ORAL PRESENTATIONS

Integration of *in vitro* culture, biotechnology and molecular biology in modern breeding of oilseed rape

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During the past 30 years, oilseed rape (*Brassica napus* L.) has become an important world crop. In Poland and in the European Union winter oilseed rape is the leading source of edible vegetable oil and high-protein meal. The increase of oilseed rape production during the last decades has been due to the considerable improvement of seed quality as well as yielding ability. The progress in the development of high yielding and good quality varieties has been achieved owing to the essential progress in the cellular and molecular biology of *Brassica napus* in the last years.

The production of haploids and doubled haploids using microspore culture has accelerated the obtainment of homozygous lines in oilseed rape breeding. The use of molecular markers in marker assisted selection and genetic transformation technique for introduction of desirable traits using doubled haploids have been improved.

Oilseed rape reveals a high level of heterosis, which is the reason that breeders are interested in the development of F1 hybrid varieties. Commercial production of F_1 seeds requires an effective cross pollination system. Molecular selection of suitable genotypes, also applied to doubled haploid lines, accelerates the production of genetically uniform lines, especially parental lines needed for the hybridization systems.

Genomics has been recently added to the wide range of techniques used for development and evaluation of new cultivars.

Brassica napus lacks many desirable traits, especially resistance to biotic and abiotic stresses, which causes considerable economic losses. In this case, asymmetric somatic hybridization has been carried out to achieve a succesful introgression of desired genes. The offspring of resynthesized oilseed rape originating from interspecific hybridization between appropriate forms of *Brassica rapa* and *Brassica oleracea* (in combination with embryo rescue culture) represents a potentially important resource to expand the genetic diversity of the narrow gene pool of *Brassica napus*.

These problems will be discussed using examples of investigations conducted at the Department of Genetics and Breeding of Oilseed Crops of the Plant Breeding and Acclimatization Institute in Poznań.

Obtaining tobacco double haploids containing different sources of resistance to PVY

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Potato virus Y (PVY), especially its necrotic strains, is a dangerous pathogen of tobacco. It causes necrosis of nerves and leaf laminas leading to a significant yield reduction. Brown vein necrosis of tobacco caused by PVY is an important problem because new isolates able to break resistance are still occurring. One of resistance sources is a wild species *Nicotiana africana* that shows resistance to all tested PVY isolates. Breeding BPA lines derived from crossing *N. tabacum* cv. BP-210 × *N. africana* are tolerant of many PVY isolates. Another source of resistance are transgenic tobacco lines – MN944 LMV (cultivar Mac Nair containing gene of lettuce mosaic virus coat protein) and AC Gayed ROKY2 (cultivar AC Gayed containing gene of PVY replicase). To increase the level of resistance by combining these sources of resistance, hybrids coming from crosses between transgenic line MN 944 LMV and line BPA as well as MN 944 LMV and AC Gayed ROKY2 were obtained. The most resistant hybrids were used for producing haploids via androgenesis. Immature anthers were put on Nitch & Nitch medium. Regenerated plants were analyzed with respect to the presence of transgenes and ploidy level. They were also inoculated with PVY. Most of the haploids showed to be resistant to the virus. Some of them were tolerant and only a few were susceptible. Doubled haploids were obtained by regeneration from stem fragments of resistant haploids. The ploidy levels of regenerated plants were estimated by flow cytometry.

The use of multi-qPCR platform and *tan1* mutant in identification of TF genes involved in somatic embryogenesis in *Arabidopsis*

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Somatic embryogenesis (SE) induced *in vitro* provides a valuable method for plant regeneration and is widely used in micropropagation and genetic transformation of plants. Thus, determination of genetic factors involved in triggering, in somatic cells, of the new, embryogenic mode of development is of practical value. The change in cell development requires a global rearrangement of the genetic program. Regulatory genes, especially transcription factors (TF), are believed to play a key role in this process. Here, we present the results of an experiment, in which the multiplex qPCR platform for *Arabidopsis* TFs was used to identify genes involved in the induction phase of SE. Two *Arabidopsis* genotypes differing in capacity for SE under *in vitro* culture were used: the *tanmei/emb2757* embryonal mutant unable to undergo SE and its parental ecotype Col-0 with a high capacity for somatic embryo formation. RNAs were isolated from freshly isolated explants and explant-derived cultures on 5th and 10th days of *in vitro* culture and the transcriptoms of the analyzed genotypes were compared in three biological replicates. The relative expression levels of 1920 known *Arabidopsis* TF genes were monitored with the use of qPCR reactions and *UBQ10* gene as standard control. It was found that only less than 6% of the analyzed TF genes were not expressed at any of the analyzed culture samples and over 78% of the TFs showed differences in activity between the compared genotype/culture time point combinations.

We found that transcriptoms of the Col-0 and mutant explants differed significantly and over 26% of the analyzed TFs displayed at least 5-fold up- or down-regulation in the compared tissues. The analysis indicated that 86 of all analyzed TFs were significantly up- or down-regulated (at least 5 fold) at different time points of embryogenic cultures derived from the compared genotypes. Among the differentially regulated genes, bHLH, MYB and MADS TF families were found to be the most frequently represented. Further experiments are conducted, in which nine of the selected genes (*At1g24625, At1g27740, At1g31040, At1g79580, At2g26320, At3g04030, At3g56970, At5g24330, At5g65050*) are analyzed in terms of their potential functions in SE.

In vitro culture of *Miscanthus sinensis* anthers

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Species of *Miscanthus* are perennial, rhizomatous grasses originating from Asia, that have the potential for very high rates of growth. Because of perenniality and C4 photosynthetic pathway, species from the genus *Miscanthus* possess several characteristics that make them favourable crops for efficient biomass production. The focus of this study was the induction of androgenesis in culture of *Miscanthus sinensis* anthers. Genetic (genotype) and non-genetic (cold pre-treatment, induction medium, date of beginning of anther culture) factors influencing androgenesis were studied. We were successful in inducting androgenesis in *M. sinensis* anther culture. Examination of acetocarmine squashes prepared from anthers from a six-week-old culture showed up to 15 nonsynchronously developing pollen-derived polycellular structures. The callus yield was strongly affected by the genotype. A beneficial influence of cold pre-treatment of spikes on the induction of androgenesis was observed. The results suggest that the high callus yield might be caused by the late initiation of culture. Initiating anther cultures at the end of the flowering season caused a significant increase in callus yield in comparison to cultures initiated in the height of flowering season. It is likely, however, that the improved efficiency of androgenesis induction in the case of *M. sinensis* anther culture initiated in autumn could be related to a positive influence of growing donor plants under the conditions of cooler and shorter days, i.e. 11-h days with temperature around 11°C and 13-h nights with temperature around 5°C.

In vitro selection of varieties and lines of *Brassica napus* L. resistant to multiple heavy metals

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Heavy metal contamination presents a serious problem worldwide. The existing methods of decontamination are usually not only expensive but also invasive to the environment. Phytoremediation – a group of methods of pollutant removal based on living plants grows in significance as a valuable alternative. Among other methods phytoextraction, the technology of extracting the contaminating agents from soils through the plant root system is the most applicable as far as heavy metal contamination is concerned. Particular traits such as: high heavy metal accumulation and translocation to aboveground parts, high biomass production and well known agricultural techniques are required for the plant to be useful for such purpose and only a limited number of plant species can be used in phytoextraction processes (Vera-Estrella et al., 2009). Therefore there is an increasing need to understand the defense mechanisms that make a plant tolerant to heavy metals as well as to directly know which plants are particularly useful. This requires testing of different species and varieties for heavy metals resistance (Nehnevajova et al. 2007). *In vitro* cultures of plants constitute a powerful tool to achieve such aim. One of the plant species known for its relatively high tolerance and accumulation is rapeseed (*Brassica napus*). In our research we tested nine *Brassica napus* cultivars towards resistance to Zn, Pb, Cu and Cd applied in combination. From each cultivar, two types of explants – hypocotyl and petiole were cultured on MS medium supplemented with hormones (0.15 mg/l NAA and 3 mg/l BAP), $AgNO_3$ (2.5 mg/l) and $ZnSO_4$, $PbNO_3$, $CuSO_4$ and $CdSO_4$ at the concentrations of 30 μM, 60 μM, 7 μM and 7 μM, respectively in order to obtain multi-heavy metal – resistant lines from regenerated plants. By measuring the shoot induction frequency and effectiveness parameters we showed that the potential to regenerate on medium supplemented with heavy metals differs significantly among the different *B. napus* cultivars.

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Enzymatic markers of rhizogenic competence in *in vitro* culture of common ice plant

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Hypocotyl explants of *Mesembryantheum crystallinum* L. were maintained on root induction medium for 1, 3, 5, 7, 10 and 14 days followed by subculture onto shoot induction medium for 14 days. Using tissue transfer experiments, rhizogenic determination was found to occur on the 5 day after explantation. Histological studies revealed the presence of clumps of meristematic cells at the time of determination whilst roots with root caps were observed after 7 days of culture. The endogenous concentration of H_2O_2 changed throughout the culture, reaching a peak on day 3, followed by a gradual decrease till day 14. The activity of catalase (CAT) decreased after day 1 and became constant till day 5. Next, a significant increase in its activity was observed, followed by a slight decrease on days 10 and 14. An increase in CAT activity was concomitant with the occurrence of its additional form CATII on the 5th day of culture. The pattern of activity of superoxide dismutases (SODs) was similar for CuZnSOD, FeSOD and MnSODI forms throughout the culture, being generally slightly higher (days 3, 5, 7) or similar (days 10 and 14) to control. On the 5th day of culture, that is at the time of induction of rhizogenic determination, an additional band of activity of MnSODII occurred. Its intensity remained constant until day 14. Fumarase activity and general respiration intensity reminded constant, with the exception of day 14 when a conspicuous increase was noted, while cytochrome c oxidase activity altered throughout the culture and corresponded with the photosynthetic activity measured as chlorophyll a fluorescence. A decrease in cytochrome c oxidase activity as well as in fluorescence parameter of chlorophyll *a* correlated with a slight increase of SOD activity on the 3rd day and CAT activity on 5th day suggesting alterations in ROS levels that preceded rhizogenesis. In summary, the occurrence of specific forms CATII and MnSODII did not seem to be directly related to metabolic changes accompanying regeneration but rather corresponded with the tight regulation of endogenous H_2O_2 level, itself possibly directly involved in the regenerative process.

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Use of biotechnology for conservation of a critically endangered population of alpine saxifrage (*Saxifraga nivalis* L.)

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Saxifraga nivalis is an arctic-alpine species that inhabits rocks in the upper sub-alpine zone. In central Europe it is known from only one location in the Karkonosze Mountains, where it grows in crevices and windings of basalt rocks in the glacial circus of Mały Śnieżny Kocioł. The population is endangered because of the restricted cross pollination, small production of viable seeds and poor vegetative reproduction (Fabiszewski, 2001). Considering the threats to the existing population, our efforts focus on successful maintenance of this population and creation of supplementary habitats to preserve the genetic pool of Polish representatives of the genus *Saxifraga*. Sterilized seeds were sown on 1/2MS medium and the cultures were kept at 25°C. After 3 months, seedlings reached 1.5 cm and their further growth was examined under various temperature regimes (4–25°C) and light intensities (20–80 μmol quanta m^{-2} s^{-1}). The impact of mineral nutrition and phytohormones on growth and propagation was also evaluated. The number of stimulated axillary buds fluctuated from 5 to 10 per rosette on the basal medium under controlled growth conditions. Comparison of plant growth at supplementary locations, i.e. in the Wrocław Botanical Garden (120 m a.s.l.) and at the Jagniątków nursery of Karkonoski National Park (650 m a.s.l.) showed that plants performed perfectly well at the higher altitude.

Autonomous endosperm induction in cultured unpollinated ovaries is strongly species dependent

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In sexually reproducing angiosperms embryo and endosperm development is initiated by double fertilization and a ratio of 2 maternal genomes to 1 paternal genome (2m:1p) is essential for endosperm formation. However, there is a group of taxa (autonomous apomicts) representing fertilization-independent embryo and endosperm development. Autonomous endosperm (AE) can be induced experimentally with the use of irradiated pollen and also through *in vitro* culture of unfertilized ovules or ovaries. These results could be very helpful in understanding the mechanisms leading to endosperm development with no involvement of paternal genome and therefore how genomic imprinting can be overcome in sexually reproducing flowering plants.

Unpollinated pistils, ovaries, ovules of several wild (*Viola tricolor, Salix viminalis, Arabidopsis thaliana* var. Columbia and Landsberg, *Alisma pantago-aquatica, Sedum acre, Gagea lutea, Anemone nemorosa, A. ranunculoides Capsella bursa-pastoris*), cultivated (*Lycopersicon esculentum* cv. Adelajda, Cunero, Havana, Maeva, Shirley; *Brassica napus* cv. Topas, var. Feliks, *Synapis alba*) and ornamental taxa (*Viola xWittrockiana, V. cornuta, Calendula officinalis, Rudbeckia bicolor*), representing annual, perennial and woody life forms were cultured on hormone-free MS medium (with different sucrose concentrations) as well as on media supplemented with plant growth regulators: 2,4-D, NAA, BAP, KIN, IAA, GA3, homoBl (homobrassinolide) in different concentrations and combinations.

Partial AE development (or endosperm like structure) was induced in unpollinated ovules on hormone-free MS medium as well as on media supplemented with growth regulators but the frequency was species not medium dependent. Lack of induction was noted in *V. tricolor, V. xWittrockiana, V. cornuta, Alisma pantago-aquatica, Sedum acre, Gagea lutea, Anemone nemorosa, Brassica napus* var. Feliks, *Synapis alba*; sporadically was observed in *Lycopersicon, Rudbeckia, Calendula, Salix Capsella bursa-pastoris* and *Anemone ranunculoides*. The highest frequency of AE formation was found in *Arabidopis thaliana* (45% of ovaries have ovules with AE) but neither cellularization nor differentiation on specific regions typical for endosperm of wild-type *Arabidopsis*, resulting from fertilization were observed.

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Liquid nitrogen efficacy in protection of genetic stability in plant material

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Cryopreservation is important for long-term conservation of plant genetic resources, especially for species producing recalcitrant seeds and crops that are propagated clonally through grafting or through vegetative cuttings, suckers, roots, tubers and bulbs. Up to now, it has been successfully used for over 100 plant species. Despite the fact that in the temperature of liquid nitrogen the cell division and metabolism are arrested, the factors associated with cryotreatment, cryostorage or plant recovery could become sources of somaclonal variability. The lecture will attempt of assess the state of knowledge concerning the influence of cryopreservation on the morphogenic capacity of tissues and genetic integrity of plants.

Assessment of genetic and epigenetic stability of the recovered plants derived from cryopreserved plant material is an important step to success of any storage protocol. Determination of the plant's "trueness-to-type" after cryopreservation can be carried out at the phenotypic, histological, cytological, biochemical and molecular levels. Several molecular markers based on different techniques (RFLP, RAPD, AFLP, MSAP, SSR) have been applied. The results suggest that the processes of cryoprotection and cryostorage have an impact on DNA methylation status and could lead to alterations in chromatin structure and changes in gene expression. However, majority of studies have reported insignificant or null influence of cryopreservation on the plant material.

Application of genetically modified trees in forest plantations

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Wide use of forest-tree products and progressive deterioration of natural forests make the development of new sources of wood supply necessary. Human activities and global climate change make forest ecosystems more prone to biotic and abiotic agents. Some of the measures taken to control them rely on achievements of biotechnology. For several years, genetic engineering has been applied to create modified trees in order to enhance their growth, quality of wood, susceptibility to pathogens and pest resistance. Efforts have concentrated mostly on *Populus* sp. and *Eucalyptus* sp. whose genomes are considered to be easily transformed *via* the traditional *Agrobacterium tumefaciens* mediated method or by particle bombardment. Other tree species, i.e. spruce, pine and birch have also been studied in the field of molecular engineering, with main focus on the introduction of cry and aro genes, conferring insect and herbicide resistance in forest-tree plantations.

The paper discusses some problems in GMO forest-tree breeding and summarizes recent achievements in the transformation of tree species, in the context of possible applications for improving and introducing novel traits into forest plantations.

Efficient plant regeneration of *Trifolium nigrescens via* somatic embryogenesis

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This study presents an efficient protocol for regeneration of *Trifolium nigrescens* Viv. plants via somatic embryogenesis (SE). The immature zygotic embryos at torpedo (TsE) and cotyledonary (CsE) stage were cultured on EC6 (TsE) and MS (CsE) solid media supplemented with auxins and cytokinins. The type of explant and the combination and concentration of plant growth regulators were the key factors for the induction of somatic embryos. SE was observed directly during culture of TsE on media supplemented with 2, 4 or 8 mg/l 2,4-D. Cytokinin (kinetin or 2iP) and auxin (2,4-D or NAA) alone did not induce somatic embryos from CsE. The combination of 2 mg/l 2iP or kinetin along with 2,4-D or NAA had beneficial effect for somatic embryo induction from CsE, but the addition of cytokinin was inhibitory for SE induction from TsE. The highest frequency of SE (up to 100%) was obtained from CsE cultured on medium supplemented with 4 mg/l 2,4-D and with 2.0 mg/l 2iP. The appearance of somatic embryos was concomitant with the inhibition of shoot and root growth of the original embryo. After 3–5 days the explants became swollen. The first somatic embryos appeared directly from upper part of the hypocotyl after 7 days of culture (TsE and CsE) and margin of cotyledons (TsE). After 10 days the explants started to produce embryogenic callus. Somatic embryos induced from TsE became necrotic after 10–18 days of culture. Most of the embryoids induced from CsE and maintained on media containing 2,4-D and kinetin or 2iP displayed several morphological abnormalities such as: fusion of embryonic axes, elongated hypocotyls, fused cotyledons or additional cotyledons. Subculturing somatic embryos on hormone-free medium resulted in their conversion into plants with an average frequency of 25%. These relatively low conversion rates clearly seemed to be related to the various morphological abnormalities induced under *in vitro* conditions. Somatic embryos obtained from CsE on media containing NAA and kinetin or 2iP had mostly normal morphology (clearly distinguishable root and shoot poles) and after transferring onto hormone-free medium regenerated into flowering plants with 64–100% frequency (depending on auxin concentration in the induction medium).

HBV core antigen (HBcAg) as a plant-based prototype of therapeutic hepatitis B vaccine and a carrier for HBV preS epitopes

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Chronic hepatitis B afflicts 400 mln people worldwide and is the main factor inducing hepatocellular carcinoma and the cirrhosis. The inefficiency of current anti-viral drugs encourages research on new methods of hepatitis B therapy using durable and highly immunogenic Capsid-Like Particles (CLPs) formed by HBV core antigen HBcAg (Lau et al., 2002). The preS epitopes are used mainly for HBV prophylaxis but they are also considered as alternative for hepatitis B therapy (Couillin et al., 1999). However preS epitopes require a carrier, e.g. HBcAg, for stabilization and immunogenic display (Murray and Shiau, 1999; Chen et al., 2004).

PreS1 and preS2 epitopes were inserted into immunodominant 'c' epitope of HBcAg, displayed on the surface of CLPs. HBcAg and HBcAg-preS1 and HBcAg-preS2 coding sequences were placed under control of CaMV 35S promoter and the transgenes were transferred into genomes of tobacco and lettuce via *Agrobacterium*-mediated transformation. Regenerated plants expressed HBcAg at a level of up to 400 μg/g FW but HBcAg-preS only at a level reaching 30–40 μg/g FW. HBcAg and HBcAg-preS proteins were stably produced in plants during plant development and in consecutive generations, as confirmed by ELISA assay and western blot analysis. Selected plant lines, verified for CLPs assembly and immunogenicity, can be potentially exploited as an initial material to produce therapeutic vaccine for chronic HBV carriers, orally administered when produced in lettuce, and injective, when produced in tobacco.

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Cryoprotective activity of thermal hysteresis protein in evergreen plants

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Thermal hysteresis proteins (THPs) inhibit the growth of ice by binding to the surface of ice crystals, preventing the addition of water molecules to cause a local depression of the freezing point. Recent findings demonstrate that apoplastic THPs from Norway spruce show anti-ice nucleation activity (Jarząbek et al., 2009), and also embryonic axes from *Acer, Fagus* and *Quercus* seeds possess specific cryoprotective activity proteins. Here, we have focused on the most active THPs from five frost-hardy conifers of North American and Eurasian boreal forest: *Abies grandis* (Douglas ex D.Don)Lindl., *Picea pungens* Engelm., *Pinus nigra* J.F.Arnold, *Pinus sylvestris* L., and *Tsuga canadensis* (L.)Carriere, and additionally from one alpine (2100 m a.s.l.) evergreen shrub *Loiseleuria procumbens* L. The objective of this study was to determine whether the thermal hysteresis proteins of frost-hardy plants influence survival at subzero temperatures by modifying the freezing process and/or by acting as cryoprotectants. Apoplastic extracts were obtained by vacuum infiltration of leaves with 5 mM ascorbic acid, and the extracts were concentrated by using a 10 kDa cut-off Ultrafree centrifugal filter device (Millipore). Proteins were analysed by SDS-polyacrylamide gel electrophoresis. Cryoprotective activity of apoplastic proteins was determined with the use of the freeze/thaw inactivation, by four cycles in liquid nitrogen (–196°C) and room temperature while the assay of lactate dehydrogenase (LDH) was performed as described by Wisniewski et al. (1999). Antifreeze activity was determined by using the droplet freezing assay (Vali, 1971).

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Influence of different culture strategies on growth and indolizidine alkaloid accumulation in *Securinega suffruticosa* shoot cultures

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Securinine is one of the indolizidine alkaloids from Securinega suffruticosa, Phyllanthaceae. It is a CNS stimulant, and GABA receptor antagonist. Moreover, this compound may be helpful in prevention and treatment of Alzheimer's disease as neuroprotector against neurotoxicity induced by β-amyloid (25–35) and can improve the quality of life in people suffering from amyotrophic lateral sclerosis (ALS) (Raj and Łuczkiewicz, 2008).

In vitro cultures allow production of biologically active compounds in a controlled manner, independent of climatic conditions and season. Considering the special properties of *S. suffruticosa* and the fact that the plant is not native to the European climate, it seemed pertinent to investigate *in vitro* cell cultures of this plant.

The aim of this work was to test different culture strategies in order to determine the influence of investigated factors on the accumulation of indolizidine alkaloids by *S. suffruticosa* shoot biomass and to create an *in vitro* plant system suitable for the production of securinine. Moreover, attempts were made to scale up the experimental culture of S. suffruticosa shoots to the bioreactor scale. Within the experiment, culture type (stationary or shaken), growth conditions (light access) and production scale (bioreactors) were modified to achieve the purpose. Moreover, the cultures were fed with precursor of biosynthesis and growth complexes (casein hydrolysate and coconut water) to change the production potential of the shoots. Murashige for *Lilium* (ML) medium was used as the basis. Phytochemical screening of all *in vitro* biomasses was carried out simultaneously. The TLC technique with densitometric detection was used for all phytochemical analyses (Raj et al., 2009).

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Bacteria in plant environment

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Plant environment contains diverse bacteria whose populations are determined, both in quantitative and qualitative terms, by conditions present in a particular biotope. Those conditions play an important role in mutual relationships between plant and bacteria as well as between bacteria and other organisms. Most of bacteria colonizing the rhizosphere, rhizoplane and phylloplane are commensals that do not have detectable influence on plant growth or physiology. Among mutualistic bacteria, the microsymbionts of leguminous plants and other bacteria promoting plant growth and yield may be distinguished. Their activity leads to an increase in bioavailability of nutrients, production of substances improving plant growth (e.g. phytohormones) and/or biological reduction of pathogens and other deleterious organisms. Endophytic bacteria colonizing healthy plant tissue offer an enormous potential for increased agronomic performance of plants. The influence of bacteria on plant vigor and health status may also involve their participation in bioremediation of soil pollutants, decomposition of organic matter and improvement of soil structure. Although bacteria with the ability to degrade toxic substances represent a small fraction of soil microorganism populations present in plant habitats, they can play an important role in plant productivity.

Of more than 5000 species of bacteria recognized, over 100 are the causal agents of plant diseases. They constitute a very important factor limiting growth and yield of cultivated plants. Some diseases, such as fire blight of apple and pear (*Erwinia amylovora*) or bacterial canker of tomato (*Clavibacter michiganensis* subsp. *michiganensis*) can devastate the plants over considerable areas making their production unprofitable for many years. Pathogenic bacteria are divided into two major groups: eubacteria possessing the cell wall and ability to grow on artificial media and bacteria without cell wall but surrounded by cell membrane only. The latter group, called mollicutes, formerly known as mycoplasma-like organisms, includes phytoplasmas and spiroplasmas.

In recent years the development of modern molecular techniques has enhanced interest in the genomics and proteomics of plant-associated bacteria. The conventional methods of their identification have been supplemented and often replaced by DNA-based analysis allowing the improvement of diagnostics and the study of bacterial phylogeny.

Somatic embryogenesis of selected coniferous tree species

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Somatic embryogenesis in combination with cryopreservation is very effective in preservation of selected genotypes and mass clonal propagation. Experiments on somatic embryogenesis in selected spruce (*Picea abies* (L.) Karst.), silver fir (*Abies alba* Mill.), and European larch (*Larix decidua* Mill.) were conducted to determine if this method of micropropagation enables forest nurseries to produce quality seedling. Mature and immature zygotic embryos or megagametophytes with immature embryos were used for embryogenic tissue initiation in spruce, silver fir and European larch. Experiments were also carried out on embryogenic callus of hybrid larch (*Larix×leptoeuropaea*) (INRA, Orleans). High frequencies of embryogenic callus formation were achieved in spruce (23-31%) and fir (29%) when mature zygotic embryo explants were used, whereas immature embryos were required in larches (36% frequency). Initiation of somatic embryogenesis from somatic embryos resulted in second and third generation of embryogenic calli. Maturation of spruce somatic embryos was achieved on BM (Gupta and Durzan, 1986) medium containing 20–40 μM ABA combined with 1 μM IBA when tissue was cultured under low intensity light for 5 weeks. For larch, the MSG (Becwar et al., 1990) medium supplemented with 20–60 μM ABA and 1 μM IBA and also cultivation of tissue under low light intensity for 3–4 weeks was the best. Somatic embryos of silver fir required 8–10 weeks of cultivation in darkness on modified MCM medium (Bornman and Jansson, 1981 after Hristoforoglu et al., 1995) supplemented with 20 μM ABA and 1 μM IBA for maturation. The present studies demonstrated the possibility of applying somatic embryogenesis for efficient propagation of coniferous trees.

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In vitro culture of endophytic fungi as a potential source of novel biologically active secondary metabolites

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An endophyte is a bacterial (including actinomycetes) or fungal microorganism, which spends the whole or part of its life cycle colonizing inter- and/or intra-cellular locations inside healthy tissues of the host plant, typically causing no apparent symptoms of disease (Hallmann et al., 1997; Tan and Zou, 2001). The endophytic population of a given species varies from several to a few hundred of bacterial and fungal strains. Endophytes can be isolated from mildly surface disinfected plant tissues and cultivated on nutrient agar (*l.c*). The relationship between the endophyte and its host plant may range from latent phytopathogenesis to mutualistic symbiosis. Almost all vascular plant species examined to date were found to harbor endophytic bacteria and/or fungi. Endophytes colonizing plant tissues usually get nutrition and protection from the host plant. In return, they profoundly enhance fitness of the host plants by producing certain functional metabolites. Primarily, endophytes can produce bioactive secondary compounds, such as alkaloids, steroids, terpenoids, sesquiterpenes, isocumarin derivatives, phenylpropanoids, lignans and others (Tan and Zou, 2001). Moreover, endophytes can influence host's interactions with other species, altering plant community composition (Tan and Zou, 2001; Rudgers et al., 2004; Clay et al., 2005). As a poorly investigated store of microorganisms 'hidden' within the host plants, endophytes are obviously a rich and reliable source of bioactive and chemically novel compounds with huge medicinal and agricultural potential. For example, the advantage of production of some important phytochemicals such as paclitaxel and huperzine A in endophytes lies in the fact that it provides an alternative strategy for easing the impact of the growing human population on plants which are needed for the preservation of biodiversity and the ecosystem (Tan and Zou 2001; Szypuła et al., 2005, 2006). The aim of our investigation was to establish the culture of *Huperzia selago* endophytes that had been isolated from shoots and to perform their phytochemical analysis. After surface disinfection, shoot fragments were cut into 2 mm pieces and plated onto 4% potato-dextrose agar (PDA). Plates were incubated at room temperature for 10 weeks or until fungal growth was observed. The isolated fungi were transferred onto solid medium and incubated in the culture room at 25°C for 5 days. In order to determine the presence of secondary metabolites in the mycelium, HPLC-UV and HPLC-MS/MS-ESI analyses were performed in the positive ion mode.

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Cytologial, cytometric and molecular characterization of gentian somatic hybrids

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The aim of the presentation is to show and summarize the results obtained with the help of various methods, of somatic hybridization between selected species of *Gentiana*.

In a breeding program, somatic hybridization can overcome sexual incompatibility through the somatic cell fusion. In contrast to sexual hybridization, after protoplast fusion all nuclear and cytoplasmic DNA from both parents are united in one individual. However, regeneration of plants from fused protoplasts is often accompanied by polyploidization and/or the elimination of genome parts of one or both fusion parents. Thus, detailed investigations are needed not only to confirm the hybrid status of regenerates, but also to exactly characterize their genome size and composition.

In interspecific somatic hybridization program involving *Gentiana*, numerous calli and plants were produced by electrofusion between *G. kurroo* Royle and *G. cruciata* L. as well as between *G. cruciata* and *G. tibetica* King protoplasts. Several methods, including flow cytometry, chromosome counting, stomatal characteristics and AFLP were applied for identification and description of the recovered somatic hybrids. Results of cytological and cytometric analyses revealed higher genome sizes and chromosome numbers of regenerates in comparison to parental species. Some evidence of polyploidy and mixoploidy as well as partial genome elimination was found. According to molecular data, hybrids showed differential levels of symmetry.

Induction of tetraploids in hop (*Humulus lupulus* L.) using *in vitro* cultures

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Hop (*Humulus lupulus* L.) is a diploid, perennial plant whose secondary metabolites are used for brewing as well as in pharmaceutical industry. The Polish hop breeding programme centres around the generation of triploid cultivars which are superior to diploids because they produce more acid resins and essential oils. In the process of breeding triploids, diploid cultivars need to be raised to the tetraploid level (Koutoulis et al., 2005).

In this study tetraploids were induced through culturing apical buds of Polish diploid cultivar Sybilla on liquid MS media containing three colchicine concentrations: 0.01%; 0.05%; 0.1%. Buds were incubated on an orbital shaker for 24h or 48h and then transferred to shoot multiplication medium. Flow cytometric analysis of ploidy level revealed that the highest induction of tetraploids was achieved with the exposure to 0.05% colchicine for 48h while the lowest with 0.01% colchicine for 48h. Out of 116 plants, 8.7% were found to be tetraploids whereas others were diploids (63.3%) and mixoploids (30.7%). Shoot internodes of mixoploids were selected and calli derived from shoots were obtained. Subculture of these calli on different regeneration media supplemented with indoleacetic acid (IAA) and several concentrations of cytokinins promoted shoot regeneration. The best caulogenic potential was achieved on medium with 0.5 mg/l IAA and 5 mg/l zeatin riboside. The regeneration rate was 42.9%. Plantlets were successfully rooted and transferred to the greenhouse. Individual plants were classified as mixoploids or tetraploids after screening by flow cytometry.

This study provided a population of tetraploids that will be used in further hop breeding program.

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Is that really a step forward in protoplast culture of lupins?

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Considering the agricultural importance of lupins, studies aiming at an expansion of the gene pool effectively contribute to the improvement of breeding programmes in the genus. Lupin species, both those commercially grown and their wild relatives, are considered to be recalcitrant to *in vitro* culture. On the other hand, some valuable characters may be successfully introduced into the plant genomes by means of such manipulations. Protoplast and single-cell culture could be suitable methods for overcoming interspecific barriers and for increasing the genetic variability of lupins.

Comprehensive studies were undertaken to determine optimal conditions for yellow lupin (*Lupinus luteus* L.) protoplast culture. The adapted procedure of isolation resulted in a high yield of protoplasts obtained from various explants. Cultivar 'Parys' proved to be the most promising material for manipulations. Various media and culture techniques were evaluated in terms of their usefulness in promoting survivability and morphogenetic responses of protoplasts. Medium solidification enhanced the development of cultures initiated from hypocotyls and cotyledons by significantly increasing the mitotic division rate. However, an unfavourable phenomenon appeared in both liquid and solid media: after first regular division daughter cells did not undergo consecutive mitoses. An important breakthrough in the development of cultures occurred in media supplemented with 0.1% activated charcoal. Overcoming the suppression of mitosis led to the formation of small aggregates from hypocotyl protoplasts. The noteworthy progress achieved here indicates that in spite of yellow lupin recalcitrance it is possible to obtain responsive cultures with a higher morphogenetic potential and thus provide new materials for exploitation in modern and sustainable agriculture.

Anti-mitotic agents in *Salix viminalis* polyploid plant induction

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The genus *Salix* contains many economically important species used for energy, chemicals and fibers. *In vitro* polyploidization system of the species can be an essential step to generate variation for selection of desirable traits. In the present study, the potential of microtubule depolymerising agents trifluralin, pronamide (KERB), oryzalin amiprophos-methyl (AMP) and colchicine for *in vitro* genome doubling in *S. viminalis* clones was investigated. The mitotic inhibitors were dissolved in 2% dimethyl sulfoxide and added to MS medium with double concentration of Fe-EDTA. Embryos, shoots apices and axillary buds of the basket willow were exposed to various concentrations of the inhibitors for 1, 2, 3 and 6 days. The control test was performed with explants cultured on MS medium supplemented with 2% dimethyl sulfoxide. All cultures were kept under continuous light (cool white fluorescence lamps; 35 μmol quanta m^{-2} s^{-1}) at 22±2°C. Flow cytometry was applied to estimate ploidy level. Artificially induced tetraploid plants from *S. viminalis* embryos were obtained on media supplemented with 0.025 and 0.05% colchicine, 0.1% trifluralin or 0.3% KERB when the treatment lasted two and three days. The highest efficiency of doubling chromosome numbers was achieved on medium supplemented with 7 % trifluralin. In cultures of shoots apices and axillary buds no tetraploids were detected. Oryzalin was very toxic for tested explants of *S. viminalis* even at a very low concentration (0.001%).

The effect of genotype, explant type and medium on the regeneration of linseed (*Linum usitatissimum* L.) in *in vitro* culture

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Linseed (*Linum*) belongs to the family *Linaceae* that comprises about 200 species. Most of them are wild and only a few, such as *L. usitatissimum* L., are cultivated. This work reports the results of studies on the regeneration capacities in *in vitro* cultures of two *Linum usitatissimum* cultivars: Modran and Selena. Cotyledon (3 different fragments), hypocotyl and hypocotyl-cotyledon explants of tested cultivars were obtained from 6-, 7- and 8-day old seedlings. They were cultured on MS basic and MS medium supplemented with 1mg l^{-1} 2,4-D, NAA and BAP.

The genotype, type of media and explant significantly affected the ability and direction of regeneration. Irrespective of the genotype and age of explants, the best formation of shoots was achieved from hypocotyl and hypocotyl-cotyledon explants cultured on MS control and MS+BAP media. The largest number of regenerated plants in the soil was obtained from 6-day old hypocotyl explants incubated on MS medium with BAP. There were no plants transferred to the soil from 8-day old hypocotyl explants of both tested linseed cultivars. The addition of 2,4-D did not stimulate regeneration from most explants except for 6-day old hypocotyl-cotyledon explants of Modran cultivar which formed shoots. The callus formation and later the roots were the best on MS medium containing NAA irrespective of age of seedlings from which they were collected. Endogenous ethylene that accumulated in the culture vessels was likely responsible for stimulation or inhibition of regeneration. With respect to cotyledon and hypocotyl-cotyledon explants it caused them to turn yellow and consequently to die. However, it stimulated callus formation on hypocotyl explants. Explants from 6- and 7-day old seedlings showed a higher regeneration efficiency than 8-day old explants. Hypocotyl-cotyledon explants showed a better regeneration if they were harvested from 7-day old seedlings compared to 6 day old seedlings and as a result, a higher number of plants were transfered to the soil.

Agrobacterium – mediated genetic transformation of *Gentiana tibetica* King

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The discovery of natural ability of bacteria from genus *Agrobacterium* to infect plants and to integrate bacterial genetic material into the plant genome opened a new chapter in contemporary plant experimental biology. For over 30 years *Agrobacterium* has been used in biotechnological laboratories all over the world (Vain, 2007) and *Agrobacterium* – mediated genetic transformation has became an indispensable genetic engineering tool.

Species from genus *Gentiana* are valued as ornamental plants and material for isolation of pharmacologically active compounds (Skrzypczak et al., 1993). Their aesthetic and medicinal quality could be increased by a genetic transformation. The aim of our work was to develop an *Agrobacterium* – mediated transformation technique of *Gentiana tibetica* and to regenerate genetically modified plants. C58C1 *Agrobacterium tumefaciens* strain containing pDraGON-G:GFP vector was used (Wróblewski et al., 2005).

On the basis of the experiments it was shown that genetic transformation of G. tibetica leaf explant cells took place only in the presence of L-glutamine in the medium. Transformation efficiency increased with its concentration. The most efficient regeneration of transgenic plants was observed on the WPM medium without any plant growth regulators.

The transgenic character of tissues was determined on the basis of β-glucuronidase histochemical reaction. GUS activity was examined in transformed leaf explants, callus tissues and regenerated plants. PCR analysis of regenerants confirmed the transgene integration into plant genome while *Agrobacterium* contamination was excluded. Randomly selected plants were checked by Southern hybridization.

Topophysis in adventitious shoot regeneration *in vitro* in *Chrysanthemum*

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Adventitious shoots originate from a single cell or several cells and therefore the adventitous shoots technique is widely applied in plant breeding e.g. for induction of mutagenesis, genetic transformation and separation of components of chimeras. The success of breeding programmes often depends on the regeneration efficiency. The aim of this study was to investigate the influence of topophysical position of explants on the efficiency of adventitious shoot regeneration.

Uniform single shoots of chrysanthemum 'Satinbleu' propagated *in vitro* and consisting of 12 nodes were divided equally into three topophysical zones: distal, central and proximal. Two leaves and two internodes were isolated from each zone. The explants were isolated from the distal end of the distal zone, the central portion of the central zone, and the proximal end of the proximal zone. Explants were cultured for 12 weeks on MS medium supplemented with 0.6 mg l^{-1} BAP and 2.0 mg l^{-1} IAA. The number of adventitious shoots and regenerating explants were recorded. Regeneration curves were plotted as a function of time.

Regeneration proceeded differently in leaves and internodes. Internodes produced almost four times more shoots than did leaves. Internodes excised from the distal and proximal zones were more efficient in shoot regeneration, while explants taken from the central zone produced the lowest number of adventitious shoots. Leaves isolated from the central zone produced eight times fewer shoots than did leaves taken from the distal zone, whereas leaves excised from the distal zone did not regenerate at all. The earliest formation of adventitious shoots was observed on internodes isolated from the distal zone, where it started in the third week and continued until the seventh week of culture and then declined. The regeneration curves for proximal internodes as well as for distal leaves were shifted by two weeks towards the regeneration curve for distal internodes.

Due to the highest adventitious shoot regeneration capacity and the earliest formation of shoots, the best explants for chrysanthemum breeding programmes are internodes excised from distal parts of *in vitro* plantlets.

The control of bacterial contaminations during *in vitro* shoot multiplication

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Multiannual tissue cultures can be contaminated by different bacteria that do not reveal themselves *via* symptoms in the medium or on the shoot explants. Such contaminations are often transferred to subsequent subcultures. During a two-year period we obtained a number of isolates belonging to 24 species of bacteria from cultures of hosta, rose, raspberry, elder, blackberry, anthurium, narcissus, apple rootstock and orchid. Their identification was based on phenotypic and molecular analyses. The most frequently isolated bacteria were *Pseudomonas putida*, *Bacillus* sp., *Staphylococcus pasteuri, Methylobacterium lusitanum* and *Serratia marcescens*. The following compounds or commercial products known to have bactericidal activity: dichloroisocyanuric acid (Aldrich), thymol oil (Riedel-deHaën), Triclosan (Irgasan, Fluka), PPM (Plant Cell Technology, Inc), Biosept 33SL (Cintamani), Biochicol 020 PC (Poly-Gumitex Farm), silver nitrate (Sigma), benzoic acid (Chempur), menthol oil (Fluka), Decaben C (Jan Dekker), acetylsalicylic acid (Duchefa) and Proclin 300 (Supelco) were added to the media on which selected isolates were inoculated.

The growth of bacterial colonies was inhibited by Biosept, thymol oil, Triclosan and PPM. A small degree of inhibition or lack of growth was observed on media with silver nitrate, Biochicol, menthol oil, benzoic acid, acetylsalicylic acid, Decabenen C and Proclin 300.

Concentrations of 0.5 and 1% were used to evaluate the phytotoxicity of the compounds. After 2–3 days on the media containing thymol oil or Triclosan, explants from all of the tested cultures died. Phytotoxic effects of Decaben C were observed for cultures of apple rootstocks, strawberry, raspberry and hosta, but it did not affect the growth and development of gerbera explants. No phytotoxicity symptoms were observed after the application of PPM and Biosept at concentrations of 0.5 and 1%.

The next step was the use of bactericides in shoot cultures artificially contaminated with the studied bacteria. Silver nitrate, Biosept, Decaben C, PPM and Proclin 300 were added to multiplication cultures of strawberry, hosta and gerbera.

The most effective treatment against all tested bacteria in gerbera cultures was Proclin 300 at 0.05%. In cultures infected with *Bacillus* sp. or *Methylobacterium lusitanum*, the growth of bacteria was inhibited by 0.3% PPM, 0.05% Decaben C, 1% $AgNO_3$ and 0.5% Biosept. In hosta cultures, only Decaben C was not effective when cultures were contaminated with *Pseudomonas putida*. No signs of bacterial presence were recorded in cultures of strawberry supplemented with Proclin 300 (0.05%), $AgNO_3$ (0.5–1%) and Biosept (0.1–0.5%). In cultures contaminated with *Methylobacterium lusitanum*, only PPM at 0.3% was effective. The bacteriostatic effect lasted for 4 weeks.

Application of nanomaterials to fight and prevent bacteria *in vitro*

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Nowadays, among the most promising nanomaterials that have revealed antibacterial properties are the nanosized metal particles and nanotubes of polypyrrole (Ppy) or protoneated polyaniline (PAni) (Xia et al., 2004; Srivastava et al., 2007). In the present study we report the effectiveness of nanosilver (NS) particles sized 5–10 nm against *Microbacterium* strains during micropropagation of *Alocasia sanderiana*. Contaminated explants were dipped in 50, 100 and 200 mg l^{-1} solutions of NS. Results of the treatment recorded after one month of culture on modified MS medium showed a high number of decontaminated explants without any adverse effect on their growth characteristics. We also investigated the activity of polyaniline fixed directly onto filter paper by electrochemical oxidative polymerization against *Microbacterium*. We showed that *A. sanderiana* cultured in liquid MS medium on bridges of filter paper coated with the conducting polymers were decontaminated and developed without any visible symptoms of bacteria in comparison to the control.

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Some remarks on the application of *in vitro* techniques to manipulation of sexual reproduction of plants

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The *in vitro* culture conditions enable researchers to control and modify the processes of pollination, fertilization and the course of embryogenesis in seed plants. Technological development and the improvement of various procedures make it possible not only to obtain a deeper knowledge about sexual reproduction but, in particular, to provide strategies to transfer this knowledge into crop improvement. Sophisticated techniques are now available to manipulate the female and male organs, thus allowing to introduce new genetic material into breeding lines.

The presented lecture will be based on investigations carried out in our laboratory. It will mainly summarize those achievements that concern wide hybridization among various species.

Application of androgenesis in basic research and breeding of cereals

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The techniques for obtaining double haploids opened up an opportunity for practical breeding to obtain, in a short time, homozygotic lines possessing alleles of genes in the recessive form. The first varieties of wheat that were obtained by anther culture appeared on the markets in China and France in the 80-ties. Later, the more sophisticated method of isolated microspore cultures has been applied. Microspore culture allows for examination of large numbers of cells in a small area and in a short period of time as well as the selection of cell lines with the desired characteristics.

On the basis of many laboratories' reports and the subsequent publications on the issue, there has emerged a hierarchy among cereal crops in terms of their morphogenetic capacity in anther cultures. In this respect, rye and oat have always been considered the most difficult to regenerate, while wheat was found to be less recalcitrant and barley and triticale the least. One can observe a growing interest in homozygotic lines among scientists and breeders. They can be used in basic research as a "clean" material and in experimental plant breeding to obtain lines whose offspring does not segregate.

On the other hand, double haploid lines are a source of variation because each microspore, which is a source of regenerated homozygotic line has a certain set of gene alleles that can appear important in the breeding process. For this reason, breeding of cereals in many countries in recent years has been based mainly on double haploids. Polish breeding companies slowly start to follow in the steps of foreign breeding companies, who have been using that method. Based on our experience we may even risk the statement that *in vitro* induced androgenesis is the most efficient biotechnological method, used in practical breeding.

Increase of bioactive compounds in flax plants overexpressing enzymes of flavonoid synthesis pathway

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Flavonoids are a group of secondary plant metabolites important for plant growth and development. They are also well known as very effective antioxidants with a major significance for human health. We obtained flavonoid-rich plants using two strategies. The first strategy involved a simultaneous overexpression of three genes of flavonoid synthesis (chalcone synthase, chalcone isomerase and dihydroflavonol reductase) and the second strategy consisted in overexpression of the broad substrate specificity enzyme glucose transferase.

Both groups of plants had increased levels of phenylopropanoid compounds important for the antioxidant potential, such as kaempferol, quercetin, phenolic acids, poli-unsaturated fatty acids and lignans. Because the constitutive promoter (CaMV 35S) was used for modification, the positive changes were observed in the entire plant body – green parts and seeds.

Flax seeds are a very good source of the plant lignan precursor secoisolariciresinol diglucoside (SDG), which is metabolised by bacteria in the colon to produce the mammalian lignans enterolacton and enterodiol. Lignans inhibit cell proliferation and growth, which makes these compounds potential anticancer agents (in hormon-sensitive cancers). In our transgenic plants a significant increase of SDG level (up to 30-fold) was noticed. A dramatic increase of ferulic acid level (about 40 fold) was also observed. The high level of lignans was also found in seedcakes – the residual material left after pressing of oil from linseed of transgenic plants. For this reason, seedcake obtained from flavonoid-rich plants should be use as a very good source of bioactive compounds.

A significantly higher level of antioxidants was also detected in oil from transgenic plants. Remarkably, such oil is rich of poli-unsaturated fatty acids and the ratio of ω6/ω3 was close to that recommended by FAO/WHO for optimal human diet. The presence of antioxidants in this oil also resulted in increased unsaturated acid stability.

Flax fibers obtained from transgenic plants on a semi-technical scale have increased contents of many bioactive phenylopropanoids such as a phenolic acids and lignans. In fibers we also identified canabidiol – a compound that can reduce pain. For this reason fabrics produced from these fibers may be recommended as wound dressing.