molecular analysis it will not be possible to by us transgenic rabbits producing in mammary gland human growth hormone and five lines of transgenic pigs for xenotransplantation purposes. In our laboratories we use cell culture of human and animal cells for biotechnology needs or as models. In animal transgenesis we usually perform several steps using molecular and cytogenetic methods at cell culture levels. Important issues in producing transgenic animals cover characteristics of donor of gene, transfection and cell transformation, detection of transgene, mapping of transgene, passing of transgene into next generation, homozygote selection and analysis of transgene activity. From cytogenetic methods the most important are banding chromosomes methods for karyotyping each transgenic individual and mapping the transgene on metaphase chromosomes using fluorescent *in situ* hybridization (FISH). It is possible to study structure of the transgene on the chromatin level using FISH on chromosomal loop domain or using 3D FISH technique. This methods is important to distinguish homozygous offspring from heterozygous and confirm the stability of transgenesis. The last step include analysis of expression of transgene using flow cytometry methods.

References

Rous P., Jones F.A. (1916) J. Exp. Med. 23: 555.

The quality of oocytes and embryos produced *in vitro* – the porcine and bovine model

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The complex procedure of the production of mammalian embryos in laboratory conditions (*in vitro*, IVP) has acquired a stable position in science and commercial applications in both, domestic animals and humans. It must be underlined that animals are of special importance as models in biomedical sciences, with a special attention paid to cattle. When it comes to domestic animals, the complex procedure consists of three steps: oocyte *in vitro* maturation – IVM, *in vitro* fertilization IVF and *in vitro* embryo culture until the blastocyst stage – IVC. The efficiency of the *in vitro* embryo production differs significantly between animal species. The less satisfactory results have been observed for the domestic dog and the horse whereas the domestic cattle is characterized by a very efficient procedure (30-40% of fertilized oocytes transform into blastocysts on the day 7 post insemination). Despite satisfactory efficiency, the quality of *in vitro* produced embryos is still significantly reduced when compared to their *in vivo* derived counterparts. Embryo quality is a complex term equivalent in this case to the term of developmental potential. The quality of oocytes and embryos is affected by a wide spectrum of factors, starting with donor related parameters (eg age, health, nutrition), intrinsic traits of the oocyte (eg transcript and protein content, correctness of the meiotic division, organelle reorganization, degenerative processes – apoptosis) and, finally, embryo related parameters (eg regularity of cleavage, compaction and cavitation, extent of apoptosis, transcript and protein content). It is also evident that oocyte and embryo culture *in vitro* may, *per se* cause some disturbances on the cellular level "forcing" the embryo to adapt to suboptimal environment. Despite the fact that preimplantation embryos show some extent of plasticity in such adaptations, this also means a need for modifying some cellular processes e.g. gene expression. Changes at the molecular level may also increase apoptosis and lead to degeneration and embryonic death. Moreover, the current state of knowledge does not allow to identify the crucial factors present *in vitro,* which significantly reduce the quality of the resulting embryos.

Research carried out by the group of Reproductive Biotechnology of the Department of Genetics and Animal Breeding (Poznan University of Life Sciences) has been focused on the identification of factors influencing the quality of *in vitro* incubated/produced oocytes and embryos of the pig and cattle. Some of the main achievements of the group include demonstrating the relationships between: 1) the IVM media composition and the sexual maturity of the donor gilt and the regularity of meiotic division, 2) donor nutrition and the composition of the follicular fluid, 3) the timing of the first zygotic cleavage and the quality of resulting bovine embryo, 4) showing the importance of the embryonic genome activation to embryo viability by demonstrating significant changes in the nuclear architecture of blastomeres within the 8-16-cell stage embryos and 5) demonstrating a limited value of the follicular components in predicting the quality of bovine oocytes.

The aim of the present talk is to characterize the current state of knowledge on the quality of oocytes and embryos with a special emphasis placed on our own achievements. A wide spectrum of laboratory procedures will be also discussed (e.g. real time PCR, immunostaining, FISH, 3D-FISH).

Epi-genes potentiate plant biodiversity

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The dynamic development of sequencing methods resulted in vast accumulation of the genomic data in a number of worldwide databases. Therefore, a great challenge for biology is to determine the function of the identified sequences. The most effective tool to realize this task is generation of genetically modified organisms in which the sequence of interest is up- or down-regulated. The GMO generating methodology is the best and the most frequently used; however, it is quite bothersome and time consuming. Another a frequently stressed drawback is the fact that the genome continuity is physically broken when new, foreign or own altered sequence is introduced. Recently, several studies have proposed a new possible epigenetic modification based on the introduction into the plant short oligonucleotide sequences (OLIGO) that are homologues to the gene of interest. In most cases OLIGO silences the target gene by interference but in a few cases target gene activation have been reported. So far, there are only few reports referring to mammalian cells whose genes were activated by oligonucleotides at the transcriptional level by the histone modification and/or DNA methylation. There has been no constructive hypothesis, which would be helpful in understanding when OLIGO reveals one of its activities. Most probably, the effect of OLIGO depends upon the targeted gene's structural part. This, however, needs further investigation such as the targeted gene expression profile, the gene-body and total genome methylation profile, as well as the expression analysis of the genes involved in the formation of the complex of OLIGO with a group of RISC and DICER proteins, methylases, demethylases, and polymerases.

Recently, we have undertaken a thorough and systematic analysis of the impact of OLIGOs targeted to main structural elements (promoter, exons, introns, 5′ and 3′ UTRs) of selected genes. Gene expression, its methylation (and the entire genome) profile, the expression profile of epi-genes such as methylase and demethylase and RNA-dependent RNA polymerase as well as proteins that are important components of RISC/DICER complexes were measured. The purpose of this analysis is to answer the question whether the OLIGO technique can be an alternative to GMO for gene function analysis and for diversifying plants' genotypes.

The preliminary data suggest that OLIGO technology is effective in the analysis of gene function as well as in the generating new types of plants; however, it still requires the precise molecular background explanation to become predictable. For example, preliminary data showed that OLIGO homologous to the target gene is able to cause opposite changes in its expression. Is the final effect of the OLIGO action dependent on its sequence, structure, or the recognized gene domain? Are cytosine residues (C) selectively methylated? Is there a specific nucleotide context of the methylated C? Is the transcriptional activity of epi-genes changed? And finally, are the changes in the methylation and transcription of the gene modified by OLIGO stably inherited? These questions remain unanswered today but the growing number of interdisciplinary research undertaking epi-genome issues guarantee clarification of the unknown in the nearest future.

The microRNA-guided regulation of tillering in barley

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MicroRNAs are small RNA molecules, usually 21 nt long, that regulate gene expression through target mRNA cleavage or repressing its translation. MicroRNAs function as regulators of plant development and response to environmental stresses. Heat stress is one of the major abiotic factors that can induce severe plant damages leading to a decrease in crop plant productivity.

We have analyzed barley microRNAs expression profile under heat stress (2-weeks old barley plants exposed 24 h to high temperature) and compared them to microRNAs from barley plants grown under control conditions. We found 15 and 7 microRNAs which are up- and down-regulated, respectively. The most profound up-regulation was found for miRNA5048a, miR165a, and microRNA444.1 a2. The last one targets transcription factor (TF) that belongs to the MADS-box TF class II. Its rice ortholog OsMADS57 is a positive regulator controlling tiller outgrowth. MADS57 binds to CArG motif present in rice *Dwarf14* gene promoter and represses the expression of *D14* negative regulator of the tillering.

Alongside the elevated level of the barley mature miR444.1a2 during heat stress, we observed dramatic decrease of its target mRNA *HvMADS57* and inhibition of tillering in heat stressed plants suggesting the existence of a similar mechanism controlling tillering in barley as in rice plants.

To elucidate interplay between heat stress regulated microRNAs and their targets we carried out barley transcriptome sequencing (RNA-seq). A miRNAs/target genes network in response to heat stress in barley will be discussed in context of barley development.

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Brachypodium distachyon – a model plant to study grass genome structure, dynamics and evolution

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Ecologically, as well as economically, Grasses (Poaceae) belong to the most important groups of plants. Members of the Poaceae, such as wheat, rice, and maize, provide a vast majority of human food worldwide and dominate the vegetation in various habitats across all continents and climate zones. However, the experimental and genomic tractability of most grass crops is frequently hindered by their large genome sizes and polyploidy. *Brachypodium distachyon*, a small annual weedy grass, emerged several years ago as a model system to study various aspects of genome organisation, functioning, and evolution in temperate cereals and grasses. B. distachyon possesses numerous attributes sought after in a model organism. These include a favourable phylogenetic position within the 'core' Poaceae species, a small (~350 Mb) genome, small physical stature, self-fertility, a short life cycle and undemanding growth requirements. It also has an ever growing repertoire of experimental resources such as germplasm collections, complete and almost fully-annotated genome sequence, large-insert genomic DNA libraries, expressed sequence tag (EST) databases, as well as efficient *Agrobacterium*-mediated transformation protocols and availability of mutant collections. The recent years have shown that B. distachyon can serve as an excellent model system to study cell wall composition and development, biomass accumulation, plant-pathogen interactions and reaction to abiotic stress. Due to the compactness of its genome and low basic chromosome number, it also provides unique opportunities to address many novel and important areas of plant molecular cytogenetics.

Advanced cytomolecular techniques, such as fluorescence in situ hybridisation (FISH) with evermore sophisticated probes coupled with state-of-the-art microscopy and digital image acquisition offer an unprecedented insight into various aspects of nuclear genome organisation and dynamics. One of the most informative cytomolecular tools is chromosome painting (CP), which enables unique and unambiguous visualization of individual chromosomes or large segments not only during cell division but also during the interphase. It can also be employed to study the karyotype evolution among closely related species and the phylogenetic relationship at the level of the chromosome. Another powerful cytomolecular technique utilises the application of specific antibodies linked with fluorochromes. Such antibodies allow selective localisation of epigenetically modified components of chromatin, such as methylated DNA and histones. They may also target various proteins that compose specific biological structures, like the synaptonemal complex, or are responsible for various enzymatic processes, for example recombination events during meiosis in plants.

This presentation summarises the present status of analyses of plant genome structure, dynamics and evolution using *B. distachyon* and some of its relatives. The on going research projects address various important scientific problems such as elucidating the mechanisms responsible for the grass karyotype evolution and divergence, deciphering the factors that determine the distribution of individual chromosomes within an interphase nucleus, or the chromosomal behaviour during meiotic cell division. Other on going investigations attempt to answer the questions whether there are hot spots of structural rearrangement in *Brachypodium* chromosomes and which epigenetic processes play a crucial role in *B. distachyon* embryo development and selective silencing of rRNA genes in *Brachypodium* allopolyploids.

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Possibilities and limitations of light microscopy in the study of the structure and function of a plant cell

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The most important for research in the field of biology is the possibility of analyzing living cells, which allows a better understanding of their structure and function. The most suitable in such an analysis are different microscopy techniques. Over the centuries light microscopy has been the basic research tool in various disciplines of natural sciences. In the analysis of life processes, the light microscopy techniques are more useful in tracking changes in living cells.

Development of the existing and a creation of the new light microscopy techniques has improved and increased the possibilities of the use of light microscopes to study living cells in greater detail. The former one was a phase contrast technique developed by F. Zernike. Phase contrast is used with an especially good effect by researchers e.g. in biology, and by cell biologists in particular. Living cells belong to the class of phase objects that are difficult to study (without staining) in an ordinary microscope. Phase contrast allows invisible optical path differences or phase shifts occurring in the object plane to be transformed into visible differences of light intensity in the image plane of microscope (Pluta, 1989). The success of the phase contrast microscope has led to a number of subsequent phase imaging methods. Among them, the best known is differential interference contrast (DIC) microscopy patented by G. Nomarski.

Since the construction of the first microscope until now light microscopy has experienced at least twice the hey-day. The first one was at the turn of the 20th century. The return to light microscopy as a useful research technique was because of two reasons: the synthesis of the first fluorochrome, and development of fluorescent microscopy. The use of fluorochromes in the study of living organisms was the milestone in increasing our knowledge of the cell function and the influence of different factors, both internal and external on cell growth and development.

The second hey-day of light microscopy started (and has continued since) with the construction of confocal microscopy and isolation of green fluorescent protein from the jellyfish *Aequoria victoria*. The popularity of the confocal microscopy is evidenced by the increase in the number of confocal systems in use today. The power of this approach lies in its ability to image structures at discrete levels within an intact biological specimen achieving increasingly higher resolutions e.g. resolving power of microscope STORM is 12 nm; (Paterczyk, 2013). The development of light microscopes themselves and their applications have accelerated in last decades, which was possible thanks to the improvements of technology, the use of computer-optimization in the design of optic systems, and computer analysis of microscopic images. The present usage of light microscopy, in particular confocal microscopy and quantum dots, will contribute to a large and rapid increase of information concerning the developmental processes and control mechanisms on the cellular and molecular level.

All mentioned above possibilities of light microscopy techniques and their use in the biological study are not without the limitations, which is obvious to anyone who works in the field of cell biology including tissue cultures. That is why possibilities and limitations of various optical-microscopic techniques related to biological samples, especially the analysis of plant cell structure and function, in particular during *in vitro* cultures, will be discussed.

References

Paterczy B. (2013) *Droga do nanoskopii*. Kosmos, 62 (2): 261-180
Pluta M. (1989) *Advanced light microscopy*. PWN, Warszawa

The patentability of GM plants in light of recent amendments to the EU law on GMOs. Some remarks on the possible effects of Directive 2015/412/EU

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This article aims to identify and analyse current tendencies in the laws governing cultivation of genetically engineered plants in the European Union, as well as to determine whether recent changes influence the possibility to patent transgenic plants or the methods of their production.

While growing in popularity around the world, transgenic plants face strong opposition within the European Union. Recent changes to the EU law governing the cultivation of GM plants are just another example of this opposition. *The Directive (EU) 2015/412 of the European Parliament and of the Council amending Directive 2001/18/EC*, provided member states with means to restrict or effectively prohibit cultivation of genetically engineered plants on their territories, even if such plants were already authorised for cultivation in the EU. The reasons countries can currently invoke in order to introduce restrictions are not related to bio-safety anymore, but rather encompass a set of political or social matters such as socioeconomic impacts, avoidance of GMO presence in other products, agricultural policy objectives, public policy etc. Hence they are to a much lesser extent based on the precautionary principle, as possible restrictions will also pertain to already examined and authorised GMOs. The restrictions no longer need to target certain transformation events but certain traits or crops as well. When it comes to the patentability of GM plants or methods of their production, the newly adopted changes seem to have limited influence, although it is not excluded that local laws adopted on the basis of the newly introduced changes could influence procedures before local patent offices or patent cancellation procedures, should those offices decide to apply the morality or *ordre public* exception to patentability. The newly adopted laws have a rather different effect though. The profitability of developing GM plants in the EU (and hence of their patenting) may become questionable if the exploitation of such inventions were prohibited in a number of the EU Member states.

Key words: GMO, European Union Law, Patentability, Patent law, Precautionary principle

New tool microscopy technique

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VS120 – the Olympus virtual slide system for digitizing slides at high magnification. The system can rapidly scan a slide in less than two minutes, creating a digital 'virtual slide' that is an exact copy of the original. This is easily created and navigated using an intuitive graphical interface. All of the VS120 system components are designed to interact seamlessly, producing a fully automated, high-speed scanning system with excellent flexibility and simple operation. All these ensure that users get the best results for each type of specimen with the minimum of effort. The system is extremely fast, scanning a 15 × 15 mm area using a 20× objective lens in around 90 seconds, while sample detail is enhanced and preserved via the high quality Olympus UIS2 optics and custom camera algorithms. Using a threshold-based method of removing small particles, the new Sample Detection Mask improves the image quality by maintaining an accurate focus when using automatic settings. In addition, the Expert and Virtual Z scan modes offer semi-automatic or manual focus settings, thus providing more flexibility. Analyzing virtual slides with multiple Z-stacks, it is possible to extract a single Z-stack of choice for further study, while the new Freehand Measurement tool can be used to generate quantifiable data from each image. To facilitate high-throughput scale-up, Olympus provides the optional VS120-L6, which can scan up to six slides automatically. For larger collections, the VS120-L100 provides 'walk away' scanning of up to 100 slides, while an inbuilt barcode scanner ensures that all slides are accurately tracked and catalogued.

Using the VS120, images can be captured regardless of the illumination technique, including bright-field, dark-field, phase-contrast, polarization, differential interference contrast (DIC) and multi-channel fluorescence via the additional fluorescence extension module. In addition, the new high resolution camera has three predefined brightfield settings that simplify and optimize the scanning of normal, dark or faintly stained samples. The system works on objectives from 2× to 100× (immersion). The created virtual slides can be navigated digitally in real-time as if the user were controlling a microscope, without the need for an instrument or even the sample. All images are ICC profile corrected to ensure perfect colour reproduction, and the system is compatible with standard and double sized slides for maximum accuracy and flexibility.

Virtual microscopy enables information from one slide to be shared simultaneously across the world, via the simple upload interface provided by a the secure Net Image Server SQL networking system. This makes the VS120 perfect for teleconsultation, global research collaboration, the archiving of slide materials and for sharing data with the classroom when teaching.

A clear advantage of an electronic storage is that slides can be found and loaded quickly and easily, enabling users to quickly reopen a slide for further investigation, access slides for reference purposes, or prepare presentations for conferences and lectures. Furthermore, there is no degradation to the sample over time – the slide image will always be as crisp and clear as the day it was recorded. This is especially advantageous when archiving old or rare samples, which can then be made available for everyone as if they had been copied.

From trait to dinner-plate: Using genomic information in plant breeding to enhance stress resistance in crops – A snapshot of how genomic technologies are used to enhance traditional plant breeding, from well-established PCR and gels to the emerging use of Next Generation Sequencing

PAUL BUTLER

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The use of genomics in crop breeding is not new. Gregor Mendel was already making breakthroughs in the area centuries ago, and produced incredible insights into gene regulation. Nowadays, genomics is crucial in plant breeding and continues to develop aggressively in response to the demands brought about by population growth and changing climate.

Drought tolerance, pest resistance and increased nutritional value are common goals as we try simultaneously to increase the yield of food from every hectare, decrease the attrition of crops due to environmental stresses and make previously non-arable land productive.

These goals manifest themselves in a demand by the growers and breeders for faster development of new cultivars. We now see growers aspiring to development cycles of 5 years compared with 10 years previously.

This talk will investigate the tools and techniques that are enabling scientists to drive forward and enable those new aggressive goals to be met.

Breakthroughs in nucleic acid extraction technology allow higher sample throughput as well as larger volumes of individual samples to be processed, important when various end-point testing methods may be necessary. Also reflecting the different sources of DNA such as seeds, leaves and sprouts.

Genotyping is a core requirement in agri-genomic research. A range of markers can be used for genotyping, both in establishing the relevant genes and identifying them in subsequent experiments. These include SSRs, RFLPs and SNPS. Genotyping tools will be discussed including PCR methods, gel analysis, micro-fluidics, micro-arrays and Next Generation sequencing technology.

The use of genetic engineering to insert genes of a different species and create Genetically Modified Organisms(GMOs), has flourished in recent decades. The Research, Development and QC of such products creates a need to identify markers to confirm the presence of GMO's. Tools for this purpose will be discussed also.

Finally, relating cellular phenotypic data such as from cellular high content imaging systems back to the genomic data and finding linkages between them is a holy grail and latest developments in this field will be discussed.

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Somatic embryogenesis of a tree fern *Cyathea delgadii* Sternb.: achievements and prospects

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The aim of this lecture is to present the latest achievements describing somatic embryogenesis in ferns. In monilophytes, the process is a newly discovered phenomenon, recognized for the first time in a tree fern *Cyathea delgadii* (Mikuła et al., 2015). This process is completely independent of a plant growth regulator in contrast to a majority of seed plants. The efficiency of somatic embryogenesis in the fern can be modified by physical and chemical factors of *in vitro* culture, and it reaches 42 somatic embryos per explant measuring 2.5 mm in length, within 2 months. Our study showed that etiolation of source sporophytes used for culture initiation is a critical factor for induction of the fern somatic embryogenesis. We also provided some morphological evidences for single cell origin of a somatic embryo and defined three morphogenetic stages of its development using light, transmission (TEM) and scanning (SEM) electron microscopic analyses. The early stage of somatic embryo formation is characterized by sequential perpendicular cell divisions of individual epidermal cells of an etiolated stipe explant. It is totally different from the system which exists in a zygotic embryogenesis of leptosporangiate ferns. However, the later stages of embryo development seem to be similar for both, the somatic and the zygotic embryogenesis. The whole process leading up to formation of somatic epidermal cell-derived sporophytes was instigated by a half strength MS medium supplemented with 1% sucrose. Light and sucrose stimulated the development of leaves and roots. Moreover, the photoperiod (16/8 h) was one of the most important external factors which affected the type of morphogenetic response and the sporophyte development. The hormone-free system of induction of somatic embryogenesis described by us for *C. delgadii* may be common for other fern species. Our study showed that this regeneration way is also possible to be induced in *Asplenium cuneifolium* (Viv.).

We believe that our achievements can help to broaden the fundamental knowledge of the process of somatic embryogenesis. Ferns could provide an excellent model for the future research on somatic cell dedifferentiation and somatic embryo formation at molecular, biochemical and physiological levels. At present, we are carrying out research on the possible role of endogenous hormones in the induction of somatic embryogenesis of *C. delgadii*. The effect of the salicylic acid, ancymidol, antypiryne, fluridone and triiodobenzoic acid, which are ethylene, gibberelin, jasmonic acid and abscisic acid biosynthesis inhibitors, and auxin polar transport inhibitor, respectively, on the response of stipe explants are being studied. To extend our knowledge of the role of symplasmic communication in somatic embryogenesis induction, the fluorochromes and confocal microscopy are used. Moreover, a two-dimensional gel electrophoresis and a mass spectrometry analysis are also employed for understanding the mechanisms underlying the induction and development of somatic embryos. The simple, effective and hormone-free system may be valuable for the mass propagation and conservation of various tree and herbaceous fern species.

Acknowledgments

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References

Mikuła A., Pożoga M., Tomiczak K., Rybczyński J. (2015) *Somatic embryogenesis in ferns – a new experimental system*. Plant Cell Rep. 34: 783-794

The influence of various sucrose concentrations, pH and hormone inhibitors on the efficiency of somatic embryogenesis in two fern species: *Cyathea delgadii* and *Asplenium cuneifolium*

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In a plant tissue culture, the growth regulators, sucrose and pH were believed to play a primary role in the initiation of various types of cells morphogenetic responses, among them somatic embryogenesis (SE). Our previously study (Mikuła et al. 2015) showed that the tree-fern – *Cyathea delgadii* (Sternb.) and herbaceous fern – *Asplenium cuneifolium* (Viv.) offer an effective model for the induction of embryogenic competence on a hormone-free medium. The phenomenon opens the door to new discoveries. Thus, the aim of the presented investigation was to find the most favorable conditions for initiation of this propagation system and to confirm that endogenous hormones play an important role in the initiation and overseeing of this process.

In order to find the optimum amount of sucrose and the pH value, the induction medium was supplemented with 1-10% sucrose and pH ranging from 5.8 to 7.8. To investigate the role of phytohormones in SE, hormone inhibitors were applied. The effect of triodobenzoic acid (TIBA), fluridone, salicylic acid (SA), ancymidol and antypiryne, which are substances altered balance of auxins, abscisic acid, ethylene, gibberellin and jasmonic acid, respectively, have been studied. Stipes of somatic embryo-derived sporophytes that had developed 2 or 3 fronds growing in the dark, were used for the culture initiation. The cultures were kept in a climatic chamber at +22 ±2 °C, in constant dark, and at a relative humidity of 35-55%. To evaluate the effectiveness of SE, the percentage of responding explants and the number of somatic embryos per responding stipe explant were assessed after two months of culture.

The analysis showed that the most suitable sucrose concentration in the culture medium for initiation of an efficient SE in *C. delgadii* was 1% while *A. cuneifolium* needed leastwise 10%. The influence of pH was not significantly different for both species and amounted to 5.8 and 6.8 for *C. delgadii* and *A. cuneifolium*, respectively. In this optimal culture conditions about 67% and 97% *C. delgadii* and *A. cuneifolium* explants were capable of producing 33 and 10 somatic embryos, respectively. Our results indicate that disturbances in auxins, ethylene and ABA level had an adverse effect on the number of somatic embryos produced by the stipe of responding explant. Inhibition of jasmonic acid and gibberelins led to the disorders in somatic embryos development. In this context, this unique system of *in vitro* propagation may be an excellent method for a mass acquisition of morphological and developmental studies of plant embryogenesis, as well as for conservation and commercial purposes of both studied fern species.

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References

Mikuła A., Pożoga M., Tomiczak K., Rybczyński J. J. (2015) *Somatic embryogenesis in ferns – a new experimental system*. Plant Cell Rep. 34: 783-794

Potential possibilities of the induction of European beech (*Fagus sylvatica*) tissue cultures from various types of explants

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Fagus sylvatica (European beech) is one of the most economically important deciduous tree species in Central Europe (Cuenca et al., 2000). Propagation by seeds is the main way of reproduction of European beech, although the seeds are difficult to store. Vegetative propagation of *F. sylvatica* by conventional methods is very difficult, too (Vieitez and San-José, 1996). Its *in vitro* propagation is not very popular, either. There are only a few known studies concerning micropropagation of this forest tree species (Vieitez and San-José, 1996; Naujoks, 2003). Because of the increased interest in recent years in possibilities of trees propagation through *in vitro* culture on a mass scale, it is worth developing an effective reproduction protocol of this economically important tree species.

The aim of this study was to investigate the efficiency of induction of somatic embryogenesis or organogenesis of beech from different types of explants in various culture conditions. Explants derived from immature, fresh seeds (collected in 2011 and 2013) and from mature seeds, stored at −10 °C and some stratified at 3 °C. They were placed on induction media with various combinations of plant growth regulators: zeatin, 2,4-D (2,4-dichlorophenoxyacetic acid) and/or BA (benzyladenine). The initial cultures were kept in the dark or in weak light (white fluorescent or blue-red LED). Limited success was achieved in the initiation of somatic embryogenesis. We obtained friable, yellow-white callus with characteristic PEM-like structures (cPEM-ls, from embryonic axes or fragments of immature embryos with embryonic axes). These structures could have been an early developmental stage of the embryogenic callus of *F. sylvatica*. This type of callus regenerated from explants incubated in the darks, mainly on a WPM medium with addition of 6.8 μM zeatin or WPM and MSG media with 9.1 μM 2,4-D and 2.2 μM BA. The highest frequency of the regeneration of callus with cPEM-ls was 5%. However, further studies are required to confirm if the observed structures are capable of development into somatic embryos. We have succeeded to induce organogenesis from both immature and mature zygotic embryos and from embryonic axes. The best results were obtained for mature zygotic embryos incubated on 1/2 WPM medium (half-strength Woody Plant Medium) with 9.1 μM 2,4-D and 2.2 μM BA. Adventitious buds were regenerated on up to 15% of the explants. The induced buds developed into shoots, enabling us to establish tissue cultures of *F. sylvatica*. The induction of organogenesis from the tested explants was more efficient than the induction of somatic embryogenesis.

Acknowledgements

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References

Cuenca B., Ballester A., Vieitez A.M. (2000) *In vitro adventitious bud regeneration from internode segments of beech.* Plant Cell Tiss. Org. Cult. 60: 213-220

Naujoks G. (2003) *Somatic embryogenesis in beech (Fagus sylvatica).* Biologia, Bratislava 58: 83-87.

Vieitez A.M., San-José M.C. (1996) *Adventitious shoot regeneration from Fagus sylvatica leaf explants in vitro.* In Vitro Cell. Dev. Biol.- Plant 32: 140-147

In vitro culture of *Pelargonium*

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Species from *Pelargonium* L'Hér. (*Geraniaceae*) genus, widely known as rose geraniums, are represented by aromatic as well as non-aromatic lines which are eagerly cultivated as popular indoor plants. In a successful commercial production of these plants, especially as an ornamental or therapeutic material, new cultivation technologies and healthy starting material are obviously crucial. *In vitro* organ and cell culture provide a perfect opportunity to overcome numerous limiting factors in plant production. It has been revealed that almost each *Pelargonium* genotype (Bakker et al., 2000) requires supplementation of specific components to the culture media. Therefore, in order to induce and sustain the morphogenetic capacity of a specific *Pelargonium* genotype, the optimization of culture conditions is really an important stage of propagation *in vitro.* The goal of this study was to establish an *in vitro* culture of two attractive *Pelargonium* × *domesticum* cultivars, that is 'Tip Top Duet' and 'Black Knight'. We were primarily focused on obtaining the well proliferative culture, and healthy looking shoots ready to be rooted. Further, epidermis in leaves of regenerated shoots, and the leaf anatomy were checked out. The idea was to evaluate the effectiveness of the applied regeneration protocol, and to gather some experimental data for future taxonomical comparisons.

Primary explants for culture initiation were excised from donor plants grown under greenhouse conditions. Nodal segments (about 10 mm long) with axillary buds were obtained from young shoots. They were surface sterilized and explanted on a set of initiation media containing 30g/l sucrose, BA and IBA, and solidified with 0.8% Difco agar. Additionally, one particular initiation medium was supplemented with 0.6 g/l activated charcoal. Some media for proliferation stage contained peptone, and they were supplemented with various concentrations of cytokinin BA and adenine. Secondary explants were excised from *in vitro* developed shoots. The micropropagation coefficient was calculated as a number of new shoots developed from one secondary explant.

During the stage of culture initiation all excised nodal segments developed into shoots. The total number of regenerated explants, however, was reduced because of contaminations. Viable 10 mm long explants were sufficient to initiate a culture, and to form new axillary buds. The regeneration efficiency was proved to be stable during passages. Microshoots obtained in every passage were rich green, up to several millimeters high, and approximately formed two leaves. The regeneration efficiency of *Pelargonium* × *domesticum* 'Tip Top Duet' was elevated in comparison to 'Black Knight'. During the proliferation stage the addition of peptone was advantageous, especially for 'Tip Top Duet' cultivar. After 14 days of culture the propagation coefficient was 26.5 for 'Black Knight' and 12.0 for 'Tip Top Duet'. The chemical composition of the medium optimal for effective shoot multiplication was elaborated. Differences in trophic requirements at different stages of *in vitro* culture have been shown. Finally, the real risk of the onset of endogenous bacterial infection was determined.

References

Bakker F.T., Culham A., Pankhurst C.E., Gibby M. (2000) *Mitochondrial and chloroplast DNA-based phylogeny of Pelargonium (Geraniaceae).* Am. J. Bot. 87(4): 727-734

Research on stimulation of protoplast development *in vitro*

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Plant protoplast develops as a result of mechanical or enzymatic removal of cell walls. In theory protoplasts are totipotent, which means that they are potentially capable of regenerating an organism. This property can emerge in specific conditions in culture, which initiate the change in the prime determination of cells. It is conditioned by the process of dedifferentiation and the change in the pattern of gene expression, which initiates the rebuilding of the wall and enable the cell to enter the renewed cycle of mitotic divisions. The emerging descendant cells, implementing various models of cytodifferentiation may differentiate into other types of cells in newly-developed organs or somatic embryos. This property makes protoplasts convenient tools in broadly understood biotechnology. In the majority of applications, the possibility of protoplast regeneration is an essential stage. Although there are some known protocols to obtain plants from protoplasts (protoplast-to-plant systems) for more than 400 species, mainly from such families as Solanaceae, Fabaceae, Gramineae, Asteraceae, Brassicaceae, Apiaceae or Rosaceae, but especially for a number of cultivated species, the efficiency of regeneration is still low or occasional. In recent years, we have seen the return to studies based on the isolation of protoplasts. In basic research, these are used in cell biology experiments, in particular those associated with the mechanism of cell differentiation. In the context of application, the manipulations made on protoplasts are used in the breeding of cultivated species to generate new genetic diversity via the fusion of protoplasts of cultivated species with related wild species or via *in vitro* selection. In plant breeding, the use of technologies based on the isolation of protoplasts must nevertheless be preceded by developing, most often highly efficient, universal, or dedicated to specific tissue, procedures for the regeneration of plants for a given species.

The main objective of the presented work was to study the stimulation of protoplast development under various conditions of *in vitro* culture. These studies were conducted chiefly on cultivated species of Apiaceae family including carrot (as a model object), parsley and coriander, but also on wild representatives of *Daucus* genus, as well as on sugar beet, commonly considered as a recalcitrant species in protoplast cultures.

Protoplast were isolated from 2-week-old *in vitro* grown plantlets or shoot cultures, embedded in alginate matrix and cultured as thin alginate layers (TAL) or extra thin alginate films (ETAF) in carrot petiole protoplast medium used as a basal medium (Maćkowska i in., 2014). The effect of alginate sterilization method (filter vs. autoclave), different systems of cell culture in alginate, and supplementation of protoplast medium with phytosulfokine and/or haemoglobin on plating efficiency and plant regeneration will be discussed.

References

Maćkowska K., Jarosz A., Grzebelus E. (2014) *Plant regeneration from leaf-derived protoplasts within the Daucus genus: effect of different conditions in alginate embedding and phytosulfokine application*. Plant Cell Tiss. Organ. Cult. 117(2): 241-252.

Doubled haploids as a material for biotechnological manipulation and a tool for rapid breeding of winter oilseed rape (*Brassica napus* L.)

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Oilseed rape (*Brassica napus* L.) is the third, after palm and soybean, most important source of vegetable oil in the world and a significant contributor to the economy of many countries. Due to its high performance in *in vitro* culture, oilseed rape is also considered one of the most suitable species for improvement through biotechnology. Cultured microspores of oilseed rape can be induced to develop into fully functional haploid embryos, instead of mature pollen grains. The ability of these cells to change their development in response to environmental stimuli is an exceptional example of totipotency in plants.

Doubled haploids (DHs) are produced by chromosome doubling of haploid plants, whereas conventional inbred lines are developed by selfing in successive generation. Haploids and DHs in *Brassica* ssp. have been produced using anther culture or isolated microspores. Since the method of isolated microspore culture became available (Lichter, 1982), this technique has continuously being improved and modified, and a number of androgenic plants from different species of the *Brassica* genus were obtained. The key to higher regeneration efficiency during androgenesis lies in the control of two crucial developmental points: the induction of microspore cell division and their commencement to embryogenic pathway. Haploids and doubled haploids of *Brassica napus* have been extensively used in genetic studies such as gene mapping, location of QTLs, marker/trait associations, genomics, and as targets for transformation. Nowadays, oilseed rape haploid induction can be efficiently combined with several other biotechnological techniques, enabling novel breeding achievements like hybrid breeding, mutation, genetic transformation and resynthesis of *Brassica napus*.

Advancements in *in vitro* androgenesis of oilseed rape permit to obtain DH populations on a larger scale, facilitating application of DH lines in genetic analysis of quantitative traits and research on environmental impact on yield and seed quality. Thanks to its homozygosity, a single DH line produces only one type of gametes which in controlled conditions can duplicate their own genotype. This allows to carry out multiple experiments with the same genotype, in various locations and years, which is not possible with a generation of segregated hybrid populations in classical breeding. Moreover, for traits controlled by polygenic inheritance, DH technology requires fewer genotypes, since there are no heterozygotes, thus no dominance effects, and no interactions of nonallelic heterozygous loci can be observed. A microspore culture is a useful tool in breeding, as homozygous lines exhibiting desired agronomic traits can be rapidly selected, creating opportunity for faster production of commercial cultivars. Cultivar Monolit (Plant Breeding Strzelce Ltd., Co.), is the first Polish winter oilseed rape variety obtained using doubled haploid technology. The breeding cycle of this variety was about four years shorter than a classical breeding program. The other Polish cultivar Brendy (Plant Breeding Smolice Ltd., Co.) was developed in a similarly short time. Currently, homozygous restorer lines are exploited in most breeding programs pursuing new hybrid varieties of oilseed rape.

Utilization of oilseed rape doubled the haploid technology in basic research and its application to improve qualitative and quantitative traits of oilseed rape will be presented in view of results obtained in the Institute of Plant Breeding and Acclimatization NRI in Poznań as well as ongoing research in the world.

References

Lichter R. (1982) Z. Pflanzenphysiol., 105: 427-434.

Identical microspore-derived plants – problem or benefit?

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The wide diversity of triticale gene pool has much potential for the future progress of conventional and hybrid breeding. There are however, certain problems, and many of those which occur in the triticale, particularly in relation to the registration of new cultivars, are connected with inequality and instability of the breeding lines. Genetic uniformity can be obtained through a number of inbreeding systems that differ in the procedure of selection but require many generations of repeated cycles. For these reasons, breeders are interested in a method which simplifies, but at the same time speeds up their work. Homozygosity can be achieved in a short time through a doubled haploid (DH) technology. The efficiency of DH lines production is critical when a system is used for practical purposes. Because each regenerated plant is derived from a single cell of a gametophytic pathway- microspore, each such line is expected to be unique and different from another. However, research with molecular markers identified a series of clones in androgenic populations of DH lines. In many laboratories the production of clonal plants via organogenesis and embryogenesis is used for rapid multiplication of morphologically and genetically uniform of valuable plant material for agriculture, horticulture and forestry. Moreover, propagation of individuals exploits totipotency of plant cells and offers a promising means for commercial mass production of pathogen-free superior clones. Although clone formation is important, especially in the context of micropropagation, clones regenerated during the process of androgenesis may have a negative effect. The presence of clones seriously reduces the efficiency of the DH approach and increases the cost and other workloads of production of useful lines. It is especially important during the creation of the mapping population due to the costs of a molecular analysis.

The main motivation for this experiment was regeneration of genetically uniform lines among androgenic plants of triticale genotyped by DArT markers. Hybrids from breeding crosses of winter hexaploid triticale from breeding companies were used as donor material. Microspore-derived plants were regenerated from selected androgenic structures regarding to protocol routinely used in the ZBiCR laboratory for the triticale. The development of selected androgenic structures was tracked. Each regenerated plant had its complete pedigree and the genetic status and each regenerant was verified with microsatellite markers. The existence of genetically uniform regenerants was identified as an unwanted result of androgenesis. The numbers of clones regenerated from individual structures were different. The clones originated by formation of polyembryos, twinembryos and callus tissue. The SSR analysis showed that all plants were obtained from microspores (none was derived from the somatic tissue of anthers). A high frequency of genetically uniform microspore-derived plants indicates the importance of precise determination of the origin of individual regenerants by molecular analysis to use them in further studies.

Induction of haploids in the genus *Secale* L. via androgenesis

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Induction of haploids via androgenesis (anther culture and isolated microspore methods) makes it possible to produce haploid and homozygous plants. As a result, the availability of homozygotes considerably facilitates selection of desirable combinations of genes from hybrid populations as these lines show no trait segregation.

The capacity for regeneration of haploid plants depends mainly on the genotype of a given crop. For this reason the optimal selection of a genotype makes it possible to increase the frequency of produced haploids, while a selection of rye breeding material in terms of the capacity for *in vitro* androgenesis may accelerate the breeding cycle of new cultivars. To date the greatest potential for efficient androgenesis has been found in species from the genera *Nicotiana*, *Datura*, *Hyoscyamus* and *Solanum*.

Important factors determining the process of androgenesis include abiotic stress in the form of low temperature of harvested ear storage. Temperature stress triggers the transition of microspore from gametophytic to sporophytic plant development. Other factors include the physiological status of the donor plant, the composition of the medium as well as culture conditions. It needs to be stressed that the diversity of factors affecting the process of androgenesis considerably hinders progress also in the work leading to the development of new cultivars.

Rye belongs to the genus *Secale* L., the class of monocotyledonous plants (Monocotyledonae), the family Gramineae and the subfamily Poaceace. It is a plant with a strongly developed root system, high resistance to low temperatures. Due to high resistance to pests, diseases and other stress factors as well as soil conditions it is a popular cereal grown in central, northern and eastern Europe. The aim of this study was to assess the capacity of plants in anther culture of 13 wild species, 5 cultivars and 9 inbred lines of winter rye, for androgenesis and regeneration.

Plants were grown in a greenhouse. After an adequate development phase was reached, ears were cut and treated with a temperature of 4 °C for different periods of time (from 2 to 30 days). Androgenesis was induced in anther cultures run according to the method consisting on using two media: C17 and 190-2. Not all tested genotypes showed the capacity to induce androgenesis in anther cultures. The greatest mean efficiency of androgenesis induction was found in *Secale cereale* ssp. *dighoricum* 5687 (0.22%) and Dańkowskie Diament (0.14%). Green and albinotic plants were regenerated in 5 out of 27 tested genotypes, with a total number of 23 regenerated plants. The greatest regeneration efficiency of green plants in anther cultures was recorded in cv. Dańkowskie Diament and Warko.

Rye is a particularly difficult plant to run *in vitro* cultures. The main aim of studies on its breeding under laboratory conditions is to develop a haploidization methodology. The most frequent problem is connected with the low percentage of regenerated plants and a high percentage of albinotic plants. Another drawback observed after the application of colchicine is a considerable percentage of spontaneously formed sterile plants in comparison to double haploids. Initial stages of androgenesis are obtained much more frequently than the regeneration of the entire haploid plant.

Efforts to obtain the largest possible number of plants regenerated from single haploid cells extend genetic variation and promote further improvement of rye cultivars, while the produced double haploid lines constitute a valuable source of plant material for breeding programs.

Albinism – common phenomenon during cereal androgenesis

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Androgenesis is a process of development of the male cells of the gametophytic pathway – microspores, leading to the formation of embryos which regenerate into plants. The plants are doubled haploids (DH) or, in other words, homozygous organisms at every allele pair. This trait makes the DH plants an attractive object of genetic and genomic research. Androgenesis is also highly useful for plant breeding as it accelerates the breeding cycle by shortening the time required to achieve homozygosity from several to one year, allowing diversification of the range of genotypes. Nevertheless, for a widespread use in the breeding practice or other biological sciences, this process must be efficient, which also means that from a single spike/panicle a many fertile plants have to be regenerated, independent of genotype. Despite several decades of research into the androgenesis phenomenon there are still many barriers that must be overcome to improve its efficiency. One of problems inherently associated with the androgenesis process is albinism.

Albinism is a phenomenon where plants are unable to produce chloroplasts and thus carry out the process of photosynthesis. Albinism occurs in androgenesis-developed plants via anther and microspore culture, in nearly every cereals, such as wheat, rye, triticale, oat or barley. Within the same species, there are usually genotypes that are more susceptible to albinism than others. In most susceptible cultivars, all obtained plants lack chloroplasts (99.7% albino plants), while in other cultivars, the fraction of albino plants is insignificant (1% albino plants). It was reported that during the regeneration process in the anther culture in barley, more albino plants occur in spring cultivars than in winter ones. This can be explained by evolutionary adaptations of winter genotypes to stress related to low temperatures, which results in a better response to androgenesis induced by low-temperature stress than that in the spring ones. However to date, there has been no clear explanation of reasons for the phenomenon of albinism. The differences between albino and green plants are found on three main areas: cytological (structural changes in plastids at different stages of androgenesis that prevent the formation of chloroplasts), plastid genome (differences in the genomes of green and albino plant plastids) and nuclear genome (nuclear genes, which are responsible for a large number of albinos in some cultivars). Many studies demonstrate that by optimising the various parameters in an androgenic *in vitro* culture, a reduction in the number of albinos is possible. The most important external factors contributing to regeneration albino plants are: the conditions created for the growth and development of donor plants, the appropriate stage of microspores, the type of reprogramming stress used, the choice of *in vitro* culture method in androgenesis and the composition of the induction and regeneration media. A comprehensive understanding of causes of albinism and changes leading to albino plant regeneration would make it possible to manipulate *in vitro* culture conditions to eliminate this undesirable phenomenon.

This presentation is based on the current knowledge and our results of research in the field of cereals androgenesis.

The study on possible mechanisms involved in gametic embryogenesis of sugar beet (*Beta vulgaris* L.)

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For breeding of new sugar beet (*Beta vulgaris* L.) lines and improving the existing ones, the doubled haploid (DH) production is of critical importance. The traditionally achieved homozygosity requires the performance of time-consuming and labor-intensive back crosses. Owing to the induced process of chromosome doubling that takes place during the early stages of haploid embryo development, fertile double haploid plants can be easily regenerated within a short period of time. Of all the haploidization techniques tested so far for sugar beet gametic embryogenesis is the only possible method. Unfertilized ovules a few days before anthesis stage are required for viable gynogenetic embryo regeneration, which originates predominantly from the egg cell. The success of haploid induction depends on the induction method, developmental stage of ovules, pre-treatment or physical factors during tissue culture. However, the influence of genotype of the donor plant on the regeneration rate should be pronounced, so the detection of genotypes with a high gynogenic response is important.

Despite a considerable advance in doubled haploid production, currently little is known about the molecular mechanisms during the induction of embryogenic pathway in particular sugar beet genotypes. From a developmental point of view, the gynogenesis is a rewarding system for understanding the process of embryo formation from single, haploid egg cell. The identification of genes that might serve as stage-specific markers of ovules embryogenesis would help in further understanding of the above mentioned process. Any progress revealing molecular determinants in gynogenesis can lead to improvements in the use of haploids and further doubled haploids during genetic studies and breeding programs. In relation to morphogenetic potential, the presence and changes in plant cell wall composition have been previously described. Especially pectins and arabinogalactan proteins (AGPs) are the major cell wall components implicated to the development and differentiation of plant cells and tissues. The above mentioned compounds are widely distributed throughout the plant kingdom and occur either in intercellular species, plasma membranes and certain cytoplasmic vesicles.

Therefore, the preliminary characterization of gametic embryogenesis at the cytological and molecular level among selected sugar beet genotypes will be discussed. A biochemical, immunocytological and molecular approaches were employed to locate and analyze the biological role of endogenic arabinogalactan proteins and to identify differences in genes expression during the regeneration of sugar beet unpollinated ovules by differential display technique. A comparison of selected breeding lines with two embryogenic potential abilities: high and low was performed. The results will broaden the knowledge about the basic events taking place during the regeneration of unpollinated ovules and will help to improve the efficiency of the above mentioned process.

Analysis of genetic components of winter oilseed rape (*Brassica napus* ssp. *oleifera*) regeneration ability under *in vitro* culture

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Winter oilseed rape (*Brassica napus* ssp. *oleifera*) is described mostly as a good source of plant material to conduct *in vitro* cultures; however the species reveals a considerable genotypic diversity concerning regeneration ability. Regeneration is a prerequisite in obtaining valuable plant material under *in vitro* conditions. Genotypes with high regeneration ratio are a priceless tool in biotechnological research, especially in genetic engineering. Despite the research progress which has been made, many important aspects of plant regeneration in *in vitro* culture are poorly known. The present study is an attempt to investigate the genetic background of regeneration of rapeseed plants by means of a classical genetic analysis. The aim of the research was an analysis of genetic components of the regeneration ability of winter oilseed rape explants (*Brassica napus* ssp. *oleifera*) incubated on an MS medium with the addition of 3.0 mg/l BAP (6-benzylaminopurine) and 0.3 mg/l NAA (1-naphthaleneacetic acid).

Plant material used in the study was 5 doubled haploid lines of winter oilseed rape (*Brassica napus* ssp. *oleifera*), showing different regeneration abilities under *in vitro* conditions. The material also consisted of the F1 hybrids obtained from diallel crosses of doubled haploids. The collected plants were used to analyze the four characteristics which make up the overall picture of the regeneration ability of rapeseed explants cultured *in vitro*. These were: the efficiency of shoot regeneration from hypocotylous explants, the number of regenerated shoots per single hypocotyl explant, the efficiency of shoot regeneration from cotyledon explants with a petiole, the number of regenerated shoots per single cotyledon explant with a petiole. On this basis, the significance of the general (GCA) and specific combining ability (SCA), the effects as well as the heritability in the broad and narrow sense were estimated.

The evaluation of the general combining ability effects revealed that the majority of GCA effects were not statistically significant. There were no positive GCA effects for any of the DH lines, while the negative effects of general combining ability were demonstrated for the effectiveness of the shoot regeneration from hypocotyl explants (lines W15 and W131), the number of shoots regenerated per a single hypocotylous explant (lines W15 and W131) and the number of regenerated shoots per single cotyledon explant with a petiole (line W131). None of the F1 hybrids revealed significant SCA effects for the analyzed traits. The values of heritability in the broad and narrow sense were calculated, which proved to be very low. For the efficiency of shoot regeneration from hypocotyl explants heritability in the broad and narrow sense amounted to 0.074 and 0.06, and for the efficiency of shoot regeneration from cotyledon explants with a petiole were 0.035 and 0.008 respectively. The number of regenerated shoots per hypocotylous explant was characterized by slightly higher values of heritability, which amounted to 0.11 in a broad sense, and 0.085 in the narrow sense, as well as the number of regenerated shoots per single cotyledon explant with a petiole, which were 0.057 and 0.037 in the broad and narrow sense respectively. The obtained results confirmed that quantitative traits associated with regeneration under *in vitro* condition, such as shoot regeneration frequency and the number of shoots per explants, are characterized by high variability, which further hindered the genetic analysis of these characteristics. Although the obtained results have generally widened our knowledge of the genetic determinants of the phenomenon, they still require further study and observation.

Application of new biotechnologies in *Brassica napus* L. resynthesis

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Oilseed rape (*Brassica napus* L., AACC, 2n = 38) originates from a spontaneous interspecific hybridization between turnip rape (*Brassica rapa* L., AA, 2n = 20), and cabbage (*Brassica oleracea* L., CC, 2n = 18). The current oilseed rape gene pool has relatively narrow genetic diversity, resulting from a limited geographical area of cultivation, selection during the formation and subsequent improvement of this species (most of the current 00-quality cultivars derive from common ancestors). For this reason, there is a need to introduce a new genetic variation to the breeding material. One strategy for broadening the genetic base of oilseed rape germplasm is to exploit the diploid progenitor species of *B. napus*, specifically *B. rapa* and *B. oleracea*. Both of these species possess extensive variability in morphology and agronomic characteristics, and represent a valuable resource for improving the pathogen and pest resistance, tolerance of abiotic stress, and heterosis Thus, the resynthesis of a new *B. napus* from interspecific crosses between the original ancestor species has been aimed to increase the genetic variation in this species. The development of new biotechnologies like *in vitro* culture (including *in vitro* pollination, embryo rescue) and molecular techniques for detection of valuable genotypes, allow a much broader and targeted approach.

In the present study, resynthesized (RS) oilseed rape was obtained by reciprocal crosses between *B. rapa* and *B. oleracea* by 1) *in vivo* pollination where 51 RS oilseed rape were obtained, and 2) by *in vitro* placental pollination where 30 RS oilseed rape were obtained. Two subspecies of *B. oleracea* and three subspecies of *B. rapa* were used in the hybridization process. In earlier work, prezygotic incompatibility barriers between genetically distant species were reduced by applying *in vitro* placental pollination, whereas postzygotic barriers were avoided by an *in vitro* culture of enlarged ovules with embryos. In this study, ovules were isolated from pistils or ovaries from 7 to 15 days after pollination (dap). Only ovules isolated after 12-15 dap were suitable for further development. The average efficiency of obtaining new oilseed rape plants from enlarged ovules was around 6.1% in *in vivo* pollination, and 6.8% in *in vitro* pollination. To confirm their hybrid genotype, all plants were tested for nuclear DNA content via flow cytometry. An analysis of leaf samples showed that the received hybrids were amphihaploid (n = 19). The number of chromosomes was doubled using colchicine. Further cytogenetic studies of these hybrids are being conducted. The phenotyping analysis of RS *Brassica napus* plants indicated their large morphological diversity (leaf shape and/or a color; flower size). For measurements of pollen fertility, pollen grains were stained with 1% acetocarmine solution. Well-filled pollen grains with stained nuclei were regarded as fertile, while unstained pollen grains were counted as sterile. Pollen fertility of the analyzed resynthesized plants ranged from 49.5% to 92.6%. Genetic similarity of resynthesized *B. napus*, their parental lines, and different cultivars of winter oilseed rape, were determined by AFLP-PCR using 10 fluorescently labeled primer combinations. The dendrogram based on AFLP markers showed that resynthesized lines of *B. napus* formed a group genetically distinct from the compared cultivars of winter oilseed rape.

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Auxin-related functions of LEAFY COTYLEDON2 gene in the induction of somatic embryogenesis in Arabidopsis

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Somatic embryogenesis (SE) induced *in vitro* exemplifies the developmental plasticity of the plant somatic cells while genes that trigger embryogenic transition in differentiated explant cells are intensively identified. A significant progress in SE studies has been made recently owing to the application of expressive genomic tools, transgenic lines and mutants of Arabidopsis, a model plant in genomics. Several transcription factor (TF) genes were indicated to be essential for SE induction and among them LEAFY COTYLEDON2 (LEC2), a master regulator of zygotic embryogenesis (ZE) in Arabidopsis. To reveal the genetic components of LEC2-dependent pathway of SE induction, explants of Arabidopsis undergoing embryogenic transition on 2,4-dichlorophenoxyacetic acid (2,4-D) -supplemented medium were studied. Gene expression profiling and mutant/transgenic line analysis indicated auxin related functions of LEC2 during SE induction. Accordingly, LEC2 was found to promote the embryogenic pathway in somatic cells through stimulation of auxin biosynthesis. Three of YUCCA (YUC) genes, YUC1, YUC4 and YUC10 which encode flavin monooxygenases involved in tryptophan depended pathway of auxin biosynthesis, were proven to be up-regulated by LEC2 in explants undergoing SE induction. In addition, GFP-monitored expression of YUC genes was detected in SE-involved regions of the explants i.e. cotyledons, shoot apical meristem and developed somatic embryos. Further support on the essential role of YUC genes in SE induction allowed to observe embryogenic capacity of yuc mutants. Two of them, yuc2 and yuc4, were found to display an impaired embryogenic potential. Relevantly to the hypothesis on LEC2-stimulated activity of auxin biosynthesis genes in SE induction, an elevated level of indole-3-acetic acid (IAA) was observed in tissues overexpressing LEC2 gene. The results also suggest that LEC2 may impact SE induction through the regulation of the components of auxin signaling pathway. Accordingly, two of AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) genes, the key regulators of auxin-response pathway, IAA30 and IAA31, were observed to be highly stimulated by LEC2 during SE. However, the relation between LEC2 and AUX/IAA genes needs further analysis.

Auxin-stimulated expression of LEC2 and a presence of Auxin Response Element (AuxRE) in LEC2 regulatory region suggest that the gene may be controlled by AUXIN RESPONSE FACTORS (ARFs), the key regulators of auxin-responsive genes. Thus, to identify ARFs engaged in SE induction, especially those involved in regulation of LEC2 activity, an expression profiling of all 22 ARF genes of Arabidopsis was conducted in an embryogenic culture. The qRT-PCR and GFP-reporter lines analysis indicated that the majority (14) of ARFs were active in tissues subjected to SE induction. Moreover, the arf mutants and overexpressor lines were evaluated in the embryogenic culture. ARFs with a significantly modulated expression in SE coupled with an impaired embryogenic response of the relevant mutant and/or overexpressor line were identified, including ARF1, ARF2, ARF3, ARF5, ARF6, ARF8 and ARF11. Among the candidate ARFs involved in SE induction, ARF5 encoding MONOPTEROS (MP) protein of a key role in ZE, was indicated. ARF5 was found to be highly up-regulated in SE and arf5 mutant displayed a distinctly reduced SE response. However, ARF5-regulated targets that control SE induction remain to be revealed.

Gibberellin metabolism pathway activity with relation to LEC2 and FUS3 transcription factors during the induction phase of *Medicago truncatula* cv. Jemalong somatic embryogenesis

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Somatic embryogenesis (SE) is a phenomenon of plant cell totipotency resulting in somatic embryos formation through an a-sexual process. There have been proposed many concepts and mechanisms of regulation which could be mediated by stress, hormones and transcription factors (TFs) as crucial elements of the puzzle. Besides a great progress in understanding the impact of exogenous hormones and plant growth regulators (PGRs) on these processes. There still remains data concerning endogenous hormone levels and their importance as regulators.

Previous studies have shown that SE of *Medicago sativa* L. requires the presence of endogenous gibberellins, or else its exogenous application during the induction has an inhibitory effect on the process. The crucial steps of GA biosynthesis in plants take place in the cytoplasm while GA_{12} pass through 13-hydroxylation and not-hydroxylation pathways resulting in bioactive gibberellins. Individual steps of biosynthesis are catalyzed by two families of oxidases, namely GA_{20}-oxidases and GA_3-oxidases. The deactivation of active gibberellins is carried out by a family of GA_2-oxidases. To date, there has been no information on endogenous gibberellin metabolites levels measured in the course of SE induction process.

Two of the B3 domain containing transcription factors LEAFY COTYLEDON 2 (LEC2) and FUSCA 3 (FUS3) are regarded as master regulators of plant zygotic embryogenesis. Previous experiments conducted on a loss of function and over-expression mutants confirmed that they are required for Arabidopsis somatic embryogenesis. Both of proteins are capable of inhibiting AtGA3ox2 gene activity through binding to specific RY promoter motif. To date, there is has been no data concerning the activity of those genes in *Medicago* somatic embryogenesis, nor any information connecting endogenous gibberellins level with gene expression during these processes. Leaves of two *Medicago truncatula* cv. Jemalong lines, M9 not-embryogenic line (NEL) and M9-10a embryogenic line (EL) were used as explants. Plant material for all analyses was sampled at five time-points of the induction process.

The results of the LC-MS analysis of 20 gibberellin metabolites indicated a 13-hydroxylation pathway as an active one in EL just after one week of induction, resulted in a 14 times higher GA_{53} and 48 times higher GA_{19} metabolites amounts when compared to NEL. The amount of active gibberellin GA_3 in EL dramatically increased starting from day 14 up to 10 times higher amount compared to NE line and until 21st day of induction remained elevated.

Expression profiling confirmed up-regulation of two early gibberellin biosynthesis genes of ent-kaurenoic acid oxidase MtrKAO2 and MtrKAO3, amount of transcripts was respectively 7 and 4 times higher in embryogenic line after one week induction. We also confirmed changes for two *Medicago truncatula* GA_3ox genes activity in EL. MtrGA3ox1 gene expression was raising up to day 7 and was 6 fold higher than in NEL after that gene were down-regulated until end of induction. Expressions of MtrGA3ox2 gene were induced only in EL starting from day 14 where expression was 14 times higher if compared to NEL and remained at the same level to the last day of induction.

A gene expression analysis of two classical transcription factors MtrLEC2 and MtrFUS3 indicated that changes in expression were observed only in the embryogenic line. Transcripts of both genes started to increase just after 7 days of induction and continued to the 21st day ended, respectively, with 1400 fold and 350 fold higher amount compared to NEL for both analyzed genes.

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Changes in cell physiology at the stage of dehydration in a cryopreservation protocol

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Safe cryopreservation of plant material in ultra-low temperature requires an establishment of appropriate procedures which include a range of activities that aim to protect the material from dehydration stress, freezing in liquid nitrogen, and thawing. Encapsulation-dehydration is the most reliable cryopreservation method for embryogenic competence of *Gentiana cruciata* cell suspension to preserve viability and embryogenic potential.

A comparative proteomic analysis was performed at every step of a dehydration process in a cryopreservation protocol which runs as follows: embryogenic cell suspension of *Gentiana cruciata* was encapsulated in 1.3% calcium alginate beads, incubated for 48 hours in each medium containing 0.3 M, 0.5 M and 0.75 M sucrose, and finally transferred to 1 M sucrose for 24 h. After this pre-treatment capsules were air dried and cooled in liquid nitrogen. Protein profiles were done after each stage of incubation in a medium supplemented with a high concentration of sucrose using a 2D-PAGE technique. Protein identification by mass spectrometry for statistically significant differences between the intensity of spots in comparable profiles was performed using a LC-MS/MS method. The total amount of 103 proteins was identified and assigned to 14 protein groups connected to a range of metabolic functions and pathways in the cell: glycolysis; gluconeogenesis; TCA cycle; pentose-phosphate pathway; fermentation; biosynthesis and metabolism of fatty acids and lipids; purine biosynthesis; amino acids metabolism; transcription, translation and degradation proteins; the use of proton gradient; cell organization; protection and defense of the cell; reaction of the reactive oxygen species; signal transduction. Various type profile changes of proteins in escalating dehydration stress were found on the obtained images – peptides with increasing, decreasing and mixed intensity profiles of expression after subsequent protocol steps.

A compilation of MS identification data and a comparison of alterations in spot profiles of particular proteins allowed an analysis of metabolic changes under this type of stress. Energy metabolism was identified to be the most sensitive function with the greatest number of variations in the intensity of spots. The balance of carbohydrate metabolism was shifted towards the production of energy via glycolysis. The Functioning of the TCA cycle was inhibited, with fermentation being favored method of respiration. A strong link between reactive enzymes was observed in the form of utilisation of carbohydrate metabolism products in other cellular processes such as metabolism of amino acids, fatty acids and lipids as well as a synthesis of cell wall components. A particularly important pathway that increased its activity under the conditions of dehydration stress was the synthesis and accumulation of starch for osmoprotection.

The physiology of gentian cells in suspension observed at the proteome level during a prolonged dehydration process of cryopreservation was reprogrammed and acquired the tolerance of dehydration and freezing. It also maintained viability and embryogenic potential after having been stored for some time in liquid nitrogen.

Epigenetic modulation of the activity of transgen and endogenous gene based on the example of chalcone synthase in flax

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Throughout human history people have struggled to obtain improved plants. The methods used to achieve such plants varied and changed over ages – originally simple selection was used, then intra- and interspecific crossing, mutagenesis and the last century has witnessed a more specific genetic modification. Changes in simple genes between selected /generated plant and wild plants were small – C replaced T or A replaced G – which is a frequent result of methylation of cytosine. The same changes will be observed between gene isoforms among species.

Nowadays we call these modifications epigenetic modification and we know that such changes will be generated by stress conditions, mutagens and, what is important, will be stable across generations. But the questions are: If genes are introduced to plant genome by genetic modification will they be modulated in the same way?" Can we generate similar stable epigenetic changes under laboratory conditions?

The phenylpropanoid pathway is one of most frequently modified metabolic pathways. Phenylpropanoids are a diverse class of natural plant compounds and consists of e.g. simple phenolics (benzenoid derivatives), flavonoids, tannins and lignins. Chalcone synthase is one of the key enzymes of this pathway. The changes in activity of this enzyme resulted not only on anthocyanin, phenolic acids and lignin level but also on fatty acid metabolism. Introduction to flax genome exogenous chalcone synthase resulted in twofold way – by an increase in gene expression or by a decrease in the exogenous and endogenous gene expression. Both changes were manifested by different methylation profile – plants with overexpression of chalcone synthase indicated a decrease in total genome methylation whereas plants with repression of chalcone synthase showed an increase of total genome methylation. Differences in specific methylation sequences (CCGG sites) in chalcone synthase genes were also observed. Such modifications concern both, endogenous and introduced chalcone synthase, and they are stable during at least five generations.

In transgenic plants differences in expression of epigenes (DNA chromomethylases and demethylases, histone acetyltransferases) were indicated. Such observation allowed us suggest epigenetic origin of observed changes in genes activity. The other problem is to generate the same epigenetic changes without genetic modification in which, the desired alteration in gene / genes expression will be performed with natural methods using oligonucleotides derived from the manipulated gene. The results of primary experiments convince such naturally modulated / improved plant generation. Plants obtained using the above method indicate similar to transgenic plants changes in chalcone synthase endogenous gene expression, total genome methylation level, specific methylation sites pattern. The results obtained on green tissue seem to be similar to the previously observed generated transgenic plants. An alternative to GMO method to get improved plants allows using the great scientific potential and application potential gathered for years of experiments with transgenic plants.

Encapsulation-dehydration technique for chrysanthemum "Lady Orange" cryopreservation

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Chrysanthemum is among the most popular species on the ornamental plants market. The number of available cultivars is constantly growing and they constitute a valuable breeding material source. A fast and easy access to high quality gene banks of a great material variety is the key for ornamental plant producers. Thus an efficient method for long-time conservation of the plant material may be extremely valuable for breeding and horticultural production. Over time several medium- and long-term plants storage methods have been developed. Traditional storage (cultivation in the ground or in the glasshouse), however, is expensive, work-intensive and threatened with loss due to pests and diseases or bad climate conditions. *In vitro* long-term storage, on the other hand, may lead to the occurrence of somaclonal variation and material loss due to human errors and development of bacterial/fungal contaminations. Today, cryopreservation is believed to be the most effective long-term storage method. Several chrysanthemum cryopreservation techniques have been developed with various levels of success. Cryopreservation usually does not influence the characteristics of the plant material. However, there are some reports about (epi)genetic disturbance after storage of chrysanthemum in liquid nitrogen. The reason is usually not the storage in liquid nitrogen itself, but the use of *in vitro* procedures which do not guarantee genetic stability, during preculture and/or at the restoration phase. Chimeras are particularly vulnerable to variation occurrence after cryostorage unless all tunica layers are properly protected. Therefore several criteria should be considered when developing a cryopreservation procedure of this species. These reports emphasize the need to monitor the stability of samples stored in liquid nitrogen by using different markers (molecular, phenotypical and/or biochemical).

The aim of this study was to determine the effect of sucrose concentration during preculture and the time of osmotic-dehydration on the efficiency of chimera chrysanthemum 'Lady Orange' shoot tips cryopreservation by encapsulation-dehydration. The recovered plants were verified at the phenotypic, biochemical and molecular levels. Shoot tips were precultured on MS medium supplemented with different sucrose concentrations of 0.09, 0.25 or 0.5 M for 14 days, encapsulated in sodium alginate and then osmotically dehydrated in sucrose gradient for 4 or 7 days. The best explant survival after cryopreservation reaching about 50% was obtained with the lowest (0.09 M) sucrose concentration, and 4-day-long osmotic dehydration. Higher sucrose concentrations slow down shoot growth, stimulate their vitrification and conduce to the regeneration via callus, while encapsulation inhibits rooting. Longer dehydration also led to increased formation of multiple shoots. The analysis of the phenotype (inflorescences and leaf colour, diameter and weight, flowering time and plant habit) and biochemical activity (pigment content in ligulate flowers and leaves) as well as the cytogenetic analysis and genetic markers confirmed the stability of the plants obtained after liquid nitrogen treatment. However, it was noted that the leaves of shoot tips cryopreserved-derived plants were smaller and had a reduced amount of chlorophyll, and their internodes were shorter when compared to the controls. Furthermore, their inflorescences often opened slower. Finally, these phenotypic changes are positive from the horticultural production point of view. This confirms the validity of utilizing cryopreservation in the protection of valuable plant material.

Morphogenic responses of Arabidopsis explants cultured *in vitro* in relation to the level of oxidative stress

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Oxidative stress is defined as an imbalance between antioxidants and oxidants activity in favor of the latter. The reactive oxygen species (ROS) are required in low concentrations for normal growth and development while under high ROS level various cellular components (proteins, lipids, carbohydrates, nucleic acids) can be damaged. ROS production is controlled by antioxidants which prevent a cell from excessive damages. The oxidative stress can be generated during natural biological processes in cells (e.g. aging) or it is induced by unfavorable environmental factors (e.g. water scarcity). Oxidative stress factors were indicated to modify embryogenic potential of *in vitro* cultured plant tissues and numerous genes related with stress responses were found among those differentially expressed in embryogenic cultures of different plants. Accordingly, it is believed that somatic embryogenesis (SE) is a specific response of plant somatic cells to oxidative stress related to *in vitro* culture conditions.

In the present study the role of oxidative stress in the mechanism governing SE induction was evaluated in the culture of Arabidopsis explants. In a culture of immature zygotic embryos (IZEs) different morphogenic responses were induced, including somatic embryo (SE), adventitious shoot (ORG) and seedling (E0) development. To modify the oxidative stress level, the culture media were supplemented with glutathione (GSH) and alloxan (AL) at various concentrations. In the presence of GSH (antioxidant) a reduced level of oxidative stress is expected. In contrast, under AL treatment (increased production of ROS) an increased level of oxidative stress in the culture is produced. The effect of these chemicals on the efficiency and productivity of SE was analyzed to evaluate the relation between the level of oxidative stress and the embryogenic potential of the culture. In addition, the impact of different oxidative stress levels on shoot (ORG) and seedling (E0) development was also analyzed.

It was observed that in explants cultured on an auxin medium both, a reduced and an increased level of the oxidative stress impaired the embryogenic response of explants. In contrast to the auxin medium, a positive impact of the reduced oxidative stress level on the embryogenic potential of the culture was observed on a hormone free medium. It was found that a low level of oxidative stress may substitute for auxin treatment required to induce SE in the control culture and somatic embryo formation was observed in explants cultured on a hormone-free medium supplemented with 0.1 mM and 0.5 mM of GSH. Moreover, GSH treated cultures induced towards ORG were found to produce an increased number of shoots per explant.

The results showed that GSH treatment of cultures distinctly affects the morphogenic potential of Arabidopsis explants, including (I) stimulation of shoot regeneration on hormone media (II) reduction of embryogenic response on auxin medium and (III) induction of SE on auxin free medium. In conclusion, the results of the study indicate a significant impact of oxidative stress level on the morphogenic potential of explants cultured *in vitro* and shows that GSH treatment can replace the auxin required for SE induction.

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The application of nanometal colloids in plant *in vitro* culture

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In vitro technique is one of the biotechnological methods enabling a creation of new valuable cultivation materials and improving a variety of plants. An essential element involved in conducting a plant *in vitro* culture is explant disinfection. It is indispensable when it comes to preserving sterile culture conditions. Sterility guarantees proper plant growth and development. It is crucial to ensure a correct way of plant material disinfection. The first stage of seed sterilization is rinsing the seeds in running water, which allows to remove dust and other smaller pollutions. The next stage is pre-sterilization with 70% ethanol, which increases the effectiveness of the final disinfectant through degreasing the surface and eliminating the air. Proper sterilization is conducted using active substances that contain active groups that act at various speed and with different effectiveness. In plant *in vitro* cultures the most frequently used disinfectants are ethanol, sodium or calcium hypochlorite, hydrogen peroxide, chloramine T and mercury (II) chloride. Moreover, commercial chemicals composed of some of the above-mentioned substances are commonly used, e.g. ACE containing disinfectant – NaClO.

The drawback of these well-known ways is the use of chemicals that can damage seed or embryo hindering germination. Frequently, certain ways of sterilization are used for seeds of only one species. The problem also seems to be complexity and multistage of treatments, e.g. seed disinfection and detoxification. Furthermore, using multistage seed sterilization, and rinsing the seed in particular, may trigger secondary infection.

Due to its specific structure and differences from metallic silver, nanosilver can be characterized by a whole array of interesting features. It manifests very strong antibacterial and antifungal properties. It has also been found that even in low concentrations (5 ppm) a bacteriostatic effect can be seen. At 10 ppm concentration, nanosilver virtually hinders the growth and development of Gram(+), Gram(−) bacteria and fungi of Trichocomecaceae such as *Aspergillus fumigatus* or *Aspergillus niger*.

The conducted experiments involved water solutions of spherical nanosilver and nanocopper in the following concentrations: mix of Ag Cu 10 ppm and 20 ppm, Ag 10 and 20 ppm, Cu 10 and 20 ppm. Preliminary sterilization with the use of 70% ethanol (1 min) was carried out as well as the proper sterilization with nanometal solutions (8 min). The sterile seeds were inoculated onto the medium excluding the phase of numerous rinsing them in redistilled water. The lack of infected seeds in subsequent repetitions gives evidence of strong antibacterial and antifungal properties of nanometals. When it comes to seed sterilization, silver nanoparticles at concentrations of 10 ppm and 20 ppm proved their 100% effectiveness, which is higher than in the case of sodium hypochlorite. The seedlings obtained owing to such modern method of sterilization are characterized by normal type, green color and typical for this species growth ratio.

Effect of light emitting diodes on the growth and development of *Gerbera jamesonii* 'Dura' in *in vitro* cultures

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Rapid advances in LED lighting technology and a higher fixture efficiency provided an expanding number of options for lighting in horticulture. Light is a key factor influencing the growth of green plants. Plants react to light mainly via photosynthetic, photomorphogenetic and phototropic responses dependent on the photon flux density (PFD), light quality, and the photoperiod. During *in vitro* growth of plants, the photosynthesis level is relatively low. The growth and development of *in vitro* cultures depend on an external supply of artificial light provided by fluorescent lighting. The use of light-emitting diodes (LEDs) as a main radiation source for plants during *in vitro* cultivation has attracted considerable interest in recent years due to low energy consumption and a scientific potential for basic research on plant physiology. However, the application of LED has some limitations and one of them is the lack of precise data about its influence on the morphogenesis and multiplications efficiency of individual plant species.

Gerbera jamesonii is one of the most commercially important ornamental plant, and *in vitro* propagation is the major technique used for multiplication of gerbera. The aim of the present studies was to test the effect of LED light sources on gerbera multiplication and to assess the physiological parameters of shoots, in comparison to plantlets cultured under standard fluorescent light. Shoot micropropagation of gerbera "Dura" was obtained on the Murashige and Skoog media supplemented with 5 μM BA and 0.5 μM NAA, 3% sucrose and 0.5% BioAgar, pH 5.7. Six different types of LED lights were tested: red 100% (R); blue 100% (B); 30% red and 70% blue (3/7 RB); 50% red and 50% blue (RB); red 40%, blue 40% and white 20% (RBW); red 49%, blue 40% and far red 2% (RBfR). Plants cultivated under conventional Philips TL-D 36W/54 lamps were used as a control. Cultures were maintained under 16-hour photoperiod, photosynthetic photon flux density (PPFD) of 35 μmol $m^{-2}s^{-1}$, temperature of 25/23 ± 1 °C (day/night) and 80% relative humidity. After 6-week cycles, biometrical observation were recorded (shoot multiplication rate, height of plants, number of leaves). Also fresh and dry mass of shoots were measured and leaf phenotyping analysis was performed by scanning all leaves and with the use of ImageJ software and LeafJ plugin (Maloof et al., 2013). The content of photosynthetically active pigments (chlorophyll a and b, and total carotenoids) was estimated according to Lichtenthaler and Buschmann (2001) spectrophotometric method.

The highest multiplication rate was noted down under the combination of red and blue light at both tested proportions (14.8-15.6 shoots per explant). Moreover, the red and blue light in the proportion of 7/3 enhanced fresh weight of plantlets. Blue light alone decreased multiplication rate of gerbera shoots (6.8 shoots per explant). The addition of far red to blue and red spectrum (RBfR) led to the reduction in leaf dry weight (6.5%) in comparison to the highest level observed under the red LED (8.1%). Monochromatic light, red and blue alike, caused reduction of the leaf blade size of gerbera. Plantlets grown under red light (R) were the highest, and had the longest petioles and the largest photosynthetic pigments content.

Acknowledgements

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References

Lichtenthaler H.K., Buschmann C. (2001) *Extraction of photosynthetic tissues. Chlorophylls and carotenoids. Measurement and characterization by UV-VIS spectroscopy*. Curr. Protoc. Food Anal. Chem. F4.2, F4.3.1, Suppl.1.

Maloof J.N., Nozue K., Mumbach M.R., Palmer C.M. (2013) LeafJ: *An ImageJ Plugin for Semi-automated Leaf Shape Measurement*. J. Vis. Exp. (71), e50028, doi:10.3791/50028.

Effect of AlgaminoPlant and Route® on anatomical, biochemical and molecular changes during acclimatization to *ex vitro* conditions of common ninebark (*Physocarpus opulifolius* (L.) Maxim.) "Dart's Gold"

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Common ninebark (*Physocarpus opulifolius* (L.) Maxim.) "Dart's Gold" is a popular ornamental shrub used in urban greenery. The most common method of its propagation is by cuttings; however, it does not always give satisfactory results due to problems with rooting. For the initiation of rhizogenesis, nurserymen use rooting powders containing synthetic auxin. Because of the planned withdrawal of these preparations from the market, producers are looking for alternative methods of reproduction. Currently, tissue cultures have become increasingly popular, and the development of this method allows the production of high quality seedlings that have commercial application. Despite this, nurserymen do not always decide to buy *in vitro* material. The reason for this is the high price of plants, as well as their difficulties in adapting to the greenhouse or field conditions.

Plants propagated *in vitro* often have a limited amount of epicuticular waxes, poor cuticles and malfunctioning stomata. In addition, photosynthesis occurs at a low level. These abnormalities can lead to physiological disorders, which is the main cause of death for micropropagated plants after their transfer to *in vivo* conditions. Acclimatization stress can be minimized by the use of biopeparations, which have a positive effect on the overall condition of the plant. Biopreparations impact plants by increasing the level of naturally occurring tolerance/resistance in plants to the stressor, and in stress-free conditions for better use of the genetically determined abilities.

The objective of this study was to determine the effect of biopreparations, AlgaminoPlant and Route®, on anatomical, biochemical and molecular changes during acclimatization to the greenhouse conditions of common ninebark "Dart's Gold". Microplantlets, rooted on MS medium supplemented with 2.0 mg · dm^{-3} IBA, 30.0 g · dm^{-3} sorbitol and solidified by 8.0 g · dm^{-3} agar, were potted into P9 containers filled with a sterile substrate composed of peat mixed with perlite in the ratio 1:1 (v/v). After that plants were sprayed with Proplant 722 SL and then with biopreparations at a concentration of 0.25% (three treatments at weekly intervals). Control plants were sprayed with fungicide and distilled water. During fourth week, the plants were put into plastic, transparent boxes covered with glass, placed in a growth chamber, and then, after nextr five months, they were removed from the boxes and transferred to the greenhouse. After this period, the plant material was taken for testing.

During the acclimatization to *ex vitro* conditions, significant changes in the anatomy of leaves and stems were observed in regenerants. It has been shown that as a result of acclimatization, the diameter of the tracheal elements in shoots increased and the ground tissue of cortex disappeared due to exfoliation of dead layers of cork. Regardless of the biopreparations used, the leaf blades parenchyma organization and thickness of the epidermis underwent changes. Spraying the plants with either algae preparation or Route® preparation significantly increased the level of photosynthetic pigments. The use of the AlgaminoPlant resulted in a reduction in the total level, reducing sugars and free amino acids. Polyphenolic acid level, regardless of the biopreparation, decreased, compared to the control treatment.

As a result of the performed RAPD molecular analysis, there was little change in the structure of DNA in the control acclimatized plants and these sprayed with Route® preparation.

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Stomata and ploidy level differentiation of plants obtained from *Actinidia deliciosa* var. *deliciosa* endosperm culture

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One of the most unique processes in the plant world is double fertilization. In angiosperms it leads to the formation of a diploid embryo and a triploid structure called endosperm. Endosperm is a tissue extremely specialized and confined to a short stage of the plant life-cycle. Depending on the species, it is used as a source of nutrients by the embryo in an early stage of its development or it plays a role as a storage of spare substances in a mature seed. It is worth noting that endosperm, because of its special features like unique origin, growth and ploidy level, is an interesting model for *in vitro* studies.

Plant organisms have different capacity for tissue proliferation and morphogenetic response under *in vitro* cultures. Mature and immature endosperm of various species has an ability to proliferate, differentiate and eventually regenerate triploid plants under *in vitro* cultures. They can be successfully obtained if only factors like adequate phase of endosperm development and proper culture conditions are taken into consideration. *In vitro* culture of endosperm is a useful method to produce plants with a higher ploidy status, especially with 3C amount of nuclear DNA. Such a process may be crucial in obtaining new varieties of plants which are commercially important. Additionally, the method is less time consuming and easier in comparison to traditional breeding methods. Nowadays, it grows in popularity for more plant species.

Actinidia deliciosa var. *deliciosa* (popularly known as a kiwifruit) is a plant widely known for its health benefits and taste. The endosperm of this species was previously studied under *in vitro* cultures (Gui et al., 1982; Machno and Przywara, 1997) and finally, Góralski et al. (2005) have successfully developed a protocol for shoot regeneration of kiwifruit from this tissue under *in vitro* cultures. However, there are no studies about morphological and histological features of plants regenerated by this way.

It is well known that structural features of stomata are often connected with the amount of nuclear DNA and thus they can be used as a diagnostic feature of specimens with different levels of ploidy (Mishra, 1997). It this paper we present a preliminary study of *Actinidia deliciosa* var. *deliciosa in vitro* regenerated plants in relation to the differentiation of their stomata and ploidy level. The 3C amounts of DNA in regenerated plants were confirmed by a flow cytometry analysis. Regenerants were compared to the plantlets with 2C level of ploidy.

References

Góralski G., Popielarska M., Ślesak H., Siwińska D., Batycka M. (2005) *Organogenesis in endosperm of Actinidia deliciosa cv. Hayward cultured in vitro*. Acta Biol. Crac., Ser.: Bot. 47(2): 121-128.

Gui Y.L., Mu S.K., XU T.Y. (1982) *Studies on morphological differentiation of endosperm plants of chinese gooseberry in vitro*. Acta Bot. Sin. 24: 216-221.

Machno D., Przywara L. (1997) *Endosperm culture of Actinidia species*. Acta Biol. Crac. Ser.: Bot. 39: 55-61.

Mishra M.K. (1997) *Stomatal characteristics at different ploidy levels of Coffea L.* Ann. Bot. 80: 689-692.

Optimization of the regeneration condition of sorghum for the purpose of bioethanol production

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The type of sorghum (*Sorghum Moench*) belongs to the grass family – *Poaceae*. This type includes annual and spring species. Sorghum has been grown in Africa (Nigeria, Egypt, Sudan), North America (the dry areas of the U.S.), Central America, South America, India and Asia (Japan, China, Korea). In countries where sorghum occurs naturally, it is widely used in the food industry for the production of flour and grits. As a plant of the C4 – type photosynthesis, sorghum is found useful in the bioenergy industry. In addition, it is used for industrial purposes and also as green fodder and hay. In Poland, sorghum is grown mainly as a complementary plant of corn harvests. In respect of economy the most important species distinguished within the *Sorghum* genus are: *S. bicolor*, *S. durra* and *S. saccharatum*, included recently to the Sorghum bicolor species.

Due to the decrease in the surface of crops such as wheat, corn and others for food and feed production, the role of sorghum as a plant with bioenergetic potential for the production of second generation bioethanol has increased. Biomass quality tests have shown that sorghum at low protein content (9.00%) in the dry weight contains many monosaccharides, especially fructose (9.75%) which is advantageous for the production of bioethanol. Crude fat content accounts for an average of 5.40% and ash content to 4.94% in the dry matter. The results of the quality assessment of sorghum biomass indicate its good suitability for obtaining bioethanol. If the high dry weight yield at low production costs in relation to other crops used for the same purpose is taken into account, the choice of sorghum appears to be fully justified. An increased efficiency of bioethanol production can be also obtained by the plant modification in order to increase the sucrose content as a preferential fermentation substrate and/or reduce the lignin content as a substance decreasing participation of fermented sugars in the biomass and adversely affecting the efficiency of the process itself. However, the genetic modifications are possible only on the basis of efficient regeneration of modified plants.

Studies of the *in vitro* culture of sorghum originally focused on callus cultures, its tolerance to salt stress, exposure to heavy metals and drought. The first paper on the regeneration of *S. bicolor* through indirect organogenesis was published in eighties of the 20th century. Current research on sorghum *in vitro* cultures is mainly focused on the development of regeneration protocols, usually by somatic embryogenesis. Despite many years of research sorghum is still perceived as a difficult plant in *in vitro* culture. This study describes an efficient and reproducible plant regeneration system developed from tissues of *Sorghum saccharatum* and *Sorghum bicolor* genotypes. Plant regeneration was achieved from epicotyls, coleoptiles and apical buds from aseptically germinated 7-day-old seedlings. The explants were cultured on MSS1 medium: MS medium supplemented with 0.5 mg/l 2,4-D, 2 mg/l BA, 3% sucrose. Although production of secondary metabolites by all type of explants inhibited their growth and development, modification of MSS1 medium with 0.05 mg/l ascorbic acid, 1 g/l proline, 1 g/l polyvinylpyrrolidone (PVP) and 0.3 g/l casein hydrolysate resulted in improvement of *in vitro* culture conditions. The highest regeneration efficiency was observed for coleoptiles (92%). Plant regeneration from epicotyls and apical buds remained at a lower level: 64% and 54%, respectively. Root induction with 90% efficiency was achieved on an MS medium containing 3% sucrose. Rooted plants were successfully acclimatized, with the survival rate reaching 85% (*Sorghum saccharatum*) and 68% (*Sorghum bicolor*).

Rumex thyrsiflorus Fingerh. – sex ratios among seedlings and explants cultured *in vitro*

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Rumex thyrsiflorus Fingerh. (thyrse sorrel) is a dioecious plant species, with a fully developed polymorphic sex chromosome system. The chromosome constitution of females is 2n = 12A + XX and of males is 2n = 12A + XY1Y2. It is closely related to *R. acetosa*, which is a model species in plant sex chromosomes studies. For this reason it is an attractive object for studies on the structure and function of sex chromosomes, sex ratio, and a comparison between the primary ratio in seeds and the secondary in populations.

Although it is expected to constrain the average primary sex ratio to a 1:1 ratio, the numbers of males per females at sexual maturity (operational sex ratios) may be biased due to the differences in germination, mortality, vegetative vigour, flowering frequency and environmental responses. In this context, there are no sufficient data on early sporophyte development, or a growth of explants cultured *in vitro* in this species.

The aim of our studies was to 1) analyse the sex ratio and viability among *R. thyrsiflorus* seedlings, using both sex-specific DNA markers and seedlings measurements, 2) examine the type of morphogenetic response of *R. thyrsiflorus* explants cultured in vitro, using histological and SEM analyses, 3) analyse the sex ratios of explants using PCR-based methods, in order to explain whether a morphogenetic response under *in vitro* conditions depends on the sex of the explants.

An efficient micropropagation protocol developed has been developed, using fragments of the hypocotyls isolated from 11-day old seedlings as explants which were cultured on the basal Murashige and Skoog's (MS) medium, supplemented with different concentrations of the following plant growth regulators: 2,4-D, BAP and TDZ. To confirm the type of morphogenesis, the material was prepared for embedding tissues in Technovit 7100 and sectioned to 5 μm with a rotary microtome. The sections were stained using periodic acid Schiff/naphthol blue black (PAS/NBB) double staining.

A Scanning Electron Microscope (SEM) analysis of morphogenic callus was also made. Callus induction and morphogenetic response of explants were observed on every type of medium. Callus was heterogenous and composed of cells varied in shape, size and vacuolization degree. Meristematic cells were small, isodiametric, with dense cytoplasm and they were located on the surface (2,4-D, BAP, TDZ) and in the internal region of the callus (TDZ).

The regeneration of plantlets occurred mainly via indirect organogenesis (adventitious shoots formation via callus). Somatic embryogenesis on media supplied with 2,4-D and BAP was also observed.

To analyse sex ratios among seedlings of R. thyrsiflorus and explants cultured *in vitro*, a PCR- based method was used. It involved DNA markers located on Y chromosomes. The following pairs of primers were used: 1) RAY – F and RAY – R, 2) UGR08 – F and UGR08 – R, resulted in obtaining products in male seedlings and explants. Additionally, amplification with primers R730 – A and R730 – B was carried out to verify the DNA quality of a template.

The results of our preliminary studies revealed that female seedlings show a higher life span and grow faster. This may provide them an advantage in the later stages of development. Female-biased sex ratios were observed among explants cultured *in vitro*, followed by a higher regeneration ability of female explants. It requires further detailed studies on some physiological factors,however, to determine different morphogenetic reactions of male and female explants under *in vitro* conditions.

Elucidating molecular control of terpenoid and phenylpropanoid biosynthesis in *Agastache rugosa* – an East Asian medicinal plant

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The physiological function and biological relevance of an immense diversity of so called secondary metabolites has been attracting interest of plant biologists for several decades. Starting from initial oversimplifications suggesting a marginal role of the plethora of compounds as by-products and a sink of primary energy and protein turnover, up to today's systemic view of metabolism as the entity on which the life of any organism is based. Specialized metabolic branches of isoprenoid synthesis are among the best examples of ubiquitous and almost universally important sources of biologically active compounds. Even so, our understanding of their regulation and controlling mechanisms of key metabolic events and fluxes is far from satisfying.

Agastache rugosa belongs to "traditional medicines" from East Asia which have recently gained popularity in Europe and America, mainly due to the composition of bioactive natural compounds in essential oils and in non-volatile fraction. However, the variability of essential oils is a major obstacle in increasing the commercial use of various aromatic plants, including *A. rugosa*. Thus, there is a significant demand for high-quality herbal drugs derived from East Asian plants.

We aimed at investigating mechanisms regulating biosynthesis of potentially bioactive terpenoids and phenylpropanoids in *A. rugosa*. Control mechanisms of these biosynthetic pathways are being elucidated at the transcriptional level. A pilot study showed significant differences in the expression of genes from the proposed phenylpropanoids biosynthesis pathway in field-grown *A. rugosa* plants of different ages. Phytochemical and gene expression studies were made with leaves of one-, two- and three-year-old *A. rugosa* plants harvested before and after flowering. The highest transcript level of phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI) was observed in one-year-old plants harvested before flowering, compared to two- and three-year-old individuals. The same result was obtained for flowering plants; however differences between transcript levels in plants of a different age were less pronounced. A phytochemical analysis showed the highest levels of rosmarinic acid – the end-product of the phenylpropanoids biosynthesis pathway – in leaves of two-year-old *A. rugosa* plants. So far, no correlation between the phytochemical profile of non-volatiles and the transcript level of genes of the phenylpropanoids biosynthesis pathway has been observed. This shows the need for a more detailed study of mechanisms regulating the biosynthesis of potentially bioactive terpenoids and phenylpropanoids in *A. rugosa*. In another experiment, *in vitro* organ cultures of *A. rugosa* grown on media supplemented with five different growth regulators were used. The plants showed a highly diversed morphology and differed in levels of non-volatiles. A gene expression analysis is being currently conducted.

Gaining better understanding of mechanisms regulating the terpenoid and phenolic biosynthetic pathways will hopefully lay a scientific foundation for development and delivery of new herb with optimised composition of bioactive natural compounds in essential oils and in non-volatile fraction.

Molecular characterization of nuclear and cytoplasmic genomes of *Solanum* × *michoacanum* (+) *S. tuberosum* somatic hybrids

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Somatic hybrids are an interesting material for model research of nucleus-cytoplasm interactions and are the sources of new nuclear and cytoplasmic combinations. Structural changes in the nuclear genome composition of the hybrids, such as chromosomal deletions, aberrations, eliminations or recombination between homologous fragments of chromosomes have often been observed after somatic fusion (Orczyk et al., 2003). That phenomena influence normal morphogenesis of somatic hybrids. Understanding the somatic hybridization process is important for efficient exploitation of its products.

The objective of this work was to characterize the nuclear and organellar genomes of somatic hybrids *Solanum* × *michoacanum* (+) *S. tuberosum* [*mch* (+) *tbr*]. For the analysis, 97 tuber-bearing interspecific somatic hybrids *mch* (+) *tbr* from five fusion combinations, 11 autofused tetraploid *mch* and fusion components were selected.

The DArT analysis was performed in Diversity Array Pty Ltd. Canberra, Australia. Two panels of hybriddization: one for wild potato species and one representing clones of *tbr* were applied. Markers, polymorphic between *mch* and *tbr* parents in every fusion combination were selected. Localization of individual DArT markers to appropriate chromosome was done based on a comparison with DArT maps of diploid potato species. 4*x mch* was used as a standard to define the rate of error of the applied method. Cytoplasm types were examined in somatic hybrids and their parental forms using molecular marker system elaborated by Hosaka and Sanetomo (2012).

5,358 DArT markers were generated in a single assay. Above 2,000 DArT markers were polymorphic between parents in each fusion combination. Above 1,500 were with known chromosomal localization and were sufficient for characterization of the structure of 12 potato chromosomes. The level of error was below 1% proving that the applied strategy was reliable. We noticed elimination of DNA of both wild and *tbr* fusion components. There were no losses of whole chromosomes.

We observed a random and non-random segregation of cpDNA and mtDNA, without any recombinations. The segregation of chloroplast and mitochondria took place at an early stage of the hybridization process. Non-random segregation was noted in two combinations, where D type of cytoplasmic DNA predominated. Statistical analysis indicated a significant positive correlation between the cytoplasmic type D and the percentage of nuclear DArT markers specific to *mch* parent. Those data suggested that D type cpDNA and mtDNA were more compatible with nuclei containing more *mch* genome.

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References

Hosaka K., Sanetomo R. (2012) *Development of rapid identification method for potato cytoplasm and its use for evaluating Japanese collections*. Theor. Appl. Genet. 125: 1237-1251.

Orczyk W., Przetakiewicz J., Nadolska-Orczyk A. (2003) *Somatic hybrids of Solanum tuberosum – application to genetics and breeding*. Plant Cell Tiss. Org. Cult. 74: 1-13.

Smyda P., Jakuczun H., Dębski K., Śliwka J., Thieme R., Nachtigall M., Wasilewicz-Flis I., Zimnoch-Guzowska E. (2013) *Development of somatic hybrids S. × michoacanum Bitter. (Rydb.) (+) S. tuberosum L. and autofused 4x S. × michoacanum plants as potential sources of late blight resistance for potato breeding*. Plant Cell Rep. 32: 1231-1241.

Activity of hygromycin phosphotransferase marker gene optimized for expression in plants for the construction of vectors for genetic modifications of grasses

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Production of biomass as a source of bioenergy and biofuels is one of main directions in contemporary plant biotechnology. Therefore, improvement of the yield or quality of dedicated biomass crops, for example miscanthus and other perennial grasses, is a subject of numerous research and developmental projects. Biotechnological methods based on genetic engineering and plant transformation are an essential complement in these efforts. Hygromycin phosphotransferase (*hpt*) is one of commonly used marker genes in molecular biology and biotechnology. Advantages of hygromycin phosphotransferase gene optimizations are particularly relevant as biotechnological tools for fundamental research and crop improvement.

Preliminary tests showed that hygromycin B (*hyg*) is an appropriate selection factor for miscanthus species (*Miscanthus* × *giganteus*, *M. sinensis*, *M. sacchariflorus*). Thus, the use of the vector with optimized *hpt* coding sequence can be a promising tool for transformation of grasses.

In order to increase the efficiency of miscanthus transformation, the coding sequence of natural *hpt* gene of bacterial origin (*Streptomyces hygroscopicus*) was optimized to potentially increase its expression in grasses, basing on codon usage characteristic for maize (*Zea mays*, Codon Usage Database, www.cazusa.or.jp). Optimized hpt coding sequence (Ohpt) was introduced in the place of the original *hpt* gene in pCAMBIA 1201 vector to achieve pCAOhpt. Both vectors were used for transformation of tobacco (*Nicotina tabacum*) as a model plant. Regenerating explants were cultured on selection media containing 10, 20 and 50 mg/l of hygromycin. Plants transformed with pCAOhpt and regenerated on media with hyg 10 and 20 mg/l, accounted for 142% and 130% of those obtained for pCAMBIA. In the case of hyg 50 mg/l, only pCAOhpt gave positive results, i.e. 4 plants were regenerated. PCR analyses confirmed the presence of *hpt* sequence in 62.5% and 66% of obtained plants, for *hyg* 10 and 20 mg/l, respectively. In the case of pCAOhpt, these ratios were 50%, 83% and 100% for *hyg* 10, 20 and 50 mg/l. These results lead to a conclusion that modified *hpt* sequence is more efficient for plant transformation. It is estimated that the Ohpt sequence increases transformation efficiency by approx. 35% as well as decreases risk of false positive transgenic plants (escapes). Further analyses confirming functionality of Ohpt, as RT-PCR and assay of enzymatic activity of hygromycin phosphotransferase are in progress.

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Biotransformations of flavanones in callus cultures

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The flavonoids were first discovered by Albert Szent-Gyorgyi, who isolated rutin from orange peel. These compounds are ubiquitous in vascular plants, they are responsible for different petal colors luring pollinators, protect plants against attack by pathogens, as well as determine the growth and development of plants.

Flavonoids are present in the diet of people in amounts of up to 1 gram per day. The sources are primarily fruits and vegetables, but also red wine, coffee, tea, or beer. In *vitro* and in *vivo* study shows that flavonoid compounds have broad biological activity: anti-inflammatory, antitumor, antidiabetic, antibacterial, antiviral and antifungal as well as protective effect against cardiovascular diseases. The pharmacological effect of flavonoids is mainly due to their antioxidant activity, ability to capture free radicals and the chelation of metal ions. The presence of two hydroxyl groups at C3′ and C4′ (ring B) and the double bond C2-C3 conjugated to the carbonyl group at C4 in ring C is essential for the antioxidant activity of flavonoids.

Biotransformations are environment-friendly alternative to chemical catalysis. Biocatalysis offers high selectivity, which allows to obtain the desired product with only a small number of intermediates. Biotransformation are also a tool that could be used to recognize the metabolic pathways. A wide variety of chemical compounds including steroids, alkaloids, coumarins, terpenoids and flavonoids can be biotransformed by plant cell cultures.

As far as we know, flavonoid biotransformations are resulting mostly in formation of glycosides. The use of plant cultures in biotransformations offers many advantages: culture plant can be grown in thelaboratory and propagated indefinitely, the plant material is homogeneous which makes the extraction easier, experiments can be carried out throughout the year, cells can accumulate substantial amounts of the desired products.

In cultures of *Eucalyptus perriniana* glucose, rhamnose and gentiobiose units were attached to naringin and naringenin, isolated from cultures of *Astragalus sieberi* enzyme catalyzed the attachment of glucose to kaempferol, quercetin, and isorhamnetin.

Plants are a rich source of secondary metabolites which are used among others in the pharmaceutical and food industries. The compounds used in medicine exhibit a broad spectrum of activity, for example: antihypertensive - ajmalicine obtained from *Catharantus roseus*, antimalarial artemisinin (*Artemisia annua*), or antitumor colchicine (*Colchium autumnale*). It has been proved that a soy callus is a great source of isoflavones, compounds used to relieve the symptoms of menopause.

The first stage of the research was to obtain callus cultures from *Phaseolus coccineus* and *Glycine max*, which in the later stages served as biocatalysts in biotransformation processes. Commercial flavanones were used as substrates. The process of biotransformation was carried out in two variants: in suspension cultures and solidified agar medium. Biotransformation products were determined using chromatographic methods (HPLC, TLC). It has been shown that the enzymatic systems of *P. coccineus* and *G. max* can lead dehydrogenation reactions resulting in flavones with high antioxidant activity. In addition, it has been proved that the tested callus cultures produce isoflavones.

The expression of HBV antigens in transgenic lettuce micropropagated *in vitro* cultures for the purpose of anti-HBV vaccine

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Plant-derived oral formulations are regarded as an attractive alternative for cost-effective and reliable mass hepatitis B vaccination programs in developing countries. Initial experiments provided positive results on immunization using freeze-dried lettuce containing the small surface antigen (S-HBsAg) of Hepatitis B Virus (HBV). Possible commercial vaccine production would require providing plant material of high and uniform antigen content. The usually observed variability of antigen expression among plants substantially limits their use for vaccine manufacturing. The solution can be vegetative propagation *in vitro* cultures of the most productive plants.

The aim of this study was to verify the effect of *in vitro* culture conditions on the expression of HBV antigens in micropropagated lettuce clones. Tests were carried out on three separate lines of varied expression levels (from hundreds ng to several tens mg/g fresh weight) for each HBV surface antigen, S-, M- and L-HBsAg. Initial plants were obtained from seeds of primary transformants and 20-40 of those were used for each line. Plants were cultured for 4-5 weeks on an SH medium supplemented with kinetin 5 mg/l and then with 0.5 mg/l. Micropropagation was carried out for eight monthly passages under 14/10 photoperiod and 4000 lux at 24/22 °C temperature regime. Successively obtained plant clones were rooted on 1/2 SH medium, transferred to soil and grown in glasshouse for HBsAg analyses. The multiplication rate approx. 5.6 was achieved, independently of the type or level of an expressed protein, and no decline was observed throughout the culture run. The most important factor – the mean level of an expressed antigenic protein – was statistically equivalent with normally developed control plants for 64-100% of clones. Conditions of an *in vitro* culture did not cause any significant variations of antigen expression between clones for 7 tested lines: ≥80% of their clones were homogenous regarding the content of a given antigen and for 2 lines this parameter accounted for >70%. These results allowed to assume that the microptopagation method developed *in vitro* does not essentially affect the expression of the transgene.

Biomass of traditionally cultivated plants and plants obtained through *in vitro* vegetative propagation proved to be homogenous, both in comparison to control plants and between clones. However, among the clonally propagated plants a visible decline was observed regarding the mean number of produced seeds when compared to control plants. Apart from possible reasons, the sum of all clones' seeds for a given line significantly outnumbered that for normal plants.

Plant material obtained via vegetative propagation *in vitro* culture was freeze-dried, according to the protocol previously devised in our laboratory. The structure and antigenicity of all three antigens – S-, M- and L-HBsAg was preserved during freeze-drying as well as long-term storage. Moreover, freeze-dried material containing S antigen was used successfully in immunization trails, which proved retained immunogenicity.

The presented results indicate that vegetative propagation *in vitro* culture enables to increase several-fold plant population without a negative impact on their growth and development, and without a transgenic protein content regardless of its type. The developed technology can be one of effective ways of providing continuously and in bulk valuable and uniform plant material for production of oral vaccine against HBV.

Expression of Hepatitis B core Antigen in lettuce and tobacco for advanced plant-derived vaccine formulations

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Plants can be widely used in biopharming i.e. production of biopharmaceuticals, including vaccines. Many plant-produced antigens induce an effective immune response without side effects. For many years research has been focused on orally administered plant-based vaccines. However, main hopes for biopharming are currently associated with antigen purification and injectable vaccines, while oral vaccines can play a complementary role, especially in the case of blood-borne pathogens. Vaccines against hepatitis B Virus (HBV) are a good example of such current approaches to plant-derived vaccines.

Currently, the commonly used anti-HBV preventive vaccines are based on the small surface antigen of the virus (S-HBsAg) produced in recombinant yeast. The antigen is characterized by an ability to self-assembly into Virus-Like Particles (VLPs), which can act both as an immunogen and an adjuvant, as well as can be used as a carrier of other antigens for epitopes. The core antigen (HBcAg) forms similar structures, here Capsid-Like Particles (CLPs). These have been shown to be effective vessels for drug delivery and epitope carriers. Much research has focused on the generation of HBcAg-CLPs displaying heterologous antigens or their epitopes to initiate an efficacious humoral and cellular immune response, where the CLPs enhanced immunogenicity of attached antigens. HBcAg-CLPs are also considered to constitute the fundamental component of a potential therapeutic vaccine against chronic form of hepatitis B.

In this study HBcAg-CLPs were expressed in transgenic tobacco and lettuce plants. Both plants were transformed with *Agrobacterium tumefaciens* EHA105 strain, carrying modified pGPTV-BAR vector with HBcAg coding sequence which transcription was regulated by 35S CaMV promoter and *bar* gene determining phosphinotricin (ppt, herbicide) resistance as a selection marker. Tobacco transformants were regenerated on an MS selection medium, initially supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA and then 1 mg/l KIN and 0,1 IAA mg/l, and with ppt 20 mg/l. Lettuce was regenerated *via* an organogenesis of the callus according to the developed method, initially on an MS medium with 0.2mg/l BAP and 0.05mg/l NAA and then on a medium composed of SH salts, B5 vitamins, 0.5mg/l KIN and 0.5mg/l zeatin.

Following expression, HBcAg-CLPs assembly was confirmed by ELISA assays and Western blot analyses. The expression level in tobacco plants reached up to about 200 μg/g FW and in lettuce 100 μg/g FW. Lettuce leaves were lyophilised at 20-22°C for 22 h and then stored for 12 months at 4, 22 and 37°C. Directly post freeze-drying 91% of HBcAg-CLPs was maintained, while the stored antigen was relatively stable only at cold temperature, finally preserving 56% of initial content. Tobacco leaves were chosen for HBcAg-CLPs purification. The particles were purified from the crude extract of tobacco leaves in three steps: initial filtration, ultracentrifugation in CsCl and/or sucrose density gradient and dialysis. The results of an ELISA test and density analysis of isopycnic ultracentrifugation indicated that the purified particles with HBcAg mainly located at the density of about 1.36 g/ml. All purification steps from the extraction to the dialysis were optimized to produce preparations of the highest possible purity and content of functional HBcAg-CLPs. The obtained results firmly indicate a possibility to conduct mouse injection and oral immunisation trials.

References

Siddharth T., Praveen C.V., Pradhyumna K.S., Rakesh T. (2009) *Plants a bioreactors for the production of vaccine antigens*. Biotechnol. Adv. 27: 449-467.

Pumpens P., Grens E. (2001) *HBV Core Particles as a Carrier for B Cell/T Cell Epitopes*. Intervirology 44: 98-114.

Michel M.-L., Mancini-Bourgine M. (2005) *Therapeutic vaccination against chronic hepatitis B virus infection*. J. Clin. Virol. 34: 108-114.

Session 1

Some aspects of symplasmic communication during somatic embryogenesis of tree fern *Cyathea delgadii*

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Somatic embryogenesis is an asexual way of plant propagation in which the somatic cells differentiate into embryos bypassing the fusion of gametes. The phenomenon mimics many of the events of sexual reproduction and, as such, represents a model for studying the morphological and molecular regulation of the process. So far, for those examinations model plants belonging to spermatophytes have been used. The last achievements showed that the tree fern *Cyathea delgadii* provides an excellent and effective pattern for both the induction and production of somatic embryos (Mikuła et al., 2015). Discoveries that single epidermal cells of *C. delgadii* stipe explants begin to differentiate into somatic embryos opened new fields of studies of the mechanisms controlling the somatic embryogenesis, including an analysis of symplasmic communication during this process. In multicellular organisms intercellular communication is a key factor for coordination of developmental processes. In plants, a system of cell cytoplasm connected by plasmodesmata (PDs) called the symplasm, is a convenient and precise way of information exchange between cells (Wróbel-Marek et al., 2015). Such exchange of signals through PD is called symplasmic communication. Molecules that pass through the PD include ions, some hormones, minerals, amino acids, and sugars but also proteins including transcription factors, and different classes of RNA, and as such, PD can participate in the coordination of plant growth and its development (Marzec and Kurczynska, 2014). It is known that cell differentiation is correlated with the changes in symplasmic communication (Zambryski and Crawford, 2000).

Explants of *C. delgadii* during the culture (methods described by Mikuła et al., 2015) were analysed with the use of low-molecular weight fluorochromes and confocal microscopy. Within the explant, cell distribution of fluorochromes was not uniform and changed spatiotemporally during the culture. The most intense fluorescence signal was detected within the cells which were engaged in somatic embryo formation. They were well distinguished from other cells of the explant. After the division of these cells, daughter cells included in the embryogenic complex showed different patterns of fluorochrome distribution. Some of these cells were characterized by an intense fluorescence signal in comparison to other cells of the complex. In a more advanced culture with well visible somatic embryos at the globular stage of development, the distribution of fluorochromes indicated that the movement of symplasmic tracers between the embryo and the explant tissues had occurred.

The obtained results show that within an explant tissue, differentiation of cells in various directions is correlated with the changes in the plasmodesmata permeability. This suggests that one of the factors controlling cell differentiation in the case of fern somatic embryogenesis, is symplasmic communication and its changes.

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References

Zambryski P., Crawford K. (2000) *Plasmodesmata: gatekeepers for cell-to-cell transport of developmental signals in plants*. Annu. Rev. Cell Dev. Biol. 16: 393-421.

Marzec M., Kurczynska E. (2014) *Importance of symplasmic communication in cell differentiation*. Plant Signaling & Behavior 9, e27931.

Mikuła A., Pożoga M., Tomiczak K., Rybczyński J.J. (2015) *Somatic embryogenesis in ferns: a new experimental system*. Plant Cell Rep. 34 (5): 783-794.

Justyna Wróbel-Marek J., Katarzyna Kulińska-Łukaszek K., Ewa Urszula Kurczyńska E.U. *Komunikacja symplastowa i jej rola w rozwoju roślin*. Post. Biol. Kom. (in press).

Adventitious shoot development in *Helianthus tuberosus* callus culture

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Jerusalem artichoke (*Helianthus tuberosus*) is a perennial species with a new environmental applications as an energetic and phytoremediation plant. The objective of the study was to develop a protocol of a high frequency callus induction for *in vitro* shoot regeneration of *H. tuberosus*, which could be applicable to plant improvement through somaclonal findings and genetic transformation.

The effects of different concentrations of auxin (NAA) and cytokinin (BAP) on indirect shoot bud induction and shoot elongation were investigated. All cultures were maintained at a temperature of 22 ± 2°C and constant light intensity (45 μmol $m^{-2}s^{-1}$). Explants were collected from rooting axillary shoots of *in vitro*-grown Jerusalem artichoke.

Callus culture was initiated from an adventitious root and shoot segments on an MS medium supplemented with BAP (3.0 mg l^{-1}) and NAA (0.5 mg l^{-1}). The induction medium contained 0.6% agar. After two weeks of culture, calli were induced from the excised part of the explants. Callus proliferation was most intensive in the root cultures and it rapidly covered the entire surface of the explant. Based on morphology, two types of callus formation were noted after four weeks of culture. A compact green hard callus derived from the stem tissue and a friable white callus developed from adventitious roots. The friable white callus, subcultured on an MS media with different concentrations of BAP, (1.0 to 4.0 mg l^{-1}) and NAA (0.5 mg l^{-1}) underwent differentiation forming shoot buds. The first shoot bud differentiation was observed in the form of greenish protuberances after three weeks of callus culture on an MS medium with BAP (2.0 mg l^{-1}). The highest frequency of shoot bud induction was observed on MS medium supplemented with BAP (4 mg l^{-1}). However, the medium with a high concentration of BAP inhibited shoot growth. Modifications of plant growth regulator concentrations in the medium supplemented with BAP (1.0 mg l^{-1}) and low concentration of NAA (0.1 mg l^{-1}) enhanced shoot elongation.

A histological analysis (Light Microscopy) and cytological observations (Transmission Electron Microscopy) confirmed *de novo* meristemoid differentiation in the callus and a unipolar structure formation. Meristematic areas consisted of small cells with a dense cytoplasm and a prominent nucleus. Histological sections of a seven-week-old morphogenic callus showed developing shoots with apical meristem flanked by leaf primordia and young leaflets. Further research in *H. tuberosus* has been conducted to optimize shoot elongation and shoot rooting media.

Indirect shoot regeneration from leaf explants of *Kalanchoe pinnata*

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Plants that are pharmacologically relevant have always had a great attention of scientists around the world. It all started with people trying to find the cure for minor wounds, food poisonings and so on. Later on, ethnopharmacologists used to isolate specific substances to prepare the exact medicine for the illness. Nowadays the world's scientific "hot spot" is to execute a cure for cancer. *Kalanchoe pinnata* (Lam.) Pers. is plant which belongs to Crassulaceae family and was originally found in Madagascar, now found mainly in India, seems to cover all of the aspects mentioned before. Including prospective cure for cancer.

The aim of the present paper was to develop an efficient protocol for adventitious shoot regenertion of *K. pinnata*. Plant material was collected from plants growing in laboratory conditions obtained from *in vitro* culture after acclimatization. Lengths of the collected leaf lamina oscillated within 7.3-9.8 cm (the average length was 8.41 cm). The leaves of *K. pinnata* were washed in water with drop of commercial detergent, then surface-disinfected in solution of 70% (v/v) ethanol (30 s) and followed by 0.25% water chloride (8 min). Disinfected leaves were washed three times in deionized autoclaved water and divided longitudinally and transversely into segments of size about 1.0 cm^2. The outer leaf margin tissue was cut off and explants were placed on MS medium supplemented with 2.0 mgl^{-1} TDZ (thidiazuron). Explants were placed in 100 ml Erlenmeyer flasks covered by aluminum foil, four explants per dish. All cultures were maintained in controlled conditions at 22 °C (± 2 °C) and under continuous light (35 μmol m^{-2} s^{-2}) provided by cool white fluorescent tubes. Experiments were repeated 3 times using a complete randomized block.

Regeneration frequency and the number of regenerating shoots per explant were recorded after 10 weeks of culture initiation. The cultured explant were collected at weekly intervals and fixed in FAA (formalin, acetic acid, ethanol alcohol) fixative. The samples were then embedded in paraffin, cut into sections using microtom and stained with safranine and fast green.

The results reveled that leaf explants become considerably swollen at the cut ends after two weeks of cultures. A histological analysis indicated that parenchyma cells dedifferentiated and underwent a division to form callus. The cell divisions were initiated in subepidermal layers of mesophyll cells. Epidermal cells did not undergo divisions. Moreover, a histological analysis showed a typical callus formation with vascular tissue and meristematic regions. The first shoot regeneration was obtained in calli after 7 weeks of culture. The number of regenerated shoots per explant ranged from 1 to 10. The average number of adventitious shoots formed per explant was 5.06. Further research was conducted to elaborate the best concentration of TDZ and also to study other plant growing factors to establish a protocol which would give the best results in regenerated shootlets per explant.

Key words: adventitious shoots, micropropagation, histology

Rhaponticum carthamoides regeneration through direct and indirect organogenesis, molecular profiles and secondary metabolite production

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Rhaponticum carthamoides (Willd.) Iljin (known as "maral root" or Russian leuzea) is an endemic, perennial, herbaceous plant belonging to the family Asteraceae. The plants originate from the mountains of South Siberia, Central Asia and China (Kokoska et al., 2002; Biskup and Lojkowska, 2009; Kokoska and Janovska, 2009). The roots and rhizome of *R. carthamoides* have been used for a long time in Siberian folk medicine in the treatment of overstrain and weakness after illness. They also have adaptogenic, antioxidant, immunomodulatory, anticancerogenic and antimicrobial properties (Kokoska and Janovska, 2009). The raw material of *R. carthamoides* is used to eliminate physical and mental weariness, and to promote muscle growth and sexual functions (Kokoska and Janovska 2009). The major chemical constituents in this plant are ecdysteroids, flavonoids, phenolic acids, triterpenoid glycosides, polyacetylenes and sesquiterpene lactones (Kokoska and Janovska, 2009). Harvesting the roots and underground parts requires the plant to be cut down, which has led to the depletion of *R. carthamoides* from its natural habitat. The development of biotechnological methods such as micropropagation is one solution which may overcome this problem.

The organogenic competence of different explants of *R. carthamoides* was investigated on MS agar medium supplemented with BA, IBA or NAA at concentrations of 0.2 and 0.5 mg l^{-1}. Adventitious shoot formation was obtained through direct organogenesis using leaves of *in vitro* cultures as explants and through indirect organogenesis when seedling explants (hypocotyl, cotyledon and root) were used for regenerative callus initiation. The shoots were rooted on a half-strength MS medium (1/2 MS) without auxin or containing IBA (0.2-2.0 mg l^{-1}). The plantlets regenerated through direct and indirect organogenesis were transferred into pots and grown in the greenhouse for 3 months.

Significant differences in morphology, accumulation of chlorogenic acid and 20-hydroxyecdysone (20-HE) as well as in genetic profile were observed. The UHPLC analysis showed that the highest level of chlorogenic acid (12 mg g^{-1} DW) was found in leaves of plants developed directly from explants, whereas leaves of plants developed *via* callus tissue accumulated the highest amount of 20-HE (7.4 mg g^{-1} DW). Its level exceeded that detected in leaves of 3-month-old plants obtained from seeds (2.4 mg g^{-1} DW). A cluster analysis indicated that genetic similarity values calculated on the basis of RAPD and ISSR data among plants regenerated through direct and indirect organogenesis to the mother plant ranged from 0.765 to 0.941 and 0.647 to 0.947, respectively.

References

Biskup E., Lojkowska E. (2009) *Evaluation of biological activities of Rhaponticum carthamoides extracts.* J. Med. Plant Res. 31: 1092-1098

Kokoska L., Janovska D. (2009) *Chemistry and pharmacology of Rhaponticum carthamoides: a review.* Phytochemistry 70: 842-855.

Kokoska L., Polesny Z., Rada V., Nepovim A., Vanek T. (2002) *Screening of some Siberian medicinal plants for antimicrobial activity.* J. Ethnopharmacol. 82: 51-53

Comparison of plant regeneration of *Miscanthus sinensis*, *M. sacchariflorus* and *M.* × *giganteus* in *in vitro* culture

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Grasses from the *Miscanthus* genus are used in many industries depending on its chemical composition. For example, to serve as a biomass source for renewable energy production. *M.* × *giganteus*, *M. sinensis* and *M. sacchariflorus* are frequently cultivated species. Giant *Miscanthus* is a sterile hybrid of diploid *M. sinensis* and tetraploid *M. sacchariflorus* and due to its sterility it can be reproduced only vegetatively via a rhizome division or via an *in vitro* culture. *Miscanthus* × *giganteu*s is a one of the plants most widely considered as a species useful for sustainable agriculture. This grass is a C4 plant and produces a large quantity of biomass under relatively low input levels. It is cultivated mainly as an energy crop; however due to its high cellulose content it may be used in the paper industry or in architecture for roofing. *Miscanthus* is used as a material for protecting screens against dust and noise. As a non-forage plant, it is also planted to clear up industrial waste areas contaminated with heavy metal ions or polycyclic aromatic hydrocarbons. Apart from this, *Miscanthus* may be grown on soil with elevated salinity, so in Asia is planted often on seashores. The lifetime of a *Miscanthus* plantation varies from 20 to 25 years, so its long-term cultivation enhances soil carbon sequestration. Additionally, products obtained from biomass of *Miscanthus* might also play a role in Carbon storage.

The aim of the presented study was to compare plant regeneration ability of three *Miscanthus* species: *M.* × *giganteus*, *M. sinensis* and *M. sacchariflorus*. Callus was induced from 0.5 cm-fragments of immature inflorescences on four induction media: 1) MS1: MS (Murashige and Skoog, 1962) + 5 mg dm^{-3} 2.4-D + 0.1 mg dm^{-3} BAP + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar; 2) MS2: MS + 6.5 mg dm^{-3} 2.4-D + 0.25 mg dm^{-3} BAP + 500 mg dm^{-3} casein hydrolysate + 30 g dm^{-3} honey + 8 g dm^{-3} agar; 3) MS3: MS + 6,5 mg dm^{-3} 2.4-D + 0.25 mg dm-3 BAP + 500 mg dm^{-3} casein hydrolysate + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar; 4) MS4: MS + 6.5 mg dm^{-3} 2,4-D + 0.25 mg dm^{-3} BAP + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar.

After 12 weeks, embryogenic calli were transferred on either of three regeneration media. 1) R1: shoot regeneration on MS + 2 mg dm^{-3} BAP + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar, while root regeneration on MS + 0.5 mg dm^{-3} NAA + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar; 2) R2: MS + 0.05 mg dm^{-3} kinetin + 30 g dm^{-3} honey + 8 g dm^{-3} agar; 3) R3: MS + 0.05 mg dm^{-3} kinetin + 30 g dm^{-3} sucrose + 8 g dm^{-3} agar.

The optimal callus induction medium for *M.* × *giganteus* and *M. sinensis* was medium M1, while for *M. sacchariflorus* was medium M3. *M.* × *giganteus* and *M. sacchariflorus* regeneration efficiency from embryogenic calli was the highest on R2 and R3 media. The use of sucrose or honey as carbon source did not influence the regeneration frequency, however our previous research has shown that honey used instead sucrose decreased the phenolic synthesis by explants on callus induction medium. Phenolic accumulation is a disadvantageous phenomenon in tissue culture, demonstrating toxic influence on callus development. The most calli of *M. sinensis* regenerated when two steps procedure (R1) were used, while it was the least efficient for plant regeneration of *M.* × *giganteus* and *M. sacchariflorus*. The obtained results indicate that for each *Miscanthus* species, a separate procedure of micropropagation should be adjusted.

Influence of growth regulators and genotype on callus induction from cotyledonary explants of *Camelina sativa* (L.) Crantz

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False flax (*Camelina sativa* L. Crantz) is the oldest oil crop grown in Europe. Polish false flax oil has been registered in the EU list of traditional specialties guaranteed under the name of "olej rydzowy". The nutritive value of false flax oil, as well as its potential use as biodiesel have resulted in a considerable, renewed interest in this partly forgotten crop. False flax oil may be used in several ways: as a source of vegetable oil with high contents of omega-3 fatty acids and as salad oil, while it is also possible to use it in the cosmetic industry to produce soap and soft detergents. It is also used in industry as biodiesel, in agriculture where it may be added as an adjuvant in sprays or used as cattle, pig and poultry feed. This plant may also be used as green manure or as a cover crop protecting soil against erosion.

The aim of the study was to assess the regeneration potential of Polish spring and winter false flax cultivars in cotyledonary *in vitro* cultures and to determine the effect of the seedling age and the applied combination of growth regulators on callus formation.

Plant material comprised 2 winter large-seeded false flax cultivars, Luna (the Polish National List of Agricultural Plant Varieties 2012) and Przybrodzka (the Polish National List of Agricultural Plant Varieties 2008), as well as 2 spring large-seeded false flax cultivars, Borowska (the Polish National List of Agricultural Plant Varieties) and Omega (the Polish National List of Agricultural Plant Varieties 2013).

Cotyledons cultures were run according to the method proposed by Zandecka-Dziubak and Łuczkiewicz (1999). Cotyledonary explants of 4 false flax cultivars were collected from seedlings grown *in vitro* on an MS basic medium (Murashige and Skoog, 1962). Cotyledons were collected from 7-, 14- and 21-day old seedlings and placed on 4 variants of the MS medium containing kinetin, 2,4-D and dicamba. Four types of induction medium were used: I – MS + 2.0 mg/l kinetin + 2.0 mg/l 2,4-D, II – MS + 2.0 mg/l kinetin + 2.0 mg/l dicamba, III – MS + 3.0 mg/l kinetin + 2.0 mg/l 2,4-D, IV – MS + 3.0 mg/l kinetin + 2.0 mg/l dicamba. Four explants were placed on one Petri dish, while their combination comprised 5 Petri dishes in 3 replications. After 4 weeks of incubation in the growth chamber at a temperature of 24°C and a 16-h photoperiod the capacity of cotyledonary explants to form callus tissue was assessed.

The efficiency of the callus tissue formation was very high and ranged from a mean of 58.9% for cv. Przybrodzka to 90% for cv. Borowska. The greatest efficiency of callus tissue formation from false flax cotyledons was recorded on an MS medium containing 3.0 mg/l kinetin and 2.0 mg/l 2,4-D at 91.6% and on MS medium with an addition of 2.0 mg/l kinetin and 2.0 mg/l 2,4-D at 95.4%.The optimal seedling age was 14 days, while the mean efficiency of callus formation on cotyledons of the tested false flax cultivars was 88.1%.

References

Zandecka-Dziubak J., Łuczkiewicz T. (1999) *Regeneracja pędów z segmentów hypokotylowych lnianki siewnej Camelina sativa L. w kulturach in vitro*. Rośl. Oleiste 20: 631-635.

Effect of 2iP and NAA on shoot induction and proliferation in micropropagated willow herb (*Chamaenerion angustifolium* (L.) Scop.)

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Chamaenerion angustifolium (L.) Scop syn. *Epilobium angustifolium* L. is a valuable medicinal plant belonging to Onagraceae family. Willow herb raw material contains abundant flavonoids and their glucosides, phenolic acids and tannins. For the last few decades *E. angustifolium* has attracted interest due to pharmacological properties of its extracts and as a potential source of drugs used in the prevention and treatment of benign prostatic hyperplasia (BPH) (Granica et al., 2014). There are only few studies on the micropropagation of *Epilobium* species. The aim of this study was to develop an efficient protocol of micropropagation for clonal repository of elite medicinal plant germplasm.

In vitro cultures have been initiated from sterilized seedlings and nodal explants of rooted plants propagated *in vitro*. Seedlings were cut for fragments of shoots, roots, leaves and shoot tips. Explants were inoculated on MS medium (Murashige and Skoog, 1962) supplemented with 2iP (0.1-2.0 mg/l), NAA (0.5-1.0 mg/l) and ascorbic acid (0.1 g/l as antioxidant agent) and 30 g/l sucrose, 8 g/l agar; pH = 5.7. Induction of shoots from nodal explants (including one axillary bud) was initiated on an MS medium with 2iP (0.1-2.0 mg/l) and vitamin C (0.1 g/l) as an independent experiment. All cultures were incubated at 25°C under a 16-h photoperiod. After 4 weeks proliferating explants were subcultured on a fresh MS medium without growth regulators for shoot elongation. Shoot number and percentage of explants producing shoots were calculated. After next two weeks separated shoots were rooted on a 1/2 MS medium with IAA (0.5 mg/l). The root explants and shoot tips were the most productive for shoots formation. The greatest number of shoots per root explant was recorded on a medium containing 2iP at concentration 1.0 mg/l and NAA – 0.1 mg/l. Shoot tips were less responsive in terms of explants percentage, but a number of 38 new shoots per one explant were noted on a medium with 2iP (2.0 mg/l) and NAA (1.0 mg/l). Most leaf explants died within four weeks and only a few generated callus. A small percentage of shoots fragments formed multiple shoots on a medium with 2iP (2.0 mg/l) and NAA (0.5 mg/l and 1.0 mg/l). Nodal explants generated fewer new shoots per explant. The maximal number of shoots (5.8) was recorded on a medium with 2iP at concentration 0.25 mg/l. The browning of tissues was observed as an effect of polyphenols exudation from cut surfaces of the explants which limited regeneration. The varied reactions of explants on 2iP were observed depending on the type of explants and the genotype. The results highlighted the differential effect of 2iP and NAA on shoot proliferation and suggest a synergistic effect of auxin and cytokinin.

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References

Granica S., Piwowarski J.P., Czerwińska M.E., Kiss A.K. (2014) *Phytochemistry, pharmacology and traditional uses of Epilobium species (Onagraceae): A review*. J. Ethnopharm. 156: 316-346.

Murashige T., Skoog F. (1962) *A revised medium for rapid growth and bioassays with tobacco tissue cultures*. Physiol Plant 15: 473-497.

Effect of the iron chelate FeEDDHA on *Staphylea pinnata* shoot multiplication and photosynthetic pigment concentration

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Iron is involved in fundamental physiological processes as an enzyme cofactor in photosynthesis, respiration, nitrogen fixation, molecular DNA synthesis, lipids and chlorophyll synthesis and growth regulators. During *in vitro* culture iron is a standard component of a sustainable growth medium. The most widely used iron form present in the Murashige Skoog medium (1962) is the chelate FeEDTA. It is a photolabile iron form quickly released and bound with phosphorus. Improving the availability of iron by the use of its other forms and optimal concentration increases the amount of chlorophyll. It can be done by the addition the photostable chelate FeEDDHA to the medium. This compound reduces chlorosis, increases proliferation, rooting, formation of adventitious shoots and somatic embryos, and improves the physiological state of cultures.

European Bladdernut (*Staphylea pinnata* L.) is a rare deciduous shrub at the northern limit of its range in Poland. Since 1957 it has been strictly protected. Optimization of *in vitro* culture will contribute to the protection of this precious species. The aim of the present study was to determine the optimum concentration of the iron chelate FeEDDHA for *Staphylea pinnata* shoot micropropagation.

In vitro plantlets of bladdernut were subcultured on the MS (1962) medium with 30 g dm^{-3} sucrose, 5 μM BAP (6-benzylaminopurine), 0.5 μM NAA (1-naphthyl acetic acid) and 5 g dm^{-3} BioAgar. The tested media were supplemented additionally (besides Fe in the standard MS) with 20, 40 or 60 mg dm^{-3} of FeEDDHA. The control medium contained Fe only from the MS medium (36.7 mg dm^{-3} FeEDHA). Shoots of bladdernut (with 3-4 leaves) were multiplied in an 8-week cycle, with a transfer to a fresh medium after 4 weeks (with 0.5-mm-thick cut off of the shoot base). The cultures were maintained under a 16 h photoperiod at PPFD of 35 mmol m^{-2} s^{-1}, temperature of 20/23 ± 1 °C (night/day) and 80% relative humidity. Observations were made twice (after two 8-week growing cycles). The multiplication rate, the height of plants, number of leaves, chlorophyll a and b and total carotenoids content (Lichtenthaler and Buschmann, 2001) were estimated.

Media with extra iron had no influence on the multiplication rate or leaf number in *Staphyela pinnata* in *in vitro* culture but affected the height and physiological quality of plants. Microshoots of bladdernut were longer in all media with FeEDDHA in comparison to the control medium. The addition of iron in a photostable chelate form (FeEDDHA) increased the concentration of all photosynthetic pigments in leaves of *Staphylea*. The amount of Chlorophyll was three times greater in the medium supplemented with 60 mg · dm^{-3} FeEDDHA in comparison to the control (standard MS). Similarly, chlorophyll b and total carotenoids were at the highest level in the medium with 60 mg · dm^{-3} FeEDDHA. The weight ratio of chlorophyll a and b to total carotenoids (a+b) / (x+c) in all FeEDDHA treatments were between 5.5 and 6.3 but the highest value was observed in the control media with a standard iron content.

Acknowledgements

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References

Lichtenthaler H.K., Buschmann C. (2001) *Extraction of photosynthetic tissues. Chlorophylls and carotenoids. Measurement and characterization by UV-VIS spectroscopy*. Curr. Protoc. Food Anal. Chem. F4.2, F4.3.1, Suppl.1.

Murashige T., Skoog F. (1962) *A revised medium for rapid growth and bioassays with tobacco tissue cultures*. Physiol. Plant. 15: 473-497.

The effect of phytosulfokine on embryo development in protoplast cultures of *Daucus carota* subspecies – histological analysis

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Somatic embryogenesis is a process in which somatic cells differentiate into a bipolar structure through a series of stages characteristic for zygotic embryo development. Factors appropriate for the induction of somatic embryogenesis in different species are usually discovered by trials and errors analyzing effects of different culture conditions. One of the ways to increase the embryogenic competence of the cells is supplementation of a culture medium with phytosulfokine (PSK). PSK, a 5-amino acid sulfated peptide identified in a conditioned medium of plant cell cultures which promotes cellular growth *in vitro via* binding to the membrane-localized PSK receptor. Existing data suggest that PSK is one of the factors initiating and enhancing embryogenic competence and plays a role in a wide range of cellular changes associated with these events. The first attempts of *Daucus* protoplast regeneration in the presence of PSK showed its beneficial effect on the mitotic division frequency and somatic embryo formation. The goal of this work was to analyse the effect of PSK on the embryogenic structure formation and cytochemical nature of cells forming these units.

The embryogenic tissues were obtained from *Daucus carota* subsp. *sativus* and subsp. *gadacei* protoplast cultures. Protoplast were isolated from 2-week-old shoot cultures, embedded in alginate solutions and cultured in the presence of phytosulfokine (100 nM) (Maćkowska et al., 2014). Histological analyses were performed after 2 months of culture. The material for sectioning was fixed in 5% buffered glutaraldehyde at room temperature, dehydratated in ethanol series and then embedded in Technovit 7100. The specimens were sectioned into 5 μm fragments with a rotary microtome and subsequently stained with 1% toluidyne blue O (TBO) for general analysis, periodic acid Schiff (PAS) for total carbohydrates detection, naphthol blue black for total protein detection, and aniline blue for callose detection.

After two months of culture the protoplast-derived callus was compact and white-yellow in colour. The globular and torpedo-shape somatic embryos were induced on the callus surface. Root structures were also noted. Histological studies of the callus showed the existence of both groups of meristem cells with a dense cytoplasm and highly vacuolated cells. In the globular and torpedo-shape embryos, a vascular tissue could be observed. The protoplast-derived embryogenic callus was rich with sugars and proteins. The presence of polysaccharides was demonstrated in the proembryogenic structures and in the somatic embryos. The analysis showed accumulation of starch grains and smaller starch grains in the embryogenic cells or structures. The cells differed in protein content. Naphthalene Black reaction detects the storage proteins recognized in the cells as protein bodies, and/or densely protein reach cytoplasm. These characteristics were observed both in PSK-treated and control cultures. Callose deposition was observed in single cells especially in the tissue developed in the presence of PSK.

Reference

Maćkowska K., Jarosz A., Grzebelus E. (2014) *Plant regeneration from leaf-derived protoplasts within the Daucus genus: effect of different conditions in alginate embedding and phytosulfokine application*. Plant Cell Tiss. Org. Cult. 117(2): 241-252.

Dependence of callus regeneration potency on the structure and presence of the suspensor of lettuce parthenogenetic proembryos cultured *in vitro*

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It is well known that the efficiency of plants regeneration from embryos *in vitro* is dependent on various factors such as genotype, media composition or culture conditions. Moreover the specified embryo development stage and its regular structure may also have a significant impact on the effect of plants development *in vitro*. Numerous reports indicate a possibility of plants regenerating from a heart-stage and, especially, from the cotyledonary stage embryos. However the *in vitro* cultures of isolated proembryos are still ineffective. In our investigations we were looking for a similar correlation between the structure of parthenogenetic proembryos and their regenerative potential for obtaining haploid plants of *Lactuca sativa* L.

In lettuce, the growth of haploid globular embryos can be relatively easily stimulated by distant pollination (conducted by a conventional method of pollination of stigma of *L. sativa* male-sterile plants with pollen grains of 18 used species) or through the application of some (7) tested chemicals on the stigmaWhen the induced embryos stopped developing very early, we implemented the embryo-rescue method. Because of the small size of proembryos, we cultured them *in vitro* jointly with the embryo sacs (surrounded by endothelium). Embryo sacs were plated in petri dishes containing one of 28 combinations of the MS media of various modifications (Murashige and Skoog, 1962). The plates were kept in the dark at room temperature (22°C) for 1 week and then transferred to the light (250 mmol m^{-2} s^{-1}) at 16°C or exposed only to the light. The growth of the globular embryos was examined during the *in vitro* cultures. For the analysis of the development of haploid embryos, ovaries and ovules were isolated 6 h after pollination (HAP) to 6 DAP, fixed in FAA, embedded in Paraplast, sectioned and stained with iron hematoxylin and counterstained with fast green FCF.

During the cultures of embryo sacs we observed that the regenerating potency of embryos was most likely dependent on their structure. Most of the formed embryos which were cultured on modified MS media did not grow further or regenerate callus, and degenerated very soon. Proembryos (obtained after chemical induction and pollination representing 16 foreign species) were mostly composed of only several to 15-20 cells and usually characterized by irregular arrangement of cells, of different size, shape or vacuolization – thus no development to form the embryo-like structures was present. Suspensors were not present or formed either, only structures composed of few and strongly vacuolized cells. In two combinations of pollination with *Helianthus annuus* L. or *Helianthus tuberosus* L. (which were also the most efficient pollinators for inducing haploids of lettuce) the presence of well developed 25-30 celled parthenogenetic proembryos (similar to the control material) with properly formed several-celled suspensor was observed. Those embryos demonstrated a high regenerative potency; as from the intensively dividing cells of morphogenic calluses after transfer on fresh media – haploid plants were regenerating.

Study of anther culture response in wheat hybrids with increased resistance to leaf rust

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Leaf rust disease is caused by a fungus *Puccinia triticina.* It infects all grain crops and wild grasses and causes a loss of about 10% of global grain production every year. Meanwhile in Poland that value can reach even 30% per year. The most environmentally sound, low cost method of controlling leaf rust is to breed and grow resistant wheat varieties. So far over 70 leaf rust resistance genes (*Lr* genes) have been identified. European varieties (including Polish) are characterized mainly by the presence of: *Lr1, Lr3, Lr10, Lr13, Lr14a, Lr17b, Lr20, Lr26* and *Lr* genes. Unfortunately rust pathogens are able to overcome the resistance by adding new virulence and the effectiveness of resistance genes depends on the composition of the pathogen population. Therefore *Lr19* genes from the wild relative *Lophopyrum ponticum* introduction into Polish cultivars would be very useful. In order to accelerate the selection process the hybrids are brought to the haploid stage by the androgenesis. Doubled haploids (DH) obtained by *in vitro* cultures are fully homozygous and can be selected with the use of molecular markers.

The aim of the study was to obtain doubled haploid lines in anther culture of winter wheat hybrids with increased resistance to leaf rust.

In the experiment hybrids from two cross combinations were used: genotype T39 × Ozon and genotype T36 × Hondia (T36 and T39 lines included *Lr 19* gene). The plant material has been obtained at Danko Plant Breeding Ltd. The control in the experiment was a variety of AC Abbey, characterized by a high regeneration ability in the anther culture.

Anothers with microspores in uninuclear stage were cultured on a C17 medium. Three combinations of growth regulators were applied: 2,4-D, 2,4-D with dicamba and 2,4-D with kinetin. Fifty anthers without filaments from a single spike were plated per dish. A total of 13 950 anthers were cultured. Regenerated calli were plated onto the regeneration medium MS supplemented by NAA and kinetin. The ploidy level of the green plants was verified by flow cytometer. Haploid plants were treated by colchicine solution to double the number of chromosomes.

Out of the 13850 anthers used, a total of 1375 calli were obtained – the average embryo-like structure frequency reached almost 10%. The embryo-like structure frequency of T36 × Hondia hybrids was almost two times higher in comparison with T39 × Ozon genotype (11.58% and 6.44%, respectively). The average green plant regeneration frequency was similar for both hybrids combination (1.49% and 1.43%, respectively) and above six times highest in the control genotype (9.00 plants per 100 plated anthers).

All three growth hormones combinations of C17 medium responded in a similar manner – the total frequency of embryo-like structure ranged from 9.30% to 10.42%. The green plants regeneration frequency was the highest on the induction medium with dicamba (total 2.02 per 100 anthers). The highest number of plants with chlorophyll defects was regenerated on the medium with kinetin (total 1.82 albinos per 100 anthers).

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Anther culture ability of spring rye (*Secale cereale* L.) cultivars

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Spring rye is a crop of minor importance in Poland, while in Europe it is grown mainly in Spain and Italy. As a result of the limited economic role of this variety practically no information is available on the potential to apply biotechnological techniques in breeding of spring rye cultivars. The aim of this study was to assess the potential of plants in anther cultures of 11 spring rye cultivars of varied provenance for androgenesis and regeneration.

The plant material comprised open pollinated spring rye cultivars: Arens Abruzzi (USA), Bojko (PL), Gator (USA), Gazelle (CAN), Karlshulder (DEU), Petka (DEU), PetkuserSommer (DEU), Priaborshi (ROM), Prolific Spring (CAN), Somro Petkus (DEU) and Strzekęcińskie (PL). Donor plants were grown under controlled greenhouse conditions. Ears containing microspores at the late nuclear stage were subjected to pre-treatment at 4 °C for 14 days. Anther cultures were run according to the method proposed by Immonnen and Tenhola-Roininen (2003). Androgenesis was induced using the C17 medium (Wędzony, 2003) in the following combinations: I – C17 + 90 000 mg/l maltose + 2.0 mg/l 2,4-D + 0.5 mg/l kinetin (control), II – C17 + 90 000 mg/l maltose + 2.0 mg/l 2,4-D, III – C17 + 90 000 mg/l maltose + 1.0 mg/l 2,4-D + 1.0 mg/l dicamba, IV – C17 + 120 000 mg/l maltose + 1.0 mg/l 2,4-D + 1.0 mg/l dicamba, V – C17 + 90 000 mg/l maltose + 50 000 mg/l Ficoll + 1.0 mg/l 2,4-D + 1.0 mg/l dicamba. All the variants of media were solidified adding 2500 mg/l gelrite.

All the tested spring rye cultivars showed capacity to induce androgenesis in anther cultures. The highest efficiency of androgenesis induction was observed in cv. Gazelle (34.13%) and Petkuser Sommer (31.77%) on the C17 medium + 90 000 mg/l maltose + 2.0 mg/l 2,4-D. Green and albinotic plants were regenerated in 8 out of the 11 tested spring rye cultivars. The greatest efficiency of regeneration of green plants in anther cultures was found in cv. Arens Abruzzi – 2.55%, Bojko – 2.21% and Priaborshi – 2.34% on the C17 medium + 90 000 mg/l maltose + 2.0 mg/l 2,4-D.

In the conducted experiment a total of 106 plants were regenerated, including 68% green plants (72 plants) and 32% albinotic plants (34 plants). Ploidy of regenerated green plants (57 plants) was tested using a flow cytometer and it was found that diploid plants predominated among the regenerates at 50.8%, followed by haploids at 47.3%, with tetraploids accounting for 1.75%. The greatest number of haploid plants was obtained in anther cultures of spring rye cv. Bojko – 10 plants, while it was lowest for cv. Somro Petkus – 3 plants. Only about 20% of green regenerants produced via anther culture were suitable for research purposes because of low survival rate and low fertility.

References

Immonen S., Tenhola-Roininen T. (2003) *Protocol for rye anther culture*. In: *Doubled Haploid Production in Crop Plants*. Eds. Maluszyński M. et al., Springer: 141-149.

Wędzony M. (2003) *Protocol for anther culture in hexaploid triticale (× TriticosecaleWittm.)*. In: *Doubled Haploid Production in Crop Plants*. Eds. Maluszyński M. et al., Springer: 141-149.

The androgenic response in anther culture of spring wheat is greater in genotypes with solid stem in contrast to genotypes with hollow stem

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Solid stemmed wheat is highly resistant to lodging and damage from sawfly larvae. The presence of solid pith impedes larval growth and migration. Furthermore, stem solidness improves the yield under water-limited conditions by large storage capacity for water-soluble carbohydrate. An introduction of solid pith genes to a well-yielding Polish varieties of wheat would be beneficial. The use of androgenesis in breeding programme can shorten the time needed for the development of new lines with solid stem. Despite the interest in androgenesis, recalcitrant genotypes exist that limit its use and many efforts are needed to increase green plant regeneration efficiency.

The aim of the study was to analyse the relationship between the filling of stem by pith of the spring wheat cultivars and their ability to regeneration in anther cultures.

The anthers of 24 spring wheat genotypes (*Triticum aestivum* L.), that differed in the filling of stem by pith, were used in the experiment. The evaluation of filling of stem by pith was carried out on a basis of a methodology applied in the USA (5-grade scale: Grade 1 – hollow stem – 0% filled, grade 2 – 25% filled by pith, grade 3 – 50% filled by pith, grade 4 – 75% filled by pith, grade 5 – 100% filled by pith). All of the examined genotypes were classified as one of the three groups: with solid (CLTR 7027, Carola, Tybalt, Fortuna, Sawtana, AC Abbey, Tioga, Leda Collection A47), medium-solid (Glenman, Rescue, Chinook, Alentejano, 431, 401, Marquis, HN ROD 513 750) and hollow stem (Arabeska, 404, Solid Straw Tuscan Varia, Americano 44D, Ruzynska II, Ostka Smolicka, Parabola, Bombona). The donor plants were grown in a field. Anthers with microspores in uninuclear stage were submitted to a temperature shock at 4°C and 8°C. After 7-14 days anthers were implanted on a C17 medium. Two combination of growth regulators were applied: 2,4-D with dicamba and 2,4-D with kinetin. Four combinations including pre-treatment and growth regulators were used. 450 anthers of each genotype were cultured in each combination.

The analysis of variance showed significant differences in the ability to androgenesis among the three groups. The impact of genotype on calli and green plants regeneration was significant. The efficiency of androgenesis induction was not always transferred into a number of green plants obtained. From a total of 1363 calli, 456 plants were regenerated, of which approximately 16% presented chlorophyll defects. There were obtained 382 calli – the average efficiency of calli formation was 0.88%. The highest frequency of green plants regeneration (13.89%) was observed in AC Abbey cultivar. Regeneration was not observed on the explants of four genotypes: CLTR 7027, Alentejano, Marquis and Bombona. There were no significant differences between two pretreatment temperatures (4°C and 8°C) on calli induction and green plant regeneration. Examination of the effect of different auxin types in the induction medium on calli formation and green plant regeneration revealed that a strongest stimulation of these processes was achieved by the C17 medium with 2,4-D and dicamba. The average efficiency of calli formation and green plant regeneration was significant greater in genotypes with a solid stem (4.13% and 1.81%, respectively) compared to hollow stem forms (2.01% and 0.51%, respectively).

Effect of donor plant treatment with 2,4-dichlorophenoxyacetic acid on the anther culture of hot *Capsicum* spp.

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The induced androgenesis allows a rapid production of the geneticly stable recombinants being the original plant initial material for the breeding of an innovative *Capsicum* spp. cultivars. Unfortunately, the pungent genotypes, the source of capsaicinoids, are very hard to obtain in the above technology. The results of the investigation on the *in vitro* androgenesis of *Capsicum* spp. show genotype properties as particularly important for the effectiveness of the process. Sweet fruited cultivars are a better source of the haploids obtained by the microspores sporophytic development; however, among these cultivars great differences across the genotypes occur. In the effective procedures of *in vitro* anther culture, a special role is played by 2.4-D (dichlorophenoxyacetic acid), as an androgenesis-inducing factor, whether or not anthers or microspores are used. In the current modifications of the existing procedures, a special attention is given to media improvement and composition or explant treatment. Interesting information on 2,4-D *in vivo* activity is provided in the experiments on gynogenic haploids. This growth regulator increases polyembryony and haploid embryos frequency when plants are treated during florescence. Androgenic low- and non-responsive lines obtained as a result of a selection within the hybrid population of *Capsicum frutescens* L. × *C. annuum* L. were used for determining the treatment effect of a donor plant with 2,4-D on the anther culture effectiveness.

Due to the plant material origin certain morphological and physiological properties of thirty fruits of each examined accession were evaluated. The analysis of the capsaicinoid content in the whole fruit involved the application of the HPLC technique. Another cultures were conducted using a method described by Dumas de Vaulx et al. (1981) for *C. annuum* L. The ploidy of the plants derived from anther culture as well as callus were expressed as the nuclear DNA content using flow cytometry. Samples were prepared following the Galbraith et al. (1983) procedure, with slight modifications.

When planning the experiment, it was an important methodological problem to determine the concentration and the 2.4D application period. Considering the protective effect of corolla petals that makes the growth regulator penetration difficult, the concentration of the solution used was ten-times higher that it is adequate for the induction medium used in the experiment. Additionally, it was considered that providing the donor plants with the growth regulator dozen-or-so-hours in advance, as compared to the explant collection time, will be justifiable in terms of the length of microsporogenesis and bud applicability for anther culture. In other words, it was assumed that disturbing the hormonal balance at the beginning of that process *in vivo* can enhance the effectiveness of androgenesis while maintaining the culture *in vitro*.

A significant increase in embryogenesis effectiveness for the explants from the treated plants showed an advantageous effect of 2,4-D application. About one half of the plants produced in the anther culture were diploids, characterized by 2C nuclear DNA content. Callus tissues were mixoploid and comprised of cells with the DNA content from 1C to 8C or from 2C to 16C. The microspores were the source of the first group of the mentioned callus. Spontaneous diploidization could be the reason for diploid plantlets formation from microspore cells.

Evaluation of the regeneration ability of *Brassica* interspecific hybrids in *in vitro* anther culture

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Anther cultures are used for obtaining haploid plants and further homozygous lines. Haploid male spores develop into plants with a single set of chromosomes originated from the paternal plant, which can be doubled to restore the fertility of plants. The formation of embryos from the spores on specific media is a well-recognized and widely described phenomenon. The literature data show that sporophytic development of a microspore can proceed in two fundamental ways, i.e. somatic embryogenesis leading to a regeneration of embryos and plants as well as undifferentiated callus formation, from which the plants can be obtained. The type of development is fairly typical of the species, and thus the obtained plants can be very useful in different genetic manipulations and plant breeding at a lower ploidy level. Anther cultures are a quick method of inducing homozygotes, so they have gained great interest of plant breeders (Chu, 1982; Nałęczyńska, 1991).

Regeneration in *in vitro* conditions depends on various external and internal factors. The optimal regenerating configuration depends not only on different media implementation and various culture conditions, but also on the genotype. Therefore, the aim of the present research was to determine the influence of a genotype on the regeneration ability in anther culture of *Brassica napus* hybrid lines and Topas cultivar.

In *in vitro* anther culture effect of genotype and medium composition on embryo development was tested. The material used in this experiment consisted of 8 lines of winter oilseed rape (*B. napus*, 2n = AACC = 38) originated from interspecific crosses of *B. napus* MS-8 line *with B. campestris* ssp. *pekinensis* (AA = 20), ssp. trilocularis (AA = 20), *B. carinata* (BBCC = 34) and *B. juncea* (AABB = 36). The cultivar of spring oilseed rape Topas was used as a control.

In all tested lines a callus formation was observed but particular lines showed differences in the number of anthers producing callus and the intensity of its growth. The highest number of anthers producing callus was observed in 2 lines from the cross MS-8 line × B. campestris ssp. pekinensis and ssp. trilocularis (effectiveness = 0.91 and 1.32%, respectively) and the lowest in Topas cultivar (0.04%). Among the eight tested lines the embryogenesis only in two lines from the cross MS-8 line × *B. carinata* i.e. 127/1/10 and 111/3/09 was observed. The effectiveness of anther cultures expressed as a percentage of embryogenic anthers to the number of anther placed on the medium was different on the particular tested media. The highest embryogenesis was observed in cultivar Topas on B5 medium with addition IAA (40.00%). Among the two embryogenic lines the best embryogenesis was observed in line 111/03/09 on a B5 medium supplemented with ABA (25.83%). The average number of embryos formed by embryogenic anthers was observed in Topas on B5 medium with IAA (5.53 embryos/embryogenic anther). In two embryogenig lines the average number of embryos formed by anthers varied from 1.38 to 1.76.

References

Chu C.C. (1982) *Plant improvement and somatic cell embryogenesis*. Academic Press New York: 199-204.
Nałęczyńska A. (1991) Hod. Rośl. Aklim. Nasienn. 35(1-2): 3-27.

The genotype reaction to, and effect of cold treatment on, the *in vitro* androgenesis of *Capsicum* spp.

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Modern taxonomy of the *Capsicum* genus recognizes two cultivated species: *C. annuum*, *C. frutescens* and its wild forms: *C. chinense*, *C. baccatum* and *C. chacoense*. These wild species, as a result of crossing with cultivated forms, may contribute to the extension of the genetic variability of pepper. The method that allows rapid stabilization resulting variability and obtainment homozygous form is androgenesis. This process depends on many factors, including the genotype, the microspore developmental stage, the composition of media and the *in vitro* culture conditions. Several reports indicate that stress, e.g. extreme temperatures, positively affect the embryogenesis.

The aim of our study was to test the influence of the cooling of anther on haploid regeneration *in vitro*. The research was carried on for eight genotypes of pepper: interspecies hybrids (*C. annuum* L. × *C. baccatum*)F1, *C. annuum* L × *C. chinense*)F1, (*C. annuum* L × *C. frutescens*)F1, II(F × A) androgenic line and species: *C. baccatum*, *C. chinense*, *C. chaccoense* and *C. frutescens*. The anther culture was maintained according to the method developed by Dumas de Vaulx et al. (1981). The tested variant were a one-day-long preculture of anther one, performed at 4 °C. The beneficial effect of the cooling of anther on embryo formation was proved for hybrid (*C. annuum* L. × *C. baccatum*)F1. The effectiveness of androgenic embryo formation observed for this genotype reached 3.3%. The most effective was an anther culture of II(F × A) DH line. It responded positively both, in controlled conditions (2.6%) and after cooling (2.3%). In the case of two hybrid forms embryo formation was observed under controlled conditions: for (*C. annuum* L. × *C. chinense*)F1 – 2% and for (*C. annuum* L × *C. frutescens*)F1 – 1.3%. A similar phenomenon was observed for *C. chinense*, the effectiveness of this process reached 1%. The anther of *C. baccatum*, *C. chaccoense* and *C. frutescens* did not form an androgenic embryo in any of the variants tested in the experiment.

Of the total number of 39 embryos produced, twenty four developed into mature plants of the right morphological structure, which were acclimatized under greenhouse conditions. The ploidy level of the obtained plants was carried out by Partec Ploidy Analyzer. The plant material for our analysis was prepared according to Galbraight's et al. method (1983). The flow cytometric analysis revealed that regenerants were diploids – twenty one plants (87.5%) and haploids – three plants (12.5%). Depending on genotype, spontaneous diploidization of microspore-derived regenerants was observed with the frequency ranging from 80 to 100%. The literature reports on the ploidy level of androgenic regenerants inform that the diploid plants of pepper can be produced in both anther and isolated microspore cultures and that most often they are the result of spontaneous doubling of a number of chromosomes at the early stages of haploid embryos development. The obtained androgenic plants seem to constitute good initial material for the genetic improvement of the *Capsicum* genus. However, in the case of diploid regenerants, a molecular analysis and biometrical assessment should be performed in the subsequent generations, to confirm their morphological homogeneity and microspore origin the most effective.

Comparison of the efficiency of haploid and doubled haploid induction in anther cultures of winter and spring forms of triticale (× Triticosecale Wittm.)

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Doubled haploid plants (DH) are a potentially efficient source of homozygous lines in triticale as compared to traditional methods of inbreeding. The practical success of doubled haploid breeding has been demonstrated by new varieties and breeding lines. DH plants of triticale can be obtained through androgenesis (anther and microspore culture) or through wide crossing with maize. Triticale haploids have been predominantly produced by anther cultures. Various factors have been investigated to increase the production of haploids and doubled haploids, for example: genotype, donor plants physiology, microspore developmental stage, pretreatment conditions, physical and chemical conditions for embryos for induction, plant regeneration and doubled-haploid production. The aim of this study was to compare the efficiency of obtaining doubled haploid lines of winter and spring triticale as a result of spontaneous chromosome doubling in anther cultures.

Spikes with anthers in the microspore stage were excised from F1 or F2 triticale hybrids and incubated for 6 days at 4 °C in a solution according to N6 medium + 2.0 mg/l 2,4-D. The anthers (around 1500 per genotype) were placed on an induction medium C17 + 0.5 mg/l kinetin + 90 g/l maltose and 2.0 mg/l 2,4-D. Anther cultures were kept at 28 °C, androgenic structures were transferred after 1 month to a regeneration medium 190-2, containing 1.0 mg/l kinetin + 0.5 mg/l NAA + 30 g/l sucrose. Plant organogenesis was induced at 22°C, with 12 h light/day. The ploidy level of androgenic plants was measured by determining the amount of nuclear DNA in the cells of the leaves, using cytometry.

Forty thousand three hundred and forty three (40 343) androgenic structures were obtained out of 34 winter triticale hybrids (average of 78.6/100 anthers, but depending on the genotype from 4.7 to 153.8). A total of 1457 green plants were regenerated (average of 2,8/100/ anthers, from 0.6 to 9.3 depending on the genotype). In contrast, 31 spring triticale hybrids resulted in 30 140 androgenic structures (average of 67.0/100 anthers, from 7.2 to 287.2 depending on the genotype) with a total of 975 regenerated green plants (average of 2.2/100 anthers, from 0.1 to 17.6 depending on the genotype).

The efficiency of obtaining spontaneous doubling of chromosomes within the winter and spring forms was also compared. A cytometry analysis of 834 androgenic winter plants found 364 (43.9%) spontaneously doubled haploids. Among the 513 spring plants 149 (29.0%) were doubled haploids. Differences were found in the frequency of occurrence of doubled haploids, depending on the genotype from 0 to 68.4% in the winter forms and from 0 to 56.4% in the spring forms.

In summary, the studied winter forms resulted in a greater efficiency of obtaining androgenic structures and green plants, as well as a higher frequency of spontaneous chromosome doubling in anther cultures, compared to the spring forms.

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The first report on protoplast culture of two calamine ecotypes: *Biscutella leavigata* L. and *Alyssum montanum* L.

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The impact of the metallurgical industry on the environment, and in particular the extraction and processing of zinc-lead ores, lead to the progressive degradation of ecosystems. Few plant species are able to survive the harsh conditions caused by adverse physiochemical properties of exploitation wastes. In waste disposal areas, acceleration of spontaneous succession of plant species tolerant to the challenging habitat conditions is desired. Very useful for rehabilitation of such areas are plant species representing local populations of metalophytes. These plants can exhibit accelerated tolerance to heavy metals after subjection to additional selection process. *In vitro* selection not only allows the assessment of large population of plants, but may also induce a new desired variation. The frequency of changes in *in vitro* cultures is much higher than the spontaneous mutation among plants grown in the field. Among *in vitro* techniques, protoplast cultures provide a starting point for multiple methods of plants genetic manipulations, in particular the induction of somaclonal variation, transformation and somatic hybridization. They are often used for *in vitro* selection as well. The uniformity of selection conditions is significantly greater than in other types of cultures allowing more precise selection. However, to exploit these method, it is necessary to develop efficient procedures for protoplasts cultivation and plant regeneration.

The aim of this study was to develop a protocol for protoplast isolation and culture of two species: *Biscutella leavigata* L. and *Alyssum montanum* L. representing calamine ecotypes. Experimental materials were established in *in vitro* cultures originating from seeds collected from the heap in Bolesław near Olkusz. *Biscutella* leaves and *Alyssum* apical shoot fragments were used as explants. Leaves without lower epidermis and finely sliced shoots were incubated in enzymatic mixture consisting of 1.25% Cellulase, 0.5% Macerozyme and 13% sorbitol. Incubation was carried out for 5 h statically or with rotation (75 rpm). Isolated protoplasts were placed in liquid or agarose solidified media: B_5A (Wiszniewska and Piwowarczyk, 2014), K8p (Kao and Michayluk, 1975) and MS medium modified acc. to Hanus-Fajerska et al. (2012). The size of isolated protoplasts, the isolation efficiency and protoplast viability were evaluated directly after isolation. The survival rate and changes in protoplast morphology were assessed on subsequent days of culture.

Protoplasts of two species differed in size. The average diameter of *Allysum* protoplasts was 26 μm, while that of *Biscutella* protoplasts was 33 μm. The efficiency of protoplast isolation from *Biscutella* leaves averaged 4.1×10^6 protoplasts/1g f. w. and was significantly higher than the efficiency of *Alyssum* protoplast isolation that was 0.7×10^6 protoplasts/1g f. w. The viability of protoplasts after isolation was high and did not differ significantly between species. It accounted for 90.8% for *Biscutella* protoplasts and for 84.4% for *Alyssum* protoplasts. After 5 days of culture, the highest vitality of protoplasts (19.0%) isolated from *Biscutella* leaves was observed in the liquid modified MS medium. *Alyssum* protoplasts showed the highest viability (21.7%) in liquid K8p medium. After 5 days of cultivation, oval and budding protoplasts were observed in cultures of both species in a liquid K8p medium, with the frequency between 5.8-7.2%. During 5 days of culture no mitotic activity was observed.

Developing an effective protoplast isolation and culture of selected metalophyte species will facilitate *in vitro* selection for tolerance to heavy metals and determination of mechanisms related to the response to heavy metal stress at the cellular level.

References

Kao K.N., Michayluk M.R. (1975) Planta 126: 105-110.
Wiszniewska A., Piwowarczyk B. (2014) Czech J. Genet. Plant Breed 50(2): 84-91.
Hanus-Fajerska E. et al. (2012) BioTechnologia 93(2): 97-101.

The influence of abscisic acid and ethylene on the organogenesis of *Lilium martagon* L. to bulb scale *in vitro* cultures in relation to proline content

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Ethylene is known to interact with abscisic acid (ABA) in many aspects of a plant growth. ABA is the major player in mediating adaptation of a plant to stress. The aim of the present experiments was to determine the effect of exogenous ABA and precursor of ethylene biosynthesis – 1-aminocyclo-propane-l-carboxylic acid (ACC) on the organogenesis in Turk's cup lily (*Lilium martagon* L.) in *in vitro* cultures. *Lilium martagon* bulbet scales explants were multiplied on the medium with a basic composition described elsewhere (Murashige and Skoog, 1962), containing 0-1.0 μM BA and 0-1.0 μM IAA. The media were supplemented with ABA (0.3 and 3.0 mg dm^{-3}) or ACC (1.0 and 2.0 mg dm^{-3}). On the other hand the nordihydroguaiaretic acid – NDGA (0.3 and 3.0 mg dm^{-3}), which is an antioxidant 5 – LO (5-lipoxygenase) compound which inhibits ABA biosynthesis, was used. The silver nitrate – AgNO3 (1.0 and 2.0 mg dm^{-3}) which is an inhibitor of ethylene action was also used.

The proline analysis was also performed (Bates,1973). It acts as an osmoprotectant under stress conditions and its level may be indicative of the stress of plants.

On a medium with a higher dose of ABA (3.0 mg dm^{-3}) a reduced number of regenerating explants was observed. The addition of a high-dose ABA also resulted in the reduction of the number of bulbs and roots that were formed. The number of the formed bulbs increased with the addition to the medium ACC and NDGA. The highest number of bulbs was noted on media that contained the above mentioned substances and the addition of 0.1 mg BA and 1.0 mg IAA. Similar results were obtained for a number of roots.

The proline concentration was higher in the bulbs (32.0-1124.0 μg/g of fresh weight material) than in the roots (4.0-451.0 μg /g of fresh weight material).

In the bulbs grown in media supplemented with a cytokinine at a higher concentration (1.0 mg dm^{-3} BA) the content of proline was lower than in the bulbs grown on media with a predominance of auxine (1.0 mg dm^{-3} IAA) and without added growth regulators. The addition of ABA resulted in increased levels of proline in bulbs grown in media supplemented with 1.0 mg dm^{-3} of auxin. On media containing a higher concentration of a cytokinine (1.0 mg dm^{-3} BA) the content of proline in tissues was decreased. The addition of ACC resulted a high concentration of proline in the roots. The addition of ABA and a higher concentration of a cytokinine resulted a lower concentration of proline in the roots.

The obtained results indicate that stress factors (ABA, ethylene) may affect the organogenesis of bulbous plants, i.e. *Lilium martagon* L.

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Reference

Bates L.S. (1973) *Rapid determination of free proline for water-stress studies*. Plant Soil 39: 205-207.

The effect of sugars on *in vitro* growth of *Leucojum aestivum* L. plants

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Leucojum aestivum is a medicinal plant which contains numerous Amaryllidaceae alkaloids. Galanthamine is one of the most important alkaloids currently used for treating Alzheimer's disease. Lycorine is another alkaloid found in *Leucojum aestivum* plants that shows anti-cancer and anti-inflammatory properties. Currently, studies are being carried out on the possibilities of using this alkaloid for medicinal purposes (Ivanov et al., 2013).

The initial sources of galanthamine were bulbs of summer snowflake obtained from their natural habitats in Bulgaria. This, however, meant, a partial extinction of the plants there. A chemical synthesis of galanthamine, on the other hand, was found to be economically unprofitable. An alternative method for obtaining secondary metabolites may be their biosynthesis in *in vitro* cultures. Somatic embryogenesis is considered to be an efficient method for micropropagation, and the plant material obtained that way may serve as a valuable source of alkaloids (Ptak et al., 2010). However, this method of micropropagation may be influenced by many factors, including sugars.

In the present research, plants of *Leucojum aestivum* developed from somatic embryos were transferred to the Murashige and Skoog (1962) liquid medium containing 5 μM zeatin. The effect of sugars on plant growth was examined by enriching the medium with: sucrose, glucose, fructose and maltose at concentrations of 3, 6 and 9%. The experiments were performed in bioreactor RITA® vessels with a temporary immersion system, as described earlier by Ptak et al. (2013), for the period of 4 weeks.

The plants of *Leucojum aestivum* cultivated on the medium enriched with 9% sucrose were characterized by the highest biomass increments (5.7 g FW). The lowest biomass increments were observed in the plants grown on the medium containing 9% maltose and glucose (0.58 and 0.69 g FW, respectively). Spectrophotometric analyses showed that the highest amount of chlorophyll *a* was in plants grown on a medium with the addition of 3% sucrose, glucose and fructose (31.35-32.7 μg/g FW). However, as regards chlorophyll *b*, its highest amounts were noted in plants grown on the medium enriched with 6% maltose. (23.12 μg/g FW). Somewhat lower amounts of chlorophyll *b* were found in plants grown on a medium with the addition of 3% sucrose, glucose and fructose. Maltose and fructose added to the medium at concentrations of 3% and 6%, respectively, affected favourably the synthesis of carotenoids in the plants (8.66, 8.65 μg/g FW, respectively). No effect of sugars on the production of phenolic compounds in *Leucojum aestivum* plant tissues was found.

The activities of antioxidant enzymes, *i.e.* superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (POD) in the obtained plant materials will be examined.

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References

Murashige T., Skoog F. (1962) *A revised medium for rapid growth and bioassays with tobacco cultures*. Physiol. Plant. 15: 473-497.

Ptak A., El Tahchy A., Wyżgolik G., Henry M., Laurain-Mattar D. (2010) *Effects of ethylene on somatic embryogenesis and galanthamine content in Leucojum aestivum L. cultures.* Plant Cell Tiss. Organ Cult. 102: 61-67.

Ptak A., Simlat M., Kwiecień M., Laurain-Mattar D. (2013) *Leucojum aestivum plants propagated in in vitro bioreactor culture and on solid media containing cytokinins*. Eng. Life Sci. 13: 261-270.

Comparative study on tissue culture induced variation identified with metAFLP and RP – HPLC in barley and triticale regenerants

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Tissue culture induced variation (TCIV) is a variation that occurs in regenerants during *in vitro* culture plant regeneration. Tissue culture is a stressful environment and may cause numerous phenotypic, genetic as well as epigenetic changes in regenerated plants. Genetic changes involve e.g. sequence alterations and changes in ploidy level, whereas epigenetic changes encompass DNA methylation, histone modification and smRNA mechanism.

The aim of the study was to quantify and compare both sequence variation and DNA methylation changes obtained with metAFLP and RP – HPLC in specially designed plant materials.

In our research four genotypes of DH donor plants of triticale cv. Bogo and five DH donor plants of barley cv. Scarlett, both derived from isolated microspore, were used for plant regeneration via androgenesis and somatic embryogenesis. MetAFLP, based on the isoschizomeric combinations of Acc65I/MseI and KpnI/MseI was applied to analyse the variation at both sequence and DNA methylation levels in restriction sites and their vicinity. Moreover, metAFLP enabled quantification of other characteristics describing plants produced from in vitro culture whereas the RP – HPLC allowed evaluation of the entire level of global cytosine methylation.

Our results obtained with metAFLP demonstrated that in barley the mean value of sequence variation, *de novo* methylation and demethylation were at a similar level (2.2%, 2.1%, 2.1%, respectively), whereas in triticale the mean sequence variation constituted of 19%, *de novo* methylation reached up to 4.5% and demethylation equaled to 5.5%. Barley and triticale regenerants differed significantly in terms of other metAFLP characteristics such as genome methylation (GM), sites affected by methylation (SAM), sites with methylated status in donor and regenerants (SMS) as well as sites with non-methylated status in donor and regenerants (SNMS). Global DNA methylation by means of an RP–HPLC technique in barley regenerants (20 %) was lower than in triticale regenerants (24.1 %). ANOVA demonstrated that both in barley as well as in triticale regenerants the regeneration mode did not affect the tissue culture induced variation, whereas differences between regenerants derived from different DH genotypes were observed. Our results demonstrate that neither barley nor triticale is genetically stable during tissue culture manipulations; however, the level of TCIV in barley is lower in comparison to triticale. The differences between barley and triticale may be due to the ploidy or/and the stability of these species.

Session 2

Role of the Polycomb Repressive Complex (PRC2) in the induction of somatic embryogenesis *Medicago truncatula* cv. Jemalong

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Plant somatic embryogenesis is a process of forming embryos from somatic cells of plant explants without the fusion of gametes. During the induction phase the development program is switched from the somatic to the embryogenic one. This process is associated with a temporary activation or deactivation of sets of genes that allow the dividing cells to progress through different development stages. There is a group of genes critical for the induction of somatic embryogenesis known as marker genes, including LEAFY COTYLEDON 1 (LEC1) and LEAFY COTYLEDON 1 – LIKE (L1L). They are required for early and late embryogenesis and give embryogenic competence to somatic cells. LEC1 and L1L activity can be regulated by epigenetic mechanisms which include histones methylation by Polycomb group proteins (PcG). PcG functions by forming multi-subunit protein complexes such as Polycomb Repressive Complex1 (PRC1) and PRC2. PRC2 is recruited to target genes and catalyses trimethylation of histone H3 lysine 27 (H3K27me3) through the SET domain. PRC1 binds to H3K27me3 and ubiquitinates histone H2A, resulting in a compacted chromatin state. The current data suggest that there are different PRC2 complexes in plants, composed of different members and regulating different developmental pathways. All complexes are composed of MULTICOPY SUPPRESSOR OF IRA 1 (MSI1), FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) and SET domain proteins e.g. CURLY LEAF (CLF), SWINGER (SWN) and one additional protein. To date, there have been no data for/no evidence of the participation of Polycomb group proteins in somatic embryogenesis. In the research we used *Medicago truncatula* as a model plant for Fabaceae family, in which no Polycomb group protein is characterized. The induction of somatic embryognesis was carried out with the use of leaf explants on SH medium supplemented with 0.5 μM 2,4-D and 1.0 μM zeatin in 28 °C, under dark conditions/in the dark? LEC1 and L1L expression was measured as a marker genes of induction of somatic embryogenesis. *Arabidopsis thaliana* sequences of PRC2 protein, i.e., CLF, SWN, MSI1, FIE and EMBRYONIC FLOWER2 (EMF2) were used to BLASTP search against *Medicago truncatula* Genome Sequencing Release Mt4.0.1. The obtained orthologous sequences were then used to construct phylogenetic trees confirming their evolutionary relationship. The confirmed gene sequences were used to design qPCR primers. Gene expression profiles during the induction of somatic embryogenesis were analyzed in two lines of *M. truncatula*, i.e., embryogenic (M9-10a) and non-embryogenic (M9) in five sampling time points. The results demonstrated that the expression level of LEC1 and L1L increased from 7th day of the induction of an embryogenic line, while in the non-embryogenic line a slight increase was observed at14th day. LEC1 shows 168-fold and L1L 8-fold higher transcription activity at 14th day of the induction phase than in the non-embryogenic line. *Medicago truncatula* genome may possess five possible PRC2 genes which are active during the induction of somatic embryogenesis. The activity of the most tested PRC2 complex proteins significantly increased at 14th day of the induction in the embryogenic line. Only the MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) shows a 2-fold higher expression in the embryogenic line than in the non-embryogenic. The results indicate that despite an increase in PRC2 gene expression there was no inhibition of LEC1 and L1L activity. There may be another epigenetic mechanism of LEC1 genes regulation independent of Polycomb group proteins.

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Involvement of S1/P1-like nucleases in a plant programmed cell death

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Programmed cell death (PCD) is an inherent process of all multicellular organisms and play fundamental role in their survival. In plants PCD plays a critical role during morphogenesis, defense responses to pathogens and other stress condition. A programmed cell death in plants is crucial for a number of developmental processes such as embryo formation, degeneration of the aleurone layer, differentiation of tracheary elements in xylem tissues, formation of root aerenchyma and epidermal trichomes, anther tapetum degeneration, floral organ abscission, remodeling of some leaf shapes and leaf senescence. Although the degradation of cellular DNA and RNA is a key step of PCD, the role and mechanism of this process are different in animals and plants. The primary function of DNA hydrolysis in a programmed cell death is to eliminate host genetic material, but it has been suggested that hydrolysis of plant nucleic acids during PCD also provides high amounts of phosphorus, nitrogen and nucleotides for growing tissues. Previous studies have demonstrated that in plants the members of the S1/P1 nuclease family are mainly responsible for nucleic acids degradation. Enzymes from this family hydrolyze single-stranded DNA and RNA, and less efficiently, double-stranded DNA. As a result of their high homology to well described fungal S1 and P1 nucleases that display catalytic activity in low pH and in the presence of zinc ions, it was assumed that the same conditions are required for plant DNA and RNA degradation. However, our research revealed that plant S1/P1 nucleases have a high diversity of the catalytic requirements. Some demonstrate activity in a neutral pH environment and in the presence of calcium or manganese ions, while low pH and zinc ions inhibit their activity. It is worth bearing in mind that different ionic and pH conditions occur in different steps of PCD and different cells compartment, so information about the catalytic requirements may lead to a stage of PCD where a degradation of nucleic acids occurs. It could help to understand the interplay between degradative deoxyribonucleases during this process as well. That is why we conducted studies aimed to determine the evolutionary and molecular causes responsible for such high diversity of catalytic requirements of plant nucleases. Our results have shown that important for ions preferences, among other factors, are posttranslational modifications such as glycosylation. Besides *Arabidopsis thaliana* S1/P1-like nucleases, we examined two S1/P1-like nucleases homologs from moss *Physcomitrella patens*, of which none exhibited activity in an acidic environment and in the presence of zinc ions, all of them were inhabited under such conditions. Our data show that S1/P1 nucleases evolved from enzymes activated in neutral pH and in the presence of manganese ions to the current diversity of catalytic capabilities. The importance of this evolution process for the biological functions of plant S1/P1-type enzymes is still being discussed.

The location of fusion protein AUX1-YFP in a transgenic callus of *Arabidopsis thaliana*

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Auxin controls a wide range of morphogenetic processes and the growth of plants. Only a small portion of endogenous auxin is able to diffuse freely through the cell membrane, while the dominant form of the anion must be transported via specific carriers. The auxin influx carriers auxin resistant 1/like aux 1 (AUX/LAX), efflux carriers pin-formed (PIN) (together with PIN-like proteins) and efflux/conditional P-glycoprotein (ABCB) are major protein families involved in auxin polar transport. The auxin influx carrier AUX1 belongs to the amino acid permease family of proton-driven transporters, and functions in the uptake of indole-3-acetic acid (IAA) into cells. LAX genes, the paralogs of AUX1, maintain auxin distribution pattern against environmental or developmental influences. In *Arabidopsis thaliana*, AtLAX3 is reported to promote the initiation of lateral root primordia by increasing a selection of cell-wall-remodeling enzymes and a wild cherry *Prunus avium* gene, PaLAX1, accelerates the uptake rate of auxin into cells and changes the distribution of free endogenous auxin. Despite the significant enrichment of knowledge about the proteins, AUX/LAX has not been studied regarding its role in the formation of callus tissue. The aim of this study was to evaluate the ability of 14-day leaves of the tested plants: wild type ecotyp Columbia (Wt-Col) of *Arabidopsis thaliana*, transgenic line – ProAUX1::AUX1-YFP and mutats aux1-22 of *Arabidopsis thaliana* for the formation of callus. The result of experiments has revealed that the presence of the construct ProAUX:AUX-YFP and the lack of functional proteins AUX1 had no effect on the increase or decrease in the fresh weight of callus compared to the control line Wt (Col). The fluorescence of the fusion protein AUX1-YFP was only found in two outer layers intensively proliferating the cells of the callus. This location of AUX1-YFP proteins suggests that they are involved in the distribution of auxin in the surface cells of *Arabidopsis thaliana* callus. Studies into the arrangement of auxin in a transgenic callus DR5rev::GUS *Arabidopsis thaliana* showed that elevated levels of auxin are present only in the outer layers of the callus tissue.

AUX1-YFP fluorescence has been identified in the membrane of the callus cells. Occasionally, it has been observed in the cytoplasm, and most often in endosomes like as PIN1-GFP fusion proteins. Thus, both PIN1 or AUX1 proteins are involved in the formation of auxin gradient in the outer layers intensively proliferating the cells of the callus. In the absence of functional proteins AUX1 are mostly likely to take over the function of other proteins belonging to the family AUX/LAX.

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Session 3

Effect of salicylic acid on the activity of antioxidant enzymes in flax *Linum usitatissimum* (L.)

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Salicylic acid (SA) is an important regulator in plant growth, development and defense responses. This signal molecule is involved in the controlling of flowering, seed germination, and thermogenesis, as well as in the disease and abiotic stress resistance. Abiotic stress factors (salinity, drought, etc) may lead to an imbalance between the formation of reactive oxygen species (ROS) and their removal, which can result in oxidative stress. Plants have developed enzymatic and non-enzymatic defense systems for detoxification of ROS. The nonenzymatic system consists of ascorbate, glutathione, cysteine, α-tocopherol, alkaloids, phenolics and carotenoids, whereas the enzymatic system includes a wide range of enzymes such as catalase (CAT), guaiacol peroxidase (GPOX), and ascorbate peroxidase (APX), among others. Salicylic acid was found to enhance the activity of antioxidant enzymes, when sprayed exogenously to the drought or the salinity stressed plants; moreover, it might act as a potential non-enzymatic antioxidant. Our preliminary studies are focused on determination of the effect of SA in the solid medium on the activity of antioxidant enzymes in the callus tissue of flax *Linum usitatissimum* L. cultivar Modran.

To analyze the activity of antioxidant enzymes in response to exogenous SA, the established callus was transferred to a solid MS medium containing 0.5 mg l^{-1} BAP and 0.1 mg l^{-1} NAA supplemented with 50 μM SA (variant 1), 100 μM SA (variant 2) or ethanol (control). All callus cultures were grown in the dark at a temperature of 24 °C for two weeks. Plant material was collected after 3, 7, and 14 days of culture. For enzyme assays, frozen callus tissue was ground, extracted with phosphate buffer, and centrifuged. In the supernatant, the activity of CAT, GPOX, and APX was measured spectrophotometrically.

The catalase activity was very low in the flax callus; moreover, in the control, its activity decreased to 36% after 14 days of cultivation in comparison with the initial one. The lowest CAT activity was measured in the callus after 7 days of treatment with 50 μM SA. GPOX activity was relatively high in comparison with the other analyzed enzymes. In the control callus, GPOX activity transiently declined but subsequently increased after 14 days of cultivation. Application of SA to the medium resulted in a decrease in GOPX activity. The decrease in GPOX activity was observed in the callus exposed to 50 μM SA, especially after 14 days of such treatment, where its activity reached only 29% of the control. At 100 μM SA, GPOX activity reached approx. 50% of the control after 3 and 14 days. APX activity decreased in all the variants during cultivation. After 7 and 14 days, there were no statistically significant differences in APX activity in the callus exposed to both SA concentrations in comparison with the controls.

The effect of SA on CAT activity is not clear. Some studies indicated that salicylic acid could inhibit CAT activity, while some others showed that SA treatment did not influence the activity, or could even induce it. In our experiment, the activity of CAT was very low; this can suggest that this enzyme is not engaged in the flax callus response to the elevated level of SA.

GPOX is found to be the most sensitive enzyme during exposition of the flax callus to SA. The activity of the enzyme decreased significantly. No major changes were observed in APX activity in callus incubated with SA. The explanation of the effect of SA on the antioxidant mechanism in the flax callus requires further research.

Importance of explant size and origin for cryopreservation of *Rosa pomifera* "Karpatia" shoot tips by a droplet vitrification method

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Roses are precious for their ornamental beauty although they also have practical applications in cosmetic, food and pharmaceutical industry. *Rosa pomifera* L. (apple rose) (syn. *Rosa villosa*) "Karpatia" was bred in the Institute of Plant Breeding in Bojnice (Slovak Republic) by J. Šimánek in 1753. It is a high-yielding max. 2 m high shrub. Its fruits (hypanthia) are large (1.5-3 cm), dark red, and characterized by a large amount of flesh containing many bioactive compounds easily assimilated by humans. They are used in medicine and food industry owing to their high content of vitamin C (3500 mg/100 g), A, E, K and B-group vitamins, abundant macro- and microelements, anthocyanins and carotenoids, tannins and organic and fatty acids. They also contain polyphenols and bioflavonoids (rutin, quercetin).

The aim of the present experiment was to assess the effect of the size of a shoot tip, PVS2 treatment time and explant origin (*in vitro*, *in situ*) on the survival and regeneration rate of the "Karpatia" rose after cryopreservation by a drop vitrification method. Explants were collected from axenic culture carried on the 100% Murashige and Skoog medium containing 3% sucrose and 3 μM BA, pH = 5.7. Two types (sizes) of explants were cut out, isolated and prepared: shoot tips L (large) (meristem covered by leaves, 5 mm) and shoot tips S (small) (leaves removed, primordia left, exposed meristem 0.2 mm), which for technical reasons were cut out with a shoot fragment 1-2 mm long. Shoot tips from *in situ* plants were isolated from winter buds in February, scales covering the bud were removed, meristem with primordium was exposed (shoot tips S). Explants were cryopreserved using the droplet vitrification method according to Pawłowska and Szewczyk-Taranek (2014), after LS, PVS2 treatment time was tested (10, 20 and 30 min). After thawing explants were placed on a medium containing 50% MS, 0.3 M sucrose and 0.5% BioAgar in the dark. The shoot tips were transferred to a rose multiplication medium after 24 h and were maintained in the dark during the first week, and the intensity of light (PPFD) was gradually increased to 30 μM m^2 s^{-1} after the second week. The survival of the shoot tips after cryopreservation was assessed 25 days after thawing, based on visual examination. The percentage of explants with developed leaves and stems represented the survival rate. The regrowth rate corresponding to the production of normal shoot from all cryopreserved explants was calculated 55 days after thawing.

For explants prepared from *in vitro* plants, the survival rate after cryopreservation was 11.6-43%, superior for shoot tips S compared to shoot tips L. However, the regeneration rate for explants derived from the axenic culture was very low, approximating several percent, independently of the shoot tip size. In contrast, the survival rate of explants obtained from *in situ* plants was high (60-84%). When PSV2 treatment time was 20 min, 72% of shoot tip explants retained ability to regenerate at 8 weeks after cryopreservation.

Acknowledgements

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References

Pawłowska B., Szewczyk-Taranek B. (2014) *Droplet vitrification cryopreservation of Rosa canina and Rosa rubiginosa using shoot tips from in situ plants*. Sci. Hortic. Amsterdam 168: 151-156.

Plant responses to a grafting process on the example of *Arabidopsis thaliana* hypocotyl during *in vitro* culture

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Micrografting of *Arabidopsis* hypocotyl is an elegant tool for studying many developmental and signaling processes, and a few approaches for efficient grafting procedures have been presented so far (Turnbull et al., 2002). These methods focus on gaining a stabile, functional graft union. Using the method described by Yin et al. (2012), 4 day old *Arabidopsis thaliana* hypocotyls were grafted, with no excision of cotyledons or adventitious roots. The aim of the current study was to analyze various reactions of grafted hypocotyls on tissue and cellular levels as a response to, among others, the mechanical stress that triggers morphological changes.

The stress reactions of grafted hypocotyls were: 1) reduced growth of scion part of the graft in comparison to control plants; 2) occurrence of anthocyanins in cotyledon and hypocotyl epidermis; 3) disappearance of chlorophyll from stock; 4) callus overproduction in the graft union area with cells of different size and shape; 5) emerging of adventitious roots. In transgenic lines (DR5rev::GFP and PIN1::GFP) extraordinary adventitious roots were formed, which had different phenotype (an increased number of root hairs and development of peculiar lateral roots) from those formed by Columbia wild type grafted hypocotyls.

A histological analysis of grafted hypocotyls revealed that some TEs (tracheary elements) developing within the callus tissue do not take part in vascular reconnection. Their organization and orientation is variable, forming different vortices which look very similar to the vortices observed in the woody plants during both *in vitro* and *in vivo* conditions, as an expression of circular polarity (Kurczyńska and Hejnowicz, 1991) and occur only in the scion, the part of the graft where accumulation of auxin takes place.

Nile Red stain showed occurrence of lipid substances in periclinal cell walls of endodermis next to the vascular cylinder in control hypocotyls. In endodermis of grafted plants lipid substances are deposited in all cell walls, not only the periclinal ones. Polyphenolic substances were present in outer periclinal cell walls of endodermis. We also found that some cells of ground tissue exhibit hypertrophic growth and eventually die, leading to the formation of large empty spaces, mostly within the stock.

The results from an immunocytochemical study show a more abundant occurrence of two arabinogalactan protein epitopes, recognized by JIM8 and JIM13 antibodies in endodermal cells and disappearance of galactan epitope recognized by LM6 antibody, in comparison to control hypocotyls. JIM16 epitope, representing an arabinogalactan protein, occurs abundantly in degenerated stock, coating the borders of dead cells. LM8 epitope (xylogalacturonan) was found in vessels and between cells of ground tissue or between scion and stock cells of grafted seedlings, whereas in control plants this epitope did not occur at all.

In conclusion: in stressed by grafting Arabidopsis cotyledons in *in vitro* conditions cells response involves changes in the direction of the auxin flow and the chemical composition of cell walls in tissues engaged in response to stress conditions.

References

Kurczyńska E.U., Hejnowicz Z. (1991) *Differentiation of circular vessels in isolated segments of Fraxinus excelsior*. Physiol. Plant 83: 275-280.

Turnbull C.G.N., Booker J.P., Leyser H.M.O. (2002) *Micrografting techniques for testing long-distance signaling in Arabidopsis*. Plant J. 32: 255-262.

Yin H. et al. (2012) *Graft-union development: a delicate process that involves cell–cell communication between scion and stock for local auxin accumulation*. J. Exp. Bot. doi: 10.1093/jxb/ers109, pp: 1-14.

Proteome responses to herbicidal stress in maize

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The most crucial function of plant cell is to respond against stress induced for self-defence. Abiotic stresses alter the levels of a number of proteins which may be soluble or structural in nature. Different families of proteins are known to be associated with plant responses to stresses by being newly synthesized, accumulating or decreasing. Among other things, these proteins are involved in signaling, translation, host-defense mechanisms, carbohydrate metabolism and amino acid metabolism.

Proteins undergo significant levels of post-translational modification of their primary sequences followed by targeted proteolysis. Therefore, while transcriptomic approaches are an important resource, functional gene expression profiles can only be achieved by proteomic analysis. Thus, quantitative analysis of gene expression at the protein level is essential for determining plant responses to stress conditions.

Herbicides, commonly known as weed killers, are compounds used to destroy or inhibit the growth of plants, especially weeds. The most popular weed killers, widely used in the maize fields, are nonselective, which means that they affect not only weed populations but influence all plants that are growing in the sprayed area. To guarantee their survival under adverse conditions, plants have developed exquisite adjustments to stresses at all levels (anatomical, morphological, cellular, biochemical and molecular).

Several years ago it was observed that some maize lines show higher sensitivity to herbicide spraying than others but molecules determining such heightened resistance remain unknown to this day. Therefore our goal is to identify molecular basics of plant's increased/decreased resistance to herbicides by analyzing changes in proteomes of two maize lines showing differential resistance to Roundup®. Glyphosate (N-(phosphonomethyl)glycine) is an active compound of Roundup® that targets 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). Roundup® is absorbed by the green plant parts and then moved, or translocated, throughout the plant's tissues. Once inside the plant it prevents the plant from manufacturing certain aromatic amino acids essential for plant growth and life. For better characterization of proteome response we have chosen two maize inbred line, of which one is sensitive to the glyphosate (S79757) and the other one is tolerant for this substance (S245). Those lines were obtained as a result of a crossbreeding program and were derived from herbicide tolerance screening test performed on 25 inbred lines selected based on genetic background, type of endosperm and importance in practical breeding.

Our methodological approach was based on two-dimensional gel electrophoresis (2DE) as it combines separation of polypeptides according to isoelectric properties and molecular weight. After obtaining 2DE gels, we compared results for treated and control plants and extracted differentially expressed proteins. The identification of those proteins was performed using mass spectrometry (MALDI Tof) and MASCOT software.

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Changes in the level of non-enzymatic antioxidants in leaves of *Phaseolus coccineus* (L.) plants under heavy metal stress

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In natural conditions plants are constantly exposed to various environmental stresses that reduce crop yields. At the molecular level, stress in plants may be the result of an imbalance between the production of reactive oxygen species and the activity of the antioxidative system. Due to their relatively high cellular concentrations, ascorbate (Asc) and glutathione (GSH) are essential components of this system. These non-enzymatic antioxidants can react chemically with almost all forms of activated O_2. The level of their reduced and oxidized forms as well as the ratio of their both forms play the main role in the maintenance of cellular homeostasis, and thus in the increasing of the tolerance under stress conditions.

Runner bean plants (*Phaseolus coccineus* L. cv. "Piękny Jaś") grown hydroponically in the nutrient solution were treated with cadmium and copper at two different stages of the primary leaf growth. Cd and Cu ions at the final concentrations of 25 and 75 μM were added to the nutrient medium immediately after the transfer of the seedlings (younger plants) and after 10 days of their growth under controlled conditions (older plants). After 12 days of the metal treatment the contents of ascorbate/dehydroascorbate (ASC/DHA) and homoglutathione (hGSH) in the primary leaves were examined and compared to their age-adequate controls. Additionally, the level of homophytochelatin (hPCs) accumulation in Cd- and Cu-treated runner bean plants was determined and compared to the control.

Differences between the level of Asc, DHA, and hGSH in the metal-treated plants in comparison with the control were observed, and were found to be strictly related to the stage of plant growth. In younger and older plants, the amount of AA decreased after Cd and Cu treatment in comparison with the control, however the level of this metabolite was clearly higher in younger plants. On the contrary, the level of DHA increased after metal treatment, which was particularly evident for Cu-treated younger plants. Therefore, the low values of ASC/DHA ratio in the case of both Cu-treated younger and older plants indicate the essential role of ascorbate in the plant response to stress caused by copper ions.

The hGSH level in the leaves of runner bean plants decreased considerably with plant age. The level of hGSH was significantly higher in Cd-treated younger plants in comparison with the controls. The lower content of hGSH was found in the primary leaves of Cu-treated younger plants as well as in older plants after Cd and Cu treatment. The accumulation of hPCs in the leaves of Cd-treated plants also decreased markedly with the age of the leaves. However, no presence of hPCs in the primary leaves after addition of copper ions was detected.

The response of runner bean plants to the heavy metal stress seems to be related to the type of metal and its concentration, to the growth stage of plants in which metal was applied to the nutrient solution, as well as to the duration of plant exposition to this stress factor.

Manipulation of vanillin synthesis genes in flax

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Plants have been used by humans for centuries as a source of pro-medical components. One of these plants which gained interest among scientists is flax. It is a source of many organic components used in the pharmaceutical industry and diet supplement production. These compounds are secondary metabolites which participate in maintaining proper functionality of many processes in plants, such as cell growth modulation, plant elongation, photoprotection, the defense mechanisms, and regulation of permeability and fluency of plasma membranes. They also have antioxidant, anti-inflammatory, antibacterial and antifungal properties. Moreover, these compounds have a beneficial effect on human health. The main groups of secondary metabolites in flax are terpenes (derived from the C5 precursor isopentenyl diphosphate, IPP), alkaloids (derived from amino acids) and phenolics (syntetized on the shikimate pathway or malonate/acetate pathway). Vanillin (4-hydroxy-3-methoxybenzaldehyde) being such metabolite is crucial in the food and cosmetic industry. Its annual worldwide consumption is estimated to be over 16,000 tons. However, only about 0.25% of it comes from plant bioproduction, whereas the rest is mostly produced in the microbial systems. The current goal is to fully describe the biosynthetic pathway of vanillin in plants. In flax, vanillin is synthesized in the benzoic pathway which is part of the phenylopropanoid pathway. In the future, the exploration of the pathway will allow creating plants with increased amount of vanillin. In this work it was analyzed how genetic changes introduced into the phenylopropanoids pathway affected vanillin synthesis. The approach involved either the up- or down-regulation of genes from the vanillin biosynthesis pathway. New plants with the overexpression of vanillin synthase (enzyme that catalyzes the chemical reaction 3-hydroxy-3-(4-hydroxy-3-methoxyphenyl)-propanoyl-CoA to vanillin and acetyl-CoA) were generated using agro-transformation. The transformation was based on the introduction of two genes from *Pseudomonas fluorescens*. The genes, known as fcs and ech, are responsible for encoding enzymes involved in bioconversion of ferulic acid to vanillin, feruloyl-CoA synthetase and enoyl-CoA hydratase/aldolase.

Also other genes encoding benzoic aldehyde dehydrogenase, BALDH; isochorismate synthase, ISO and 3-ketoacyl-CoA thiolase, KET were analysed as they may be involved in the vanillin synthesis pathway. These genes were silenced using the biolistic methods. The resulting plants were subjected to further analysis of gene activity and marked metabolites level.

A new method of modification was used: the antisense oligonucleotide technique (OLIGO). The use of single-stranded DNA fragments (OLIGO) allows changes in the expression of homologous genes. This non-vector technique is based on infiltration or acquisition of short, 12-21 bp sequences of single-stranded DNA (ssOLIGO) in sense or antisense orientation, complementary to coding and/or regulatory sequences of the modified DNA. The resulting duplex OLIGO-mRNA is a signal for inhibiting (siRNA) or activating (RNAa) mechanisms. Genes which were silenced by these methods are: cinnamate-4-hydroxylase, C4H, 4- coumarate- CoA ligase- 4CL and phenylalanine ammonia lyase- PAL.

On the basis of the results, a gene(s) most effective in the manipulation of vanillin synthesis will be chosen for plant generation.

Effect of PEG 4000 on maturation, germination and conversion frequency of *Picea abies* and *P. omorika* somatic embryos

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Polyethylene glycol (PEG) is used for improving the efficiency of somatic embryogenesis and the quality of somatic seedlings of conifers species. In some studies, the positive development of somatic embryos was obtained after application to the maturation medium of PEG of molecular weight 4000 (Attree et al., 1990, 1995). PEG of this molecular weight cannot readily penetrate the cell walls of plants (Carpita et al., 1979), and it works on plant tissues as natural water stress.

This study aimed to improve the number and the quality of *Picea abies* and *P. omorika* somatic embryos, their germination and conversion using PEG 4000. Embryogenic tissues of *Picea abies* (line A and B) and *P. omorika* (line O) were placed on the LM medium, supplemented with 20 μM ABA (abscisic acid), 1 μM IBA (indolile-3-butyric acid), 34 g/l sucrose and 0. 5 or 7.5% PEG 4000. Cultures were maintained at temp. 25 ± 1 °C, at a light intensity 7.5 W/m^2 (40 W mercury-discharge lamps), and 16-h day photoperiod. After maturation period (5 to 6 weeks) the number of produced embryos and the number of cotyledonary somatic embryos was assessed. Next, cotyledonary embryos of both spruce species were transferred on the Margara germination medium, supplemented with sucrose (10 g/l) for 4 weeks (2 weeks in the dark, and for the next 2 weeks in the light). Finally, the germination and conversion frequencies were evaluated.

No unequivocal positive or negative effect of PEG 4000 on the maturation of somatic embryos of *P. abies* and *P. omorika* was found. Embryogenic tissues of both spruce species showed a tendency for the production of the highest number of somatic embryos in the presence PEG 4000, at 5%. Also at this concentration of PEG 4000 the highest number of cotyledonary somatic embryos was observed. The presence of PEG 4000 in the maturation medium stimulated further growth of hypocotyls in the germinating embryos only, whereas no effect on the root growth was found. As a result, a lack of synchronization in the development of both plant organs was observed. Thus the maximal conversion frequency of somatic embryos into seedlings accounted for 2.4% only for *P. abies* (line B), treated with PEG 4000, at 7.5% during maturation.

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References

Attree S.M., Tautorus T.E., Dunstan D.I., Fowke L.C. (1990) *Somatic embryo maturation, germination and soil establishment of plants of black and white spruce (Picea mariana and Picea glauca).* Can. J. Bot. 68: 583-2589.

Attree S.M., Pomeroy M.K., Fowke L.C. (1995) *Development of white spruce (Picea glauca (Moench) Voss) somatic embryos during culture with abscisic acid and osmoticum and their tolerance to drying and frozen storage.* J. Exp. Bot. 46: 433-439.

Carpita N., Sabularse D., Montezinos D., Delmer D. (1979) *Determination of the pore size of cell walls of living plant cells.* Science 205: 114-117.

Changes in DNA methylation in maize under herbicide stress conditions

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Biological organisms constantly exposed to environmental stimuli (stresses) are capable of establishing mechanisms of protection and adaptation. Because of their sedentary life style, plants are restricted to tolerance, resistance, and avoidance mechanisms only and thus require efficient short-term strategies based on the manipulation of the existing genetic information. Recent studies have indicated that the regulation of stress-responsive genes often depends on chromatin remodeling, which is governed by processes often associated with epigenetic regulation (DNA methylation, histone variants, post-translational modifications). It has also been hypothesized that stresses could reshape a genome at the epigenetic level via transpose activation. The fraction of maize genome that appears to be repetitive sums to 85%, and there is a significant change in methylation level in plants showing differential resistance to herbicides.

DNA methylation and chromatin modifications has been shown to be involved in gene silencing at both transcriptional and posttranscriptional levels. Transcriptional gene silencing is associated with hypermethylation of promoter sequences, while post-transcriptional gene silencing is linked with hypermethylation of transcribed or coding sequences. DNA methylation (both asymmetric and symmetric) plays a crucial role in the regulation of gene expression, in the activity of transposable elements, in the defense against foreign DNA, and even in the inheritance of specific gene expression patterns.

It has been observed in the fields that some maize lines display a higher resistance to herbicides than others but to this day the molecular mechanisms of such resistance has remained unknown. The link between stress exposure and sequence specific changes in DNA methylation has been hypothetical until recently, when it was shown that stresses can induce changes in gene expression through hypomethylation or hypermethylation of DNA.

To reveal more detailed changes in DNA methylation in two maize lines displaying different susceptibility to RoundUp® we used Methylation Sensitive Amplified Polymorphism (MSAP). It is a technique where isoschizomers *Hpa* II and *Msp* I are used to determine the differences in DNA methylation due to enzyme's differential sensitivity to DNA methylation. We observed differences in methylation profiles between the two tested lines under herbicide stress conditions. Differentiating DNA bands were eluted from PAA gels and sequenced. An analysis of 197 DNA fragments using Blastn and Maize GDB databases allowed us to divide them into several groups representing genes encoding for transporter proteins, transferases, methyl-transferases, genes involved in stress responses but also transposons. For more detailed analyses (DNA methylation, chromatin modifications) we chose 4 genes encoding for proteins that could potentially be involved in herbicide stress response.

Based on results obtained using the MSAP technique we were also able to analyze global levels of methylation and how they change in response to herbicide spraying.

Mechanical stress-induced reorganization of the root stem cell niche of Arabidopsis seedlings cultured *in vitro*

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The mechanical stress is one of the critical signals affecting the root functioning at different levels from individual cells to the whole root system. Many fundamental processes, such as turgor regulation, cell expansion, lateral root organogenesis and tropic responses are controlled and modulated via mechanical forces-mediated signaling pathways (Monshausen and Haswell, 2013). Experiments with mechanical stress locally applied to maize root apices (Potocka et al., 2011) proved a strong impact of a mechanical factor on the cellular organization of the root apical meristem (RAM). In the experiment, root apices were forced to grow through a narrowing that triggered ectopic cell divisions in the quiescent centre (QC) and caused a transformation of the meristem from closed to open. However, due to relatively large QC in this species and to the lack of the cell type-specific marker lines, precise tracking of cell fates within the root meristem is rather difficult.

The objective of the current study was to analyse an impact of continuously applied mechanical stimulus on the root meristem of the model plant *Arabidopsis thaliana*. The highly organized RAM of Arabidopsis contains the stem cell niche, comprising four rarely dividing quiescent centre cells surrounded by mitotically active initial cells for the different tissues (Dolan et al., 1993). To mimic the natural soil conditions in experimental assay and to cause mechanical perturbation during root growth, seedlings of wild type and chosen transgenic lines were cultured *in vitro* in tubes filled with granulated agar medium. Confocal microscopic examination of roots stained with propidium iodide showed changes in the organization of the RAM. The most significant findings were: an increased mitotic activity of the quiescent centre cells, atypical divisions of the initials and irregular arrangement of cells in the root cap columella. In our study, the differentiation status of distal stem cells (columella initials) was monitored with the J2341 enhancer trap line and Lugol staining method. Moreover, an analysis of DR5rev::GFP and DR5::GUS reporters revealed changes in auxin distribution in root tips of mechanically stimulated seedlings. An attempt was also made to find out if the cell wall chemical components might be involved in the mechanosensing processes and the maintenance of the root stem cell niche.

The obtained results allow drawing the following conclusions: the external mechanical stress disturbs the precisely specified cell division pattern of the RAM and alters auxin distribution in the region. The results are analysed and discussed in reference to the ability of stem cell niche to adapt to stressful environmental conditions, in this case to the mechanical impedance of the medium.

References

Dolan L., Janmaat K., Willemsen V., Linstead P., Poethig S., Roberts K., Scheres B. (1993) *Cellular organisation of the Arabidopsis thaliana root*. Development 119: 71-84.

Monshausen G.B., Haswell E.S. (2013) *A force of nature: molecular mechanisms of mechanoperception in plants*. J. Exp. Bot. 64: 4663-4680.

Potocka I., Szymanowska-Pułka J., Karczewski J., Nakielski J. (2011) *Effect of mechanical stress on Zea root apex. I. Mechanical stress leads to the switch from closed to open meristem organization*. J. Exp. Bot. 62: 4583-4593.

Carrot seed germination and plant growth in salt stress condition

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Carrot (*Daucus carota* L.) is the most important crop of the Apiaceae family. Initially, carrot was used as a medicinal plant as it exhibits nutritional and health benefits. Now carrot is a worldwide grown and distributed root vegetable mainly owing to its high content of α- and β-carotene which are converted to retinol (vitamin A) in humans. Carrot is rated as salt sensitive crop, increase of soil salinity for every unit above 1.0 dS/m causes 14% root yield decline.

Currently, more than 800 million hectares of land all over the world are salt affected. Currently, 20% of irrigated land and 2% of land farmed by dryland are salt affected. Saline soil has a high salt concentration and is classified as such when the electrical conductivity (EC) is above 4 dS/m, which is equivalent to 40 mM of NaCl. The presence of excess salt in soil inhibits plant growth by osmotic or water-deficit (phase 1) and by salt-specific or ion-excess effect of salinity (phase 2). Phase 1 growth inhibition is caused by high concentrations of salts remaining in rhizosphere and preventing water uptake that finally leads to the reduction of leaf and root growth. During this phase there is no incorporation of salt ions to plant tissues. The toxic effect of salt accumulated in plant tissues is observed only in the second phase, when salt ions are taken by plants and cumulate mostly in old leaves, which eventually die.

The main aim of the commenced work was to verify the hypothesis that salt tolerance in carrot is genetically determined, and to develop a laboratory assay suitable for the evaluation of plant response to salinity that might be useful in further analyses of gene expression. For this purpose, we evaluated the ability of salt tolerant and susceptible carrots to seed germination and seedling growth when exposed to salt rich medium. Seeds of a salt tolerant carrot were obtained from DLB-A plants growing in salt affected areas located in Iran. The salt susceptible population was a Polish breeding line. Seeds or germinated seedlings were placed on Murashige and Skoog mineral medium including vitamins and containing additionally 50-200 mM NaCl. Seed germination was assessed four times every seven days. The effect of salt on seedlings and then plant development was determined after four weeks of growth in *in vitro* conditions by measuring the length and weight of plant parts developing above and below cotyledons.

In control conditions (no NaCl treatment) 85-100% of seeds germinated during 28 days, and 97% of the developed seedlings were morphologically normal. NaCl treatment of 100 mM adversely affected seed germination and plant growth in salt sensitive plants. NaCl treatment also often restricted seedling development only to radicle occurrence and reduced the percentage of morphologically normal seedlings, and these effects became more pronounced at higher NaCl concentrations. In contrast, the DLB-A landrace from Iran, showed tolerance to NaCl. In the presence of 150 mM NaCl in the medium, up to 80% of normal seedlings developed and the weight and length of the plants did not differ significantly from control plants. These results show that genetic determinants of salt tolerance exist in carrot natural resources that can be used for research on the genetic mechanisms controlling this phenomenon and that can be potentially exploited for breeding purposes.

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In vitro cultures of *Populus* × *canescens* inoculated with various strains of ectomycorrhizal *Paxillus involutus* fungi under lead ion stress

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Ectomycorrhizal (ECM) fungi, important partners of plants, are most often found in a symbiosis with tracheophytes. Nearly all tree species common in Poland establish such a symbiosis obligatorily, usually with a very high level of root colonization. Therefore, the ECM symbiosis plays a very important role in physiological responses of trees to environmental conditions and should not be neglected, particularly in the analyses of poplar response to abiotic stresses.

It is well known that the ECM symbiosis generally enhances growth parameters of the poplar host as well as its resistance to various stress conditions, e.g. due to improvement of the plant nutrient and water uptake. In return, plants provide the fungal cells with carbohydrates.

When considering lead stress, it is commonly assumed that host tissues are protected against it by the fungal mantle (a structure formed by ECM fungi on the root tips), which accumulates lead molecules in its cells, primarily cell walls and vacuoles. However, interactions between the two living organisms in the presence of heavy metals are varied. The available reports present the final protective effect differently, as it depends on numerous conditions, primarily the characteristics of particular symbiotic partners.

In this work, we present the results of an *in vitro* experiment performed on poplar, *Populus* × *canescens*, inoculated with two strains of *Paxillus involutus* in control conditions and in the presence of $Pb(NO_3)_2$. The inoculated poplar plants were grown in an agar medium, without a decrease in carbohydrate concentration (symbiosis was not promoted/forced). *In vitro* ECM lines used in this experiment were established from fruit bodies collected in a lead-polluted smelter surrounding and in control areas. In six strains originating from the KGHM smelter buffer zone and six strains from the Zwierzyniec forest, tolerance indices against 75 mM Pb^{2+} ions were assessed. Subsequently, strains displaying the lowest and highest tolerance to lead ions were used for poplar inoculation.

Six weeks after inoculation, biometric features of leaves (Winfolia software), stems and roots (Winrhizo software) as well as the mineral composition of organs were analyzed. The obtained data were compared with the colonization percentage in root systems.

The strains showed significantly different levels of poplar root mycorrhization. When considering the count of typical mycorrhizal tips, poplar root tips mycorrhized with the first strain (originating from the contaminated plot and showing the highest tolerance to lead ions) accounted for over 20% of the root system, while mycorrhization with the second strain (originating form the control area) was recorded in less than 3% of poplar roots.

The differences were not only clearly reflected in the biometric features of roots, but also visible in leaf biometrics. In this poster, we show how the presence of lead ions affects the establishment of symbiosis and how the observed phenotypic changes are linked to changes in the mineral content of inoculated poplar seedlings. Additionally, we shortly discuss the tolerance of fungal strains in the context of their origin and duration of cultivation of the *in vitro* ECM lines in control conditions (without lead ions).

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In vitro cultures of sclerophyts

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Difficulties in the reproduction of ornamental Asparagus using traditional asexual and sexual methods mean that there is a need for a more sophisticated method of propagation. Tissue culture offers a potential for unlimited production of clones and gives the possibility of transfering genes from any sources. *Asparagus* L. is the main genus of the *Asparagaceae* Juss. family and belongs to xerophytes (and more precisely to sclerophytes) living (in native regions) in dry, warm or hot climates. They have developed various structural adaptations against excessive water loss by evaporation. The plants have small, reduced leaves and stems can simulate leaves – cladodes (phylloclades or cladophylls). They are often produced in the axils of a true leaf which may be reduced to a scale leaf. The tip of an asparagus spears has three main components, shoot ground tissue, buds and bracts, all of which have some chloroplasts containing cells and vascular connections (King and Davis, 1992).

The morphology and taxonomy of the genus Asparagus (belonging to Monocotyledons group), and the xeromorphic habits are essential to explants and *in vitro*-methods choice. Phylogeny *per se* promotes recalcitrance problems. Monocotyledonous shoot meristems are basal in origin, and their vascular tissue lacks cambium and meristematic cell types that are particularly responding in cultivation conditions (Benson, 2000). In monocots, the selection of appropriate explant material is more important than the plant genotype (Uma et al., 2012).

Two methods have been tried for *A. densiflorus* – one where lateral buds excised from elongating spears were cultured and a new plant was obtained from each bud. In the other method, slices of internodes were stimulated to form a callus, and after that plantlets were regenerated from the callus through indirect organogenesis or somatic embryogenesis.

Three cultivars of *Asparagus densiflorus* (Kunth) Jessop: "Sprengeri", "Meyerii" and "Myriocladus" were used as asparagus genotypes. Plants were grown in a greenhouse (*ex vivo* explant) or controlled environment cabinets (*ex vitro*). The explants were taken from 2-3 year old adult plants and from *in vitro*-grown seedlings and they were a source of explants for organogenesis, callus tissue, indirect organogenesis and embryogenesis.

A limiting factor in obtaining a higher multiplications rate is sclerophyll features of asparagus plants. Only certain genotypes and explants produced embryogenic callus, and their response was different in the number of embryos produced and conversion to plants.

References

Benson E.E. (2000) *In vitro plant recalcitrance: an introduction*. In Vitro Cel. Develop. Biol. Plant 36: 141-148.

King G.A., Davis K.M. (1992) *Identification, cDNA cloning, and analysis of mRNAs having altered expression in tips of harvested asparagus spears*. Plant Physiol. 100: 1661-1669.

Uma S., Lakshimi S., Saraswathi M.S., Akbar A. (2012) *Plant regeneration through somatic embryogenesis from immature and mature zygotic embryos of Musa acuminata ssp. burmannica*. In Vitro Cel. Develop. Biol. Plant 48: 539-545.

Activity of some elements of antioxidant system in legume protoplast after enzymatic isolation

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During isolation process protoplasts encounter stress conditions and wounding-related reactions, leading to alteration in the oxygen balance. There is opinion that homeostatic mechanisms of oxygen metabolism may not be maintained and overproduction of active forms of oxygen occurs (Roubelakis-Angelakis, 1993). Oxidative stress may be partly involved in the determination of regeneration fate of the protoplasts. Increasing accumulation of toxic oxygen during isolation has been indicated as a cause of protoplast recalcitrance (Sinimis et al., 1994; Xu et al., 2013).

In vitro manipulations on numerous legume plants are regarded as difficult and ineffective (Kuchuk et al., 2000). Previous studies on protoplast culture of grasspea and yellow lupin revealed their limited morphogenic response, manifesting by the disturbances in cell wall reconstitution, arrested divisions and decreasing viability (Wiszniewska and Piwowarczyk, 2014). Limited regenerating ability may be a result of oxidative damage and insufficient antioxidant activity in protoplast. However, the role of oxidative stress generated at the stage of isolation has not been elucidated to date in relation to the recalcitrance of grasspea and lupin protoplasts.

Therefore, we have tested the effect of solution used for enzymatic isolation on the antioxidant system in freshly isolated protoplasts of grasspea (*Lathyrus sativus* L.) and yellow lupin (*Lupinus luteus* L.). Protoplasts were isolated from leaves of grasspea and cotyledons of yellow lupin seedlings grown *in vitro*. Both species were represented by two cultivars. Enzymes were dissolved in three solutions: ultrapure water (Milipore), CPW salt solution (Frearson et al., 1973), and C solution (Sonntag et al., 2009). Purified protoplasts were subjected to the analyses of peroxidase activity, radical scavenging activity, and determination of phenolic profile.

The activity of peroxidase in yellow lupin protoplasts was significantly higher than in grasspea. It ranged from 14.5 to 38.8 U/min/g f.w. and from 3.2 to 17.0 U/min/g f.w in lupin and grasspea, respectively. In grasspea the highest POD activity was determined in protoplasts isolated in CPW solution, while in lupin the response differed between genotypes. Protoplast isolated in CPW salt solution have the highest content of phenolic compounds, ranging from 28.8-40.1 mg/g f.w. in lupin and 32.0-58.0 mg/g f.w. in grasspea. In comparison, in protoplast isolated in two other solutions the level of phenolics was lower. Also the content of phenolic acids, flavonols and anthocyanins was the highest in protoplasts isolated in CPW salt solution. Moreover, in protoplast obtained during isolation in CPW radical scavenging activity was the highest in all tested genotypes, as expressed by 375.8-701.8 and 207.3-341.7 mg DPPH scavenged by 1g f.w. in lupin and grasspea protoplasts, respectively.

The results confirm that isolation environment affects the level and activity of selected components belonging to cell antioxidant system in legume protoplasts. It is an important information in relation to recalcitrance of these plants in in vitro conditions, since events occurring at the stage of isolation may affect further development of protoplasts in the culture.

References

Roubelakis-Angelakis K.A. (1993) In: *Morphogenesis in plants. Molecular approaches*. Eds. Roubelakis-Angelakis K.A., Van K.T.T. Springer, New York: 201-219.

Siminis C.I. et al. (1994) Plant Physiol 105: 1375-1383.

Kuchuk N. et al. (2000) In: *Carbohydrates in grain legume seeds: improving nutritional quality and agronomic characteristics*. Ed. Hedley C.L. CABI Publishing, Wallingford: 145-207.

Xu X. et al. (2013) Plant Cell Tiss. Org. Cult. 114(2): 161-169.

Wiszniewska A., Piwowarczyk B. (2014) Czech J. Gen. Plant Breed. 50(2): 84-91.

Frearson E.M. et. al. (1973) Dev. Biol. 33: 130-137.

Sonntag K. et al. (2009) Plant Cell Tiss. Org. Cult.

Session 4

In vitro cultures of aquatic plants

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The history of aquaristics dates back to the beginning of the 20th century when people started to learn how to keep fish in aquariums. Fish was the main interest then. For many years aquatic plants were a modest supplement in fish keeping. A revolution started when a Japanese photographer Takashi Amano created a new trend in aquaristics – a nature aquarium – creating landscapes inspired by nature. Nowadays people around the whole world are big enthusiasts of his idea. A nature aquarium style requires usage of plants in big amounts, rocks and wood to create beautiful projects. People compete with one another to create the most beautiful landscapes and win prestigious contests. Hundreds of plant species and cultivars currently available in the market still cannot fully satisfy aquascapers – people creating underwater landscapes. Regular expeditions to tropical jungles are organized and new plant species are collected. The fastest way of propagation is *in vitro* culture. Traditional pot plants can be found in shops but more often axenic *in vitro* cultures of aquatic plants are becoming an alternative. Plants are kept in plastic containers with a solid medium just like in a laboratory.

Axenic *in vitro* cultures of aquatic plants are a source of the highest quality plant material. The development of aquascaping and "nature aquarium" trend has created new needs. Aquascapers use in their projects more and more plants and projects are now more sophisticated. People look for well nourished plants free from snails, algae, fish parasites, etc. All of these demands are fulfilled by axenic *in vitro* cultures of plants. Acclimation of plants from *in vitro* cultures is very easy. A beginning aquarium enthusiast can easily achieve 100% of successfully acclimatized plants. Most of aquatic plants live in tropical regions of the globe with wet and dry seasons. During a dry season the water level comes down and reveals submerged plants. These plants have to start to live in the emerged form. Plants have to totally change their structure. Stems are more rigid, trichomes occur or the leaf structure is changed. In *in vitro* cultures plants are not submerged but thanks to 100% humidity, the form of plants is very similar to the submerged ones. After planting plants from *in vitro* cultures do not have to transform from the emerged form to the submerged, they do not lose leaves and do not lose energy on transformation. That is one of the key factors why acclimation is so easy. Plants from *in vitro* cultures are so well nourished that they start to grow fast just a few hours after planting. Pot plants cultivated in greenhouses in an emerged form do not have such good acclimation and fast growth.

What is more when choosing plants from *in vitro* cultures people get many plants at a reasonable price. The way from the producer to the final customer is shortened because there is no need to acclimatize plants in greenhouses. *In vitro* cultures of plants are also free from pesticides commonly used in greenhouse production. This is especially important when sensitive to chemicals shrimps are kept in an aquarium.

The sale of *in vitro* cultures of aquatic plants, thanks to all advantages have an increasing numbers of enthusiasts and are said to be the future of plant aquaristics.

The initial results of experiments of *in vitro* propagation of gooseberry (*Ribes grossularia* L.)

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The popularity and increasing importance of gooseberry prompted initiation of a breeding program at the Institute of Horticulture in Skierniewice. *In vitro* cultures can help in the processes of breeding and obtaining a new variability of the plant and in the propagation of new cultivars introduced to the market. This was the reason of commencing an *in vitro* culture of this plant in our Institute. Literature reports on the micropropagation of gooseberry are scarce and they point to difficulties related to individual genotypes. The aim of our experiments at this early stage was to identify and select individual components of the medium to provide optimal conditions for the growth and proliferation *in vitro*.

The first major problem that occurred was hyperhydration/vitrification in shoot cultures of all fifteen studied genotypes. Hyperhydration of explants is a common problem that hampers the micropropagation. The hyperhydrated tissues are characterized by reduced dry weight, reduced lignin, and cellulose content, high activity of enzymes from a group of dehydrogenases, low content of calcium, manganese, sodium, high content of potassium and chlorophyll, reduced ethylene biosynthesis and disrupted the polar auxin transport in comparison with non-hyper hydrous explants. Counteraction usually concerns reducing the concentration of cytokinin in a medium, increasing sucrose, and the solidifying agent contents, modification of the concentration of mineral salts, bearing in mind the interaction between medium components.

Agar, its kind and concentration, significantly affects the intensity of the growth and morphogenesis due to the availability of media components. With an increasing concentration of agar, the water potential of the medium, the dry and fresh weight, but also the number of shoots decreased. Gerlite, which in comparison to agar does not include impurities of phenolics and sulphur compounds, can, by absorbing some of the medium elements change the ionic balance of the medium and instantly affect micropropagation of some genotypes.

In order to eliminate the hyperhydricity of gooseberry, microshoots were cultured on an MS medium containing a complete or reduced to half concentration of nitrogen. Much better results were achieved by reducing nitrogen to 1/2 because in most genotypes, the vitrification has been inhibited.

For all gooseberry genotypes, the effect of the kind and concentration of cytokinins was studied. On a medium containing BAP, a high number of axillary, durable green shoots were produced but in successive subcultures, their elongation was retarded and instead dense conglomerates of rosette shoots, difficult to separate were formed. The addition of kinetin caused shoot lengthening, but at the same time a majority of them became brown. A simultaneous addition of both cytokinins to the culture medium resulted in the hyperhydricity of shoots, even with a reduced concentration of nitrogen in the medium. Currently, the work is underway to study the effects of other cytokinins and eliminating the phenomena of their accumulation in gooseberry cultures.

The third studied factor was a kind of agar brand. Plant (Duchefa) and Bacto agars as well as Gerlite (Duchefa) were evaluated. Explants were cultured on an MS medium without growth regulators. The replacement of agar with Gelrite resulted in the best quality of gooseberry shoots in the first subculture but in the following subcultures hyperhydricity increased instantly.

The experiments on optimizing the above-mentioned factors are continued.

Micropropagation of three Polish raspberry cultivars in a tissue culture

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Poland is one of the main producers of raspberries in the European Union (raspberry production in 2014 totaled 124 thousand tons). Thus it is very important to use high quality plant material to be planted on in tunnels and open fields. Micropropagation is a very efficient method for large scale production of many plants species, including raspberry. This technique is optimal for producing and mass propagating disease-free and virus–free plants. The purpose of this study was to establish the optimal conditions for *in vitro* micropropagation of three raspberry cultivars: primocane Polana, floricane Radziejowa and Sokolica. Before the experiment, the initiation plants were tested for viruses and planted in pots, next they grew in a specially adapted greenhouse which prevented the transmission of viruses (by pollen). The flowers were cut, and young canes with ancillary buds were used for culture initiation. The plant material was properly sterilized in ethanol and sodium hypochlorite, and then rinsed three times with sterile water. After sterilization, explants were transferred to a laminar flow cabinet and with the help of a microscope meristem were isolated. Later the explants were placed on an initiation medium (MS with half strength of macronutrients) supplemented with 0.4 mg/l BA and 0.1 mg/l IBA and cultured for 3-4 weeks. The media were prepared from separate stocks of macronutrients, micronutrients, chelate iron, vitamins, amino acids, ascorbic acid and growth regulators in different concentrations with sucrose and agar. Before autoclaving, pH of the medium was adjusted at 5.7. The media were boiled and purred to 250 ml glass jars, 30 ml to each after media were autoclaved at 1.5 atm and 121 °C. The cultures were maintained in a special growing chamber with the temperature about 23 °C and fluorescent light 2000 lux for 16 h daily. Stabilized microcuttings were transferred to an MS medium (Murashige and Skoog, 1962) supplemented with BAP in various concentrations (0,4-1 mg/l). The best multiplication rate for three cultivars was obtained on an MS medium with 08 mg/l IBA and the ratio being 1 : 2.5 or 1 : 3 (from one plant after multiplication it was 2.5 or 3 depending on which cultivars were obtained). Shoots 2 cm or longer were placed on five variants of a rooting medium basing on AN (Anderson) or MS with different combinations and concentrations of IBA or NAA. The study showed that investigated raspberries were rooted depending on the cultivars. For Polana cv. the optimal rooting media consisted of an MS medium with 1.0 mg/l NAA and 0.3 mg/l IBA, Radziejowa cv. preferred a medium with 1.0 mg/l NAA, and Sokolica MS medium with 1.2 mg/l NAA. Rooted microcuttings were washed under running tap water and planted to a standard peat substrate in mini pots under plastic foil in a mini tunnel. In the course of 4 weeks the foil was being progressively removed and the finally acclimatized plants were transferred to a greenhouse.

Reference

Murashige T., Skoog F. (1962) *A revised medium for rapid growth and bio assays with tobacco tissue cultures*. Physiol. Plant. 15: 473-497.

Adventitious shoot regeneration from leaf explants of *Malus domestica* Borkh. *in vitro*

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One of the factors influencing successful mitotic polyploidization *in vitro* is high efficiency of direct shoot regeneration from the explants such as fragments of leaves, roots, stems or flower buds. In the case of *Malus domestica*, leaves collected from *in vitro* shoot cultures are used most often. Therefore, in the first step of the research on apple polyploidization, the procedure of efficient *in vitro* shoot regeneration from leaves were optimized. Five cultivars were used for the study: 'Free Redstar', 'Gala Must', 'Sander', 'Redchief' and 'Pristina'. Leaves were collected from *in vitro* shoot cultures pretreated with TDZ or BAP, i.e. the shoots were cultured during four weeks on a modified Murashige and Skoog (1962) (MS) medium containing one of these growth regulators (PGRs) each at the concentration of 1 mg/l. The leaves taken from these preliminary cultures were then incubated in darkness for four weeks on the induction medium - modified MS medium supplemented with 1 mg/l TDZ or 4 mg/l BAP in combination with 0.5 mg/l NAA. Subsequently, for shoot elongation, the leaf explants with adventitious shoot buds were cultured for another four weeks on MS medium supplemented with 1 mg/l BAP, 0.1 mg/l IBA and 0.5 mg/l GA_3. Number of shoots formed on leaf explants was noted at the end of experiment.

The plant growth regulators, TDZ and BAP used either for the pretreatments or the induction medium significantly influenced shoot regeneration. Also the cultivars differ considerably in their regenerative capacity. After four weeks of leaf incubation on induction medium, the strongest response was observed in the cultivar 'Sander' for explants derived from shoots pretreated with BAP and then incubated on the medium containing TDZ (100% of leaf explants formed callus and its growth was the most intense). However this cultivar had the lowest shoot production capacity. In the best case, 27% leaves produced on average 2.5 shoots per explant when BAP was used for the pretreatment and TDZ in the induction medium. Compared to 'Sander', 'Free Redstar' characterized with significantly higher regenerative capacity of leaves. The best results (51% leaves produced on average 3.4 shoots per explant) were obtained for BAP pretreatment and TDZ application in the induction medium. The highest percent of leaves producing shoots (ca. 90%) with the largest number of shoot per explant (ca. 15) were recorded for the cultivars 'Gala Must' and 'Redchief', when the leaves derived from TDZ pretreatment and this PGR was used also in the induction medium. In these cultivars, however, only a half of the shoot numbers were obtained when the leaf explants were derived from BAP pretreatment and incubated on induction medium with TDZ. Shoot regeneration was not observed ('Gala Must') or was sporadic ('Redchief') when BAP was used for both the pretreatment and induction medium. Instead, the best shoot regeneration efficiency of 'Pristina' was observed when BAP was used both for the pretreatment and induction medium.

Cytological analysis showed that the nature of regeneration was mixed with prevailing regeneration of adventitious shoots, although some somatic embryos were also visible especially when TDZ was used in the induction medium. The results indicate that the shoot regeneration procedure should be adjusted separately for each apple cultivar.

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In vitro shoots regeneration and plantlets acclimatization of *Stevia rebaudiana* Bertoni ssp. Sweety

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Stevia rebaudiana comes from subtropical areas of South America. In Poland it can be grown in the ground as an annual plant. Stevia is called "a sweet plant" because it contains a non-caloric natural sugar which is more than 300 times sweeter than sucrose (Lemus-Mondaca et al., 2012; Jitendra et al., 2012). In this case, steviol glycosides are an alternative to sugar derived from sugar beet or cane. Stevia extracts have hypoglycemic properties and do not cause sudden changes in blood glucose level, and those extracts show antibacterial, antifungal and anti-inflammatory activities (Goettemoeller and Lucke, 2010). Stevia is a good source of proteins, carbohydrates and fiber. The leaves of stevia are also a rich in flavonoids, so they show a strong antioxidant capacity and natural probiotic – inulin was isolated from the root (Bugaj et al., 2013; Zayova et al., 2013). Traditional cultivation of this species on a commercial scale is difficult because of the low viability of seeds and poor germination.

One of the methods of obtaining reproductive material are techniques of plant tissue and cell cultures. Techniques using the apical meristems and axillary buds *in vitro* shoot regeneration enable us to produce genetically uniform individuals regardless of the season. Thus the aim of the present investigation was to optimize the process of obtaining microsplantlets of *Stevia rebaudiana* under *in vitro* culture conditions and describes the method for adjusting the rooted plants to *ex vitro*.

Numerous shoots were obtained with sterile single-nodal shoots fragments in the presence of phytohormones on the synthetic media. They underwent a process of elongation and rooting. The highest efficiency of shoots proliferation was observed on a medium containing 0.5 mg dm^{-3} 6- BAP (benzylaminopurine), while the shoot length, number of leaves per shoot and size of the leaf blade were best when there was 0.5 mg dm^{-3} GA_3 (gibberellic acid) in the medium. The process of rhizogenesis intensified the content in the substrate 0.5 mg dm^{-3} IBA (indole -3-butyric acid). Under the influence of this phytohormone the roots were the most numerous and were characterized by the greatest length. Thus prepared microseedling were acclimated to greenhouse conditions. At the stage of acclimatization of the obtained microseedling 25% salt solution MS was used for irrigation, which in comparison to the usage of distilled water increased the yield of the process from 46% to 70%.

References

Bugaj B., Leszczyńska T., Pysz M., Kopeć A., Pacholarz J., Pysz-Izdebska K. (2013) *Charakterystyka i prozdrowotne właściwości Stevia rebaudiana Bertoni*. Żywność. Nauka. Technologia. Jakość 3(88): 27-38.

Goettemoeller J., Lucke K. (2010) *Stewia. Uprawa i wykorzystanie*. Wydawnictwo Kubajak, Krzeszowice.

Jitendra M., Monika S., Ratan S.D., Priyanka G., Priyanka S., Kiran D.J. (2012) *Micropropagation of an antidiabetic plant – Stevia rebaudiana Bertoni, (Natural Sweetener) in Hadoti Region of South-East Rajasthan, India*. J. Biol. Sci. 1(3): 37-42.

Lemus-Mondaca R., Vega-Gálvez A., Zura-Bravo L., Ah-Hen K. (2012) *Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects*. Food Chem. 132(3): 1121-1132.

Zoyova E., Stancheva I., Geneva M., Petrova M., Dimitrova L. (2013) *Antioxidant activity of in vitro propagated Stevia rebaudiana Bertoni plants of different origins*. Turk. J. Biol. 37: 106-113.

Micropropagation of monoescious fibrous hemp based on experiments with a Polish variety Bialobrzeskie

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Cannabis sativa L. is a source of cannabinoids, the compounds of which show a high clinical potential. However, the level of their synthesis is very much dependent on the environmental conditions. Therefore there is need to develop micropropagation procedures that would allow efficient production of a high quality raw material through ensuring a genetically homogeneous pool of plants that grow under constant environmental conditions. The aim of the study was to develop a protocol for an efficient initiation of apical shoot cultures of hemp and for efficient micropropagation of the plants.

The cultures conditions in our research were modified so that the growth dynamics as well as plant propagation could be affected. The experiment was carried out with sowing material of *Cannabis sativa* L., Bialobrzeskie variety, from the Gene Bank of Institute of Natural Fibres and Medicinal Plants in Poznan. Prior to the experiment, the seed purity and germination capacity were assessed. Pure seeds accounted for 94.19% (SD 8.52), of which germinated seeds were 62.88% (SD 18.9). The germinated seeds were used for obtaining sterile parent plants and starting cultures. Cultures of apical buds with the use of two types of media: Murashige Skoog (MS) and Daria. Each medium was used in two variants, with different set of plant growth regulators: (KI) with addition of 0.1 mg/l TDZ, (KII) with addition of 0.1 mg/l TDZ and 2.5 mg/l GA_3. The scheme of the experiment included 2-week cultures on MS/Daria KI medium, followed by a passage without separation of explants and 2-week cultures on MS/Daria KII medium. After 4 weeks the explants were transferred to MS/Daria KI medium and then the multiplication rate was calculated for hemp.

As a result of running the hemp apical bud culture in a cycle of MS KI and KII media growth of the explants was observed with 100% efficiency. However, for Daria KI medium the value reached 90% (SD 14.14), but in KII cultures it dropped to 14% (SD 18.97). After 4 weeks of running the cultures another transfer of apical buds was done to KI medium type in order to evaluate the explant quality and to check the possibilities of running a n-2 subculture. The best quality explants were obtained on an MS medium, where 94% (SD 13.14) of explants were characterized by proper growth and only 2% (SD 6.16) of explants vitrified. During the experiment not only the explant quality was of high importance but also the multiplication rate (MR). The best results were achieved on MS KII medium, where the multiplication rate reached 4.73 (SD 0.99). For Daria KII medium the value was only 1.95 (SD 0.45). Micropropagation of hemp (Bialobrzeskie variety) was the most efficient on a rich MS medium, where MR was twice as high as in case of Daria medium. Additionally, higher MR was obtained in the second subculture, which indicates a possibility of running a cyclic micropropagation.

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Callus cultures of *Harpagophytum procumbens* (Burch.) DC. ex Meisn. – production of secondary metabolites and antioxidant activity

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Harpagophytum procumbens (Burch.) DC. ex Meisn. (Pedaliaceae), commonly known as Devil's claw, is native to the Kalahari Desert region of southern Africa. The dry extract of root tubers has demonstrated anti-inflammatory and analgesic effects in animal models of inflammation and in human trials of osteoarthritis and rheumatic diseases such as arthrosis and lower back pain (McGregor et al., 2005). Clinical studies have shown a reduction of pain sensation and an improvement in mobility and flexibility. The anti-inflammatory and analgesic benefits exerted by *Harpagophytum* can be attributed to, at least in part, its antioxidant properties (Grant et al., 2009). *In vitro* studies have shown that harpagoside, the major iridoid of H. *procumbens*, can reduce nitric oxide (NO) release in lipopolysaccharide (LPS) stimulated RAW 264.7 cells (Huang et al., 2006; Inaba et al., 2010). In this study, three cotyledon-derived callus lines of *H. procumbens* on agar-solidified Schenk and Hildebrandt (SH) medium supplemented with 0.2 mg l^{-1} NAA (α-naphthaleneacetic acid) and 1.0 mg l^{-1} BAP (6-benzylaminopurine) or with picloram at the concentration of 0.5 or 2.0 mg l^{-1} were established. They were maintained in three different culture vessels: culture tubes, Erlenmeyer flasks and Magenta vessels. Hormonal treatment and type of culture vessel influenced callus growth and morphology. The highest biomass (64- to 130-fold increase in fresh and dry weights) within 4 weeks was achieved for calli cultured in Erlenmeyer flasks. Harpagoside, harpagide, verbascoside and isoverbascoside were identified in the callus lines and the ability to produce these compounds was maintained for over 3 years. *H. procumbens* calli were also analyzed by colorimetric methods for total contents of phenolics, flavonoids and anthocyanins.

An evaluation of antioxidant activities showed that the extract of callus cultured on an SH medium with NAA and BAP had the highest antiradical activity against DPPH and ABTS and a capacity to reduce metal ions (Ferric Reducing Antioxidant Power – FRAP). The same extracts were also characterized by the highest total content of phenolics and flavonoids.

References

Grant L., McBean D.E., Fyfe L., Warnock M. (2009) *The inhibition of free radical generation by preparations of Harpagophytum procumbens in vitro*. Phytother. Res. 23: 104-110.

Huang T.H., Tran V.H., Duke R.K., Tan S., Chrubasik S., Roufogalis B.D., Duke C.C. (2006) *Harpagoside suppresses lipopolysaccharide-induced iNOS and COX-2 expression through inhibition of NF-κB activation*. J. Ethnopharma 104: 149-155.

Inaba K., Murata K., Naruto S., Matsuda H. (2010) *Inhibitory effects of devil's claw (secondary root of Harpagophytum procumbens) extract and harpagoside on cytokine production in mouse macrophages*. J. Nat. Med. 64: 219-222.

McGregor G., Fiebich B., Wartenberg A., Brien S., Lewith G., Wegener T. (2005) *Devil's claw (Harpagophytum procumbens): an anti-inflammatory herb with therapeutic potential*. Phytochem. Rev. 4: 47-53.

In vitro cultures of *Eryngium alpinum* L. – an endangered and protected species as a source of plantlets and biomass rich in bioactive compounds

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Eryngium alpinum L. (Apiaceae) is a perennial, rare species native to Austria, Liechtenstein, Croatia, France, Switzerland, Italy, and Slovenia. There is a decline in the number of mature individuals, the quality of its habitat and the extent of the area of occupancy. The threats are mainly due to human activities. Spring grazing by domestic animals or meadows harvested by farmers could have an impact on the reproductive success (Andrello et al., 2012). The species is listed in Annex II of the Habitats Directive and under Appendix I of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention) and it is protected by the European Habitat Directive of Natura 2000, the national Red Lists of protected species and it is considered vulnerable by IUCN. The roots of alpine eryngo, the main species investigated in the study of Le Claire, are known to contain chlorogenic acid, R-(+)-rosmarinic acid and its derivative R-(+)-3′-O-β-D-glucopyranosyl rosmarinic acids (Crowden et al., 1969). The roots demonstrated radical scavenging properties toward DPPH radical in a TLC autographic assay (Le Claire et al., 2005). The identification of two flavonoids, quercetin and kaempferol in the leaves was described (Crowden et al., 1969). The main components of the essential oils obtained by hydrodistillation of the aerial parts were the oxygenated sesquiterpenes (Dunkic et al., 2013) To our knowledge, there is no know traditional use of this species.

The Global Strategy for Plant Conservation promotes the development of both *in situ* and *ex situ* conservation methods of rare and vulnerable species. The aim of the study was to develop a protocol for *E. alpinum in vitro* clonal propagation through axillary bud proliferation. The protocol allows harvesting of high quality raw material from micropropagated plants without a reduction in the medicinal quality and quantity of bioactive compounds. Different duration of fruits and seeds surface disinfection using commercial bleaches were used to assess the aseptic *in vitro* cultures of alpine eryngo. The tested medium variants used for *in vitro* culture induction and micropropagation were based on MS or LS formula supplemented with different growth regulators (cytokinines and auxins) concentrations. The highest survival efficiency was obtained on 1/2 MS medium when 0.1 mg l^{-1} BA (6-benzyladenine) and 0.01 mg l^{-1} IAA (indole-3-acetic acid) were added. Thin-layer chromatography (TLC) analysis indicated that multiple shoots and roots from *in vitro*-derived plants are able to produce phenolic acids (chlorogenic acid and rosmarinic acid) and flavonoids as those present in the plants from botanical gardens and described in the literature. The use of *in vitro* cultures of alpine eryngo allows conducting the phytochemical analysis and testing further biological activities of this species without a reduction of plant material from natural sites.

References

Andrello M., Bizoux J.P., Barbet-Massin M., Gaudeul M., Nicole F., Till-Bottraud I. (2012) *Effects of management regimes and extreme climatic events on plant population viability in Eryngium alpinum.* Biol Conser 147: 99-106.

Le Claire E., Schwaiger S., Banaigs B., Stuppner H., Gafner F. (2005) *Distribution of a new rosmarinic acid derivative E. alpinum L. and other Apiaceae*. J. Agri. Food Chem. 53: 4367-4372.

Crowden R.K., Harborne J.B., Heywood V.H. (1969) *Chemosystematics of the Umbelliferae – A general survey*. Phytochem. 8: 1963-1984.

Dunkic V., Vuko E., Bezic N., Kremer D., Ruscic M. (2013) *Composition an antiviral activity of the essential oils of Eryngium alpinum and E. amethystinum*. Chem. Biodiv. 10: 1894-1902.

In vitro regeneration of *Plantago media* L. from shoot tips and other seedling explants

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Plantago media, a hoary plantain Plantaginaceae, is a perennial, nearly cosmopolitan species occurring in dry grasslands in Europe and Asia. It is considered a medicinal plant, but to a lesser extent than the related species – *P. lanceolata* and *P. major* (van der Art et al., 1992). To the best of our knowledge, the only report on *in vitro* cultures of *P. media* concerned callus culture. Many species of the genus *Plantago* were a subject of *in vitro* culture investigations (Fons et al., 2008; Makowczyńska and Andrzejewska-Golec, 2009) and the present study represents a continuation of the scientific interest in this field of our department (Budzianowska et al., 2004; Budzianowska and Budzianowski, 2009).

The aim of this study was *in vitro* propagation of *P. media*. Such propagation has never been performed before. Seeds of *P. media* obtained from the Botanical Garden of the University of Kiel were used to establish the soil cultivation at the Department of Medicinal and Cosmetic Raw Materials, University of Medicinal Sciences in Poznań. The seeds, which were collected from the developed plants in July 2012, served as a starting material for the establishment of *in vitro* cultures. The sterile seedlings were obtained on a half-strength Murashige and Skoog (MS) medium without growth regulators. Shoot tips, cotyledons, leaf and root fragments and hypocotyls of 1-7 week-old seedlings were used as explants. Those explants were incubated on four variants of the MS medium supplemented with growth regulators: with BAP only or BAP with IAA or NAA. The culture was maintained for 6 weeks and formed shoots were transferred twice at 6-week intervals. For each passage the shoot propagation index was estimated. Propagation of *P. media* was performed from shoot tips and by direct organogenesis on various explants.

The age of seedlings had no influence on the induction of shoot propagation. The highest propagation index was obtained by propagation from shoot tips but a higher number of explants from seedling indicates that the most efficient method of propagation of *P. media* is shoot regeneration from root fragments.

References

van der Art P.J.M. et al. (1992) *General biology of Plantago*. In: *Plantago: a multidisciplinary study*. Ed. Kuiper et al. Ecological Studies 89. Springer-Verlag, Berlin: 4-19.

Fons F. et al. (2008) *Culture of Plantago species as bioactive components resources: a 20-year review and recent applications*. Acta Biol. Gallica 155: 277-300.

Makowczyńska J., Andrzejewska-Golec E. (2009) *Micropropagation of Plantago maritima L. – a vanishing species in Poland*. Acta Soc. Bot. Pol. 78: 13-18.

Budzianowska A. et al. (2004) *Phenylethanoid glucosides from in vitro propagated plants and callus cultures of Plantago lanceolata L.* Planta Med. 70: 834-840.

Budzianowska A., Budzianowski J. (2009) *Phenolic compounds in shoot and callus cultures of Plantago ovata Forssk*. Acta Biol. Cracov. Ser. Bot. 51 suppl. 1: 34.

Regeneration of sorghum in cultures of isolated embryos

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High efficiency of plant regeneration can be achieved by indirect organogenesis and somatic embryogenesis. Indirect regeneration involves forming of organs or embryos from plant somatic cells. The course of the process includes induction of a callus tissue, acquiring competence to induce buds and then for their elongation and rooting, and finally acclimatization of the regenerated plants. It is believed that the age of initial explants and adjusting factors of *in vitro* culture can be of vital importance for dedifferentiation of the cells of the initiated callus to the meristematic state and thus for endowing them with organogenic properties for plant regeneration. The main reason beyond the lack of competences for *in vitro* regeneration often seems to be the age of the initial explants used. Therefore, in the test on the induction of callus tissue and assessment of its regeneration potential in a Sorghum genus, an isolated embryo culture was applied. The study aimed to improve the efficiency in plant regeneration of two sorghum varieties (Rona 1 and Santos) using embryos isolated from immature seeds.

Sorghum panicles on the 10-14th day of flowering were used for starting the isolated steralised embryo cultures. Embryos with caryopsis (embryo axis with scutellum) were isolated from immature sterile seeds. The isolated embryos of about 1-2 mm were placed on 6 types of Murashige Skoog media (MS) with different concentrations of 2.4-D and KIN or BA with addition of nutritional substances. The experiment was so designed that two subcultures were run at the same type of media and lasted for 3 weeks. Next the cultures were transferred to a regenerative medium – MS containing BA (2.0 mg/l) with an addition of honey. Those cultures were run for another 3 weeks, then the regenerated buds were transferred to a rooting medium 1/2 MS with an addition of IAA (0.5 mg/l), where explants were grown for 4-5 weeks.

In isolated embryo cultures for Rona 1 variety, the best results in the induction of a callus culture were obtained on Emb-5 medium, where 80% (SD 12.64) of explants formed callus during the 1st subculture. The share of callusing explants in the 2nd subculture was the highest on Emb-1 medium (64%, SD 18.97). The best callus survivability was achieved with Emb-2 medium. For Santos variety the highest callus proliferation ability was observed for cultures on Emb-4 and Emb-6 media. In the 1st subculture the value was 86.67% (SD 5.77; 11.55), and in the 2nd subculture 73.33% (SD 20.82; 23.09), respectively. The survivability was the best on an Emb-5 medium.

Further stages of the study involved testing the explant regeneration. Positive results were achieved only for Rona 1 variety, whereas Santos variety did not show regeneration capacity. The regenerative potential of the isolated embryo culture for Rona 1 was 40.07% (SD 9.27). Among the regenerated explants, 6.59% of them (SD 1.48) formed buds at the proliferation capacity that equaled 5.78 (SD 5.61). The regenerated buds were rooted and acclimatized under *ex vitro* conditions with the efficiency at 78.52% (SD 11.4).

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The influence of the term of excision and the explant type on *Fritillaria persica* L. regeneration *in vitro*

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Fritillaria persica L is a valuable and interesting bulb plant used in different types of landscape compositions. The plant forms big bulbs and features a low multiplication rate. Hence it is important to evaluate more effective propagation methods for this species. The most often used explants for micropropagation of bulbous plants are buds excised from bulbs or scales with a piece of heel. Disinfection of bulbs is very difficult and ineffective. Studies conducted by Witomska (2002) on micropropagation of *Fritillaria imperialis* L. showed that the explants might be pieces of shoots with or without leaves excised from young shoots 20 cm long.

The aim of the conducted study was to evaluate the best term of explants excision and to define their regeneration ability on media with addition of different cytokinins and auxins.

The explants were shoots 15 cm long obtained at the end of March, as well as ones with leaves obtained in the middle of April. The 2-scale pieces of bulbs with heels obtained in April were also used. Shoots and cut bulbs were disinfected with 0.2% Amistar 250 SC fungicide for an hour, then in 1% solution of NaOCl for 30 min. The explants (0.5 cm thick leafless shoots, 0.5 thick leafy shoots, the base of the leaf separated from the shoot and bulb scales with a piece of shoot) were placed individually in tubes on MS media supplemented with 2.0 mg dm^{-3} BA + NAA in a concentration of 0.1, 0.5 and 1.0 mg dm^{-3} or BA 1.0 mg dm^{-3} and NAA 0.1 mg dm^{-3}.

The fungicide used for disinfection allowed to obtaine 98% contamination free explants from the above ground part of the plant, while in the case of bulbs the effectiveness of disinfection was less than 50% and the pieces of bulbs with heels regenerated very weakly and occurred completely useless for initiation. The term of the explants excision had a crucial impact on their regeneration. Only young shoots without leaves, obtained at the end of March, formed a callus and bulbs on all media. Older shoots, obtained in the middle of April, did not regenerate on the media tested. The regeneration ability of different types of explants on 4 media was tested and showed that all parts of shoots as well as leaves separated from shoots could regenerate and the form callus or bulblets. After 4 weeks of growth the best regeneration was noted on leafy shoots. On media containing 1.0-2.0 mg dm^{-3} BA and 0.1 mg dm^{-3} NAA 67% of explants regenerated forming 3.9-4.2 of bulblets. On the MS media supplemented with 2.0 mg dm^{-3} BA and 1.0 mg dm^{-3} NAA the best regeneration was observed when leafless shoots were used (50%) and leafy shoots (73%) which formed from 1 to 7 bulblets (3.4 on average). 40% of leaf bases formed callus on this medium. The leaves separated from shoots regenerated the best on the medium supplemented with 2.0 mg dm^{-3} BA and 0.5 mg dm^{-3} NAA (60%) as after 4 weeks 1 to 3 bulblets were obtained from the callus. On the above media 83% of regenerating explants were from the leafy shoots, which formed from 1 to 8 bulblets (3.8 on average). The most callus tissue and small bulblets were formed on the young leafless parts of the shoot, which regenerated the best on media containing 0.5-1.0 mg dm^{-3} NAA and 2 mg dm^{-3} BA. The pieces of young shoots with leaves regenerated the quickest and they formed 0.5 cm high bulblets on all media tested. The most bulblets were formed on MS media supplemented with 2 mg dm^{-3} BA and 0.5-1.0 mg dm^{-3} NAA. The slowest regeneration was observed in the case of leaves separated from shoots, which at first formed a callus and bulblets appeared later. The leafless parts of shoots formed a lot of callus tissue on which many small bulblets were formed.

References

Witomska M. (2002) Wpływ wybranych czynników na regenerację in vitro szachownicy cesarskiej – *Fritillaria imperialis* L. Rozprawa habilitacyjna. SGGW, Warszawa.

Protoplast culture of *Brassica* vegetables

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Brassica is the one of the most widely grown plant genus in the world. It consists of vegetables which are rich in nutrients and vitamins, valuable for the human diet. For many years attempts have been made to obtain cultivars of *Brassica* vegetables resistant to disease, for example clubroot. Clubroot is the most dangerous disease that afflict *Brassica* crops, and its eradication is very difficult. Cultivation of resistant varieties would be beneficial for the *Brassica* vegetables producers, plant breeders and consumers. The use of new biotechnological methods in plant breeding programs is a groundbreaking opportunity. By using *in vitro* culture techniques, in particular cultures of protoplasts, it is possible to obtain somatic hybrids which combine desirable traits of parental species. In this process an essential step is to elaborate an efficient *in vitro* method of protoplasts isolation and their regeneration into fertile plants. Because vegetables of the *Brassica* genus are highly morphologically variable, optimization of protoplast isolation and culture conditions must be adjusted to each species and sometimes even cultivars.

The aim of this study was to verify whether the protoplasts isolation method developed at our Department, effective for white cabbage (*Brassica oleracea* var. *capitata* f. *alba*) can be used successfully to maintain cultures for other *Brassica* vegetables.

In this study protoplasts were isolated from leaves of cauliflower (*Brassica oleracea* var. *botrytis*) "Bora", Brussels sprouts (*Brassica oleracea* var. *gemmifera*) "Casiopea", kale *(Brassica oleracea* var. *sebelica*) "Verheul" and red cabbage (*Brassica oleracea* var. *capitata* f. *rubra*) "Reball F1". The control consisted of two cultivars of white cabbage "Kamienna Głowa" and "Kilaton F1". Protoplast were isolated enzymatically (the filter-sterilized enzyme solution consisted of 0.5% Onozuka R-10 celulase, 0.1% pectolyase Y-23, 3 mM CaCl2 and 0.4 M mannitol, 18 h incubation) from leaves of 4-week-old plants grown *in vitro*. The isolated protoplasts were purified by filtering, centrifugation and centrifugation of the two-phase system and washing. After determining their density, the protoplasts were embedded in an calcium alginate layer.

The final culture density was 4×10^5 protoplasts per 1 ml of a culture medium. Immobilized protoplasts were cultured in a liquid culture medium based on Kao and Michayluk, supplemented with 100 mg l^{-1} myo-inositol, 74 g l^{-1} glucose, 250 mg l^{-1} casein hydrolysate, 0.2 mg l^{-1} zeatin and 0.1 mg l^{-1} 2,4-D.

The applied method allowed us to obtain protoplasts from all tested objects. The yield of protoplasts from 1g of fresh tissue and protoplast viability were determined. During the protoplast culture, mitotic activity of protoplasts was observed. The initial research shown that the highest yield of protoplasts after isolation was observed in cauliflower and Brussels sprouts. Additionally, cauliflower as well as kale, had relatively high protoplast viability and mitotic activity.

The influence of L-phenylalanine on the accumulation of flavonoids and phenolic acids in *Vitex agnus castus* (Verbenaceae) shoot cultures

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Vitex agnus castus L. (Verbenaceae), also known as a Chaste Tree or Monk's Pepper, is a medicinal plant originating from the Mediterranean region. The healing properties of this plant have been known since the ancient times. Its fruits are used in the treatment of the premenstrual syndrome, corpus luteum insufficiency, abnormal menstrual cycle, or menopause while leaves show an antibacterial, antioxidant and antiangiogenic activity (Rani and Sharma, 2013). Phenolic acids and flavonoids are plant secondary metabolites with a wide spectrum of pharmacological properties including an antioxidant activity. Agni casti fructus has its monograph in the 8th edition of the European Pharmacopoeia and in the 10th edition of the Polish Pharmacopoeia.

Besides plants growing in the open air, tissue cultures can be an alternative source of the secondary metabolites. The yield of their accumulation in *in vitro* cultures can be increased by numerous biotechnological methods, including culture medium supplementation with different precursors. The purpose of this study was to investigate the influence of L-phenylalanine (L-Phe; the precursor of the important metabolic pathways leading to the biosynthesis of numerous plant secondary metabolites) on the accumulation of phenolic acids and flavonoids in *Vitex agnus castus* shoot cultures.

Agitated shoot cultures were established in Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with BAP (1 mg/l), NAA (0.5 mg/l) and GA3 (0.25 mg/l). Three and four week cultures were supplemented with L-Phe (0.8, 1.6 or 2.4 g/l). Control cultures without L-Phe were prepared and they were treated as a reference. The biomass was collected after one or four days after supplementation, weighted, frozen and lyophilised. Flavonoids and phenolic acids were qualitatively and quantitatively analysed in methanolic extracts from the biomass (before and after acid hydrolysis) using the HPLC method (Ellnain-Wojtaszek and Zgórka, 1999) L-Phe in all three concentrations did not affect the inhibitory effect on the growth of the shoot culture. Some phenolic acids (e.g. protocatechuic, chlorogenic, p-hydroxybenzoic, caffeic acids) and flavonoids (e.g. cinaroside, luteolin, apigenin) were determined in the extracts. The content of the compounds ranged from 0.1 to 139.7 mg/100 d.w. and from 0.2 to 295.4 mg/100 g d.w. (phenolic acids and flavonoids, respectively). The medium supplementation with L-Phe influenced the strong increase of the accumulation of phenolic acids and flavonoids. The best option was to add the 1.6 g of L-Phe to the four-week agitated shoot culture.

The results demonstrate a possibility of increasing accumulation of the pharmacologically important secondary metabolites in *Vitex agnus castus* shoot cultures using medium supplementation with the precursor L-phenylalanine.

References

Rani A., Sharma A. (2013) *The genus Vitex. A review*. Phcog. Rev 7: 188-198.

Murashige T., Skoog F. (1962) *A revised medium for rapid growth and bio assays with tobacco tissue cultures*. Physiol. Plant 15: 473-497.

Ellnain-Wojtaszek M., Zgórka G. (1999) *High-performance liquid chromatography and thin-layer chromatography of phenolic acids from Ginkgo biloba L. leaves collected within vegetative period*. J. Liq. Chrom. Rel. Technol. 22: 1457-1471.

Impact of 3-hydroxybutyrate on the expression of plant histone deacetylases, methylases and crucial genes of the phenylpropanoid pathway

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The 3-hydroxybutyrate (3-OHB) is the main component of ketone bodies in mammalian cells, which might serve as an energy source during the lack of a sufficient amount of glucose in the blood. The studies has shown that even a slight increase in 3-OHB concentration can be significant in certain physiological states because it may modulate important signal cascades that are involved in the cell growth, proliferation and defense against oxidative stress. 3-OHB is characterized by a good penetration ability through the plasma membrane, as well as rapid permeation into the peripheral tissues and good dissolution in water and blood. The metabolism of this compound in the target cells can change the intercellular balance of acetyl-CoA, succinyl-CoA and NAD, the components of many metabolic pathways. Despite the important role in the metabolism, this compound takes part in cellular signaling. It serves as a ligand for at least two surface cell receptors. Intriguing is also the fact that, the 3-hydroxybutyrate is an endogenous inhibitor of the histone deacetylases, which increases the level of their acetylation. Acetylation leads to the suppression of the bonds between the histone tails and the DNA, which influences the conformation changes of the chromatin structure. Such relaxation of chromatin enhances access of the DNA for the transcription factors and enzymes, thus it is important for the regulation of the genes expression. Apparently, 3-OHB in mammalian cells is not only an important metabolite, but also a significant regulatory molecule.

In the available literature there is no detailed information on the synthesis and function of 3-OHB in plants. It is only known that in plant cells enzymes needed for the synthesis of this compound are present: acetoacetyl-CoA thiolase (EC 2.3.1.9) and a reductase, beta-ketoacyl-CoA reductase (EC 1.1.1.36). In our research we decided to analyze the impact of this compound on the gene expression of the plant histone deacetylases and methylases. We determined if it influences the histone deacetylases in plant cells in a similar way as in the mammalian cells and whether the changes at the acetylation level will influence the organization of the transcriptome.

Another investigated aspect is the content of the metabolites in the flax phenylpropanoid pathway and the expression of the enzymes of this pathway. The previous research concerning plants with overexpression of one 3-OHB synthesis gene has shown an increase in the amount of phenolic acids which are synthesized in this pathway. In the phenylpropanoid pathway, a vast group of plant secondary metabolites that play many important functions in plant is synthetized. These metabolites act as i) substances defending against patogens and as pigments in fruits, flowers and seeds; ii) compounds guarding a plant against various stress factors; iii) effective insects deterrent and very active; iv) natural antioxidants. Those compounds, mainly due to their antioxidant properties show a positive impact on the human health. Thus, it would be beneficial to increase their level in consumable plants. Considering the regulatory function of the 3-hydroxybutyrate in the studied aspect we would like to activate phenylpropanoids pathway by using 3-OHB.

The target of our research is approximation of the biological function of this compound in plants and its influence on the possible changes on the genome, transcriptome and metabolome levels. Particularly important is the determination of the role of 3-OHB in the process of plant histone modification, which could extend the knowledge on broadly defined epigenetics.

The content of anthocyanin in potato plants in an *in vitro* culture on the media with different humic products

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The list of plants which produce anthocyanin compounds in *in vitro* cultures of various cells, tissues and organs contains about 30 species. The most common manufacturers of anthocyanins are: *Daucus carota, Vitis vinifera, Perilla frutescens, Aralia cordata and Fragaria pineapple.*

The impact of plant hormones in the medium on the content of anthocyanins in plants differs. So far, studies have shown that auxin stimulated the accumulation of anthocyanin pigments. However, callus cultures *Oxalis linearis* anthocyanin production was promoted by cytokinin and inhibited by auxin, for example NAA and 2,4-D. Humic substances (HS), may result in different morphological, physiological and biochemical effects in higher plants, and they may also affect the production of plant pigments.

The effect of different products containing humic substances (HS), namely: I) humus fertilizer Vermicompost universal, II) active universal humus Humus, III) Vermicompost humus Extra-natural universal, IV) Unifor humus Bio organic-mineral fertilizer, V) Vermicompost humus Extra-natural green flowers, VI) humus Aktivit PM Life activator positive micro-organisms; on the content of anthocyanins in different potato varieties was studied. The following potato varieties were used in the: 1) Aster (very early), 2) Augusta (early), 3) Irga (mean early), 4) Denar (very early), 5) Owacja (early), 6) Tajfun (mean early). Humic substances were added to the MS medium and later the content of anthocyanins in plants was assayed.

When added to the medium humic substances different effects of these compounds on the content of anthocyanins as compared with the control plants were observed. Stimulation was observed in varieties of Augusta, Irga and Denar, whereas in varieties Aster, Tajfun and Owacja, inhibition was observed. The plant body also differed in the level of humic compounds content. The average content of HS was higher for Augusta, Irga, Aster varieties than other one Tajfun, Denar, Owacja Humic acids are water-soluble organic acids naturally present in the soil. It is possible that these heterogeneous molecules (HS) can affect the stimulation or inhibition of various processes including a process of anthocyanins productions. The reason for this could be the variety of potato types and the products used containing humic compounds. Attention should be paid to their concentration and richness of different lateral substituents present in the compounds of humus. The basic structural features of humic acids that may affect a variety of processes include:

1) the aromatic skeleton – consisting of benzene rings connected to each other through various ester bonds, carbon-carbon or carbon-nitrogen;
2) the oxygen-containing functional groups – which have a negative charge such as: A) carboxy (COOH); B) phenolic OH (phenols) – hydroxyl group (OH) attached to a benzene ring; C) ketone (C = O) – ketones, attached to the benzene ring;
3) the amine (NH_2) – which has a positive charge.

All of these chemical groups are present in humic substances and they may have a beneficial effect on the manufacturing process of anthocyanins and phenolic groups. Despite initial setbacks with the marketing of potato, varieties with colored pulp are now increasingly seeking to grow. The potato varieties having a naturally high content of anthocyanins and different color of the pulp and peeled tubers are in great demand. Examples of such variaties may be: Terra Rosa, Amarosa and Purple Fiesta tested and used in the United States.

Impact of exogenous gibberellic acid on carotenoid composition in carrot cells *in vitro*

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Plant cells may accumulate a range of different pigments. Among them the most common are chlorophylls, carotenoids and flavonoids. The first two groups are accumulated in plastids, while flavonoids are deposited in vacuoles. Carotenoids can occur in photosynthetic active tissues where they accompany chlorophylls in chloroplasts as well as in photosynthetic inactive plant tissues and organs where they are sequestered in chromoplasts. Carrot is an example of a relatively small group of plants that accumulate carotenoids in their photosynthetic inactive storage organs. Carrot carotenoids are sequestered in the taproot where they can occur in a crystalline form and as complexes with lipids and proteins.

The amount and type of accumulated carotenoids in cells are determined by biosynthetic pathway, factors affecting its gene expression and chromoplast development. Thus carotenoid accumulation is a complex process depending on the plant developmental stage and plastid biogenesis that may be affected by environmental factors like light exposure or by metabolite flux and signal molecules. Growth regulators may function as important factors in carotenoid biosynthesis and accumulation. Among them, gibberellins are considered to be regulators mediating plastid development and expression of carotenoid genes; thus they may affect carotenoid sequestration in chromoplasts. The impact of gibberellic acid (GA_3) on carotenoid accumulation and other plant pigments like anthocyanins has already been examined. The results indicated that in vitro GA_3 supplementation to a medium enhanced accumulation of carotenoids in cucumber corolla (Vainstein et al. 1994). Moreover, exogenous GA_3 increased expression of ipi-1, ipi-2 and lyc carotenoid pathway genes in *Haematococcus pluvialis*, a chlorophyta rich in astaxanthin (Gao et al., 2013).

Callus or a cell suspension culture *in vitro* are easy for handling and can be considered as model systems for research on genetic control of biosynthetic pathways. This approach is of particular significance in a crop like carrot that accumulates carotenoids in roots, hence any tissue specific analyses are destructive to plant and require a long time of plant growth. The aim of our research was to determine the effect of exogenous GA_3 on carrot cells and callus in their ability to accumulate carotenoids. For this purpose we acquired stable callus cultures from two carrot varieties, Koral and Amsterdamska, and a breeding population 0493B. Koral callus was also used to obtain a cell suspension culture. GA_3 was supplemented to the culture medium in concentrations ranging between 0-80 mg l^{-1}. After one month of callus growth and two months of suspension culture, materials were lyophilized and directed to UPLC (Ultra Performance Liquid Chromatography) analysis to assess carotenoid compound composition and quantification. Initial results showed differences in the amounts of individual carotenoids depending on GA_3 treatment. Moreover, the ratio between the carotenoid compound contents was also affected. The results may also be used to determine optimal culture conditions for elevated sequestration of carotenoids, which is essential for future assessment of genetic determinants in a carrot carotenoid biosynthesis.

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References

Vainstein A., Halevy A.H., Smirra I., Vishnevetsky M. (1994) Plant Physiol. 104: 321-326.

Gao Z., Meng C., Gao H., Li Y., Zhang Xi., Xu D., Zhou S., Liu B., Su Y., Ye N. (2013) Indian J. Biochem. Biophys. 50: 548-553.

Agrobacterium-mediated genetic transformation of *Miscanthus sinensis* and *Miscanthus* × *giganteus*. Preliminary studies

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Miscanthus is a genus of about 20 species of perennial giant grasses which have a great potential for biomass yield (up to 30 t/ha) in countries with moderate climate. This biomass can be used as a renewable feedstock for conversion into bioenergy or biofuels such as bioethanol. Biotechnological methods based on genetic engineering and plant transformation can be an essential complement of projects with the aim to improve miscanthus biomass yield or quality.

The aim of this research was to develop an *Agrobacterium-mediated* method of genetic transformation for *Miscanthus* species.

In the first round of the studies, three genotypes of *M. sinensis* (MS-1, MS-16 and MS-17) and two genotypes of *M.* × *giganteus* (MG and D-116) were transformed with *Agrobacterium tumefaciens* hypervirulent strains: EHA105, AgL0 and AgL1. Preliminary tests showed that hygromycin B is an appropriate selection factor for miscanthus species. Therefore all *Agrobacterium* strains carried pCAMBIA1201 vector, containing within the T-DNA the hygromycin phosphotransferase gene (*hpt*) as a selection marker and *uid* gene coding β-glucuronidase (GUS) as a reporter gene, both under control of 35S RNA CaMV promoter. About 10-week-old embryogenic calli (50 per variant) from immature inflorescences were used as plant material for *Agrobacterium* inoculation. Callus induction was conducted on an MS as a basal medium supplemented with L-proline (0.5 mg/l), L-glutamine (0.5 mg/l), casein (1.0 mg/l) and 2,4-D (2.0 mg/l). Plant regeneration was performed initially on the same medium but with 2,4-D (5.0 mg/l) and BAP (0.5 mg/l) and later on 190-2 medium. All media were supplemented with 2.5 mg/l of hygromycin as a selection agent. Finally 12 putative transgenic plants, 5 for MS-17 and 7 for MG, were obtained. These were micropropagated up to 5 clones, which were rooted and transferred to soil. Clones were analysed using PCR for the detection of transgenes, both *hpt* and *uid*. Probable T-DNA genomic integration was confirmed in all clones for 5 transformants. The transformation efficiency was about 1 to 2%. Further analyses confirming transgenic character of obtained plants, as assay of GUS or *hpt* activity, Southern blot etc. are in progress.

In the next round, the transformation of genotypes MS-17, MG and *M. sacchariflorus* "Robustus" was performed using Agrobacterium tumefaciens strains: EHA 105, AgL0, AgL1 and C58C1 carrying pCAHGA vector. This vector was derived from pCAMBIA and comprised the coding sequence of *hpt* gene optimised according to codon usage in grasses and placed under the control of the ubiquitin gene promoter – ubiquitin 1 (ubq) from *Zea mays* (GenBank JX947345.1) and the GUS coding sequence under of actin gene – actin 1 (Act1) from *Oryza sativ*a (GenBank: S44221.1). This time, 6-week-old embryogenic calli were used for transformation. Despite an optimised vector and, as it was assumed, a more appropriate starting material, no putative transformants were obtained. All of inoculated calli turned brown and eventually died on the selection medium. It may be concluded that the effectiveness of miscanthus transformation is strongly affected by the maturity grade of the used callus. Basing on the obtained results, we plan to repeat miscanthus Agrobacterium-mediated transformation experiments using callus of various ages. We also plan to perform biolistic procedures.

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The influence of precursors and elicitor feeding on lignans accumulation in cultures of *Taxus* × *media* roots carrying a taxadiene synthase transgene

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Lignans – dimeric phenylpropanoids, showed a great number of pharmacological effects such as antibacterial, antifungal, antiviral, antioxidant, anticancer, and anti-inflammatory (MacRae and Towers, 1984; Rios et al., 2002). Lignans are formed by dimerization of two coniferyl alcohol units, derived from L-phenylalanine, to give pinoresinol (Suzuki et al., 2007) and other lignans. *Taxus* × *media* roots carrying taxadiene transgene, the enzyme which committed the first step in taxane biosynthesis were (Sykłowska-Baranek et al., 2015) investigated and the following lignans were determined: (+) pinoresinol, (racemic) lariciresionol, (–) matairesinol, (racemic) secoisolariciresinol and (–) hydroxymatairesinol. Transgenic roots were cultivated in hormone-free liquid DCR-M medium (Sykłowska-Baranek et al., 2009) and supplemented on day 28 with coniferyl alcohol (1, 10 or 100 uM), coniferyl alcohol and elicitor – methyl jasmonate (100 μM) or coniferyl alcohol, methyl jasmonate and L-phenylalanine (100 μM). The samples were harvested after 1 and 2 weeks of cultivation in presence of precursors and/or elicitor. Roots were lyophilized and subjected to HPLC-UV-DAD analysis according to the method described elsewhere (Schmitt and Petersen, 2002).

Supplementing the culture with coniferyl alcohol or coniferyl alcohol and methyl jasmonate resulted in a significant biomass reduction in comparison to the control culture, up to 68%.

In the control culture secoisolariciresinol and lariciresinol (as glucoside) were determined with the highest yield on day 28, 2.35 and 1.43 ug/g DW, respectively. The addition of an elicitor and/or precursors resulted in accumulation of secoisolari-ciresinol as free lignan while lariciresinol was present as free compounds or glucoside. The supplementation o media with methyl jasmonate induced production of free hydroxymatariesinol.

Among all examined lignans, the most abundant was hydroxymatairesinol. Its highest content was detected in roots treated 2 weeks with coniferyl alcohol 1 uM and methyl jasmonate and accounted for 37.9 μg/g DW.

References

Giner R.M., Prieto J.M. (2002) *New findings on the bioactivity of lignans*. Stud. Nat. Prod. Chem. – Bioact. Nat. Prod. G 26: 83-292.

MacRae W.D., Towers G.H.N. (1984) *Biological activities of lignans*. Phytochem. 23: 1207-1220.

Schmitt J., Petersen M. (2002) *Influence of methyl jasmonate and coniferyl alcohol on pinoresinol and matairesinol accumulation in a Forsythia × intermedia suspension culture*. Plant Cell Rep. 20: 885-890.

Suzuki S., Umezawa T. (2007) *Biosynthesis of lignans and norlignans*. J. Wood Sci. 53: 273-284.

Sykłowska-Baranek K., Pilarek M., Bonfill M., Kafel K., Pietrosiuk A. (2015) *Perfluorodecalin-supported system enhances taxane production in hairy root cultures of Taxus x media var. Hicksii carrying a taxadiene synthase transgene*. Plant Cell Tiss. Org. Cult. 120: 1051-1059.

Sykłowska-Baranek K., Pietrosiuk A., Kokoszka A., Furmanowa M. (2009) *Enhancement of taxane production in hairy root culture of Taxus x media var. Hicksii*. J. Plant Physiol. 166: 1950-1954.

The influence of culture conditions on shikonin derivatives accumulation in nontransformed and transformed roots of *Rindera graeca* (A. DC.) Boiss. et Heldr. (A. DC.)

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Rindera graeca Boiss. & Heldr. (Boraginaceae) is an endemic plant of South-East Europe and Mediterranean Basin, growing in rocky places of Greece. This species listed in the WCMC Plants Database as "Rare". It grows on stony slopes with shallow soils, loose screes and humus-deficient soils within the main range 1700-2200 m.

The potential of two nontransformed root lines (RgKN and RgKN-NOA) and four transformed root lines (RgKT3, RgKT7, RgKT17 and RgKT18) for shikonin derivatives production was investigated. To obtain transformed root cultures the *Agrobacterium rhizogenes* ATCC 15834 strain was used (Sykłowska-Baranek et al., 2008). To examine growth parameters and shikonin derivatives production the following hormone-free liquid media were tested: LS (Linsmaier and Skoog, 1965) and four modification of DCR medium (Gupta and Durzan, 1985) with reduced to levels of salts. After four-week cultivation the roots were transferred to M9 (Fujita et al., 1981) production medium. Lyophilized roots and post-culture media were extracted with n-hexane followed by ethyl acetate till they became discolored. Next the extracts were examined using HPLC-UV-DAD technique.

The highest fresh biomass increase was observed when roots were cultivated in DCR medium and ranging from 7-fold (RgKN-NOA) to 47-fold (RgKT18). Shikonin itself was not detected in all investigated samples, however, nontransformed and transformed roots produced unidentified shikonin derivatives. The highest number of shikonin derivatives – four, were detected in ethyl acetate extracts prepared form RgKN and RgKT17 root lines maintained in M9 medium followed cultivation in DCR/2X medium (DCR medium containing 1/2 of macro-, microelements and vitamins). Afterwards all resulted extracts were subjected to TLC chromatography which allowed to determined and pick up an orange compound. The structure of this orange compound was examined and it proved to be a novel naphthoquinone derivative the structure of which was published recently [Jeziorek et al. 2012]. The HPLC-UV-DAD analysis indicated that the orange compound is present mainly in cultures of RgKN and RgKT7 root lines.

References

Fujita Y., Hara Y., Suga C., Morimoto T. (1981) *Production of shikonin derivatives by cell suspension cultures of Lithospermum erythrorhizon. II. A new medium for the production of shikonin derivatives*. Plant Cell Rep. 1: 61-63.

Gupta P.K., Durzan D.J. (1985) *Shoot multiplication from mature trees of Douglas fir (Pseudotsuga menziesii) and sugar pine (Pinus lambertiana)*. Plant Cell Rep. 4: 177-179.

Jeziorek M., Damianakos H., Pietrosiuk A., Kawiak A., Laudy A.E., Jodłowska J., Sykłowska-Baranek K., Chinou I. (2012) *Phenolic compounds from in vitro cultures of Rindera graeca Boiss. & Heldr.* 8th Joint Meeting of AFERP, ASP, GA, PSE & SIF. New York (USA), 28. 07. – 01. 08. 2012, Planta Med.: PI371.

Linsmaier E.F., Skoog F. (1965) *Organic growth factor requirements of tobacco tissue cultures*. Physiol. Plant. 18(1): 100-127.

Sykłowska-Baranek K., Pietrosiuk A., Kuźma Ł., Chinou I., Kongel M., Jeziorek M. (2008) *Establishment of Rindera graeca transgenic root culture as a source of shikonin derivatives*. 7th Join Meeting of AFERP, ASP, GA, PSE & SIF. Natural Products with Pharmaceutical, Nutraceutical, Cosmetic and Agrochemical Interest. Atheens, Greece, 3-8. 08. 2008. Planta Med. 74: 1161 (abstracts: PG-54).

Manipulation with the expression of genes of lignin and pectin synthesis pathways for optimal ratio of biopolymers of flax cell wall

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Common flax (*Linum usitatissimum*) is a dicotyledonous annual crop used by humans for about 10 thousand years. It is a source of seeds used in the production of oil rich in bioactive compounds and fibres used in textile, building or paper industries. Initially, the cultivated flax was used for oil and fibre production at the same time. Today, two main types of flax can be distinguished: oil flax (for oil production) and fibrous flax (for fibre production). The two types differ in the thickness and length of the stem, the number of branches or the number of seedcapsules and seeds in a single capsule. Currently, the oil type is cultivated much more often than the fibrous flax. The flax fibre paled into insignificance because of its physical properties (hardness and stiffness) when compared with the synthetic or cotton fibre. Nevertheless, flax fibre is rich in phenolic acids and flavonoids which possess anti-allergic, antiviral and anti-inflammatory properties. Owing to this property, flax fibres have found application in medicine. The main components of cell walls in flax are biopolymers: cellulose (b1-4-glucose)$_n$, hemicellulose (composed of glucose, xylose, mannose, galactose, uronic acids), lignin (composed of phenolic derivatives (coniferyl alcohol, synaptic alcohol, coumaric alcohol) linked with ether bonds, carbon-carbon bonds, di-phenyl bonds or phenyl-ether bonds) and pectin (composed of the main chain of uronic acid molecules (galacturonic acid) and side sugar chains (rhamnose, arabinose, xylose, galactose). Lignins and pectins are the most biologically active and have the strongest influence on the quality of the fibre. High contents of those polymers in the cell wall contributes to unfavourable prolongation of retting time during fibre production and increases the stiffness of the final product. At the same time lignin and its monomers and dimers are a group of natural plant compounds beneficial for human health, while cellulose constitutes a potential source of energy as an alternative to the decreasing resources of fossil fuels. Cultivation of flax containing a proper ratio of biopolymers would be of interest.

The aim of the study is manipulation with lignin and pectin synthesis in order to obtain an optimal proportion of biopolymers, beneficial for application by human in different branches of industry, by silencing of selected genes of lignin and pectin pathways: formaldehyde dehydrogenase (GFD), SFH, pectin methylesterases (PME1, PME3, PME5), cinnamic alcohol dehydrogenase (CAD), phenylalanine ammonia lyase (PAL), FD, UDP-glucuronosyltransferase (UDPE), Cinnamoyl-CoA reductase (CCR), caffeate O-methyltransferase (COMT) and Acetyl-CoA synthetase (COAL). For this purpose, transient transformation using a gene gun was used. The obtained plants were analysed.

Hairy root cultures of marigold (*Calendula officinalis*) as a source of oleanolic acid glycosides

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Hairy root cultures of many plants have been reported as biological matrices for a large scale production of valuable natural compounds, including triterpenoid saponins. In our laboratory, marigold (*Calendula officinalis* L.) hairy root culture (WC16 line) was established by transformation with the use of wild-type *Agrobacterium rhizogenes* strain ATCC 15835. In standard conditions hairy roots were then maintained in liquid 1/2 Murashige and Skoog medium on a rotary shaker at 120 rpm in the dark and subcultured every 3-4 weeks. This culture has the ability to synthesize oleanolic acid glycosides (saponins) – up to 16.8 mg/g of hairy root d.w., and to release these compounds to the surrounding medium – up to 0.41 mg/l.

Triterpenoids are generally divided into two main groups, steroids considered as primary metabolites and triterpenes (including a large class of pentacyclic compounds like oleanolic acid) classified as secondary metabolites playing various ecological functions and involved in chemical plant protection. Enhancement of the synthesis of secondary metabolites in *in vitro* cultures can be achieved by various strategies including selection of cell lines with high productivity, optimization of the medium and culture conditions, application of genetic engineering, biotransformation and elicitation. We have tested some of these possibilities, e.g. modifications of the medium composition (particularly the forms of nitrogen source) as well as various methods of elicitation with biotic (jasmonic acid, chitosan) and abiotic (heavy metals) factors, in order to increase the production of oleanolic acid glycosides in marigold hairy root cultures. Changing the mineral nitrogen source to only NH_4NO_3 or only KNO_3 resulted in about 4-times increase in oleanolic acid content whereas increasing edamine content produced different results depending on the mineral composition, although in every variant it significantly decreased the culture's growth rate. Among elicitors, the treatment with jasmonic acid appeared to be one of the most promising methods. At the concentration range of 100-150 μM jasmonic acid increased about 20-times both the yield of oleanolic acid glycosides in hairy roots and the amount of these compounds released to the medium. Simultaneously, jasmonic acid decreased 2.5-times the content of sterols in hairy roots. This phenomenon can be explained by the fact that sterols and pentacyclic triterpenes synthetic pathways are competitive for a common precursor – linear 2,3-oxidosqualene.

Jasmonic acid is a signaling molecule that modulates various physiological processes in plants, including the protection against pathogen and insect attack by the induction or the increase of the biosynthesis of defensive secondary metabolites. Jasmonic acid and its derivatives (e.g. methyl ester) have been shown to increase the saponin production in several medicinal plants including *Panax ginseng*, *Glycyrrhiza glabra*, *Bupleurum falcatu*, *Medicago truncatula*. It can be assumed that the stimulation of saponin production may be mediated by the up-regulation of the enzymes involved in the biosynthesis of the triterpene aglycone, like squalene synthase, squalene epoxidase and subsequent triterpene synthases (e.g. β-amyrin synthase), however, also by redirection of the metabolic flux into nonsterol pathway of triterpenoid biosynthesis. Our results showed that marigold hairy roots are a suitable model for metabolic studies on various aspects of regulation of biosynthesis of tritepenoids.

Agrobacterium-mediated transformation of tobacco and sorghum with a genetic construct containing genes encoding enzymes in the sucrose synthesis pathway

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Recently, owing to fast development of molecular biology and the availability of genetic engineering tools, a wider range of genetically modified organisms (GMO) has emerged. Increasing the efficiency of bioethanol production can be achieved by genetic modification of plants. The use of conventional energy sources can be reduced through the application of plant biomass as a material for bioethanol production. Then, bioethanol is used as a component of petrol in car engines. A plant with a high potential in the context of the application of modern bioenergetics is sorghum. It is characterized by low water and soil requirements, as well as high efficiency of biomass production.

Sucrose is the main precursor of the organic compounds used in the metabolism of the plant during its growth and development. Its role is not only limited to providing products of metabolism. Sucrose acts as a signal and regulatory molecule, too. Sucrose is a prefered substrate of alcoholic fermentation. Overexpression of key enzymes involved in sucrose metabolism can lead to changes in the proportions of the sugars for the benefit to produced sucrose. *Agrobacterium tumefaciens* -mediated transformation has been to date the most commonly used method for obtaining transgenic plants. *Agrobacterium* -mediated transformation is the result of a precise interaction of plasmid genes and clear genes of bacteria in response to specific signals derived from plant cells. The overall advantages of using Agrobacterium-mediated transformation over other transformation methods are reduction in transgene copy number and an intact and stable integration of the transgene (newly introduced gene) into the plant genome.

In order to increase the sucrose content we developed and prepared a genetic construct allowing overexpression of the genes encoding enzyme in the sucrose synthesis pathway as a sucrose-phosphate phosphatase (SPP, EC 3.1.3.24), UDP-glucose pyrophosphorylase (UGPase , EC 2.7.7.9) and sucrose synthase (SUSY, EC 2.4.1.13). The genetic construct was transformed into *Agrobacterium tumefaciens* using the electroporation method. The initial steps involving *Agrobacterium* infection, cocultivation, and an early culture/selection of tobacco were made. The surviving plants were transferred into a regeneration medium.

The genetic constructs containing SPP, SUSY and UGPase genes will also be used for *Agrobacterium*-mediated transformation of sorghum. Transgenic plants should be characterized by overexpression of the SPP, SUSY and UGPase genes, which will probably lead to an increase in the sucrose content in sorghum.

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Biomass accumulation and diterpenoids biosynthesis in *Salvia austriaca* transformed roots grown in a bioreactor

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Salvia austriaca Jacq. (Lamiaceae) is a herbaceous perennial plant growing in countries of Eastern Europe (Clebsch, 2003). It is known that this plant is rich in triterpenoic acids (Janicsák et al., 2006), essential oil (Kintzios, 2000) and abietane diterpenoids (Nagy et al., 1999).

From *in vitro S. austriaca* shoots, via genetic transformation by strain A4 *Agrobacterium rhizogenes*, the hairy roots culture were obtained (Kuźma et al., 2011). These roots were grown on a rotary shaker in Erlenmeyer flasks supplemented with liquid SH (Shenk and Hildebrandt, 1972) medium. The transformed roots are capable of biosynthesising abietane-type diterpenoids: taxodione, 15-deoxyfuerstione (Kuźma et al., 2011), 7-2′-oxohexyl-taxodione (Kuźma et al., 2012) and taxodone (unpublished data). These secondary metabolites are known of high biological activity, e.g. antibacterial and cytotoxic properties (Kuźma et al., 2012a, 2012b).

The aim of this work was to enhance the culture scale from flasks to a nutrient-sprinkle bioreactor. The *S. austriaca* hairy roots were cultivated for 50 days in 5-liter nutrient-sprinkle bioreactor supplemented with 1 L of liquid SH medium. After this culture period the fresh and dry biomass of the roots increased 7.5- i 6-fold, respectively. The content of abietane diterpenoids (taxodione, taxodone, 15-deoxyfuerstione and 7-2'-oksohexyl-taxodione) in the culture grown in a bioreactor was determined by the UHPLC method (Kuźma and Wysokińska, 2014). The total diterpenoid content in the 50-day-old *S. austriaca* hairy roots was about 5 mg g-1 dry weight. Taxodone was biosynthesized in the highest amounts. Its content was about 4 mg g-1 dry weight. In those roots, the diterpenoid productivity was almost 62 mg per liter of the culture.

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References

Clebsch B., Barner C.D. (2003) *Salvia austriaca Jacquin*. In: *The New Book of Salvias. Sages for every garden*. Ed. Clebsch B. Timber Press: 39-40.

Giannouli A.L., Kintzios S.E. (2000) Essential oils of *Salvia* ssp: Examples of intraspecific and seasonal variations. In: *Sage. The genus Salvia*. Ed. Kintzios S.E. Harwood Academic Publishers: 69-71.

Janicsák G., Veres K., Kakasy A.Z., Máthé I. (2006) *Study of the oleanolic and ursolic acid contents of some species of the Lamiaceae*. Biochem. System Ecol. 34: 392-396.

Kuźma Ł., Kisiel W., Królicka A., Wysokińska H. (2011) *Genetic transformation of Salvia austriaca by Agrobacterium rhizogenes and diterpenoid isolation*. Die Pharmazie 66: 904-907.

Kuźma Ł., Wysokińska H., Różalski M., Budzyńska A., Więckowska-Szakiel M., Sadowska B., Paszkiewicz M., Kisiel W., Różalska B. (2012) *Antimicrobial and anti-biofilm properties of new taxodione derivative from hairy roots of Salvia austriaca*. Phytomed. 19: 1285-1287.

Kuźma Ł., Wysokińska H., Różalski M., Krajewska U., Kisiel W. (2012) *An unusual taxodione derivative from hairy roots of Salvia austriaca*. Fitoterapia 83: 770-773.

Kuźma Ł., Wysokińska H. (2014) *UPLC-DAD Determination of taxodione content in hairy roots of Salvia austriaca Jacq*. Acta Chromatograph. 26: 671-681.

Nagy G., Güther G., Máthé I., Blunden G., Yang M., Crabb T.A. (1999) *Diterpenoids from Salvia glutinosa, S. austriaca, S. tomentosa and S. verticillata roots*. Phytochem. 52: 1105-1109.

Schenk R.U, Hildebrandt A.C. (1972) *Medium and techniques for induction a growth of monocotyledonous and dicotyledonous plant cell cultures*. Can. J. Bot. 50: 199-204.

Plant *in vitro* cultures as a tool in functional studies of ABC (ATP-binding-cassette) transporters

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The ABC (ATP-binding cassette) transporters constitute one of the largest and most evolutionarily conserved multigene family and are recognized as being crucial for plant development as well as interactions with the environment. Participation of ABC proteins in various plant physiological processes is highlighted by translocation of compounds from diverse groups, for instance: 1) phytohormones; 2) secondary metabolites with anti-microbial properties; 3) surface lipids; and 4) signaling compounds necessary in symbiotic relationships (Kang et al., 2011). The current knowledge about ABC transport functions in plants is gained mostly from studies in *Arabidopsis thaliana*. Although Arabidopsis is the best-characterized model dicot plant with powerful genetic and genomic resources (e.g. T-DNA inserted mutant lines), it does not allow research on, for instance, symbiotic relationships between plants and e.g. arbuscular mycorrhizal (AM) fungi or nitrogen-fixing bacteria.

An interesting and relatively poorly understood group of membrane transporters modulating symbiotic interactions are legume ABC transporters. Despite their putative role in the symbiotic associations, defense responses and the interplay between them, to date, only a few legume ABC transporters have been characterized at the functional level (Banasiak et al. 2013, Zhang et al. 2010, Sugiyama et al. 2008).

Genetic transformation is an invaluable tool for molecular, genetic, biochemical and physiological studies. One of the most popular method to deliver gene constructs into the plant genome is *Agrobacterium*-mediated DNA transfer. Transformation with *A. rhizogenes* has become a powerful tool for gene functional and root biology studies. It represents applicable system to study endosymbiotic associations in transgenically modified roots. The so-called composite plants, generated by inoculating wild-type seedlings with *A. rhizogenes*, which consist of transgenic roots and WT shoots, offers an unique chance to decipher the role of e.g. membrane transporters in symbiosis. In addition, hairy roots can be established as a culture without aboveground parts, clonally propagated and used for metabolomics analysis. In a model legume *Medicago truncatula*, we have successfully used a aforementioned transgenic root material to characterize: 1) promoter activity, 2) symbiotic and pathogenic interactions, and to 3) RNAi silencing of several genes encoding membrane ABC transporters (Banasiak et al. 2013).

Additionally, the stable transgene integration into the genome is possible due to *A. tumefaciens* mediated transformation. This allowed us to obtain transgenic *N. tabacum* BY-2 calli heterologously overexpressing genes encoding selected ABC transporters. The latter material has been propagated as the suspension cell culture, and used for transport experiments deciphering ABC protein activity and/or substrate specificity.

References

Kang J., Park J., Choi H., Burla B., Kretzschmar T., Lee Y., Martinoia E. (2011) *Plant ABC Transporters*. Arabidopsis Book 9: e0153.

Banasiak J., Biala W., Staszkow A., Swarcewicz B., Kepczynska E., Figlerowicz M., Jasinski M. (2013) *A Medicago truncatula ABC transporter belonging to subfamily G modulates the level of isoflavonoids*. J. Exp. Bot. 64: 1005-1015.

Zhang Q., Blaylock L.A., Harrison M.J. (2010) *Two Medicago truncatula half-ABC transporters are essential for arbuscule development in arbuscular mycorrhizal symbiosis*. Plant Cell 22: 1483-1497.

Sugiyama A., Shitan N., Yazaki K. (2008) *Signaling from soybean roots to rhizobium: An ATP-binding cassette-type transporter mediates genistein secretion*. Plant Signal. Behav. 3: 38-40.

Antioxidant and DNA repair stimulating effect of extracts from *Leonurus sibiricus* against an induced oxidative stress and DNA damage in CHO cells

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Leonurus sibiricus commonly known as "Chinese Motherwort" has important biological and pharmacological activities, such as anti-bacterial, anti-inflammatory, anti-carcinogenic and anti-oxidant (Islam et al., 2005; Ahmed et al., 2006; Shin et al., 2009; Lee et al., 2010). Secondary metabolites from this plant are an important source of polyphenolic acids and flavonoids, which possesses potent antioxidant, radical scavenger properties and reduction of intracellular reactive oxygens species (Singh and Jialal, 2006; Lee et al., 2010). Antioxidants can delay, inhibit, or prevent oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. Oxidative stress is an imbalanced state where excessive quantities of reactive oxygen and/or nitrogen species (ROS/RNS, e.g., superoxide anion, hydrogen peroxide, hydroxyl radical, peroxynitrite) overcome endogenous antioxidant capacity, leading to the oxidation of a variety of biomacromolecules, such as enzymes, proteins, DNA and lipids (Ames et al., 1993). Biological activities of this plant have not yet been fully studied.

This is the first report which reveals the protective and DNA repair stimulating abilities of *L. sibiricus* root, the aerial parts of a micropropagated plant and seed-derived plant extracts in Chinese hamster ovary (CHO) cells exposed to an oxidative agent. Four tested extracts showed a better ability to repair oxidative DNA damage and protective effect against oxidant-induced DNA damage. The extract from an *in vitro*-derived plant demonstrated significantly stronger properties than the seed-derived plant extract. Moreover, preincubation of the CHO cells with 0.5 mg/ml-extracts showed an increase in the expression level of antioxidant genes such as: catalaze (CAT), superoxide dismutase (SOD2) and GPx (glutathione peroxidas) genes. Our studies showed that the *L. sibiricus* may possess antioxidant properties that protect CHO cells from oxidative stress.

References

Ahmed F., Islam M.A., Rahman M.M. (2006) *Antibacterial activity of Leonurus sibiricus aerial parts*. Fitoterapia 77: 316-317.

Ames B.N., Shigenaga M.K., Hagen T.M. (1993) *Oxidants, antioxidants, and the degenerative diseases of aging*. Proc. Natl. Acad. Sci. USA, 90: 7915-7922.

Islam M.A., Ahmed F., Das A.K., Bachar S.C. (2005) *Analgesic and anti-inflammatory activity of Leonurus sibiricus*. Fitoterapia 76: 359-362.

Lee M.J., Lee H.S., Park S.D., Moon H.I., Park W.H. (2010) *Leonurus sibiricus herb extract suppresses oxidative stress and ameliorates hypercholesterolemia in C57BL/6 mice and TNF-alpha induced expression of adhesion molecules and lectin-like oxidized LDL receptor-1 in human umbilical vein endothelial cells*., Biosci. Biotech. Bioch. 74: 279-284.

Narukawa Y., Niimura A., Noguchi H., Tamura H., Kiuchi F. (2014) *New diterpenoids with estrogen sulfotransferase inhibitory activity from Leonurus sibiricus L.* J. Nat. Med. 68: 125-131.

Shin H.Y., Kim S.H., Kang S.M., Chang I.J., Kim S.Y., Jeon H., Leem K.H., Park W.H., Lim J.P., Shin T.Y. (2009) *Anti-inflammatory activity of Motherwort (Leonurus sibiricus L.)*. Immunopharm. Immunotoxi. 31(2): 209-213.

Wu H., Fronczek F.R., Ferreira D., Burand Ch.L., Zjawiony J.K. (2011) *Labdane diterpenoids from Leonurus sibiricus*. J. Nat. Prod. 74(4): 832-836.

Application of selected medicinal plants such as chamomile and kalanchoe from *in vitro* culture for production of recombinant proteins

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Despite the importance of medicinal plants in pharmaceutical and cosmetic industry, efficient transformation procedures for those plants have been rarely established. The goal of this research was to test the efficiency of regeneration and transformation properties of chamomile *(Matricaria chamomilla*) and kalanchoe (*Kalanchoe daigremontiana*) that are believed to be plants for future production of biopharmaceuticals. Griffithsin (GRFT) exhibits an antimicrobial activity and is one of the most promising HIV entry inhibitors. It is capable of inactivating a virus even at picomolar concentrations. Here, we have combined the natural antibacterial, antivirus and antifungal properties of kalanchoe and chamomile plants as examples of medicinal plants used for decades in obstetric-gynecologic practice with genetic bioengineering to obtain a novel quality product. A synthetic, optimized GRFT gene was cloned into binary vectors: PBINPlus and pGreen0029 under the control of RbcS1 and enhanced 35S CaMV promoter (derived from PRTL2 plasmid), respectively and used for the expression of GRFT protein in kalanchoe and chamomile plants. Proteins were targeted to endoplasmic reticulum (ER) when signal peptide (SP) and KDEL C-terminal ER retention signal were present or to cytoplasm without signaling. The functionality of GRFT plant expression cassettes was tested in transiently transfected *Nicotiana benthamiana* plants. The Western Blot analysis with anti c-myc antibodies revealed a protein band of expected molecular weight of ~15 kDA in the case of proteins targeted to cytoplasm. Two additional bands of higher molecular weight of ~18 and ~22 kDA, most probably postranslationally glycosylated, were detected in proteins targeted to ER. Several parameters were tested and optimized to achieve an efficient stable transformation of chamomile and kalanchoe plants. For this purpose the influence of the seedling age, type of explants, addition to medium of hormones in different concentrations, the density of *Agrobacterium tumefaciens* (strain LBA4404) culture at OD550, as well the length of co-cultivation and post-cultivation periods on the regeneration ability of transformed explants were tested. Hypocotyls, leaves and cotyledons of young chamomile plants and plantlets growing *in vitro* were gently wounded and co-cultured with different concentrations of *Agrobacterium* for 2-5days.To test the effect of a delay period after co-cultivation, explants were placed on a medium without selection for 0-12 days and then transferred onto media with kanamycin. The highest percentage of transformed chamomile regenerants (99.6%) was observed for hypocotyls. Leaf crenate margins of young kalanchoe plants and plantlets growing *in vitro* were gently wounded and co-cultured with different concentrations of Agrobacterium for 2-3 days. To test the effect of delay period after co-cultivation transformed kalanchoe explants were placed on a medium without selection for 4,8,10,12 and 14 days and then transferred onto media with kanamycin. The regeneration results for transformed kalanchoe leaves and plantlets were the best (78%) in the case of 12 days delay period without a selection agent. The first putative transgenic chamomile and kalanchoe plants that produced roots on kanamycin-containing medium were confirmed by a PCR analysis for the presence of the GRFT expression cassette. Mature plants did not show morphological abnormalities and demonstrated normal growth abilities compared to control non-transformed plants.

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Phenolics in undifferentiated tissue cultures of *Cichorieae*

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The tribe *Cichoriaeae* of *Asteraceae* comprises several genera of economic importance, e.g. *Lactuca* and *Cichorium*. Plants of the tribe reportedly contain anti-inflammatory and antinociceptive sesquiterpene lactones (Wesołowska et al., 2006) which are concentrated in a milky latex. A number of phenolic compounds, of importance as functional constituents of food, was also isolated from the plants. Among them were caffeic acid conjugates of antiviral and hepatoprotective activities (McDougall et al., 1988; Basnet et al., 1996).

Undifferentiated tissue cultures of *Lactuca virosa* L., *Cichorium intybus* L. and *Lactuca aculeata* Boiss were examined in respect of sesquiterpene lactone and phenolics composition and contents. Except for *L. virosa* callus and cell suspension cultures, sesquiterpene lactones were absent from the analyzed plant material. The tissues, however, retained the capability to synthesize phenolic metabolites, especially those of hydroxycinnamic acid type and lignan type. Chlorogenic acid (5-CQA) and 3,5-dicaffeoylquinic acid (3,5-DCQA) were the major hydroxycinnamates found in *L. virosa* and *C. intybus* callus cultures. Their contents were 0.15-0.31% and 0.49-0.99%, respectively, calculated on the dry weight basis. *L. virosa* calli synthesized also caftaric (CTA, 0.01%) and cichoric (DCTA, 0.06%) acids, whereas only a low amount of DCTA (0.01%) was detected in the callus culture of chicory (Stojakowska et al., 2012; Malarz et al., 2013). Unlike the above mentioned cultures, the callus culture of *L. aculeata* accumulated mainly 1,5-dicaffeoylquinic acid (1,5-DCQA) and methyl caffeate. Calli of *L. virosa* and *L. aculeata*, as well as a suspension culture of *L. virosa* produced neolignans – derivatives of dihydrodehydrodiconiferyl alcohol (Stojakowska et al., 2000). Moreover, neolignans – derivatives of biologically active balanophonin (Jang et al., 2003) were isolated from the callus culture of *L. aculeata*. The compounds were not previously reported to be constituents of either lettuce or chicory plants. Furofuran lignans, but no neolignans, were present in calli of chicory (Malarz et al., 2005). Despite a close systematic relationship of the examined species, their undifferentiated tissue cultures showed a remarkable variation in secondary metabolite patterns.

References

Wesołowska A., Nikiforuk A., Michalska K., Kisiel W., Chojnacka-Wójcik E. (2006) J. Ethnopharmacol. 107: 254-258.

McDougall B., King P.J., Wu B-W., Hostomsky Z., Reinecke M.G., Robinson W.E. Jr. (1998) Antimicrob. Agents Chemother. 42: 140-146.

Basnet P., Matsushige K., Hase K., Kadota S., Namba T. (1996) Biol. Pharm. Bull. 19: 1479-1484.

Stojakowska A., Malarz J., Szewczyk A., Kisiel W. (2012) Acta Physiol. Plant 34: 291-298.

Malarz J., Stojakowska A., Kisiel W. (2013) Appl. Biochem. Biotechnol. 171: 1589-1601.

Stojakowska A., Kisiel W. (2000) Pol. J .Chem. 74: 153-155.

Jang D.S., Park E.J., Hawthorne M.E., Vigo J.S., Graham J.G., Cabieses F., Santarsiero B.D., Mesecar A.D., Fong H.H.S., Mehta R.G., Pezzuto J.M., Kinghorn A.D. (2003) J. Nat. Prod. 66: 583-587.

Malarz J., Stojakowska A., Szneler E., Kisiel W. (2005) Plant Cell Rep. 24: 246-249.

Application of *in vitro* culture techniques in the conservation of orchids in Gdańsk Pomerania

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46 orchid species belonging to 22 genera are known to inhabit wild sites in Poland. If we consider only the Gdańsk Pomerania region, then 11 species are already extinct and 16 are on the verge of extinction.

The objective of this study was to develop a long term storage method for preservation and conservation of some of the most endangered Polish orchid species employing cryopreservation.

The project focuses on the following species: *Cypripedium calceolus*, *Dactylorhiza incarnata*, *Dactylorhiza maculata, Dactylorhiza majalis, Epipactis palustris*, *Epipogium aphyllum*, *Goodyera repens*, *Liparis loeselii*, *Platanthera bifolia*. Our main efforts concentrated on two species (*C. calceolus* and *L. loeselii*) considered as the most threatened in Poland and in Europe.

The study on *C. calceolus* includes estimation of genetic diversity among wild populations in Gdańsk Pomerania, hand pollination and collection of seeds ensuring high genetic diversity of *in vitro* raised seedlings. 15 microsatellite markers have been developed and characterized (Minasiewicz and Znaniecka, 2014). Microsatellite and plastid markers were used to determine the genetic variability of the Pomeranian population of *C. calceolus*. We found that the populations retain high level of clonal (R = 0.86-1) and genetic diversity (He = 0.572) however they are highly differentiated even at small geographic distances (FST = 0.132; $P < 0.001$ for nuclear SSR and FST = 0.581; $P < 0.001$ for plastid DNA), which results from genetic drift and a low level of gene flow. The observed population structure is most likely a consequence of the extinction of approximately 80 % of population of this species in the region over the last 130 years due to habitat loss and fragmentation.

Trials with *in situ* planting of seedlings obtained by the developed *in vitro* culture methods were established in the nature reserve Dolina Kulawy. Seedlings were obtained only by germination of immature seeds, mature seeds did not germinate (Znaniecka and Łojkowska, 2004). For the same reason, it is not advisable to cryopreserve mature *C. calceolus* seeds. Currently, cryopreservation of protocorms and immature seeds is under development. Preliminary results of *C. calceolus* micropropagation have been obtained. Seedlings germinated via *in vitro* asymbiotic germination of immature seeds served as a source of material for micropropagation. Two types of explants were tested: root and protocorm sections. Preliminary results indicated that protocorm-like bodies (PLB) can be obtained from 1.5-2 mm long protocorm sections and 0.7-0.8 mm root fragments. The highest coefficient of micropropagation rate was obtained on a modified 1/5 MS medium supplemented with 2.0 mg/l TDZ and 2.0 mg/l NAA. 900 PLBs were obtained from, on an average, a single protocorm explant and only 12 PLBs per root explant.

In the case of the remaining eight orchid species the seed bank was established with about 200 accessions and the methods for seed cryopreservation and *in vitro* germination were developed. Seed samples were collected from at least two localities per species. Seeds were dried over $CaCl_2 \cdot 6H_2O$ and stored at: −196 °C; 4 °C and 20 °C for up to 3 years. Seed viability was assessed by two histochemical staining procedures (TTC and FDA). Two methods of seed germination were applied: asybiotic and symbiotic. In the case of symbiotic germination, pelotons of *Tulasnella* sp. isolated from plants growing in natural habitats and cultured *in vitro* were used.

References

Minasiewicz J., Znaniecka J. (2014) Conserv. Genet. Resour. 6: 527-529.
Znaniecka J., Łojkowska E. (2004) Bull. Bot. Gard. Museums Collect. 13: 69-73.

Effect of sucrose and nitrogen salts levels in the medium on the endogenous carbohydrate content in the *Helleborus niger* L. and *Helleborus purpurascens* Waldst. et Kit. shoots propagated *in vitro*

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The *Helleborus niger* L. and *H. purpurascens* Waldst. et Kit. belong to the family Ranunculaceae. The genus *Helleborus* comprises about 20 species, which are distributed over different parts of Europe, West and East Asia. *H. purpurascens* is a rare and endangered species of Polish flora, protected by law. In Poland it can only be found in the Western Bieszczady. *H. niger* is important in commercial horticultural production as a garden perennial, blooming in winter and early spring. It is also important as a pot plant and as cut flowers. Relatively little tissue culture research has been published on the micropropagation of hellebores (Syringe, 2002; Poupet et al., 2006; Dhooghe et al., 2007; Beruto and Curir, 2009; Beruto et al., 2013; Gabryszewska, 2013, 2014).

The objective of the study was to investigate the influence of various sucrose (10. 30 and 80 g l^{-1}) and nitrogen salts (25%, 50%, 100% according to the MS medium) levels in the medium on the endogenous carbohydrate content in the *H. niger* and *H. purpurascens* shoots propagated *in vitro*.

The axillary shoot multiplication was stimulated by combinations of various cytokinin (2iP, BAP and kinetin - each at a concentration of 1.0 mg l^{-1}) and GA3 2.5 mg l^{-1} added to Murashige and Skoog basal medium. During the multiplication stage, the carbohydrates detected in the hellebores shoots were starch, glucose and fructose. Sucrose was not detected in the shoots of both hellebores species propagated in vitro. Starch was the major carbohydrate accumulated in the shoots of both hellebores species. In general, the *H. purpurascens* shoots accumulated more starch compared to *H. niger*. A highest starch content of 18.4 mg g^{-1} DW was found in the *H. purpurascens* shoots grown on the medium with 80 g l^{-1} sucrose and nitrogen salts at 100% (according to the MS medium). Increased levels of sucrose in the medium stimulated accumulation of starch in the shoots. Additionally, starch content was found to be correlated with differences in the rate multiplication and morphology of hellebores shoots. Glucose and fructose also accumulated in the hellebores shoots, but to a lesser extent than starch. As the nitrogen salts level in the media was increased, the content of glucose slowly decreased in the shoots of both species.

References

Beruto M., Curir P. (2009) *Effects of chilling and hormonal supply on rooting and in vivo establishment of micropropagated plantlets of Helleborus spp.* Acta Hort. 813, 365-372.

Beruto M., Viglione S., Bisignano A. (2013) *Micropropagation of Helleborus through axillary budding.* Methods Mol. Biol. 994: 259-267.

Dhooghe E., Van Labeke M. (2007) *In vitro propagation of Helleborus species.* Plant Cell Tiss. Organ Cult. 91: 75-177.

Gabryszewska E. (2013) *Rozmnażanie in vitro ciemiernika białego (Helleborus niger L.). Oferta wdrożeniowa.* Konferencja wdrożeniowa Nauka - Praktyce „Innowacyjne technologie dla polskiego ogrodnictwa". Skierniewice, 10 grudnia 2013, 95-96.

Gabryszewska E. (2014) *Rozmnażanie in vitro Helleborus purpurascens Waldst. et Kit.* Wiad. Bot., 58(1-2): 1-16.

Poupet R., Cardin L., Henri A., Onesto J.P. (2006) *Healthy in vitro propagation by meristem tip culture of Helleborus niger's selected clone for cut flower.* Acta Hort. 725: 301-310.

Syringe M. (2002) *In vitro cloning of Helleborus niger.* Plant Cell Rep. 20: 895-900.

In vitro root cultures of *Lychnis flos-cuculi* L. as a potential source of triterpenoid saponins and ecdysteroids

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Ragged Robin (*Lychnis flos-cuculi* L.) is a perennial plant belonging to Caryophyllaceae family. This taxon contains valuable compounds with potentially useful pharmacological activity, such as ecdysteroids, triterpenoid saponins and polyphenols – flavonoids and phenolic acids (Maliński et al., 2014; Tomczyket al., 2008). Ecdysteroids are steroid compounds, widely distributed in living organisms and fulfilling diverse functions. In arthropods they serve as hormones, in plants they are a part of biochemical defense against insects. The effect of ecdysteroids on mammal organisms is multidirectional and encompasses anabolic, adaptogenic, antioxidant and anti-inflammatory activities (Lafont et al., 2003). Triterpenoid saponins, depending on their structure, act in diverse ways – derivatives of hederagenin and gypsogenin present in L. flos-cuculi may be attributed to expectorant, antifungal or cytotoxic activities (Sparg et al., 2004). However, the saponins present in the species have not been fully described, which justifies further phytochemical research.

L. flos-cuculi has been introduced to *in vitro* cultures in order to obtain plant material and conduct detailed phytochemical analysis without exploitation of its natural habitat (Thiem et al., 2013) A micropropagation protocol has been developed, which led to the establishment of shoot cultures, callus cultures and adventitious root cultures in liquid media.

In vitro cultures were grown on Murashige-Skoog (MS) medium with addition of plant growth regulators. Shoots were multiplied on MS medium with 1.0 mg/l BAP and 0.1 mg/l IAA. The root biomass was obtained by induction of rhizogenesis of shoots placed in an MS or a half-strength MS medium solidified with different amounts of agar; with or without auxins (IAA, IBA, NAA). It was observed that the most effective method to obtain biomass is to root the shoots in a liquid MS medium with 1.0 mg/l IAA. Whole plant cultures in liquid media were noted to develop more roots than cultures derived from root fragments. Depending on the choice of plant growth regulators, the root cultures differed in morphology, growth rate and tendency to grow callus. The biomass was harvested for phytochemical analysis.

The preliminary phytochemical investigation performed by TLC confirmed the presence of secondary metabolites in plant material from *in vitro* cultures. Organs particularly rich in the characteristic compounds, especially ecdysteroids and triterpenoid saponins, are roots both from wild plants and *in vitro*-derived plantlets. Organ cultures are known for their genetic stability, higher than cell suspension cultures, and the ability to synthesize compounds characteristic for the species, especially those of complex structure. Though it has been noted that the callus is able to produce triterpenoid saponins, the biosynthesis of ecdysteroids is minimal. Since both classes of compounds share common steps of the biosynthetic pathway, an elicitation strategy or precursor feeding of the callus cultures is planned in order to stimulate the synthesis of ecdysteroids.

References

Lafont R., Dinan L. (2003) *Practical uses for ecdysteroids in mammals including humans: an update*. J. Insect Sci. 3: 7.

Maliński M.P., Michalska A.D., Tomczykowa M., Tomczyk M., Thiem B. (2014) *Ragged Robin – (Lychnis flos-cuculi) – a plant with potential medicinal value*. Rev. Bras. Farmacogn. 24(6): 722-730.

Tomczyk M. (2008) *Preliminary phytochemical investigation of Lychnis flos-cuculi herbs*. J. Nat. Med. (Tokyo) 62: 473-475.

Sparg S.G., Light M.E., van Staden J. (2004) *Biological activities and distribution of plant saponins*. J. Ethnopharmacol. 94: 219-243.

Thiem B., Maliński M., Tomczyk M. (2013) *In vitro propagation of Lychnis flos-cuculi L., a plant with potential medicinal value*. Acta Biol. Cracov. Ser. Bot. 55: 66.

Comparison of epigenetic changes induced by particular phytohormones and oligonucleotide sequences in flax

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The remarkable revolution of the past two decades in the field of molecular biology has been acknowledgement of epigenetics as an equally significant source of organisms variability as the DNA sequence modifications. Epigenetic changes play an especially important role in plants which must perpetually adjust to surrounding environmental conditions. Stresses such as: drought, UV radiation, attack of insects or pathogens, leave a mark in the plant memory in the form of epigenetic changes that enable a quick response of an organism in the event of subsequent environmental changes. There exist three ways of encoding information as epigenetic modifications, which are called "three pillars of epigenetics". Those three pillars are: changes at the RNA level, methylation of cytosines in the DNA sequence, and modifications of chromatin (e.g. post-translational changes of histones). It has been shown that epigenetic changes might be retained during cell divisions and passed on to the next generation. Consequently, they are a promising tool for the induction of interesting features and for plant improvement without the need of genetic transformation or mutagenesis.

In contrast to mammals, plants do not have an immune system and the products of secondary metabolism constitute the first line of their defense in the face of danger, i.e. during pathogen attack. The important secondary metabolites with broad biological properties in flax are flavonoids. The key enzyme involved in the synthesis of flavonoids is chalcone synthase (CHS). Gene encoding CHS is extremely sensitive to stressing factors, which may be reflected in the modified level of the transcript. Some phytohormones can also play an important role in the plant reaction to stress conditions. Jasmonates, abscisic and salicylic acid, apart from the regulation of the plant growth and development, take part in the plant immunological response. These compounds act as signaling molecules the concentration of which increases rapidly in the tissues exposed to stress.

In this study, the plant that has been used was flax (*Linum usitatissimum* L.), which has a large potential in the pharmaceutical, cosmetic and food industries. The broad range of possibilities for flax application convinces researchers of the worthiness of improving its properties by molecular biology methods. Currently, some attempts are being made to induce epigenetic changes in plants to allow them a better response to stress.

The main purpose of this study was induction and comparison of the epigenetic changes occurring in a chalcone synthase gene of flax. During the experiment the, flax plants grown in *in vitro* cultures were treated with the particular phytohormones (abscisic acid, salicylic acid, jasmonates) and short oligonucleotide sequences homologous to the corresponding sequence of the CHS gene. Considering the "three pillars of epigenetics", in the obtained plants expression of the CHS gene, the total methylation of the genomic DNA and methylation of the selected sites CCGG in the CHS gene were determined. As a result of the analysis of sites located in particular gene regions, the cytosines that were stably demethylated (in promoter and intron regions) and characterized by varied percentage of methylation (in coding sequence) in comparison to controls, were distinguished. The expression of genes involved in the processes related to the histone proteins modifications, DNA methylation and demethylation was also examined.

The performed experiments form a promising basis for the development of an effective method of generating desired modifications in plants without the need to introduce changes in their genome.

Antisense oligodeoxynucleotide treatment as a new method of gene expression manipulation in flax

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Terpenoids, also known as isoprenoids, are a group of natural products which enclose primary and secondary metabolites. Terpenoids are the most varied class of plant natural products, and among them there are carotenoids, tocopherols, gibberellins and sterols. They have a wide range of functions for plant growth and development. Terpenoids are strong antioxidants, thus they play an important role in photoprotection of photosystems and in the regulation of plasma membrane fluidity. Moreover, terpenoids have antibacterial, antifungal and anti-cancer properties and are therefore most desirable in the human diet. Further, because of their antioxidative properties, they increase the stability of edible plant oils.

Flax (*Linum usitatissimum*) is a crop valued for its fibre and seeds as a source of oil. Flax oil contains high quantities of polyunsaturated fatty acids, essential for the human diet, though very prone to oxidation. The presence of antioxidants such as terpenoids in flax oil can decrease its susceptibility to the oxidative degradation. Although flax is a crop with an active terpenoid pathway, the desirable compounds from this pathway are in small quantities in this plant. Therefore, a study on the terpenoid pathway and its manipulation (by genetic engineering) is important for a better understanding of the metabolic flux and future application in food, medicine and pharmaceutical industries. The best method to investigate a gene role is transgenesis. Manipulations using agro- or biolistic transformations are time-consuming, require specialized equipment and are connected with GMO plant production.

Oligodeoxynucleotides (AO, ASO) are short, single stranded DNA molecules, complementary to target mRNA and can be used for gene expression modulation. An application of ASO leads to changes of the target gene expression in short time, which creates a possibility to manipulate the expression levels of various genes from a pathway of interest. Antisense oligodeoxynucleotides were shown to be efficient in animal systems, however, their effect on gene expression in plants is poorly understood.

We have designed ASOs for different genes coding for the enzymes of the terpenoid pathway (such as: isopentenyl diphosphate isomerase – IPPI, phytoene synthase – PSY, carotenoid isomerase – CRTISO, zeta-carotene desaturase – ZDS, tocopherol cyclase – VTE1) and used them for gene expression manipulation. It turned out that the antisense oligodeoxynucleotides method can be more effective than the traditional methods of transient gene manipulation, such as e.g. a biolistic transformation. Antisense oligodeoxynucleotide treatment allows us to obtain the silencing of the gene expression even by 90% in comparison to the control plants. The features of the used ASOs were determined, including for instance the secondary structure of oligodeoxynucleotides, accessibility of target mRNA and the GC to AT ratio. The final characteristics of the efficiently working ASOs will be presented. The obtained results will allow future designing of efficient manipulations of different pathways in various plant systems.

Interspecific hybridization between selected *Brassica napus* and *Brassica rapa* ssp. *chinensis* genotypes through embryo rescue and their evaluation for crossability

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Amphidiploidal rapeseed as a very important oil plant became a widely cultivated crop in many countries in the world. The big increase in rapeseed acreage is causing some new problems connected with yielding and cultivation of this species. Moreover, forms with improved traits are very much in demand. Thus interspecific crossing is a valuable tool for widening the variability of useful traits e.g. seed quality and resistance to some diseases such as clubroot caused by *Plasmodiophora brassicae*. For breeding Brassicas resistant to clubroot, attempts of transferring resistant genes (CR) through interspecific hybridization have been reported (Diederichsen et al., 2009; Niemann et al., 2015). However, interspecific hybridization between tetraploid and diploid *Brassica* species is difficult and failures occur at many stages starting from pollination (e.g. incompatibility) to embryo development (post-zygotic barriers).

The aim of this study was to evaluate the crossability between tested genotypes and obtaining hybrid plants which could be the potential sources of resistance to clubroot.

At this work results of crossing of five winter rape (AACC = 38) cultivars i.e. Jet Nauf, Lisek, Skrzeszowicki, Californium and Zhongshuang with three accessions (A – PI430485 98CI, B – Pak Choi 08 007569, C – Chinense Cabbage 08 006169) from the *Brassica rapa* ssp. *chinensis* are being presented. Altogether from all cross combinations obtained 126 siliques containing in total 946 ovules from which isolated embryos were incubated *in vitro*. In this way from all cross combinations 535 embryos were placed on the media. The highest effectiveness of interspecific hybridization expressed by fertility was observed in *B. rapa* ssp. *chinensis* (B) × *B. napus* Zhongshuang (71.79%). The lower effectiveness was being observed in combinations, where amphidiploid *B. napus* forms were maternal components i.e. Skrzeszowicki, Lisek and Jet neuf, (0 to 7.78 %), respectively. Finally, 294 plants (which accounted for 54.0%, with reference to 535 incubated embryos) were rooted in the soil. Crossability was evaluated on the basis of the pollen germination index (PGI) according to Kaneko et. al. (2009). Unilateral interspecific incompatibility occurred in the crosses between all analyzed *B. napus* cultivars and *B. rapa* ssp. *chinensis* (*B. napus* – maternal form). Quite a different situation was observed in the case of crosses in which *B. rapa* ssp. *chinensis* plants were used as a maternal cultivar and *B. napus* cultivar as a pollen donor. In this cases the intensity of pollen tubes growth was high and PGI confirmed the unilateral cross compatibility between *B. rapa* × *B. napus*.

References

Diederichsen E., Frauen M., Linders E.G.A., Hatakeyama K., Hirai M. (2009) J. Plant Growth Regul. 28: 265-281.

Kaneko Y., Bang S.W., Matsuzawa Y. (2009) [in:] *Biol Breed Crucifers*, Ed. Gupta S.K.: 207-247.

Niemann J., Kaczmarek J., Wojciechowski A., Olejniczak J., Jędryczka M. (2015) Progr. Plant Protec. (Post. Ochr. Roślin) 55(1): 87-91.

Metabolism of biologically active sulfur compounds in broccoli *in vitro* callus 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. The biggest 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 sulfide leads to a cascade of enzymatic steps of the sulfur-containing amino acids synthesis. 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. Biological activity of sulfur compounds makes them essential for the human diet, and their deficiency may cause many health complications. Thus plants are the most important source of sulfur organic compounds like cysteine or lipoic acid. It constitutes economic interest of sulfur assimilation and sulfur amino acid biosynthesis in higher plants. Additionally the sulfide/disulfide redox system is retained as detoxification mechanisms for reactive oxygen species and signal transduction mechanisms in plant.

The aim of the presented studies was to conduct a comprehensive study on the effect of sulfate supplementation on the level of active sulfur compounds and their metabolism in callus cultures of Brassica cretica ssp. botrytis.

In the first stage, callus cultures were initiated from seedlings of *Brassica cretica* ssp. *botrytis* (L.). Cultures with a high viability and proliferation rate were obtained by selecting media containing an appropriate set of growth regulators. Callus cultures were maintained on Murashige-Skoog (MS) medium containing two sets of plant growth regulators (2.0 mg/l BAP, 0.2 mg/l NAA, 1.0 mg/l 2,4-D and 2.0 mg/l BAP, 0.1 mg/l 2,4-D) supplemented with different amounts of sulfate (0, 0.5, 1.5, 3.0 and 5.0 mM) under constant artificial light (ca. 4 W/m2), at 24 ± 2°C, and 4 week- long subcultures.

The levels of the following compounds were measured: non-protein sulfhydryl groups, glutathione, cysteine, cystine, sulfane sulfur, reactive oxygen species and malondialdehyde (a product of lipid peroxidation). In addition, activities of enzymes involved in thiols and sulfane sulfur metabolism were assayed (rhodanese, cystathionase and β-cyanoalanine synthase).

The studies demonstrated that supplementation of sulfur compounds has a significant impact on the development of the cultures. Sulfate ions are taken from the medium by the callus tissue and efficiently metabolized to thiols and sulfane sulfur containing compounds. At higher concentrations NPSH, glutathione, cysteine and sulfane sulfur levels were significantly elevated. Activities of rhodanese and β-cyanoalanine synthase were also increased. Medium sulfate supplementation protects cells against reactive oxygen species, too. We demonstrated the effect of different compositions of growth regulators on tested parameters.

In conclusion, sulfate is a good precursor of cysteine, utilized for glutathione biosynthesis, which was indicated by its capability to elevate non-protein sulfhydryl groups level and leading to formation of sulfane sulfur-containing compounds. Callus cultures are better than the shoot cultures experimental model to modulate thiols and other sulfur compounds content in plants.

Green synthesis of silver nanoparticles with antimicrobial activity using an extract of *Drosera capensis*

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Drosera capensis var. *alba* (Droseraceae), commonly known as the Cape sundew, is a carnivorous plant native to the Cape of South Africa. The plants of *Drosera* genus are a natural source of pharmacologically important compounds (eg. naphthoquinones, glucosides, flavonoids, phenolic acids) with biological activities, i.e. antimicrobial, antimycobacterial, antifungal or anticancer.

Silver nanoparticles (AgNPs) are three-dimensional structures below 100 nm in diameter formed by reduction of silver ions to Ag^{o} clusters stabilized using coating ligands. The antimicrobial activity of AgNPs towards multiple fungal and bacteria species has been observed, regardless of their susceptibility or resistance to common drugs.

The aim of this study was to synthesize AgNPs using aqueous extracts from *D. capensis* plants and evaluate their antimicrobial activity against plant pathogenic bacteria: *Pectobacterium carotovorum* ssp. *atrosepticum, Dickeya dadantii* and *Pseudomonas syringae* pv. *morsprunorum*.

D. capensis var. *alba* plantlets were grown in a liquid 1/2 MS medium with 2 % sucrose, pH 5.6 (at a temperature of 20-22°C under white fluorescent light with a 16 h photoperiod (White cool fluorescent light, Philips, TLD 58W/84o, 30-35 mmol × m^{-2} × s^{-1}). For extract preparation 5 grams of fresh weight of *D. capensis* was taken from a 4 week old *in vitro* culture and extracted using distilled water. The extraction was performed using microwave irradiation (2 minutes). Next the extract was shaken for 15 minutes and filtered using 0.2 μm mixed cellulose esters (MCE) syringe filter. The extract was kept at 4°C for further use but no longer than 7 days. For silver nanoparticle synthesis 190 ml of water, 30 mg of silver nitrate and 10 ml of *D. capensis* extract was added to a 250 ml borosilicate bottle. Polyvinylpyrrolidone (AgNPs_PVP) or sodium citrate (AgNPs_Sc) were used as capping agents. The mixtures were incubated in the dark at 55°C for 4 hours without shaking and filtered through 0.2 μm mixed cellulose esters (MCE) syringe filter. Nanoparticles were concentrated using a centrifuge at 14 000 rcf (relative centrifugal force) for 20 minutes. The supernatant was discarded and the nanoparticles were re-suspended in 1 ml deionized water.

The Minimal Bactericidal Concentrations (MBC – a concentration which reduces the number of microorganisms by 99.9% or 3 logarithms) of AgNPs_PVP and AgNPs_Sc required to achieve the desired antimicrobial effect in a planktonic culture of plant pathogenic bacteria were determined using the Broth Microdilution Method. The antimicrobial properties of AgNPs were tested within the concentration values of 2 to 100 μl/ml.

Both tested silver nanoparticles show antimicrobial activity against a broad spectrum of tested plant pathogenic bacteria: *P. carotovorum* ssp. *atrosepticum, D. dadantii* and *P. syringae* pv. *morsprunorum*; MBC was 6 μl/1 ml.

In vitro production of huperzine A and other *Lycopodium* alkaloids in *Huperzia selago* through a prothallus culture under the influence of different additives

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This study presents a protocol for a fast and effective in vitro axenic culture of *Huperzia selago* (L.) Bernh. ex Schrank et Mart. (=*Lycopodium selago* L., fir club moss, Huperziaceae Rothm) gametophytes, which is a source of huperzine A (HupA, selagine) and other alkaloids. These compounds have a considerable therapeutic potential and are being extensively investigated for uses as treatment for certain diseases. (Szypuła et al. 2005, 2011, Czapski et al. 2014). The major therapeutic interest described for HupA is treatment of acetylcholine-deficit dementia, including Alzheimer's disease (Szypuła 2013). Our previous results, indicated for the first time that alkaloids from *H. selago* possess antioxidative properties and scavenge free radicals as well as prevent lipid and protein oxidation, presenting the desired mechanism of action in neurodegenerative dis-orders [3]. These alkaloids might be a promising source of lead compounds for drug discovery in the therapies for Alzheimer's and Parkinson's diseases.

H. selago is the only European species which contains HupA (Szypuła et al. 2005, Czapski et al. 2014, Szypuła 2013). Studies have shown that the plant is a rich source of HupA, much more abundant than the Chinese club moss *Huperzia serrata*. Up to date, studies on *in vitro* cultures have been conducted with a few club moss species only. However, there are only a few protocols for propagation of sporophytes of club mosses containing HupA, but there are no protocols for the culture of prothallus in the literature.

In the present study an efficient *in vitro H. selago* prothallus induction system was established on different Moore (Mr) media as modified by Friberg and Wetmore (1957), W/S (Whittier and Strochowa, 2007), Kn (Knudson, 1922) and 1/2 MS (Murashige and Skoog, 1962) with or without growth regulators, supplemented with 2.5 or 5 g/l glucose and different concentrations (0.05-1.4 μM) of plant growth regulators i.e. auxins (IBA, 2,4D and NAA), cytokinins (kinetin) and gibberellic acid (GA3) in the dark. The effect of various auxins and cytokinins or gibberellic acid on the growth of gametophytes was investigated. The accumulation of Hu-pA and other *Lycopodium* alkaloids i.e. huperzine B (HupB) and lycopodine (Lyc) was studied using HPLC-DAD and LC-ESI-MS/MS methods.

W/S, Mr and Kn media supplemented with 0.05 μM IBA and 1.4 μM kinetin supported pro-thallus growth and its proliferation. HPLC analysis of alkaloid extracts obtained from game-tophytes revealed an accumulation of HupA, HupB and other alkaloids. The established *in vitro* prothallus induction system in *Huperzia selago* can be utilized for biomass production of pharmaceutically important alkaloids such as huperzine A.

References

Szypuła W., Pietrosiuk A., Suchocki P., Olszowska O., Furmanowa M., Kazimierska O. (2005) Plant Sci. 168: 1443-1452.
Szypuła W.J., Kiss A.K, Pietrosiuk A., Świst M., Danikiewicz W., Olszowska O. (2011) Acta Chromatogr. 23: 339-352.
Czapski G.A, Szypuła W.J., Kudlik M., Wileńska B., Kania M., Danikiewicz W. (2014) Folia Neuropathol. 52: 394-406.
Szypuła W. (2013) Acta Bot Siles. 9: 25-56.
Freeberg J.A, Wetmore R.H. (1957) Phytomorphol 7: 204-217.
Whittier D.P., Storchova H. (2007) Am. Fern. J. 97(3): 149-154.
Knudson L. (1922) Bot. Gaz. 73: 1-25.
Murashige T., Skoog F. (1962) Plant Physiol. 15: 473-497.

The endogenous phenolic compounds during bulb formation in *Lachenalia* sp. *in vitro* cultures under different lighting conditions

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Lachenalia sp. is considered to be a South African bulbous plant with a promising potential for commercialisation in the global floriculture market. In the present experiment, the formation of bulbs was studied in *Lachenalia* sp. *in vitro* adventitous shoot cultures of two cultivars that vary in bulbing ability, i.e. "Rupert" (bulbing) and "Ronina" (nonbulbing), under different lighting environment (white, blue, red, darkness) in view of the phenolic compounds content. Shoots were cultivated on Murashige and Skoog (1962) medium with an addition of sucrose 3% and growth substances: BA and NAA (2.5 μM and 0.5 μM). Phenolic acids were estimated by HPLC while the total phenolic content was measured spectrophotometrically (Singelton et al., 1999).

We observed that a similar number of explants from both cultivars that were cultivated in the dark formed similar amounts of adventitious bulbs while the red, blue and white light strongly reduced the number of lachenalia "Ronina" explants forming bulbs. The content of phenolic compounds was different in terms of quantity and quality in the newly formed lachenalia bulbs. The total phenolics content ranged from about 0.5 mg/g DW (for "Ronina" exposed to red light) to 2 mg/g DW (for dark-grown "Rupert"). Most of the examined conjugated phenolic acids (cinamic, p-coumaric, caffeic, ferulic, sinapic, chlorogenic) occurred in bulbs at a higher concentration in the white and blue light in comparison to the red light or in the dark. A negative correlation has been shown between high amounts of phenolics and the bulb regeneration ability. No chlorogenic acid or its low concentration was detected in scales of newly formed lachenalia bulbs. Both, the conjugated and the free chlorogenic acids, were present in "Ronina" cultures in all lighting environment except darkness, which promoted formation of adventitious bulbs. These investigations are a completely innovative attempt of describing the bulbing ability process of lachenalia in relation to the content of endogenous phenolic compounds.

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References

Bach A., Kapczynska A., Dziurka K., Dziurka M. (2015) *Phenolic compound and carbohydrates in relation to bulb formation in Lachenalia 'Ronina' and 'Rupert' in vitro cultures under different lighting environments*. Sci. Hort. 188: 23-29.

Singleton V.L., Orthofer R., Lamuela-Raventos R.M. (1999) *Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent*. Meth. Enzymol. 299: 152-178.

Methods of eliminating contaminations in tissue cultures on the example of selected ornamental plant species

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The tissue, organ and cell cultures are widely used for research or commercial production of plants and secondary metabolites. No matter what the reason for establishing and running a culture *in vitro*, the most important is that cultures are free from any biological contamination during all the stages of micropropagation, even if stored for a long time. The methods of initiating aseptic cultures have been described for many plant species, although the contamination might appear at later stages. A secondary contamination might result from the imperfect laboratory practice (environmental bacteria) or from the plants themselves (Cassels, 2012). It has been proven that many plants are colonized intercellularly by fungi or bacteria which may pass into a culture, especially if the growth conditions, such as high nutrients and sugar content as well as moderate or high temperature, are favorable for their development.

The research has been undertaken to evaluate the available methods of eliminating contamination that occurs in well established tissue cultures. The research material consisted of two ornamental plants, *Clematis* sp. and *Strelitzia reginae*, which had been cultured in tissue culture and had been visually free from any contaminations for at least ten passages before the fungal of bacterial contamination occurred on the surface of the media.

The clematis tissue cultures had visual bacterial contaminations on the surface of the media as well as at the bases of the plants. The shoots were removed from the media and the surface was sterilized using three different methods. They were: a) soaked in water with an addition of a detergent, then disinfected in 1% solution of NaOCl and dipped in the $HgCl_2$, b) soaked first in water with a detergent then in a mixture of Gentamicine and Streptomicine and then disinfected in 1% solution of NaOCl, c) dipped in $HgCl_2$. Next, the shoots were placed on the MS media with 2.5 mg dm^{-3} 2iP and 0.5 mg dm^{-3} IAA. The effectiveness of disinfection depended on the method applied. The most contamination of free shoots (100%) was obtained when they had been disinfected with NaOCl and $HgCl_2$, and the shoots regenerated very poorly (12.1%) and many of them had the symptoms of necrosis (87.8%). The best results were obtained when the shoots had been dipped only in $HgCl_2$ for 1-2 seconds. It allowed to obtain 93.6% of contamination free shoots of which 52.5% regenerated.

Contamination in *Strelitzia reginae* tissue cultures appeared after about one year of cultivation. The contaminated shoots were taken out from the media and disinfected in two ways: a) soaking in water with a detergent, then soaking in Topsin solution, surface disinfected with 2% NaOCl for 20 minutes or b) dipped in $HgCl_2$ for 30 seconds. The shoot pieces were then placed in the MS media supplemented with 1.5 mg dm^{-3} 2.4-D, 0.5 mg dm^{-3} BA and 4 g dm^{-3} of activated charcoal. The better results when obtained when shoots were dipped in $HgCl_2$, as it allowed to obtain 81% of contamination free shoots and only 12% remained contaminated with bacteria. Soaking shoots in NaOCl allowed to obtain 0.9% of contamination free shoots while 82% had visual bacteria on the surface of the media and 16% got brown and died.

Tissue cultures of both plant species remained visually contamination-free for at least 10 cycles after the secondary disinfection.

Obtainment of transgenic porcine fibroblast cell lines for the purpose of xenotransplantation

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Pigs as a source of grafts for xenotransplantation can help to overcome the rapidly growing shortage of human donors. However, the considerable phylogenetic distance between humans and the pig causes tremendous immunological problems after transplantation, thus genetic modifications need to be introduced to the porcine genome, with the aim of reducing xenotransplant immunogenicity. The genetic engineering techniques and the achievements of a transgenesis have created a possibility of producing a genetically modified swine with an organ that could be used to save the lives of human beings.

Somatic cell nuclear transfer (SCNT) is an attractive route for genetic modification of pigs. Two of the advantages of SCNT is a lack of generation of mosaic phenotypes and a possibility of pre-selection of donor cells with regard to transgene expression. In the SCNT technique, efficient nucleic acid delivery into somatic cells such as fibroblasts is of particular importance. A wide range of biological, chemical and physical methods for nucleic acid transfer have been applied in SCNT (Rogers et al., 2008; Watanabe et al., 2005; Nakayama, 2003). The improvement of the transgenic process can be achieved by efficient and safe system of transgene delivery into cells. The use of magnetofection can greatly improve the transfection efficacy. Magnetofection uses a magnetic force acting on gene vectors that are associated with magnetic particles. The attractive characteristics of magnetofection are its ability to overcome physical limitations to gene delivery, improvement of the kinetics of the delivery process and a significant reduction of applied vector doses for effective gene expression (Plank et al., 2003; Mykhaylyk et al., 2007).

The aim of this study was to obtain transgenic porcine fibroblast cell lines by the novel method of magnetofection or standard lipofection using DreamFectTM Gold (OZ BIOSCIENCES). This transgenic cell line may be used in future as a nuclei source for somatic cell nuclear transfer for breeding new transgenic animals for the purpose of xenotransplantation.

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References

Rogers C.S., Hao Y., Rokhlina T., Samuel M. et al. (2008) *Production of CFTR-null and CFTR-DeltaF508 heterozygous pigs by adeno-associated virus-mediated gene targeting and somatic cell nuclear transfer*. J. Clin. Invest. 18(4): 1571-1577.

Watanabe S., Iwamoto M., et al. (2005) *A novel method for the production of transgenic cloned pigs: electroporation-mediated gene transfer to non-cultured cells and subsequent selection with puromycin*. Biol. Reprod. 72(2): 309-315.

Nakayama A., Sato M., Shinohara M., , et al. (2003) *Efficient transfection of primarily cultured porcine embryonic fibroblasts using the Amaxa Nucleofection system*. Cloning Stem Cells 9(4): 523-534.

Plank C., Schillinger U., Scherer F., Bergemann C., Remy J.S., Krotz F., et al. (2003) *The magnetofection method: using magnetic force to enhance gene delivery*. Biol. Chem. 384: 737-747.

Mykhaylyk O., Antequera Y.S., Vlaskou D., Plank C. (2007) *Generation of magnetic nonviral gene transfer agents and magnetofection in vitro*. Nat. Protoc. 2(10): 2391-2411.