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Adoption of genetically modified plants in the European Union and in Poland

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Sustainable development is considered to be one of the most difficult goals to achieve and yet it is a significant challenge for agricultural production worldwide. The use of biotechnology can increase yields under conditions that restrict the use of plants' biological potential. Biotechnology is currently a part of the European Union concept of bioeconomy. The development of economy through a rational use of plant resources depends on many factors, including an optimal use of human capital and an adoption of balanced regulations. Hope for an increase in the food production level adequate to population growth lies in increasing productivity per area unit. Conventional mechanisms for raising yields are strongly limited. The development of modern biotechnology offers new promising opportunities of using biological potential. The improvement of plant resistance to biotic and abiotic environmental factors by means of biotechnological methods has opened great opportunities for increasing crop productivity without an additional energy input. The number of countries that grow genetically improved crops in 2011 reached 29. While the area of GM crops is growing annually by about 10 million hectares, the discussion about potential environmental and health risks and benefits of this new technology is still ongoing. The potential of plant biotechnology is not fully utilized. Its use requires *inter alia* balanced regulations for prevention of environmental and economic damages. The EU and its member states have developed a biosafety strategy. A precautionary approach has become the central tenet of decision-making related to GMOs. The precautionary principle gives risk managers an option to act in a responsible manner, even in the face of scientific uncertainty. To apply precautionary principle means to take measures adequate to the possible threats. Taking the precautionary approach neither means to introduce a total ban nor excessive permissiveness. The "case by case" principle requires to treat each GM case individually. International commitments taken by Poland create the need for a law on GMOs, which would be in line with international standards.

One of the barriers to the use of GMOs is the fear of violation of the economic interests of conventional and organic farmers. Coexistence rules should be designed to protect those interests. Such regulations have not been introduced in Poland so far. Therefore it is necessary to carry out research on the outcrossing and its environmental implications. We have conducted such studies on corn, canola and triticale. Here, in this paper, we focus on triticale. Our results indicate that outcrossing in the examined species is possible, but to a limited extent, and at a short distance from the pollinator (11 m is enough to ensure outcrossing level below 0,9%).

It is evident that the policy and the law will in future be important factors for the development of agriculture and biotechnology. However, the new technology should be founded on the basis of well conducted research.

Flax biotechnology

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Flax (*Linum usitatissimum*) is an annual plant widely distributed in the temperate climate zone. It has a long history of cultivation, and a great significance in medicine and industry. The three main products obtained from flax are the fibres produced from the stem, the linseed and the oil extracted from the seeds. The properties of flax seeds and oil have been widely examined and have been proven to have many beneficial properties for human health. Flax oil is the richest plant source of linoleic and linolenic fatty acids that are essential for human health. Unfortunately, essential fatty acids are highly susceptible to oxidation and therefore flax oil has a very short shelf life. Traditionally flax fibres are mainly used in the textile industry, paper production, isolation materials and biocomposite production. However, in recent years there is a growing interest in biomolecular application of flax products.

The basis of these actions is several types of crops produced through biotechnology and the generation of raw materials having entirely new quality in comparison to the current value of raw flax.

Flax with antioxidants. This new type of flax, which fibres and seeds naturally synthesise strong antioxidants, is intended for biomedical uses. Good example of such modification is repression of lycopene β-cyclase resulted in increase of carotenoids content – group of hydrophobic antioxidants. On the other hand the simultaneous overexpression of chalcone synthase, chalcone isomerase and dihydroflavonol reductase resulted in overproduction of flavonoids – hydrophilic antioxidants. The same effect will be obtained by overexpression of glucosyl transferase. In addition to preventing fat rancidity, hydrolysable antioxidants have beneficial effects on human health. Consumed together with essential unsaturated fatty acids can reduce the risk of various diseases.

Residues from this two types of new flax seeds contain antioxidants which have a protective role in various types and stages of cancer, autoimmune disease and both types of diabetes.

New quality of flax fibres. Flax is also source of valuable fibres with a wide range of possible applications. Flax fibres are very flexible, stronger than cotton but less elastic. Genetic modification has been also taken to improve quality of flax fibres. New flax is equipped with biodegradable, plastic fibres (polyhydroxybutyrate – PHB) and uses for biomedical and technical applications. Bio-plastic fibres are the form of cellulose polymers chemically linked with polymers of polyhydroxybutyrate (bio-plastic) produced during the growth of plant.

Another modification that could influence fibre quality is retting improvement. Chemical composition of stems influences retting process; the higher pectin and lignin level the longer exposure to fungal and bacterial enzymes is needed. The possible solution to this problem would be reduction of pectin and lignin synthesis in flax.

For this reason transgenic flax plant with increased polygalacturonase and rhamnogalacturonase expression were generated. This modyfication resulted in a significant reduction in the pectin content accompanied by a significantly higher retting efficiency. The transgenic flax with silenced cynnamic alcohol dehydrogenase gene has lower lignin content and remarkable decrease in pectin and hemicelluloses levels. These data may have potential commercial meaning in improving retting process and also mechanical properties of flax fibres.

All presented modifications mostly resulted in increase in yield of transgenic plants and their higher resistance to *Fusarium culmorum/oxysporum* the most common flax pathogen.

Plant extracts and silver nanoparticles for treatment of burn wound infections

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Due to the growing problem of human pathogens that are becoming increasingly resistant to antibiotics, new, alternative methods of fighting those pathogens are being searched for.

An effective solution of this problem could be the use of natural, plant-derived substances in combination with novel nanotechnology products – silver nanoparticles (AgNPs).

AgNPs are particles of metallic silver in different shapes and sizes (from 1 to 100 nm). They show antibacterial, antifungal, and antiviral activity due to their high surface-volume ratio. The small sizes of AgNPs allow them to penetrate microorganisms through interactions with the cell membrane. After entering bacterial cells, AgNPs impact the respiratory chain, proteins containing sulfur, and compounds containing phosphorus. Moreover, they disturb the processes of replication and gene expression by binding to DNA and RNA, and change the three-dimensional structure of proteins by binding to disulfide bridges. The gradually released silver cations react with negatively charged molecules located inside the cell and on its surface (Feng et al., 2000).

Drosera plants have been used in medicine for centuries for treatment of asthma and pertussis. Extracts obtained from their tissues show, among others, antibacterial and antifungal activity (Juniper et al., 1989). Insectivorous plants are the source of many secondary metabolites, mainly naphthoquinones (plumbagin, ramentaceon) and flavonoids (quercetin, myricetin). A combination of extracts from insectivorous plants: *Drosera*: *Drosera binata, Drosera aliciae,* and *Drosera cayenensis;* with AgNP Ag_55_NMe3 of 5.5 nm (ProChimia Surfaces Limited Liability Company in Gdynia), at concentration of $6.74 \cdot 10^{14}$ NPs/ml, is used to increase the antibacterial and antifungal activity of those agents. A simultaneous reduction of the Minimum Bactericidal Concentration (MBC) of both of those agents can alleviate the potential adverse effects on human or animal cells. The aim of this project is to develop an efficient method for fighting *Staphylococcus aureus* and *Candida albicans* – dangerous pathogens which infect burn wounds. The research is being conducted by using planctonic and biofilm cultures. It is believed that in biofilm pathogens can survive even a 1000-fold higher concentration of antibiotics and antimicrobial drugs than in planctonic cultures. In order to study the interactions between different antimicrobials, the Checkerboard Titration Method was used. For each combination of two compounds the Fractional Bactericidal Index (FBC) was calculated. Based on the obtained FBC the following types of effects can be identified: synergistic (FBC < 0.5), additive (1 > FBC > 0.5), neutral (2 > FBC > 1), or antagonistic (FBC > 2).

The research in using combinations of AgNPs and extracts from insectivorous plants can serve as a basis for development of a new method for treatment of patients suffering from burn wounds and other kinds of skin damage infected by *S. aureus* and *C. albicans*.

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Protoplast electrofusion of wild species *Solanum* x *michoacanum* (Bitter.) Rydb. and cultivated potato *S. tuberosum* L.

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The objective of this work was to produce interspecific somatic hybrids resistant to late blight caused by *Phytophthora infestans* (Mont.) de Bary by means of protoplast fusion of the wild potato species *Solanum* x *michoacanum* (2*x*, 1EBN, source of resistance) and *S. tuberosum* (2*x*, 2EBN; 4*x*, 4EBN). *S.* x *michoacanum* is known as a source of resistance to *P. infestans*, but it is reproductively isolated from *S. tuberosum* due to the differences in EBN (Endosperm Balance Number) value. In Plant Breeding and Acclimatization Institute – the National Research Institute, Młochów Research Center (IHAR-PIB, Młochów) diploid clones of *S.* x *michoacanum* resistant to *P. infestans* were selected.

In cooperation with Julius Kühn Institute (JKI), Germany, somatic hybrids were produced by protoplast electrofusion of two accessions of *S.* x *michoacanum,* with three diploid clones of *S. tuberosum* selected in IHAR-PIB and the cultivar Rywal, which are susceptible to late blight. Protoplast isolation and fusion were done in JKI. Protoplasts were isolated from ca 1g of leaflets of 3-4 weeks old *in vitro* plants. Cellular walls were digested by solution of 0.2% macerozyme and 0.8% cellulase. The purified protoplasts of parents were mixed at a ratio 1:1 and 200 µl of mix was placed in a lamellar fusion chamber. Fusion was achieved by applying first 18-20 V an AC-field of 800 KHz for aligning the protoplasts, followed by two DC pulses of 120 V amplitude and 15 µs duration with a pause of 2 s and AC-field of 10-20 s.

For eight genotype combinations somatic hybridization was achieved and viable calluses and shoots were obtained. After protoplast electrofusion a modified VKM-medium was added to the suspension of fused protoplasts. The cultures were kept in a culture chamber at 25 °C in the dark. The forming microcalluses were transferred to Cul-medium and exposed to 16 h/day illumination (fluorescent light intensity: 55.5 µmol/m^2/s^1) at 25 °C. After four weeks, calluses were transferred on the regeneration medium RJM. After 4-6 weeks first shoots were obtained. Only one shoot from one callus was excised and rooted on MS-medium. In total, 18.775 calli were produced from post-fused products in eight combinations. From those calluses 1.482 *in vitro* plants were regenerated. The most vigorous plants were multiplied *in vitro* and transferred to the greenhouse. Phenotypes of vigorous greenhouse-grown plants were characterized in terms of their phenotypic and physiological traits. The ploidy level was evaluated by counting of chloroplast number in the guard cells and by the use of flow cytometry. Pollen fertility was estimated using an indirect lactofuchsin method, based on the percentage of regularly shaped and stained pollen grains. For identification of somatic hybrids molecular markers (CAPS, RAPD, SSR) were applied.

Based on morphological and cytological observations and molecular markers 228 somatic hybrids were selected.

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Production of carrot somatic hybrids with introduced male-sterile cytoplasm

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Cytoplasmic male sterility (CMS) is a maternally inherited trait which has been observed in many plant species. The CMS plants fail to produce functional pollen without loosing female fertility. CMS occurs spontaneously or can be induced either by intra- or inter-specific crosses as a consequence of incompatibility between the nucleus and the cytoplasm. In carrot, two different CMS types have been reported, the petaloid and the brown anther type. In the petaloid plants anthers are transformed into petals or petal-like structures. This source of CMS (the S_p cytoplasm) is commonly used for practical hybrid seed production. The donor-recipient protoplast fusion can be used to transfer the CMS trait from male-sterile to male-fertile accessions.

The aim of the presented work was to produce asymmetric somatic hybrids of carrot with the petaloid type of CMS. For the purpose of cybridization, leaf protoplasts of the CMS donor were irradiated with UV for nuclear genome fragmentation and fused with hypocotyl protoplasts of the male-fertile recipient whose cytoplasm was inactivated with sublethal doses of iodoacetic acid (IOA). The leaf protoplasts were isolated from 3 to 4 week-old *in vitro* grown plantlets of two CMS breeding lines (2874A and 2163A) while the hypocotyl protoplasts from aseptic 2 week-old seedlings of cultivar Dolanka. Tissue digestion and protoplast isolation were performed according to the protocol described by Grzebelus et al. (2012). In order to find precise conditions for inactivation of nuclear and organellar genomes, different doses of UV (125-8000 J/m^2) and IOA (0.2-1.0 mM) were tested, respectively. Suitable conditions for both treatments were determined after the analysis of protoplast viability in the first two days of culture and cell colony formation in the 10th and 20th day of culture. Protoplast fusion was induced with 40 % polyethylene glycol (PEG). The resulting cultures were evaluated with respect to their plating efficiency. Ploidy analysis, PCR-based cytoplasm identification and morphological observations of flowers were performed to verify the hybrid status of the derived plants.

The leaf protoplasts UV-irradiated with doses higher than 1500 J/m^2 were unable to divide and proliferate. Similarly, no cell division was observed after the treatment of protoplasts with IOA at concentrations above 0.2 mM. Based on these results, prior to cell fusion, the protoplasts were treated with either 2000 or 4000 J/m^2 UV dose and with either 0.4 or 0.6 mM IOA. The plating efficiency in the 3 week-old cultures varied from 4 to 62%. Plants were regenerated from the resulting callus macrocolonies through somatic embryogenesis. Most of them were diploids (56 %) but also numerous tetraploids (42%) and single pentaploids and hexaploids were observed. Out of the 503 regenerated plants 27 yielded the PCR product characteristic for the S_p cytoplasm. Within these plants 12 had petaloid flowers.

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The use of meristems for the transformation of common sage (*Salvia officinalis*)

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Common sage is a valuable herbal plant with bactericidal properties used for rinsing the oral cavity and throat, both during infections (purulent inflammation of gums, thrush, tonsillitis) and as a preventive hygiene measure. Our study aimed at the development of a sage transformation procedure with the use of apical and axillary buds and also isolated meristems. The study was supposed to be the basis for the development of a recombined, orally-administered vaccine against caries, which would be produced in plants. The transgenesis of sage was conducted with *Agrobacterium tumefaciens* that contained pBI-CRSA gene construct with central *pac* gene region coding I/II surface antigen of *Streptococcus mutans* i.e. the key factor responsible for the onset of caries in humans. I/II antigen can be a valuable component of the vaccine against caries as it triggers immunological response. The experimental part of the study involved development of the conditions of running an *in vitro* culture of sage, carrying out the transformation, determining the conditions of selecting the plants and testing the correctness of the transgenesis with the use of a PCR technique. The transformation of apical and axillary buds was done for two or three-week-old multiplants and seedlings. Moreover, the study included a trial to transform and regenerate plants from isolated meristems. The optimum growth of plants was achieved from apical and axillary buds, while the propagation of the plants was successful on MS (Murashige and Skoog, 1962) medium supplemented with BA at the concentration of 0.3 mg/l. A selection method with the use of kanamycin was developed for sage explants after the transformation. The lethal concentration for non-transformed sage plants was 200 mg/l. After completing the transformation of apical and axillary buds, one explant was isolated and then propagated. The propagation culture was run on MS medium supplemented with BA, which medium contained the following antibiotics: kanamycin as a selective agent and timentin (250 mg/l) as an agent inhibiting bacterial growth. The explant grown on the selective medium served for DNA isolation. Molecular analyses showed that the gene construct did not integrate with genomic DNA in all the tested samples. The use of apical and axillary buds in the transformation process turned out to be difficult and inefficient. These explants contain both meristem and the leaf buds, which results in a reduced access of *Agrobacterium* to the meristem tissue that forms the plant. When isolated meristems were transformed, they were pricked directly with a needle soaked in *Agrobacterium* suspension. This was aimed to improve bacterial access to the meristemic cells and thus the chances of successful transformation. The experiment did not lead to obtaining plants with a stable transgene integration. However, the observations of the control explant cultures are important as they represented the growth capacity of isolated meristems.

Induction of *Dracocephalum forrestii* W.W. Smith hairy roots by *Agrobacterium rhizogenes*

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Dracocephalum forrestii W.W Smitth called "zhiyanggu" in Tibetan is a perennial plant belonging to the Lamiaceae family. This species grows in the northwest mountain regions of Yunnan province, China. For a long time it has been used as astringent, antipyretic and diuretic agent in traditional Tibetan medicine (Li et al., 2007). The ethanolic extract of the whole plant of *Dracocephalum forrestii* has shown a significant antioxidant activity in *in vitro* tests (Li et al., 2009). The main groups of medicinally important constituents are flavonoids (luteolin, apigenin, narirutin), triterpenoids (betulin, betulinic acid, ursolic acid), lignans (sisymbrifolin, acanthoside B), phenolic acids (lithospermic acid, rosmarinic acid, caffeic acid) and phenylethanoids.

In the present study the initiation of *Dracocephalum forrestii* transformed root culture by infection with A4 (plasmid pRiA4) agropine *Agrobacterium rhizogenes* strain has been described. Explants (leaves, nodes and internodes) obtained from a 4-week-old shoot cultures were infected by wounding them with sterile needle immersed in the bacterial culture and by co-cultivation for 30, 60 or 90 min in Erlenmeyer flask containing 30 ml of a bacterial suspension (OD_{600} = 0.5-0.8). Before infection the bacteria were grown on YMB agar or a liquid medium at 26°C for 48 hours. The inoculated and control explants were incubated in the dark at 26°C on MS (Murashige & Skoog) (Murashige and Skoog, 1962) agar (0.7%) medium without growth regulators. The experiment was repeated three times. For each treatment 30-40 explants were used. As a control, 30-non infected explants of each type were cultured under identical conditions. Roots appeared at the wounded site and after infection by co-cultivation after 2-3 weeks. After 7 weeks the percentage of explants forming roots was evaluated.

The best explants for inducing hairy roots proved to be internodes. The percentage of explants forming roots was 81.6% when they were infected by wounding with a sterile needle and only 25% after co-cultivation for 60 min with a bacterial suspension. The obtained roots were excised from explants and transferred individually into 100 ml Erlenmeyer flasks containing 30 ml of half-strength B5 liquid medium (1/2B5) with ampicillin (500 mg l^{-1}) for the elimination of bacteria. The cultures were maintained in the dark on a rotary shaker at 100 rpm. After seven subcultures (one week each), the concentration of ampicillin was reduced to 250 mg l^{-1}. After fifth successive passages, ampicillin was eliminated from the medium and axenic root cultures were obtained. The PCR method will be used to confirm the root clone transformation. After the confirmation of transformation, the fast growing clones will be selected for further investigation.

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Hairy roots of *Rhaponticum carthamoides* (Willd.) Iljin

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Rhaponticum carthamoides (Willd.) Iljin (family *Asteraceae*) is an endemic, perennial, herbaceous plant originating in the mountains of South Siberia, Middle Asia and Mongolia (Kokoska and Janowska, 2009). This plant species is naturally found in high-mountain meadows, in tundra brushwood and in the glades of coniferous forests (Petkov et al., 1984). In the recent decades *R. carthamoides* has been cultivated as a medicinal plant in various regions of Central and Eastern Europe. *R. carthamoides* is commonly known as a "maral root" or Russian leuzea and has been used for the long time in folk Siberian medicine in case of overstrain and weakness after illness (Biskup and Lojkowska, 2009). The raw materials from *R. carthamoides* are rhizomes and roots. The extract from the roots and rhizomes has been shown to possess wide spectra of biological activities e.g.: adaptogenic, antioxidant, immunomodulatory, anticancerogenic and antimicrobial. It is used to eliminate physical and mental weariness, promote muscle growth, proteosynthesis and sexual function (Kokoska and Janowska, 2009; Biskup and Lojkowska, 2009). This plant contains different classes of secondary metabolites: ecdysteroids, flavonoids, phenolic acids, triterpenoid glycosides, polyacetylenes and sesquiterpene lactones. Ecdysteroids (with 20-hydroxyecdysone) are the main group of compounds (Kokoska and Janowska, 2009).

The aim of our work was the establishment of *R. carthamoides* hairy roots. Hairy roots were initiated by transformation of leaves from 4-week-old *in vitro* growing shoots using *Agrobacterium rhizogenes* (strain A4). The explants were wounded with a sterile needle immersed in the bacterial culture. After 2 weeks, adventitious roots started to emerge from the infected sites of explants. Axenic root cultures were obtained after 10 subcultures. Out of four root lines, the one which showed fast growth in hormone-free full strength liquid Gamborg (B5) medium and produced more lateral roots was used for further investigations. Integration of DNA into *R. carthamoides* genome was confirmed by PCR method. We also investigated the optimal conditions for the growth of RC3 transformed root clone. The hairy roots were cultured in liquid Gamborg (B5), Schenk and Hildebrandt (SH) and Woody Plant (WP) media with full- and half-strength macro- and microelements without growth regulators. All the media contained 3% sucrose. The cultures were maintained both in the dark or under the light conditions (photoperiod of 16/8h light/dark, with a light intensity of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$), on a rotary shaker at 80 rpm. A preliminary study showed that in all tested media, the maximum accumulation of biomass was observed after 5 weeks of growth. After 35th day of culture the fresh and dry weights were estimated. The most intense growth was observed on a WP medium with full concentration of nutrients, under the periodic light. The roots reached about 8g/flask (80 ml medium) of the fresh weight. The value represented an increment of 17 times of the inoculum biomass. The lowest biomass of hairy roots was observed in 1/2 SH and 1/2 B5 medium. The fresh weight increased only 5 times from 0.5g/flask to 3g/flask (80 ml medium). The transformed roots turned green when cultured under the light conditions. The roots cultured on the all media, in the dark and under light conditions were able to regenerate green buds. Spontaneous buds regeneration (approximately 5 buds per flask) was observed in about 60% of the cultures grown in 1/2 SH and 1/2 B5 medium, in the dark. For propagation, the regenerated buds were detached from hairy roots, transferred individually to culture tubes containing Murashige and Skoog agar (0.7%) medium without growth regulators or supplemented with 0.1 mg l^{-1} indole-3-cetic acid (IAA) and 0.2 mg l^{-1} 6-benzyladenine (BA), and cultured under a 16/8h light/ dark photoperiod.

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Crossing transgenic and non-transgenic flax plants to obtain new fiber and oil forms

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Flax (*Linum usitatissimum*) is an annual plant widely distributed in the Mediterranean and temperate climate zone and is a valuable source of oil and fiber.

The objective of this study was to achieve transgenic forms of oil flax and double transgenic types of fiber flax using a traditional genetic technique. As initial material for crossings, transgenic forms of fiber flax included PGI, RHA and CAD as well as transgenic forms of oil flax W92, obtained in previous studies were used. Overexpression of the first two genes (coding enzymes polygalacturonase and rhamnogalacturonase) originating from *Aspergillus aculeatus* resulted in a reduction of pectin content in fiber (Musialak et al., 2008). Silenced gene *cad* (cinnamyl alcohol dehydrogenase) resulted in a reduced synthesis of lignins. Both modifications improved fiber quality; in other words, their presence in flax facilitated the retting process. In the case of oil flax W92 overexpression of three genes from *Petunia hybrida* coding chalcone synthase (*chs*), chalcone isomerase (*chi*) and dihydroflavonol reductase (*dfr*) resulted in an increase in flavonoids and phenolic acids content in green parts as well as seeds? (Lorenc-Kukuła et al., 2005). The following crossing combinations were made: PGI x CAD, CAD x PGI, RHA x CAD, CAD x RHA for fiber flax and Szafir x W92.40, W92.40 x Szafir, Szafir x W92.72, Linola x W92.86, W92.86 x Linola, Linola x W92.72 for oil flax.

Having emasculated the flower of the mother plant one day before blooming hand pollination followed. The stigmata were dusted with donor pollen. The stage of flower development was estimated for 10 (mother) and 12 (father form) in the 12 stages scale according to Schewe (2011). Prepared in this manner flowers were covered with isolators made of paper bags. Reciprocal crosses in the number of 555 were made, resulting in 384 obtained seeds. Only 155 seeds had developed normally, the rest were dry and immature. The averege seed number per capsule for all crossings was about 4, whereas flax has a potential to produce up to 10 seeds. From the 97 obtained capsules 15 were seedless. The offspring were scored for seed development and survival under glasshouse conditions. The germination rate ranged from 33% for W92.86 x Linola to 100% for PGI x CAD. Seed color was also estimated, indicating that in F1 the color of seed was the same as in the mother plant.

To confirm that the crossing was successful a? PCR was conducted. In crossings with CAD two primers were designed: one, first specific for *cad* gene fragment and one for Pdk intron (element of pHellsgate). Primers specific for *npt II* were used for selection after pollination with W92 and to confirm transgenity of PGI and RHA of mother plants.

To avoid losing the unique plant material, the tested offspring were kept under *in vitro* conditions. After surface sterilization (1% sodium hyperchlorite with drop of detergent, for 20 minutes) explants were planted onto MS medium containing 20 g sucrose with 4 g of agar together with 1 g of gerlite as solidifying agents. At least 10 copies of the individual crossing and parental forms were collected in this manner. Plants were kept under a 16h-light and 8h- darkness regime.

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Identification of phenolic components in a genetically modified flax by UPLC-PDA-MS method

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Flax (*Linum usitatissimum*) is an annual plant grown widely in the Mediterranean and the temperate climate zone, with a long history of cultivation and a great significance in medicine and industry. A unique feature of flax is the possibility of whole plant to be fully exploited with almost no waste products.

The main products obtained from flax are fiber and oil. However, its by-products such as a seedcakes left after oil pressing and flax shives are also a source of valuable components. For this reason, flax has quite a significant potential for biotechnological application. To increase the valuable qualities of flax products, the flax genome has been genetically modified, with the specific aims to improve the plant's pathogen resistance, taste and nutritional properties, and to produce pharmaceuticals and other compounds mainly from the phenylopropanoid and terpenoid pathway. Traditionally, flax fibers have been mainly used in the textile industry, paper production, isolation materials and biocomposite production. However, in the recent years, there has been a growing interest in biomedical applications of flax products. One of such modifications was made by introducing polihydroxybutyrate (PHB) synthesis genes into flax genome. This modification resulted in fibers with much improved biomechanical properties, which were successfully used in preparations of composites and wound dressings. The metabolomic analysis by GS-MS of *in vitro* grown plants revealed that genetic modifications resulted in changes in phenylopropanoid modification. For this reason the phenolics content of various flax products was studied in more detail in order to determine the impact of modifications on phenylopropanoid metabolism and to establish the usefulness of flax products for PHB overexpresing plants for biomedical applications. The UPLC coupled with a PDA and MS was a method of our choice as it provides good separation without destroying the components, thus allowing a glucoside analysis. The analysis was performed on Waters UPLC coupled with a photodiode detector and Xevo Q- TOF MS. The analysis was done on seeds, seedcakes, whole flax stems, straw, fibers, fabric and flax shives. In seeds, the majority of phenolic components were found to be bound in lignan macromolecule, although the presence of free phenolic acids and flavonoids was also detected. No major changes in the composition of the phenolic components in seeds was noted. The flax fibers contained very little of soluble phenolic components, and those were mainly ferulic and coumaric acids and vanillin and apigenin C-glucosides (vitexin and isovitexin). The majority of the phenolic components in a flax fibre were bound by ester bonds and could be released by NaOH treatment. Considerable amounts of a ferulic and coumaric acid vanillin were detected in flax fibres and also in a unprocessed flax fabric. However the traditional industrial fabric processing removes most of the valuable components. The PHB overexpresing flax contains higher quantities of phenolic components than control flax, which together with a PHB contents makes it a very suitable material for a wound dressing production.

The flax shives, by-product of fiber production, are also a very good source of the same components and can be used for isolation of antioxidants. Whole unretted stems contain, besides ester-bound compounds, also a large quantity of free phenolics that are mainly chlorogenic as well as other caffeic, ferulic and coumaric acid derivatives and C-glucosylated forms of a apigenin and luteolin (vitexin, isovitexin and izoorientin). The transgenic plants contain larger quantities of a chlorogenic and caffeic acid and a catechin derivative.

Silencing CAD gene in flax: an insight into metabolite flux within the phenylpropanoid pathway in modified flax plants

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The phenylpropanoid pathway is a complex network that delivers a wide variety of secondary metabolites such as hydroxycinnamic acids, anthocyans, phenolic compounds, flavonoids, flavones, lignans, and covers a number of straight biosynthesis paths including that of lignin. The phenylpropanoid compounds contribute to plant defense against pathogens and stress response. Apart from protecting the plant, they significantly affect plant qualities such as texture, flavor, color, and processing characteristics. It must be stressed that all transformations in the phenylpropanoid pathway are not linear and there are many transitions between particular steps. Not all of them are already known. A possible redirecting of carbon subunits flux in the phenylpropanoid pathway can provide potential benefit for plants.

Lignin is synthesized as a branch of the phenylpropanoid pathway, the final enzyme in monolignol synthesis is CAD (cinnamyl alcohol dehydrogenase), which catalyzes the transformation of p-coumaroyl, feruloyl and sinapyl aldehyde into their alcohol equivalents.

Playing a crucial role in providing the mechanical strength to the plant cell wall, most of lignin is found in vascular tissues. Acting as a mechanical barrier, lignin participates in plant defense against pathogens. However, it elongates the retting time, and by this it deteriorates fibre hue, texture and cohesion. Thus, lowering the lignin level by silencing the CAD gene may lead to the improvement of the fibre quality and properties.

The subject of this study was to evaluate how repressing CAD gene in fibrous flax (*Linum usitatissimum* L. cv. Nike) affects the secondary metabolites synthesis within the phenylpropanoid pathway. The research aimed to examine mature flax plants cultivated in a filed among with flax crop, the fibre and seed.

Flax plants with CAD gene silenced by RNAi method were produced in our laboratory. Field trial with two chosen transgenic lines, CAD27 and CAD33, were conducted to obtain the crop. Mature stems, fibre and seeds were subjected to extractions with adequate solvents (methanol, strong alkali, chloroform) to release all possible phenylpropanoids. The identification and quantification of substances were done with the UPLC method and Q-TOF MS. Since anthocyans and flavanones, as well as other phenolic compounds are found in fibre in traces amount, too low to detect them using the UPLC method, the total colorimetric measurements were carried out.

The data obtained showed that two transgenic lines had differently reacted to the introduced modification. CAD27 line showed a decrease in the anthocyans level with no changes in phenolic compounds, whereas CAD33 reversely, had no changes in the anthocyans amount, but revealed a strong increase in the phenolics.

The overall phenylpropanoids composition had changed in the transgenic plants, although the shifts observed in carbon subunits were not spectacular. Nevertheless, it can be stated that the metabolite flux within the phenylpropanoid pathway does exist.

Effect of some signal molecules on PAL activity and taxane production in *Taxus x media var. Hicksii* transgenic root cultures

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Paclitaxel (Taxol, Bristol-Myers Squibb, New Brunswick, NJ) is a diterpenoid secondary metabolite present in various *Taxus* species (Taxaceae). It is a very potent antitumor compound widely used in the therapy of various cancers types, such as: breast, ovarian, lung, head and neck cancers as well as AIDS-related Kaposi's sarcoma.

In vitro cell cultures are an excellent and renewable source of paclitaxel (PXT) and other taxanes. Many different strategies have been developed to enhance paclitaxel production in yew tissue and cell cultures, including precursor and elicitor applications.

In the present work we studied the effects of three signal molecules: methyl jasmonate (100 μM), sodium nitroprusside (SNP 10 μM) as a NO donor and salicylic acid (SA 144,8 μM = 20 mg/l) on phenylalanine ammonium-lyase (PAL) activity and taxane production in hairy root cultures of *Taxus x media var. Hicksii*.

It has been reported that jasmonate and salicylate are endogenous signal molecules, which not only elicit plant resistance to pathogens and herbivores but also exogenously induce secondary metabolism pathways (Wang et al., 2004) .

PAL, the first enzyme in the phenylpropanoid pathway, was induced by various biotic and abiotic factors, such as methyl jasmonate. It has also been reported that elicitor-induced PAL activity was potentiated by the NO donor (Wang et al., 2004) .

Transgenic root cultures of *Taxus x media var. Hicksii* obtained *via* transformation with *Agrobacterium rhizogenes* LBA 9402 were carried out in modified hormone-free liquid DCR-M medium (Sykłowska-Baranek et al., 2009), on gyratory shaker at 105 rpm, in darkness and at 25°C.

The medium was supplemented with L-phenylalanine (100 μM, PHE) and/or SNP, SA and methyl jasmonate (MJ) after autoclaving. Hairy roots cultivated in the medium without any elicitor but with PHE were considered as a control cultures. Samples were taken after 12h, 24h, 48h, 1 week and 2 weeks.

No taxanes were detected in hairy roots growing in control cultures and in roots elicited only with SA. The combination of SA with SNP or MJ resulted in PXT production in roots growing in their presence for 12 h (15 μg/g DW) and 1 week (38 μg/g DW), respectively. The highest content of PXT – 323,16 μg/g DW, was determined in roots maintained in a medium with the addition of SNP and MJ after two weeks of culture.

PAL activity was rapidly induced in all types of cultures within 24 h after elicitation. However, in the most elicitor treatments used there was no correlation between PAL activity and taxane accumulation in root tissues. The one exception was SNP and MJ application when the increasing PAL activity had coincided with the increasing PXT yield.

Our results are consistent with previously published data on the amplifying role of NO donor on PAL activity by PXT production.

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Taxodione accumulation in various lines of *Salvia austriaca* transformed roots

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Salvia austriaca Jacq. (austrian sage), is a medicinal herbaceous plant native of high altitudes across Russia and Eastern Europe (Clebsch and Barner, 2003). It has been reported that the roots of this species produce abietane diterpenoids (Nagy et al., 1999). Some of them, such as taxodione, have biological activities. It also demonstrates antibacterial, cytotoxic and antitumour activities (Kuźma et al., 2011). This compound was earlier found in *Salvia austriaca* hairy roots (Kuźma et al., 2011). As a result of the genetic transformation by *Agrobacterium rhizogenes* (strain A4), seven lines of *S. austriaca* hairy roots were obtained. The transformation of these roots was confirmed by a PCR method by detection of *rol* B and *rol* C genes of *Agrobacterium* in the root cells (Kuźma et al., 2011). The roots were cultured in *in vitro* conditions in 300 ml Erlenmeyer flasks containing 80 ml of sterile growth regulator-free 1/2 B5 liquid medium in the light (16h/8h light/dark photoperiod), at the temperature 26°C ± 2°C. In this study, biomass accumulation (fresh and dry weight) and taxodione level in the obtained root lines have been presented. Taxodione content was determined by ultra performance liquid chromatography method (UPLC). The quantitative analysis was performed using the Agilent Technologies UPLC system Infinity 1290, including a binary solvent pump equipped with a degaser, autosampler connected to the thermostat, column compartment with thermostat, and UV diode array detector (DAD), connected to the computer equipped with the Chemstation LC-3D software. A Zorbax Eclipse Plus Rapid Resolution C18 column (50 mm × 2.1 mm, 1.8 μm particle size, Agilent Technologies) with a filter (diameter 5 mm, pore size 0.3 μm, Agilent Technologies) located in stainless steel guard (In Line 1290 Infinity, Agilent Technologies) were used. The column temperature was maintained at 20°C. Separation of the plant roots extracts was achieved using a gradient mobile phase consisting of 0.1% (v/v) formic acid in water (A) and acetonitrile (B). The taxodione content in seven investigated root lines ranged between 0.52 and 0.7 mg g^{-1} dry weight. The highest taxodione amounts biosynthesized the line C7 of the roots (0.7 mg g^{-1} dry weight in the 30th day of culture). The content of this compound grew until the 45th day of culture. This line had also a high biomass level (6.2 g fresh and 0.52 g dry weight per flask, after 30 days of the growth). Taking into account the high level of dry weight and taxodione content, the 30-day-old hairy root line C7 had the largest production of this compound calculated in mg per liter of culture and was almost 9 mg. On the basis of the obtained results, line C7 of the genetically modified roots of *Salvia austriaca* could be a very good source of a very important, strong and biologically active abietane-type diterpenoid – taxodione.

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A methodical approach to HMW DNA transformation of cucumber (*Cucumis sativus* L.)

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The high molecular weight of DNA (HMW DNA) is a nuclear DNA of a least 50 kb in size and it contains the entire "blocks of genes", that allow the introduction of a long sequences with a cumulative effect (QTLe). For this reason, HMW DNA is ideal for the analysis of large fragments, the large insert library construction (BAC, BIBAC, TAC, YAC or fosmid libraries) and long template sequencing. Large DNA fragments are important tools for advanced plant genomics research including *Zea mays*, *Oryza sativa*, *Triticum aestivum*, *Hordeum vulgare* and *Arabidopsis thaliana*. Large-insert libraries have been used for positional cloning, physical mapping, comparative genomics and next-generation sequencing. To facilitate the positional cloning and the transfer of complete large inserts into plants via *Agrobacterium thumefaciens*, we used a BAC library of cucumber (*Cucumis sativus* L.). We converted BAC clone into TAC to make a transformation by the *Agrobacterium*. However, TAC clones with large inserts are not easy to transform.

In our hands (Gutman et al., 2008), a BAC library was constructed with HMW DNA isolated from the B10 monoecious line of cucumber (*Cucumis sativus* L.). The BAC library is characterized by very good parameters: the average length of clones is 135 kbp and the genome coverage > 12.7 x a number of clones in the library is 34 560). Based on the nucleotide sequences of the BAC clones, we genetically identified and selected by homology a 007 BAC clone. The localized 007 BAC clone showed homology to i.e. *M* (Monoecious) gene related to the sex determination process in cucumber. Cucumber is one of the main model plant for studying sex determination as it represents different sex types. An elucidation of the function of the analyzed clone/clones is needed to assign its/their specific function/functions, thereby preparing a physical map of the function.

To HMW DNA transformation of cucumber was performed for the 007 BAC clone transferred into TAC vector. The construct TAC_007 was introduced via electroporation into *Agrobacterium*. We checked the stability of the construct in the cells of *Agrobacterium*. The enzyme restriction and the PCR were used to verify the presence of the studied insert. We have made the first attempt to the transformation of cucumber suspension culture.

Effect of compounds inhibiting DNA synthesis and senescence on endopolyploidy in sugar beet (*Beta vulgaris* L.) seedlings

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Endoreduplication is an alternative form of the mitotic cell cycle in somatic tissues, in which the repeated rounds of nuclear DNA replication occur without subsequent mitosis and cell division. During this process cells amplify their DNA without chromatin condensation, chromosome segregation, and cytokinesis. An increase in DNA content in the nuclei through endoreduplication leads to endopolyploidy and then to polysomaty of tissue/organ. Although endoreduplication is widespread in plants, particularly in angiosperms, its biological significance is still unclear. It is suggested that endoreduplication promotes cell enlargement to facilitate the rapid growth and maturation of the tissue/organ. As endoreduplication occurs in tissues displaying high metabolic activity, it is likely that it provides a mechanism to increase the level of gene expression. There are also suggestions concerning the relation between endoreduplication and programmed cell death; in some tissues endoreduplication is followed by programmed cell death (e.g. endosperm, suspensor, tracheary elements). In the present study, two compounds that inhibit DNA synthesis, camptothecin and geraniol, and two senescence inhibitors, paclobutrazole and butalated hydroxytoluene, were applied exogenously to sugar beet seedlings. Those compounds are considered as inducers and inhibitors of programmed cell death, respectively.

The aim of this study was to investigate the effect of camptothecin, geraniol, paclobutrazole, and butalated hydroxytoluene on endoreduplication in the root, hypocotyl, and cotyledons of the seedlings at the stage of unfolded cotyledons. Seeds of diploid sugar beet cultivar "Arthur" were sterilized and placed on the Murashige and Skoog (MS) medium supplemented with one of the compounds: 5 μM camtothecin, 10 μM geraniol, 10 μM paclobutrazole or 20 μM butalated hydroxytoluene. Seedlings were grown in a growth chamber in 16/8 hours day/light photoperiod at 20/18 °C. After 16 days of *in vitro* culture the seedlings were dissected into root, hypocotyl and cotyledon and analyzed by flow cytometry. Each organ was chopped with a sharp razor blade in a plastic Petri dish with 1 ml nuclei-isolation buffer (0.1 M Tris–HCl, 2.5 mM $MgCl_2 \times 6H_2O$, 85 mM NaCl, 0.1%, v/v, Triton X-100; pH 7.0), supplemented with 4′,6-diamidino-2-phenylindole (DAPI; 2 mg/ml) for DNA staining. After chopping, the suspension was passed through a 50 mm mesh nylon filter and analyzed using a Partec CCA (Partec GmbH, Münster, Germany) flow cytometer, equipped with an HBO lamp. For each sample, the DNA content in 5000-7000 nuclei was measured. Analyses were performed on 10 replicates, using a logarithmic amplification of the signal. Histograms were evaluated using the DPAC v. 2.2 programme. The percentage of the nuclei of each particular DNA content, the mean C-value, and the 4C+8C+16+32C/2C ratio were calculated. The results were estimated using a one-way analysis of variance and a Duncan's test ($P = 0.05$).

The obtained results revealed changes in seedling morphology and endopolyploidy pattern depending on the applied compound and seedling organ. Camtothecin had the strongest effect on endopolyploidy, which increased its level in the root and hypocotyl, and decreased in the cotyledon. Geraniol increased the mean C-value in the root but did not influence endoreduplication pattern in the hypocotyl and cotyledon, while paclobutrazole decreased it in those two organs. Butalated hydroxytoluene, however, increased the mean C-value as well as the 4C+8C+16C+32C/2C ratio in the root and the mean C-value in the cotyledon. The possible relationship between the mechanisms affecting the programmed cell death and endoreduplication pattern in sugar beet seedlings during their early growth will be discussed.

Polyploid induction of *Salix viminalis* L. in nodal bud culture

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Polyploidy plays an important role in plant evolution and constitutes an important mechanism of creating genetic variability. Polyploids can arise spontaneously in nature or induce artificially by some chemicals such as colchicine, trifluralin, oryzalin and amiprophos-methyl. Ploidy manipulation is considered as a valuable tool in genetic improvement of economically important plants. Artificial polyploidy generally enhances the vigor of determinate plant parts and may be favorable where vegetative organs and biomass constitute the economic product (Dhawan and Lavania, 1996).

Salix viminalis and its clones are one of the most important bioenergy woody crops cultivated in Europe for biomass production.

The aim of this study was to develop an artificial chromosome doubling method for selected *S. viminalis* clones in nodal bud culture.

Plant cultures of *S. viminalis* three clones (8; 46; 53) were established from cryo-preserved seeds. Explants were cultured on a modified MS basal medium (Murashige and Skoog, 1962) with double concentration of Fe-EDTA (MS2xFe). The cultures were grown in a continuous light from cool-white fluorescent tubes (35 μmol m^{-2} s^{-1}) at a temperature of 22°C ± 2°C. Plants from the stock cultures were transferred to a fresh medium at 5 week intervals. To establish a polyploidisation protocol, pretreatment conditions of *in vitro* plant cultures and different colchicine concentrations were tested.

Before cutting, *in vitro* plants were stored in a refrigerator at +4°C for 48 and 96h in order to halt growth and synchronize mitotic cell divisions. Nodal segments with a single lateral bud were cut from pre-treated plants and subjected to polyploidisation. Explants were treated with colchicine at different concentrations (0.025; 0.05; 0.075%) for 48, 72 and 96h. Eighteen nodal sections were used in each treatment. The experiments were repeated twice.

After treatment, all the treated nodal segments were transferred to MS2Fe medium without colchicine. The ploidy levels were determined by flow cytometry for control plants and plants developed after colchicine treatments. The effect of colchicine concentration and time of exposure on the explant survival was assessed 6 weeks after treatment.

The results indicate that treating nodal segments with a single bud in a medium supplemented with colchicine at a concentration of 0.05% for 72 and 96h produced tetraploid plants. The highest percentage (11%) of polyploids was induced after 72h of the treatment. The highest concentration (0.075%) of colchicine resulted in a decreased survival rate (9.2) of explants. The best survival rate (85%) was observed when explants were treated for the shortest time (48h) at the lowest concentration of colchicine (0.025%). However, no polyploid plants were induced. *In vitro* induction of tetraploid *S. viminalis* plants was successful with all clones.

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Application of rhizomania resistance-related molecular markers for the evaluation of selected wild accessions form the genus *Beta* and sugar beet (*Beta vulgaris* L.) cultivars

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Major directions in the breeding of sugar beet, such as: high sugar yield, monogermity, as well as cytoplasmic male sterility system are thought to be responsible for a significantly narrowed genetic pool of this crop. Wild species of the genus *Beta* may constitute, in turn, an attractive reservoir of many valuable genes, including resistance-determining genes, which has been successfully exploited in the breeding programmes. Wild beets belonging to the collection of the Plant Breeding and Acclimatization Institute – National Research Institute have not been characterized in this context thus far.

The aim of this study was to perform molecular identification of selected wild *Beta* accessions from the collection of the PBAI-NRI, as well as reference sugar beet cultivars, using RAPD, ISSR marker systems and specific rhizomania-resistance segregating sequences previously described in the literature. Three wild accessions (*B. maritima*, *B. procumbens*, *B. macrorhiza*) and two cultivars ("Japola", "Lessing") were included in the experiment. DNA isolation from leaves was according to Davis. PCR cycling conditions and reaction mixtures were optimized for each of the studied marker systems. Amplification products were resolved in an ethidium bromide-stained 1.5% agarose gel, visualized under UV light (UV TRANSILLUMINATOR 2000, BIO-RAD) and recorded (Gel Doc^{TM} 2000 Gel Documentation System). For comparisons of the percentages of polymorphic bands, the Mann-Whitney U test was carried out.

As a result of PCRs it was possible to unequivocally distinguish each of the analyzed accessions, based on their molecular band profiles. Genetic diversity, expressed as a percentage of polymorphic loci, ranged as follows: 79.1, 73.7 and 62.4% for *B. macrorhiza*; 68.4, 57.8 and 57% for *B. maritima*; 43.1, 34.6 and 37% for "Japola"; 21.2, 36.7 and 39.3% for *B. procumbens*; 29.8, 23.5 and 28.8% for "Lessing", as revealed by RAPD, ISSR and rhizomania-segregating primers, respectively. There was a statistically significantly higher number of polymorphic bands in wild beets as compared to sugar beet cultivars. Specific rhizomania-resistance segregating sequences allowed us to identify some potentially useful products, especially in the group of wild beets, in which at least 17 such sequences were obtained. The value was considerably lower for cultivars and amounted to 4 products.

The marker systems employed in our study turned out to be useful for verification of the accession identity. Statistical differences in the number of polymorphic bands between wild and cultivated beets may reflect the narrowness of a gene pool in the last mentioned group. It is also supported by the significantly higher proportion of rhizomania-related sequences in wild beets, concomitantly indicating the originality of particular resistance sources under investigation. Although some rhizomania-related products were found, their co-segregation with resistance is yet to be established in the subsequent generations of plants.

The influence of tyrosine on salidroside production in *in vitro* cultures of *Rhodiola kirilowii*

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*Rhodiola kirilowii (*Regiel et Maximowicz) from *Crassulaceae* family is a species growing in China, and in Asian mountain regions. In Poland this species is cultivated only in the Medicinal Plants Garden of the Institute of Natural Fibers and Medicinal Plants in Poznań. For many years *R. kirilowii* has been used in Chinese medicine, and known for its cardiovascular antioxidative, and anti-anoxia properties. The main active compounds of the plant are phenolic glicosides: salidroside, its aglycone – tyrosol, daucosterol, beta-sitosterol, cyanogenic glycoside – lotaustralin, rhodiocyanoside A, arbutin, epigallocatechin (Pietrosiuk et al., 2002). Salidroside can be synthesized chemically, but the yield is very low, and the pharmaceutical activity of the resultant product is not comparable to the natural compound isolated from the plant (Ma et al., 2008).

The biosynthetic pathway of tyrosol and its regulation are not well understood, yet. Two different biosynthetic pathways of tyrosol are proposed. According to the first one, tyrosol is presumably produced by decarboxylase from its precursor p-coumaric acid which is mainly derived from L-phenylalanine. According to the other, tyramine, a supposed precursor of tyrosol, may be synthesized from tyrosine (Ma et al., 2008).

The aim of the study was to perform biotransformation of tyrosine to tyrosol. Glucosylation of tyrosol is thought to be the final step in salidroside biosynthesis. Plant cell cultures are capable of performing biotransformation reactions. Biotransformation offers a great potential to generate novel products or to produce known compounds more efficiently (Giri et al., 2001).

Hairy root cultures of *R. kirilowii* were obtained by genetic transformation *via Agrobacterium rhizogenes* LBA 9402. The roots were cultivated in a modified hormone-free Grupta and Durzan liquid medium (DCR-M). For tyrosol production, tyrosine at a concentration of 1.0 mM was added to the medium as follows: 1) on the first day of growth cycle; 2) on the exponential stage of growth (day 15-16 of cycle settled of the base of biomass curve). All samples were collected within 72 h. Each experiment was done in triplicate. All root cultures were carried out in Erlenmeyer flasks, on a rotary shaker (105 rpm), at a temperature of 24°C, in the dark. As control, samples without precursor addition were applied. The quantitative and qualitative analyses of the samples were performed using a HPLC-DAD-UV-VIS technique.

The results demonstrated that hairy roots of *R. kirilowii* growing in a medium without any supplementation were unable to produce either tyrosol, or salidroside. However, hairy roots were able to perform biotransformation of tyrosine to tyrosol, and they eventually produced salidroside. Both tyrosol and salidroside were present also in the post culture media, but in very low amounts. After medium supplementation on the first day of growth cycle, the highest content of salidroside reached 1.5 mg/g DW in roots. A higher accumulation of salidroside (2.6 mg/g DW) was achieved when tyrosine was added to the medium on the 15th day of culture.

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Application of 2-chloroethylphosphonic acid as a regulator of sterility in male and female flowers of *Cannabis sativa* L.

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Broadening of hemp utilization has changed the requirements for the raw material and put the pressure on breeders for new cultivars. It has also directed work of breeders, agronomists and technologists toward obtaining the raw material with clearly defined technological parameters. Our research was conducted at the INF&MP and Institute of Bast Crops in Glukhiv, Ukraine and we investigated the effect of doses and application time of ethrel on male sterility in hemp.

A pot experiment was established with monoecious hemp cultivar Beniko. The sterilization of male flowers was performed by applying (spraying) three doses of water solution of gametocide: 1000 mg dm^{-3}, 2500 mg dm^{-3} and 5000 mg dm^{-3} at different times: I – before bud formation, II – before bud formation and when first flowers were visible, III – when first flowers were visible. The development stages of hemp were closely observed during the growing period. When male flowers were formed, the pollen and flowers were collected to analyze the pollen vigor and morphological changes in male flowers.

The plants sprayed with the lowest dose of ethrel (1000 mg dm^{-3}) had the highest number of female flowers with only single male flowers visible. Single male flowers were also found in plants sprayed with 2500 mg dm^{-3} of ethrel but some morphological changes of flowers were observed. The 5000 mg dm^{-3} solution caused leaf fall-off and single male flowers necrosis. The reduction of pollen vigor was observed at the dose 2500 mg dm^{-3} in the first and second application time and in the third application time at all doses. Some disturbances of meiotic divisions were observed in pollen maternal cells.

Attempts of crossing monoecious hemp in field conditions were made. The maternal forms were cultivars Fedora 17, Felina 34, Ferimon 42, while the paternal one was Zolotonosha 27. The first spraying of maternal plants (1500 mg dm^{-3}) was conducted before bud formation. The next two sprayings were conducted after the ethrel from the first spraying stopped working. They were differentiated upon the degree of sex change of male flowers. The ethrel concentration was 4500 mg dm^{-3}. In the field experiment, three gametocide sprayings were applied. Depending on the cultivar, no male flowers were found in 98.2-99.1% plants. In a negligible number of plants, resembling the monoecious and feminized male plants, sterile male flowers were present. Single plants in maternal cultivars that had not fully changed the sex were removed manually. In our studies repeated crossing of the first generation of the following hybrids: Kompolti x Zolotonosha 13, Kompolti x Zolotonosha 15, Kaukaskaya x Zolotonosha 15 with cultivars Zolotonosha and Beniko was conducted to sterile male flowers. To achieve this, the hybrids were sprayed twice at 4500 mg dm^{-3} of ethrel. The spraying was performed at the time when a small number of plants of monoecious maternal form was available. A total of 1225 plants were under observation, and 99.2% of female plants and 0.8% of plants with male plants transformed into female ones were found. In a progeny of hybrids that were not sprayed with ethrel and planted in a separate nursery, the monoecious and feminized plants constituted 3.8%.

Plant production of HBV Virus-Like Particles (VLPs) carrying HIV-1 epitopes for purposes of a potential bivalent vaccine

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Co-infection of Hepatitis B Virus (HBV) and Human Immunodeficiency Virus (HIV) is often observed. Both viruses seriously deteriorate the patient's condition and accelerate liver cirrhosis, which is the main reason of death of co-infected persons. HBV carriers are 10-60% of HIV-positive patients. Therefore it is postulated to develop a bivalent vaccine against HIV-and HBV co-infection. A plant-based oral vaccine is considered to be one of the possible specimens, particularly recommendable and dedicated to people from poor regions who are most endangered and prone to contract the disease.

Two approaches were taken up to make a plant-based anti-HBV/HIV bivalent vaccine: 1) construction of an immunogenic protein consisting of HIV and HBV antigens and 2) formation of chimaeric, i.e. displaying both antigens, Virus-Like Particles (VLPs), that are crucial structures to elicit immune response. At first, 8 relatively conservative HIV epitopes, encoded by *gag* and *pol* genes, were selected and joined together to form an artificial HIV antigen called "polyepitope" (PE). The PE was connected to an HBV small surface antigen (S-HBsAg) into a fusion antigen, PE-S. PE-S was expressed in mammalian cells at a moderate level, although it was partially assembled into chimaeric and immunogenic VLPs (Gonzalez et al. 2009).

The formation of stable chimaeric VLPs was considered to increase the use of the plant expression system. The initial construct (1×PE-S) was modified to adapt the plant gene expression machinery (enhanced PE-S, e1PE-S) or to ensure the sole expression of the fusion protein, excluding the leaking synthesis of S-HBsAg. These constructs were used for tobacco primary transformation and re-transformation. In the second case, chimaeric VLPs were to be formed by PE-S and S-HBsAg already existing in transgenic plant cells.

The plant production scale of PE-S antigen ranged from 0.1 up to several tens μg/g fresh weight (FW) and depended on the transformation variant. In general, PE-S was synthesized at a higher level in plants obtained after primary transformation, while in re-transformants even "endogenic" S-HBsAg expression dropped. Similarly, theoretically optimized constructs e1PE-S and e1Δ2PE-S did not increase the production scale of PE-S. These tendencies appeared even more clearly in progeny transformants. Probably, re-transformation and/or increased mRNA synthesis led to more intensive gene silencing.

Nevertheless, the conducted experiments made it possible to select an optimal variant for PE-S plant production and tobacco lines for further research. ELISA assays and western blots confirmed the synthesis of both PE-S components fused together. Superior lines expressed PE-S up to 30 μg/g FW, partially in VLP form (max. 1.5 μg/g FW). Those lines are planned to be exploited for elaboration of the lyophilisation methodology as the first step of the conversion of plant tissue into an oral vaccine and further for immunization trials.

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The course of acclimatization of chosen protected species depending on leaves morphology and anatomy

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Plants of three protected species (Rozp. Min. Środ. z dn. 9. 07. 2004): *Hacquetia epipactis* L, *Echium russicum* L., and *Primula farinosa* L. were tissue culture propagated, rooted and planted into the pots kept in chambers in gradually decreased high humidity. *Hacquetia epipactis*, a perennial plant of undergrowth in oak-hornbeam forest, has deeply lobed leaves with naked both sides. Bird's-eye primrose (*P. farinosa* L.) grows in mountain zone on midforest clearing of swamp nature with full light access. Its leaves are covered with white farina. *E. russicum*, perennial species of xerthermic communities, has thick and rigid leaves covered on abaxial and adaxial sides with simple hairs.

The features of leaf morphology and anatomy that could affect acclimatization were studied for *in vitro* and *ex vitro* grown plants. Leaves of wild growing hacquetia were covered with cuticle and deprived of any other epidermis products. Similar morphology had leaves developed *in vitro* except for the plants propagated on kinetin which leaves were curled up along the leaf nerves and were dull-green. Leaves of *in vitro* propagated *E. russicum* plant had similar hairs as *ex vitro* specimens, however shorter and of disordered arrangement. The underside of *P. farinosa* leaves from habitat conditions covered with farina composed of stellate-like structures. Leaves grown *in vitro* in most cases were deprived of farina, but if it was present had shape of globula on stem and could be observed on both sides of leaf.

Twenty eight hacquetia plants were planted into mixture of perlite and substrate (1:2) at the beginning of March in 2011 year. All of them (100%) survived six weeks of acclimatization and at the beginning of June the most vigorous eighteen plants were transplanted into permanent place at university collection (after three months of pot growing). Only thirteen (72%) out of them survived the heavy winter of 2011/2012 and first six plants flowered. Additional thirty plants, including specimens propagated on media supplemented with kinetin, were planted in the spring of 2012. 90% of hacquetia planted in 2012 survived the first six weeks of acclimatization. The plants propagated on kinetin had problems to adopt to new environment as all theirs leaves developed *in vitro* died back and although the new leaves appeared, the plants grew slower and were smaller in comparison to the others. *P. farinosa* was planted into peat substrate and peat with $CaCO_3$ (1:1) and *E. russicum* into peat substrate and perlite (1:1), the survivability was respectively 85% and 82%.

The main reason of mortality within plants during the first six weeks of acclimatization were stem diseases and was not connected with leaf anatomy and morphology.

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In vitro culture of immature embryos in production of single seed descent lines in pea and lupine

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A single seed descent technique (SSD) is frequently used in breeding of self-pollinated cereals and other species to obtain homozygous lines. It is a modified method of a classical ramsh and consists in a random choice of one seed from each individual plant in each generation (the number of individuals is dependent on the assumed number of lines to obtain), starting from F_2 hybrids. In F_5 or more advanced generations, all seeds from individual plants are harvested. Progeny of a single plant is treated as a SSD line. Currently, SSD populations are frequently used in genetic and genomic research as an alternative to doubled haploid populations. Both systems allow to obtain homozygous lines in a relatively short time, but variation of DH lines is the result of one or two rounds of recombination, whereas SSD lines represent a variation caused by 5 or more rounds. The production of SSD lines connected with an *in vitro* culture of immature embryos that has been developed for barley allows to shorten the breeding process by about 3 years (Surma et al. 2011).

A similar approach was applied in the present studies, the aim of which was to shorten the time needed for obtaining homozygous lines in pea (*Pisum sativum* L.) and lupine (*Lupinus angustifolius* L.). In the experiment two cultivars of pea (Eureka and Cysterski) and two cultivars of lupine (Sonet and Kadryl) were examined. Immature embryos of pea and lupine were dissected 20-50 days after pollination. Immature embryos were cultured on B5 (Gamborg et al. 1968) and MS (Murashige and Skoog 1962) with modifications that were connected with nitrogen concentration and agar dose. Additionally, different temperature regimes (between 12-22°C) were applied during the first week of culture. Each treatment was repeated three times and each replication consisted of 10 glass tubes. Seeds were sterilized with 70% ethanol for 3 min. and 1.5% Javel for 5 min. It was found that an optimal term for embryo dissection is ca. 25 and 50-60 days for pea and lupine, respectively. Among the applied media, MS with reduced amount of agar appeared to be the best for embryo development both for pea and lupine. A lower temperature (12-15°C) results in the acceleration of embryo development, especially in lupine. After 20-30 days of culture plants were potted and moved to a greenhouse. The results of the present studies suggest that an *in vitro* culture of immature embryos may be a useful tool for the production of homozygous lines by a single seed descent technique.

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Analysis of a terpenoid components in a flax fibre and their influence on a human fibroblast gene expression

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Flax is an important crop plant grown mainly for its fiber and seeds, which are also rich in omega-3 fatty acids and valuable antioxidants derived mainly from the phenylpropanoid and terpenoid pathways, the latter including carotenoids, tocochromanols and sterols. Many of those components found in flax have been recently shown to positively influence human health. A unique feature of flax is the possibility to exploit the whole plant, with almost no waste products, which justifies the name given to it by Linnaeus: *Linum usitatissimum*, meaning "useful flax". The long fibers are used in the textile industry, and the short fibers in paper production, isolating materials and biocomposite production. The wooden shives released during flax scutching can be used for phenolic components isolation or serve as an energy source. However in the recent time there is a growing interest in biomedical applications of flax products influenced mostly by genetic modifications to increase the anioxidants content or to introduce new components such as polyhydroxybutyrate.

The possibilities for a biomedical application of a flax fibre arises from its composition. Unlike cotton, which is mostly cellulose, the flax fibres contain hemicelluloses, mostly xylan (approximately 15%), lignins (2-5%), pectins (1-15%), phenolic acids (0.1%), and waxes (2-5%). Recent preclinical studies have shown a positive influence of flax fibres when applied as a wound dressing in the treatment of wounds of different etiology. This effect is surely connected to the special structure of the fibre and the ROS scavenging activity of the phenolic type hydrophilic antioxidants. However, the investigation of fibre hydrophobic components also indicates the presence of anti-inflammatory molecules. Although the presence of several classes of terpenoids in flax were described in literature and the presence of a fully functional pathway was proved in our laboratory by cloning key genes of a synthesis pathway, very little is known about the terpenoid presence in flax fibres. Through the HPLC and GS-FID/GC-MS analysis we determined that flax fibers contain high quantities a squalene and sterols. The major sterol found in flax fibers is beta-sitosterol, although campersterol, campestanol, avernasterol and small quantities of des-methylsterols were also detected. From carotenoids, identically as in the case of flax oil, only luteine was found to be present in flax fibres No tocopherol or tocotrienols were detected. Also the terpenophenolic components identified as cannabidiol was detected in a a flax fibres. These unique chemicals have not been as far identified in any other plants than *Cannabis*, where its anti-inflammatory and analgesic activity has been widely proven. Although terpenes vary greatly in their chemical structure, they are strong antioxidants and can influence many cell signaling pathways, particularly those involved in cancer and inflammation.

We proved previously that the hydrophobic components isolated form a flax fibre have a positive influence on a quenching of the inflammation process in dermal human fibroblasts. The chemical composition of a preparation used for treatments was determined and compared with the data gene expression profile conducted by a microarray technique using Affymetrix Human Genome U133A 2.0 Array. It was found that the 210 genes were expressed differentially after the flax hydrophobic preparation treatment. 130 of them were identical to that responded to CBD standard. Of those, 106 genes were activated and the expression of 104 genes was repressed. The affected genes may be grouped into several types of inflammation, G protein signaling, growth factors cell cycle and steroid metabolism.