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Widening the genetic pool of oilseed rape through the *Brassica napus* L. resynthesis

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Oilseed rape (*Brassica napus* L. 2n = 38, genome AACC) is a natural amphidiploid which originated from several independent spontaneous hybridization processes between two diploid species, turnip rape (*Brassica rapa* L., 2n = 20, genome AA) and cabbage (*Brassica oleracea* L., 2n = 18, CC). The limited geographic range of oilseed rape combined with intensive quality breeding (00 quality – low glucosinolates and zero erucic acid content) has led to a narrow genetic basis in/of? the species. However, the diploid progenitors are highly polymorphic and offer a wide genetic variability which can be exploited for oilseed rape improvement. The possibility to create new *Brassica napus* forms from the interspecific crosses between *Brassica rapa* and *Brassica oleracea*, resynthesized oilseed rape forms are used for increasing the genetic variation in oilseed rape. At present, resynthesized *Brassica. napus* can be developed with the help of *in vitro* techniques. For such interspecific hybridization, a variety of biotechnological tools, such as for example embryo rescue techniques, is used to circumvent incompatibility barriers. The RS oilseed rape displays poor performance for many agronomic traits and shows inferior seed yield as compared to current breeding material. However, after the crossing of resynthetic *B.napus* with 00-quality oilseed rape and haploidization of obtained F_1 hybrid it is possible to select doubled haploids (DH) with improved seeds yield and quality and displaying significant genetic distance to present cultivars. In some cases RS oilseed rape forms have resulted in a successful release of cultivars carrying novel genes.

In this study resynthesized oilseed rape was obtained as a result of crosses between *Brassica. rapa* ssp. *chinensis* var. *chinensis* (pak choy), and *Brassica oleracea* ssp. *acephala* var. *sabellica* (curly kale) using embryo rescue technique. Morphological and nuclear DNA cytometric analyses of obtained RS plants have confirmed their hybrid phenotype and genotype. Molecular analysis carried out using RAPD molecular primers showed that RS plants are distinct from *Brassic napus* varieties of winter oilseed rape that are bred and cultivated nowadays. As expected, the RS lines obtained in the study were characterized by a high content of erucic acid in oil and high glucosinolates in seed meal because the phenotype of a hybrid depends on the quality of the parental form. These resynthesized oilseed rape lines were used as donors of pollen in the crossing lines of double low quality winter oilseed rape. Using *in vitro* isolated microspore culture, androgenic plants were developed from F_1 hybrids. All obtained semi-RS DH lines were biochemically analyzed with regard to 00 quality of seeds. There were selected semi-RS winter oilseed rape DH lines with very low or zero erucic acid content and low amount of glucosinolates.

Resynthesized *Brassica napus* extends the range of genetic variability of winter oilseed rape.

Androgenesis *in vitro* in vegetable plants and its potential for use in breeding

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Hybrid varieties have taken over the vegetables market in Poland and around the world. They have several advantages, which makes them better than the stabilized varieties. They include mainly yield, the size and quality, and also the degree of crop uniformity. To be able to breed such varieties, parental homozygous lines are needed. However, obtaining them by conventional methods is difficult and above all time-consuming, and besides they are then not completely homozygous. The use of androgenesis *in vitro* reduces the time needed to obtain homozygous plants several times, which helps to speed up the breeding process.

Our team at the Institute of Horticulture (formerly the Institute of Vegetable Crops) has focused on the use of anther cultures and isolated microspore cultures for obtaining doubled haploids in vegetable plants and with methods of evaluating them, during the last few years. Experiments have been carried out on the effects of various factors on the effectiveness of androgenetic embryogenesis. These included the genotype, that is, the species, the varieties and breeding lines, and even individual plants within the varieties or lines, microspore development stage, thermal shocks of low and high temperatures applied to donor plants, inflorescences or anthers laid out on culture media, conditions for growing donor plants and ways of forming them, media for the induction of androgenesis, and the time at which to collect flower buds. Work has also been carried out on the regeneration of plants from androgenetic embryos and methods for their evaluation.

Several factors that markedly improve the effectiveness of androgenesis have been found.

Effective ways to regenerate plants from androgenetic embryos have been developed. In the case of the carrot, a method for the regeneration of carrot plants from embryos obtained in anther cultures has been registered in the Polish Patent Office, Patent No. 208426. Ploidy of the plants obtained was evaluated using cytological methods by counting the chromosomes in root growth tips and indirectly by determining the quantity of nuclear DNA by flow cytometry. The convergence of the results obtained with both methods allowed us to recommend an easier, faster and cheaper method of flow cytometry for the assessment of ploidy.

To determine the homozygosity of the plants obtained through androgenesis, modern molecular methods have been used, such as isoenzymes. Thus the method for assessing the plants derived by androgenesis has been simplified, and characteristics have been found that allow a preliminary assessment of ploidy.

Technologies for the production of homozygous plants of white head cabbage, Brussels sprouts and carrot have been developed. We are currently working on the beet as well. The resulting homozygous plants have been used by breeders cooperating with us to create new hybrid varieties of vegetables. In the register of cultivars there are the first three Polish varieties of white head cabbage, Julia F_1, Judyta F_1, and Jowita F_1, derived from androgenetic plants. Varieties of Brussels sprouts obtained from such material are in the final year of registration trials. As for the carrot, homozygous lines obtained by androgenesis are currently used to create hybrids.

Broadening the variability of quality traits in rapeseed through interspecific hybridization

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Interspecific hybridization is an important tool to transfer characters across species and develop synthetic amphidiploids. Therefore it has been widely applied for improving *Brassicas*. Rapeseed (*Brassica napus*) with Canola quality, i.e. low-glucosinolate, low-erucic varieties, nowadays represent one of the major sources of vegetable oil. The value of rapeseed for food and feed uses can be further improved by increasing desirable traits, e.g. oil content, and reducing undesired characteristic, e.g. fiber content and anti-nutritional compounds. Among these, glucosinolates are the most important anti-nutritional compounds. The optimal content of glucosinolates in seeds considered harmless at currently cultivated varieties of oilseed rape is shaped in the range of 10-20 $\mu M/g^{-1}$. For many years, an increased interest has also been observed among the *Brassica* researches in lowering the sinapine content that lowers the nutritional value of rapeseed meal (Wojciechowski at al., 1994, Velasco at al., 1998, zum Felde 2005). One of the most effective methods for increasing the variability of traits affecting the quality of rapeseed products is interspecific hybridization. However, information on the genetic variation created through such crosses is meagre. For this reason the aim of our study was to determine whether interspecific crossing can help to increase the range of variability of traits connected with the higher value of rapeseed. Also, an attempt was made to investigate the influence of environment on those studied traits. That is why in our experiment first, crossess between male sterile line of *Brassica napus* F8 generation and *B. campestris* ssp. sarson, Yellow sarson; *B. campestris* ssp. pekinensis; *B. carinata* and *B. juncea* were attempted. Next, new interspecific hybrids were produced from among the above mentioned genotypes using embryo rescue techniques. Then, hybrid seeds of 96 lines derived from crosses were tested for fiber and glucosinolates content using near–infrared reflectance spectroscopy (NIRS). Moreover, gas chromatography and spectrophotometric measurements of sinapine and tocopherols content in those seeds were made. The hybridization efficiency was expressed by the number of the obtained embryos to the mean number of well developed ovules.

The results indicate that the analyzed hybrid lines differed in their glucosinolates, fiber and sinapine content. From among all hybrid combinations, valuable lines from the point of rape utility features could be selected. Especially important for plant breeders may be lines with reduced glucosinolates content in the seed. Because of the fact that the cultivar or a line might be considered as a useful rapeseed to include in a diet as it contains low specific glucosinolates, hybrid lines investigated in this work seem to be promising in future breeding programs. The environmental effect on the tested quality traits was observed. Comparing two locations, fields in Poznań and Dłoń for glucosinolate and sinapine content, the higher range of variability was in Dłoń.

Biolistic transformation of cucumber haploid suspension culture

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Recently, the methodological basics of establishment and application of haploid meristematic and embryogenic cucumber suspension cultures in plant biotechnology have been elaborated in the Department of Plant Genetic, Breeding and Biotechnology (Dobrzyńska, 2011), in consequence of which a versatile (i.e. genotype non-specific), integrated technology package, based on the use of haploid liquid culture of meristematic clumps (LCMC) stable in terms of ploidy level has been developed (Burza and Dobrzyńska, 2011).

The aim of this study was to identify the key parameters of the biolistic transformation procedure of cucumber haploid suspension culture.

Experiments were conducted on a haploid plants $Izyd_{150}$ derived from prof. K. Niemirowicz-Szczytt collection. Suspension cultures were carried out in the dark (or in the light conditions in the case of LCMC) on modified 1/2 MS media (Murashige and Skoog, 1962) in 100 ml Erlenmeyer flasks. After a period of stabilization they were passaged every 14 days.

Meristematic tissue derived from haploid LCMC was bombarded with plasmid [encoding for the β-glucuronidase (uidA (GUS)) and hygromycin (*HPTII*) genes] p-CAMBIAVIII-1381Z(Cs-XTH3)-coated (0.6, 1.0, 1.6 μm diameter) gold particles using Biolistic® PDS-1000/He device (Biorad Laboratories, Hercules, CA, US). Bombardment conditions included a Helium pressure of 1800 psi, 9 cm target distance, a gap distance of 10 mm, and a vacuum of 28 mm of Hg according to preliminary experiments performed in our laboratory. Combinations of proliferation media, osmotic preconditioning of cells (0.4 M mannitol as osmoticum), physical bombardment parameters and parameters for transgenic tissue selection were evaluated by monitoring the transient expression of the *uid* A reporter gene driven by the developmentally specific Cs-XTH3 promoter. Xyloglucan endotransglucosylase/hydrolases (XTHs) are a class of enzymes that mediate the construction and restructure of the cellulose/xyloglucan framework by splitting and reconnecting xyloglucan molecule cross-linking among cellulose microfibrils. The number of blue spots was counted 48 h post-bombardment using a binocular stereoscope.

The most important parameters of biolistic transformation procedure including: the conditions for the proliferation of haploid LCMC as a source of meristematic explants undergoing transformation, their size and time spent on the medium for deplasmolysis and conditions for selection of transgenic tissue were optimized. The correctness of the preparation of plasmid DNA and activity of Cs-XTH3 promoter was confirmed by analyzing transient/stable expression of *uid* A reporter gene. The problem of the necessity to pass in the process of transgenic tissue selection through the stage of cell suspension culture with a high potential for the division of free cells, correlated with the loss of morphogenetic potential, was solved by developing a simple method of releasing it (utilising standard medium for initiation of cucmber embriogenic culture). Testing the most promising experimental combinations has led to confirmation of the stable expression of *hpt* marker gene under control of the constitutive promoter CaMV35S. In order to improve expression efficiency a modification of the transformation procedure is postulated. The modification covers: (a) improvement of the efficiency of the penetration of mikroprojectiles into cells, (b) use of hygromycin-resistant culture for co-culture and (c) use of green autofluorescence of cells subjected to the selection pressure as a transformation marker.

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Androgenesis – a method for using biodiversity in wheat improvement

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Wheat and its relatives have evolved through divergence at the diploid level, followed by evolutionary convergence via polyploidy. This type of reticulate evolution has led to immense variability in the wheat tribe. According to the hypothesis of pivotal-differential evolution, one genome of an allopolyploid such as wheat remains stable during evolution, and serves as the "pivotal" genome. The other genomes become modified (i.e., differential), partially due to crossing with other sympatric polyploid species which have a common pivotal genome. The acceptance of this hypothesis has not only academic implications, but also influences the way plant breeders design and implement their crossing programs.

The species related within the family *Poaceae* constitute a pool of many desirable traits which can be used for improving *T. aestivum* L. In our studies conducted earlier we used the species of (2x, 4x) *Triticum* L., (2x, 4x) *Secale* L., (2x, 4x, 6x) *Aegilops* L., (2x, 4x) *Hordeum* L., (2x) *Agropyron* Gaertn., (4x) *Lolium* L., (2x) *Elymus* L. and developed many improvements in both winter and spring lines of *T. aestivum* L. This study presents 28 introgressive winter lines derived by the interspecific and intergeneric hybridization of *T. aestivum* L. with *T. durum* Desf., *T. timopheevii* Zhuk., *Lolium perenne* L. and *Aegilops speltoides* Taush. They were improved (in comparison to the cultivars and breeding materials) in respect of some spike and grain characters: the spike length (12.5-21.4 cm), the number of spikelet per spike (22.0-30.0), the number of kernels per spike (55.0-98.2), the kernel weight per spike (2.5-4.1 g), 1000-kernel weight (45.0-51.7 g), the number of kernels per spikelet (2.5-3.7), the spike density (19.0-22.4 %), the number of days to heading – earliness 7 days, the protein content (13.7-16.7 %), the sedimentation-SDS (76.2-87.0 cm^3), the falling number (280-503 s.), the quality-seed group E, the preharvest seed sprouting (12.3-0 % germinated kernels).

The androgenesis was used to develop the DH plants of these lines. Donor material of 28 winter wheat lines was grown in the field. The developmental stage of microspores was checked and immature spikes containing anthers with the microspores at the mid to late uninucleate stage were excised and pretreated in darkness at 4-5 °C for 14-21 days. For androgenesis induction we used C-17 medium, then calli were transferred on 190-2 regeneration medium. Other *in vitro* culture conditions and growth conditions of regenerated plants were the same as previously described.

The general results of *in vitro* culture will be presented on the poster. The analyses of variance have shown significant differences between the genotypes used for two characters: callus induction per 100 anthers and green plant regeneration per 100 anthers. A total of 56,414 anthers were plated and 2322 calli (4.12 %) were formed from them. Finally, 334 green plants (0.59 %) were obtained. The highest frequency of callus induction (55.49%) and green plant regeneration (5.04%) occurred in (ChS-Mirable)CHD661 genotype. The regenerated 334 DH lines are under further investigation.

Research on the gynogenesis of bulb onion (*Allium cepa* L.)

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Introduction

The research in the induction of gynogenesis of onion has been carried out in our laboratory for 15 years (Michalik et al., 1997). Gynogenic embryos were produced by flower buds cultures. The results obtained so far indicate that the effectiveness of gynogenesis depends on the genotype and media composition. Unfortunately Polish onion breeding lines have a low ability of embryo formation. That is why the research in the modification of media composition in order to increase the efficiency of gynogenesis is still carried out.

Materials and methods

During three years of research (2009-2011) gynogenesis was used in order to obtain haploids and double haploids (DH) of onion. Flower buds of 28 different breeding lines were used for the gynogenesis induction. From March to May donor plants were grown in a greenhouse, and two weeks before flowering in a growth chamber (14°C and 16 h light/8 h dark). To confirm the gametic origin of diploid regenerants, only heterozygotic plants in *Pgi* locus were used (Barański, 2000). Unpollinated flower buds 3-4 mm length were sterilized and plated on A1 medium followed by R medium (Michalik et al., 2000). In order to increase the effectiveness of gynogenesis, the media were supplemented with poliamins and TZD cytokinin in the following combinations: 1) A1 medium + 2 mM/l putrescine and R medium + 0,1 mM/l spermidine, 2) A1 medium + 2 mg/l TDZ and R medium + 2 mg/l TDZ, 3) control – A1/R media without modifications.

The effectiveness of developing a female gametophyte was expressed as the number of the obtained embryos per the number of flower buds in a culture without infection.

Results

In total, about 38,500 ovules were plated on the induction media from which 884 (2.3%) embryos developed. The embryos were being observed from the 75th to 145th day of culture. The effectiveness of embryo formation depended on the genotype and varied from 0% to 11%. The effectiveness of most of the breeding lines (86%) was at the level of 0.1%-5%. One of the maternal forms did not produce any embryos, while the other formed 11 embryos per 100 of plated ovules. Regardless of the genotype, the media composition had an influence on the effectiveness of gynogenesis. The addition of poliamins 1) did not influence the effectiveness of gynogenesis which was at the control level (2%). The addition of TZD 2) increased the effectiveness of gynogenesis to 4%. Differences in the morphology of the obtained gynogenic embryos were observed. The normal morphology was observed in 60% of the embryos while the other had deformed organs or were vitreous. The addition of TDZ reduced the number of morphological aberrations of the embryos.

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Induction of androgenesis and regeneration in spring genotypes of barley

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Doubled haploid (DH) plants are homozygotic for all traits. DH can be obtained by means of androgenesis, gynogenesis, and chromosome elimination. In barley, androgenesis, which can be obtained by anther culture and microspore culture, is one of the most frequently used system of DH production. During androgenesis under specific *in vitro* conditions microspores can be induced to switch their development pathway from gametofic to sporofitic, resulting in the formation of androgenic embryos. These embryos germinate into haploid or doubled- haploid plants.

DH plants are very useful for plant breeding. They accelerate the breeding cycle by shortening the time required to attain homozygosity. DH can used in basic research as "clean" material and in experimental plant breeding for crossings, testing and selection of suitable recombinants to obtain lines whose offspring does not segregate. Moreover, DH improve the effectiveness of selection for genome mapping (QTL), gene isolation, cloning and transformation. However, low potential of many genotypes, high ratio of regenerated albino plants, and low level of embryo conversion limit the widespread application of that method.

The objective of the study was to develop a method that would help to achieve an increased number of green plants and reduce the number of albino plants obtained in anther culture. Only high efficiency of plant regeneration makes this system practical for accelerated cultivar development in barley breeding programs. Our experiments were carried out on ten genotypes of spring H. *vulgare*. The efficiency of androgenesis in terms of the number of green plants and green/albino ratio was assessed in four variants: two pretreatment condictions – anthers in 0.4 M mannitol solutions with 10 μM $CuSO_4$ for 4 days in 4 °C and spikes in water for 21 days in 4 °C, and two different media – liquid KBP (Kumlehn, 2006) medium and solid N6 (Chu, 1978) medium. The ploidy levels of the microspore-derived plants were determined by flow cytometry.

The results confirmed the genotype as being the major determinant of androgenesis efficiency. We also demonstrated that the use of N6 medium rises the number of produced green plants. The application of copper sulfate in both, the pretreatment solution and the induction medium had a beneficial effect on green plant efficiency. In this experiment more than 1300 green plants were regenerated. The approach resulted in the regeneration of up to 50 green plants per single spike. The frequency of albino plants was about 78%.

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Effective increase of microspore embryogenesis in barley by the modification of starvation medium

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Androgenesis is a process of plant development from the male gametophyte. This process can occur by anther or isolated microspore cultured *in vitro*. Normally, microspores are programmed to maturate into pollen. However, after stress treatment it is possible to change their developmental pattern. Reprogramming of microspores and the optimal conditions of *in vitro* culture result in embryo formation and plantlet regeneration. Doubled haploids, generated spontaneously or induced, can be used for basic research and commercial applications. During induction microspores undergo cytological and ultrastructural alternations which provide a star-like morphology and a change of the pattern of cytokinesis. Besides morphological changes, induction of microspore embryogenesis leads to changes of the gene expression pattern. Many factors such as genotype, developmental stage of microspore, culture medium and culture conditions affect microspore embryogenesis. The type of treatment applied to microspores in order to reprogramme the developmental pathway has a major impact on the efficiency of microspore embryogenesis. Generally, the most common stresses applied to induce embryogenesis are cold and heat treatment and starvation. The most frequent pretreatments used for barley (*Hordeum vulgare* L.) microspore embryogenesis induction are cold treatment, starvation or a combination of both.

The aim of this study was to determine whether the modification of SMB1 (Starvation Medium Barley 1) (Coronado et al., 2005) increases the efficiency of microspore embryogenesis induction. In this study the efficiency of androgenesis was tested using eight genotypes of spring barley cultivars. Microspores in mid-late to late uni-nucleate stage were used for isolated microspore culture. Combinations of two pretreatments were applied: the cold pretreatment and starvation of microspores with a modified SMB1 medium. The SMB1 medium composition was modified by addition of sugars, macroelements or amino acids in different concentrations. The increase in the induction of microspores embryogenesis was shown when the medium was enriched with 5 mM glucose, 25 mM glycerol or 10 mM ammonium nitrate. The efficiency of microspore embryogenesis was determined based on the number of formed embryo and green plantlets regeneration. These results allowed to introduce modifications to the standard protocol of isolated microspore *in vitro* culture (Coronado et al., 2005; Kumlehn et al., 2006)

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Cucumber (*Cucumis sativus* L.) microspore isolation and *in vitro* culture conditions

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Research on cucumber haploids is directed at obtaining homozygous lines suitable for breeding. The main and most popular method of cucumber double haploid production is pollination with irradiated pollen followed by haploid genome duplication. It is also possible to obtain haploids and double haploids of cucumber with the use of anther and ovary culture. Little is known about the use of microspore culture for this purpose. Here we present the preliminary results of a study on the establishment of a protocol for androgenic plants regeneration – microspores isolation and culture conditions.

Four F1 cultivars (Marcel, Junak, Sonate and Sremski) grown in computer-controlled conditions in a glasshouse were used as donor plants. For microspore isolation and culture two liquid media were used: ECIM medium (according to Song et al. 2007; MS with 30 g/l sucrose, 0.5 mg/l 2,4-D, 1 mg/l kinetin, 0.5 mg/l BAP) or NLN medium (according to Lichter 1982; 130 g/l sucrose). The pH of both was 5.8.

Flower buds containing microspores at the uninucleate stage were collected in the morning, put into jars clad in wet cotton and kept in the dark at 4°C for 24 hours. The microspore developmental stage was determined by DAPI staining. After cold pretreatment the buds were surface sterilized (70% ethanol – 1 minute, 0.56% commercial bleach – 15 minutes, sterile water – 5, 10 and 15 minutes).

The microspore isolation procedure was carried out in two ways. In the first method, buds were pounded in a small amount of the medium directly after sterilization. The second method comprised an extra step in which anthers were excised from the buds before pounding. This extra step resulted in a notably reduced amount of other tissue fragments in the microspore suspension. The resulting pulp was diluted and poured through a nylon sieve (diameter of 70 micrometers). The microspores were purified by centrifugation (500 rpm, 15 minutes) and suspension in a fresh medium three times. The Fuchs-Rosenthal chamber was used to determine the density of culture at 50, 100 and 150 thousands of microspores per 1 ml of the medium. Microspore culture (8 ml per one 60-mm Petri dish) was carried out in the dark at 25°C.

Information on cucumber microspore culture in the literature is scarce, and the results are mostly unsatisfactory. In our experiments a protocol for successful microspore isolation was established. The culture was not contaminated with anther or flower buds tissue and it remained sterile. In addition, the culture was homogeneous in terms of the developmental stage of microspores. Nevertheless, neither dividing microspores nor developing embryos were observed during ten months of culture on two media tested.

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Examination of ability to androgenesis of spring wheat genotypes resistant to *Fusarium*

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Wheat is one of the major cereals crops cultivated worldwide and in Poland. *Fusarium* spp. is a devastating disease in all wheat-growing countries. *Fusarium* head blight (FHB) is also one of the most destructive wheat diseases. Infection reduced kernel weight and size what resulted in the yield decreasing. The kernel contamination by mycotoxins is harmfull for humans and livestock. A better way to reduce the damage and contamination is by growing resistant varieties. That is why spring wheat genotypes which are sources of resistance have unfavorable agronomic traits. After crossing a release of DH lines from F1 plants is a best way for accelerated selection. In the first step the regeneration ability of microspores in anther culture of parental genotypes was examined.

The aim of this study was to analyze the capacity of plants to regenerate in anther cultures and to generate double haploids of spring wheat genotypes that may potentially constitute input material for resistance breeding directed against fungi from the genus *Fusarium*.

In the experiment the ability to regeneration in *in vitro* anther culture of genotypes which are the source of resistance to *Fusarium*: Chinese varieties Sumai 3 and Ning 8331, Japanese Norin 52, Brazilian Frontana and 8475-59 and high yielding Polish varieties Łagwa, Waluta and Zadra were evaluated. Spikes from the plants were dissected and subjected to a temperature of 4 °C for temperature shock for androgenesis initiations. The shock period continued 7 and 14 days. Anthers with microspores being in medium or late uninuclear stage were implanted on C17 medium Two combinations of growth regulators were applied: 2,4-D with kinetin and 2,4-D with dicamba. A total of 19,200 anthers were cultured. After 8 weeks explants were moved onto the regeneration medium MS with an addition of NAA and kinetin. The ploidy of the obtained plants was verified by flow cytometr. Haploid plants were treated by colchicines and potted.

As a result, an anther culture 440 callus were formed The highest number of calli – 106 was obtained from the Polish variety Łagwa (effectiveness of regeneration 4.42%). Among genotypes resistant to *Fusarium,* explants of cv. Ning 8331 formed the greatest number of calli (67) at a regeneration rate of 2.79%. The efficiency of callus formation in Polish cultivars was on average over 3-fold higher than that of genotypes resistant to *Fusarium*. The most effective medium that stimulated the forming of callus was C17 with 2,4-D and dicamba after one week of a temperature shock.

In the experiment 352 plants were obtained, including 48 (14%) albinotic plants. Plants were generated from 6 genotypes out of 8 tested. The highest number of green plants – 134 was received from the Polish variety Łagwa (effectiveness of regeneration 5.58%) and resistance variety Frontana – 104 green plants (effectiveness of regeneration 4.33%). From that genotype the highest number of green plants (137) was regenerated on C17 medium with 2,4-D and dicamba addition after one week of a temperature shock (effectiveness of regeneration 2.85%). After examinining of the ploidy level, 132 haploids plants were selected and treated by colchicine. 54 DH lines were obtained from which 283 kernels were collected.

Comparison of methods for obtaining doubled haploids of carrot

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The aim of the experiments was to compare the methods of obtaining doubled haploids of carrot in anther cultures and isolated microspore cultures. Under study were the stages of the process: the induction of androgenesis, regeneration of plants and their adaptation, the assessment of ploidy and homozygosity of the plants obtained. The experiments were conducted to study the effects of various factors on the effectiveness of embryogenesis in anther cultures and isolated microspore cultures.

The strong influence of the genotype on the effectiveness of embryogenesis was confirmed in both anther cultures and isolated microspore cultures.

In the tested cultivars, a dependence of the microspore development stage on the size of the flower bud and the genotype was found. The most embryogenic were the buds containing mostly microspores at the uninuclear stage. Growing donor plants in a growth chamber raised the efficiency of androgenesis for all the tested genotypes in the case of anther cultures, while in the case of isolated microspore cultures it was a factor needed to obtain sterile cultures because they were more susceptible to contamination.

Donor plants in which the root was made to develop one main shoot and lateral shoots of the first order terminated in umbels produced the largest number of embryos. Among the media for the induction of androgenesis, the B_5 medium with 0.1 mg NAA, 0.1 mg of 2,4-D, 100 mg l-serine, 500 mg l-glutamine, 150 mg $CaCl_2$ x $6H_2O$, and 100 g sucrose proved to be the best for both types of culture. Using thermal shocks, the highest efficiency of androgenesis was achieved by subjecting umbels to 4 °C for 3 days. Placing anthers at 35 °C for 24 h also increased the efficiency of androgenesis.

Plants were successfully regenerated from the embryos obtained. The best medium for the regeneration of plants from both types of cultures proved to be the B_5 medium without hormones or amino acids, containing 20 g/l sucrose. After the first passage, significantly more complete plants formed from the embryos obtained in anther cultures.

The cultivar and also the method of obtaining androgenetic embryos were found to influence the process of adaptation. Plants regenerated from embryos obtained in anther cultures had a higher survival rate. Cytometric evaluation of ploidy of the plants obtained revealed that it depended on the type of culture. Among the regenerated plants about 11% and 42% haploids derived from anther and isolated microspore cultures, respectively.

The percentage share of PGI homozygotes in the population of androgenetic plants obtained in anther cultures depended on the cultivar, while in the case of AAT no such dependence was found. Among the plants from isolated microspore cultures, regardless of the cultivar, about 90% of them were homozygous for PGI and almost 100% for AAT.

The efficiency of anther cultures was generally higher at the induction stage of embryogenesis, plant regeneration and adaptation. The plants obtained by this method mostly had the amount of nuclear DNA equal to 2x the chromosomes, and the proportion of homozygotes in the tested population was dependent on the cultivar. Assessment of the plants derived through androgenesis in isolated microspore cultures showed a smaller share of plants with the amount of nuclear DNA equal to 2x, but, regardless of the cultivar, a greater percentage of homozygotes in the population was obtained for both isoenzymes tested.

Effect of selected factors on the androgenesis in microspore cultures of white cabbage (*Brassica oleracea* L. var. *capitata*)

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Introduction

In modern plant breeding programs the wide application of DH lines – highly homozygous and genetically stabile plant materials is observed. DH lines replace the long process of inbreeding and shorten breeding work, leading to the production of new cultivars. Androgenesis in *B. napus* is easy, while in *B. oleracea* the effectiveness of obtaining of DH lines is low because Polish breeding materials have a low ability of gametic embriogenesis.

Aim of the study

The aim of the research was to investigate how the cooling of buds and medium composition influence the effectiveness of androgenesis.

Materials and methods

In the experiment 13 different Polish breeding lines of white cabbage were used. The cultures of isolated microspores were induced from buds collected directly from donor plants as well as from buds that after collecting were cooled for 2-4 days at the temperature of 4°C. The induction of androgenesis was carried out according to Hansen (2003). The NLN 13 induction medium was modified by adding one of the chemical components: 1 M active carbon, 0.1 mM glutathione, 1 mM galacturonic acid, 2 mM putrescine + 0.1 mM spermidine. The control was NLN 13 medium without modifications. Dishes with isolated microspores were subjected to thermal shock at 34°C for 48 h. After 3-4 weeks of the culture maintained at 25°C, androgenic embryos started to develop.

Results

The studies confirmed that the effectiveness of androgenesis in white cabbage depended on the genotype. The most embriogenic was the genotype no. 1 (185 embryos/100 buds). In the remaining genotypes that produced embryos the effectiveness varied from 1 to 67 embryos per 100 buds. Three breeding lines were recalcitrant for the androgenesis induction. The mean effectiveness for the whole experiment was 41 embryos per 100 buds. We also proved that the cooling of buds before the isolation did not increase the effectiveness of androgenesis as compared with the effectiveness induced from the buds collected directly from the donor plants. Moreover the interaction between the genotype and induction medium was observed. We also noticed a different reaction of the genotypes to media modification. The largest number of embryos (448 per 100 buds) was observed for genotype no. 1 on NLN 13 medium supplemented with glutathione. The addition of active carbon increased the effectiveness of androgenesis in genotype no. 53. The poliamins (putrescine and spermidine) in the induction media were advantageous for genotype no. 56. The addition of galacturonic acid inhibited the developing of androgenic embryos for every accession.

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Development of induced haploid embryos and plants of *Lactuca sativa* L.

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Haploidization is an example of one of the biotechnological methods which can be used to improve many of the useful traits in plant breeding. Through producing pure lines of plants we can obtain new varieties of economically important species, which can be characterized for example, by better nutritional quality, application of male sterility systems, accelerated growth and biomass production, greater resistance to pathogens or increased tolerance to stress environmental factors.

To find an effective haploidization method in lettuce (*Lactuca sativa*), we made an attempt to induce development of haploid embryos and plants by treatmenting stigmas chemically with 8 different factors or *in vivo* cross pollination with 25 species (mainly belonging to the *Asteraceae* family). The most effective pollinators in the induction were *Helianthus tuberosum* (frequency of embryos around 19%), *Helianthus annuus* (16%), *Oenothera biennis* and *Spathiphyllum wallisii* (15%). Among the used chemical inductors, the most effective were respectively: 0.05% Dicamba (15%), 0.05% Picloram (14%), and 0.05% 2,4-D (13%). After cross pollination with *H. annuus* and *H. tuberosum* well developed pollen tubes were observed on stigmas already 1h after pollination. Three hours later, long pollen tubes penetrated stigmas and styles of pistils of lettuce. Around 6h after pollination, pollen tubes with two male gametes entered the micropyle region of embryo sacs of *L. sativa*. One of the male gametes was often observed in the vicinity of the secondary cells, but the fertilization process was not ascertained. Only globular and very rarely heart stage embryos were observed in embryo sacs after crossing. Endosperm which surround the developing embryos was characterized by many abnormalities like – fusion of nuclei, multinucleoli and strong vacuolization. In one case, after crossing *L.sativa* with *H.tuberosum*, a the probable hybrid number of chromosomes ($3n = 18 + 51$) in dividing endosperm cells was calculated. After the chemical treatment of stigmas all embryos developed only into several cell stage. No signs of endosperm development were observed after using chemicals. In all the analysed embryos (obtained after interspecific crossing or chemical treatment), only a haploid number of chromosomes was found in dividing cells ($n = 9$). Embryo sacs with haploid globular embryos, isolated from pistils after crossing with *H. annuus* and *H. tuberosum* were cultured in Petri dishes, on MS media with various growth regulators composition. From these cultures, morphogenic callus tissue were obtained after 10-12 days. Haploid plants of lettuce were regenerated from callus tissue on MS media modified by 1 mg/l kinetin addition after 40 days of culture. Obtained plants were transferred to water and later to soil. The stages of pollination and embryogenesis were observed under light and fluorescent microscope in freshly squashed (stained with carmine acetate or aniline blue) and permanent slides (stained with haematoxylin and fast green). A caryological analysis of embryos which verified their haploidy was conducted by counting the chromosomes in dividing cells. The ploidy level of regenerated plants was confirmed by using a flow cytometry analysis of young leaves.

Diploidization and assesment of the ploidy level of *Capsicum annuum* L. androgenic regenerants

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Development of haploid embryo from a single microspore in controlled conditions is called *in vitro* androgenesis. Among the obtained regenerants appear haploid and diploid forms. Literature data concerning androgenic regenerants of pepper report that diploid plants result from the spontaneous doubling of chromosomes in the early stages of embryo development. Their share depends on the genotype of donor plants. In large-fruited peppers a higher percentage of diploid forms is observed, while in the case of varieties with smaller fruits among androgenic embryos predominate haploids. In order to obtain fully homozygous lines it is necessary to double the number of chromosomes. Homozygous DH lines derived from this process are used in breeding of many crop species, including pepper. The aim of this study was diploidization of haploid plants obtained through *in vitro* anther culture of *Capsicum annuum* L. hybrids: (905 × "Sono")F1, (905 × "Mino")F_1, (405 × "Luba")F_1, (ATZ × "Sono")F_1. Pepper anther culture was based on the modified procedure of Dumas de Vaulx et al. (1981). The anthers were cultured on the CP medium containing 0.01 mg·dm^{-3} 2,4-D (2,4-dichloro-phenoxyacetic acid) and 0.01 mg·dm^{-3} KIN (kinetin). For the first 8 days another cultures were incubated in the dark at the temperature of +35°C. Then the dishes were exposed to a 12-hour photoperiod, at the temperature of +25°C. After 14 days anthers were transferred onto R_1 medium (0.1 mg·dm^{-3} KIN). In all the performed experiments silver nitrate (5 mg·dm^{-3}) was added to the CP induction medium. The embryos occurring in anther cultures were transferred onto the V_3 medium without growth regulators. The ploidy of the plants obtained in anther cultures was determined with the use of flow cytometry, based on the measurements of DNA content in the analyzed cells. The samples for a cytometric analysis were prepared following Galbraith et al. (1983) procedure, slightly modified. The leaves of *C. annuum* line, ATZ1 ($2n = 2x = 24$) were used as a reference standard in the analysis. Plant tissues were chopped with a sharp razor blade in a plastic Petri dish containing 1 ml of nucleus-isolation buffer (0.1 M Tris, 2.5 mM $MgCl_2 \times 6H_2O$, 85 mM NaCl, 0.1% (v/v) Triton X-100; pH 7.0) supplemented with fluorochrome 4,6′-diamidino-2-phenylindole (DAPI, 2 μg/ml). For each sample, measurements of fluorescence intensities were performed for 3000-5000 nuclei using a Partec CCA flow cytometer (Münster, Germany), equipped with mercury UV lamp. Histograms were analyzed using DPAC v. 2.2 software (Partec, Münster, Germany). For diploidization haploid plants were placed for 6 days on MS medium supplemented with colchicine at a concentration of 400 mg/l. DNA ploidy level of the obtained regenerants was estimated by flow cytometry. The analysis showed that approximately 50% of the obtained regenerants were mixoploid. The share of haploid and diploid plants varied depending on the genotype and resulted from 20 to 30%. Occasionally, tetraploid forms were detected. Haploid regenerants were subsequently treated *in vitro* with colchicine, added to MS medium at concentration of 200 mg/l for 6, 9 and 12 days. The ploidy level of colchicine-treated regenerants was estimated cytometrically. Additionally, microscope observations of chromosomes in root tips were also performed using a squash technique. Root tips (3-4 mm) were excised and pretreated in cold water for 24 h at 4°C to accumulate cells in metaphase. They were fixed in cold Carnoy fixative at 4°C for 48 h. The fixative was prepared by mixing 3 volume of ethanol and 1 of glacial acetic acid just before using. After rinsing twice with distilled water, root tips were stained for 24 h in 2% of Aceto-orcein. Then about 1 mm the root of tips were cut and transferred on a slide and squashed in beneath a cover slip. The largest group of the analyzed plants consisted of mixoploids. The effectiveness of diploidization using colchicine was 10% in total for all the treated plants.

Evaluation of genetic diversity in doubled haploids and maternal lines of sugar beet (*Beta vulgaris* L.)

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The development of inbred lines from heterozygous parents in sugar beet (*Beta vulgaris* L.) with the use of conventional breeding methods requires a lot of time and work. To enhance the efficiency and shorten the time of homozygous plants production, the induction of haploid plants followed by doubling of chromosomes with the use of unpollinated ovules tissue culture is needed. It should be mentioned that doubled haploid (DH) lines driven from gynogenesis obtain 100% homozygosity among all *loci*, which is advisable in hybrid production, but not possible to obtain through conventional methods. Due to the highest heterosis effect, components used for hybrid production should be varied, so the evaluation of genetic variability with the use of molecular markers would help to indicate the parental lines of larger genetic distance for creating F_1 hybrids in sugar beet.

In the present work, the evaluation of genetic diversity and uniformity among DH genotypes obtained from unpollinated ovules tissue culture and maternal lines is reported.

Materials represented three types of sugar beet: Z-type, ZN-type and N-type. Studies concerned 15 different maternal lines and 38 doubled haploid lines, part of which were obtained form different ovaries of the same maternal plant of sugar beet. DH lines were examined for: ploidy level using flow cytometry of leaf tissue, morphology differences, and genetic variability with the use of RAPD and ISSR markers. Total DNA of the above mentioned plant material was extracted from leaf tissue using the protocol described by Davis. The concentration, purity and integrity of DNA were determined spectrophotometrically and electrophoretically. Based on the experience of previous analyses, most polymorphic RAPD and ISSR primers were used for PCR reactions the products of which were separated on 1.5% agarose gels, stained with ethidium bromide and visualized under UV light. For the degree of genetic diversity between DH and maternal lines all polymorphic and monomorphic bands were scored as present (1) or absent (0) and the similarity coefficient matrix was performed according to Nei and Li coefficient. Dendrograms based on UPGMA method were used to estimate the relationships among the analyzed materials. The results were elaborated statistically with Statistica 7.0 software.

The analysis of RAPD and ISSR profiles of DH regenerants revealed several missing bands compared with donor plants, but the appearance of a novel bands was not detected. Of the total bands that scored 90% were polymorphic for RAPD markers ranging in size from 280 to 1797 bp, whereas for ISSR markers 87% the bands were polymorphic ranging in size from 229 to 1571 bp. The value of the similarity coefficient estimated for doubled haploids and their maternal lines ranged between 0.77 – 0.97 for RAPD and 0.82 – 0.96 for ISSR markers. The differences at the molecular level among DH lines probably indicated the presence of gametoclonal variation, which should be verified in further studies.

Studies on early development of microspore-derived embryos of *Brassica napus* cv. Markus and Feliks cultured *in vitro*

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Agriculture nowadays is focused on increasing the productivity and quality of crops with the use of an advanced technology of doubled haploids. Male gametophytic cells which are totipotent are successfully used in this method of stress-stimulated immature pollen grains to acquire embryos and plants provided from them.

Brassica napus is a well known model species in the fundamental research of microspore embryogenesis. The technique for obtaining traditional androgenic embryos of rape goes with success, but it is difficult to receive androgenic embryos which mimic zygotic embryogenesis. Donor plants used in our experiments were spring oilseed rapes – the population of cultivars Feliks and Markus giving the highest yield in 2011 of open pollinated varieties registered in Poland. Experiments were carried as sustainable microspore cultures followed by a physiological shock: cultivation of donor plants at low temperature (10°C); incubation of isolated microspores in moderately high temperatures.

Androgenesis occurred in both cultivars and in all shock variants (12 and 24h). Two kinds of androgenic embryos were distinguished: proper embryos without suspensor (Markus and Feliks) and suspensor-bearing embryos (Feliks). Embryos without suspensor derived from microspores which were symmetrically divided. The asymmetrical divisions of 1-nucleate microspores lead to a generation of embryos supplied with several-celled suspensors, which developed up to late-torpedo stage embryos.

Our results confirmed that moderate stress induces the development of suspensors in androgenic embryogenesis (Supena et al., 2008). Additionally, the application of a very short temperature shock (for only 12 h at 32.5°C) allows to obtain significantly higher frequency of androgenic embryos which mimic zygotic embryogenesis.

Cytoskeleton plays a crucial role in the polarization of microspore in the androgenic switch, leading heat-treated microspores to an asymmetric division. In further development cytoskeleton sets the apical-basal axis of suspensor-bearing embryos (Baluška et al., 2001; Dubas et al., 2012).

Our preliminary immunocytochemical results visualize the organization of microspore-derived embryos' cytoskeleton (co-location of microtubules and microfilaments) using indirect epifluorescence microscopy.

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In vitro pollination as a breeding tool in wide hybridization of *Brassica* sp.

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Distant crossing is an important tool for expansion of variability and for resynthesis of rapeseed (*Brassica napus* L.) from diploid species, such as turnip rape (*Brassica rapa* L.) and cabbage (*Brassica oleracea* L.). Due to a large number of closely related species of the genus *Brassica,* transfer by distant crossing of new features to oilseed rape breeding material is possible. The development of resynthetic *Brassica napus* (oilseed rape) lines has provided an important basis – germplasm – for further improvements of seed yield, disease and pest resistance as well as seed quality traits. The successful *Brassica napus* resynthesis consists on manual crossing of *Brassica rapa* x *Brassica oleracea*, and the application of embryo rescue culture. While cabbage was chosen as maternal parent for crossing with turnip rape positive effects were not always obtained. The genetic incompatibility often prevents an effective crossing between two of these species and thus the formation of hybrids. *In vitro* techniques are only useful and necessary if a desired genetic combination cannot be obtained efficiently by conventional crossing methods. This incompatibility can be overcome by *in vitro* pollination of isolated ovule or pollination on an ovule of pistil after the removal of the style. After that, by the use of *in vitro* embryo rescue culture, the hybrid plants should be obtained with suitable efficiency. Before starting experiments on *in vitro* pollination, many aspects of the floral biology of the species used must be understood. These include viability of pollen grains, viability of the female gametophytes, pollen germination and growth, fertilization, embryo and endosperm development. Effects of the medium on the process of *in vitro* fertilization and embryogenesis seems very important too. The medium plays a very important role in the culture at the stage of early embryogenesis.

The preliminary results of *in vitro* pollination of *Brassica oleracea* L. var. *acephala* subsp. *lanciniata* x *Brassica rapa* and development of resynthesized (RS) *Brassica napus* have been presented. For the study in aseptic conditions the cabbage stigma was cut off jointly with the style and the ovary was opened. Pollen grains of turnip rape were placed directly on the surface of the exposed ovules. These pollinated ovaries were placed on a basal MS medium in glass tubes. Four days after pollination, a pollen tube growth as well as its penetration of the style and ovule were observed. Enlarged ovules were isolated from the ovary and transferred to MS medium with an addition of kinetin and indole-3-acetic, coconut milk and 2% sucrose. When the explants had turned green, they were transferred onto MS medium with kinetin. The shoots were rooted and plantlets were transferred to soil. The four-, five-leaf plantlets were treated with colchicine for doubling of chromosome number. The embryological and cytometrical analysis of nuclear DNA of plants obtained from the interspecies crosses between *Brassica oleracea* and *Brassica rapa* confirmed their hybrid character – resynthesized oilseed rape.

Obtaining and cytogenetic identification of tetraploid hybrids *Lolium* spp. × *Festuca pratensis*

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The genera *Lolium* (ryegrass) and *Festuca* (fescue) include most of the major temperate forage grasses. They offer a range of complementary agronomic traits, such as the high nutritive value of ryegrasses and the persistency and stress tolerance of fescues, which can be combined in intergeneric hybrids. Some of the most important species of the *Lolium-Festuca* complex, used in both research and breeding programs, are: meadow fescue (*F. pratensis* Huds., 2n = 2x = 14), perennial ryegrass (*L. perenne* L., 2n = 2x = 14) and Italian ryegrass (*L. multiflorum* Lam., 2n = 2x = 14). In the *Lolium-Festuca* complex, intergeneric hybrids may be obtained by conventional crossing technique, especially in combinations where the seeds are well developed and germinate. It is much more difficult to obtain hybrids from crosses between tetraploid *Lolium* spp. and *F. pratensis* because of post-zygotic incompatibilities that cause abnormal development of endosperm and its subsequent degeneration, which then inhibits seed germination. To overcome this obstacle, *in vitro* cultures of immature embryos are usually used to regenerate hybrid plants.

The main aims of this study were: 1) to obtain intergeneric, reciprocal hybrids derived from crosses of autotetraploid (2n = 4x = 28) forms of *L. perenne* and *L. multiflorum* with autotetraploid (2n = 4x = 28) forms of *F. pratensis*, 2) to identify the obtained hybrids cytogenetically and to discriminate *Lolium*- and *Festuca*-originated chromosomes in hybrid genomes using genomic *in situ* hybridization (GISH), and 3) to detect the number and location of rDNA sequences (5S and 35S rDNA) as well as to identify rDNA-marked chromosomes using fluorescence *in situ* hybridization (FISH). Four cross combinations were made: *L. perenne* (4x) × *F. pratensis* (4x), *F. pratensis* (4x) × *L. perenne* (4x), *L. multiflorum* (4x) × *F. pratensis* (4x), and *F. pratensis* (4x) × *L. multiflorum* (4x). Immature embryos (excised 12-16 days after pollination) were cultured on Gamborg's B_5 medium.

The crossability level (the number of obtained hybrids in relation to the total number of emasculated and pollinated flowers) in all crosses was very low ranging from 0.1% to 1.5%. Hybrids derived from combinations in which *F. pratensis* was used as a maternal component featured a slightly higher level of crossability than hybrids derived from reciprocal combinations. In total, 47 F_1 hybrids were obtained, including: *L. perenne* × *F. pratensis* – 3 hybrids, *F. pratensis* × *L. perenne* – 23 hybrids, *L. multiflorum* × *F. pratensis* – 7 hybrids, and *F. pratensis* × *L. multiflorum* – 14 hybrids. Out of the 47 F_1 plants studied cytologically, forty were tetraploids (2n = 28), and seven were aneuploids (2n = 26, 27, 29, and 30). Ther GISH analysis showed that all of the obtained F_1 plants were intergeneric hybrids. All tetraploid hybrids had 14 *Festuca* and 14 *Lolium* chromosomes, and in aneuploids 12-13 *Lolium* and 13-16 *Festuca* chromosomes were observed. rDNA-FISH revealed variability in the number and distribution of 5S and 35S rDNA loci among both cultivars and hybrids of all four combinations. Some of the hybrids contained unexpected rDNA loci patterns, which seemed to have been caused by chromosome rearrangements and transposon-mediated rDNA mobility.

The effect of antimitotic agents on chromosome doubling of triticale produced via androgenesis

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The production of doubled haploids (DH) by *in vitro* androgenesis is the most often used method for the development of homozygous lines from heterozygous material. It is a time-saving alternative to a long, multi-generation breeding cycle. The generated material can be selected for important traits and transfer to the next generations. DH lines improve not only the efficiency of cultivar development but can also be helpful in genetic and molecular studies. Moreover, they constitute useful tool for various biotechnological applications such as the *in vitro* selection and mutagenesis.

The main condition for the use of regenerated plants is their ability to produce offspring. Standard procedures create an opportunity to obtain fertile plants without any additional procedures, which saves time and, at the same time, avoids using chemicals that may be toxic to both plants and humans. Although in *in vitro* cultures many microspores are capable of spontaneous doubling of their chromosome numbers in the first or second mitotic division (through the fusion of nuclei), in plants such as triticale, most regenerants are haploid. Finding an efficient method of doubling the number of chromosomes is one of the basic conditions for effective use of regenerated androgenetic plants. To increase the number of useful regenerants, the low frequency of spontaneous doubling in triticale demands an additional duplication system. Colchicine, a substance commonly used *in vitro* for this purpose is associated with many problems. One of its undesirable effects is the occurrence of mixoploids and chimeras. Moreover, the survival rate of treated plants is reduced by the phytotoxic effect of colchicine aaplied in doses effective at doubling. An alternative to colchicine may be in anti-mitotic substances-herbicides (oryzalin, trifluralin, pronamide, amiprofosmethyl – APM) which due to their much larger affinity for plant tubulin compared to colchicine may show a similar level of chromosome doubling at lower concentrations.

The experiments present the effects of several antimitotic compounds on the rate of chromosome doubling in triticale plants (X Triticosecale Wittmack) produced via androgenesis in anther culture. Experiments were done on two winter triticale cv. Mungis and cv. Bogo. In this study the antimitotic agents were added to the media at various stages of haploid production (on the callus stage, on 1-2 cm plantlets), and at different concentrations (1 μM and 10 μM) and compared to the traditionally used *in vivo* colchicine. Moreover, the effect of doubling agents on the survival rate of regenerants and their morphology has been examined. Ploidy was tested by flow cytometry. This results were compared with the ability of plants to form caryopses. The spontaneous diploidization rate was 19%. The highest regeneration rate was obtained after the use of trifluralin and APM, whereas the most harmful effect on the development and growth of plants was for pronamide (growth retardation and abnormal morfology). Colchicine *in vivo* treated haploid in majority survived the treatment and were diploid.