Genetic diversity of wheat (*Triticum aestivum* L.) plants-regenerants produced by anther culture

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The study of genetic diversity of wheat (*Triticum aestivum* L.) plants-regenerants produced by anther culture method from hybrids involved in the Latvian wheat breeding programme was performed. Flow cytometry was used to test ploidy of 3×10^3 cells of each green plant-regenerant, and universal retrotransposon based iPBS (inter primer binding sites) method were used to establish genetic diversity of plants-regenerants. Progenies of 13 genetically distant hybrids were involved in the study. Most of plants-regenerants have leaves with mixoploid cells. Seeds were formed only by plants-regenerants that had cells with ($2n = 6x$) ploidy. Majority of fertile plants-regenerants have more than 40 % of hexaploid ($6x$) cells. The percentage of 6x cells in plants-regenerants and diversity in cell ploidy demonstrated association with mother plant (hybrid) genotype. Percent of spontaneous diplodization was also genotype-dependent. In this experiment colchicine treatment had no significant influence on outcome of fertile plants- regenerants. New dominant allele in a hybrid plant was found in comparison with both parents what indicated possible retrotransposon moving. Genetic diversity of the plants-regenerants obtained in anther culture is a combination of parent's allele segregation and somaclonal variation.

Key words: anther culture; DH-lines; wheat breeding; ploidy; flow cytometry; iPBS.

Генетическое разнообразие растений-регенерантов пшеницы (*Triticum aestivum* L.), созданных методом культуры пыльников

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Изучено генетическое разнообразие растений-регенерантов пшеницы (*Triticum aestivum* L.), которые созданы методом культуры пыльников из гибридов, используемых в селекции этой культуры в Латвии. Метод проточной цитометрии применяли для определения плоидности каждого зеленого растения-регенеранта. В свою очередь, универсальным iPBS (inter primer binding sites) методом, основанном на анализе характерных последовательностей ретротранспозонов, определяли генетическое разнообразие этих растений. Анализу подвергали потомство 13 гибридов различного происхождения. В листьях большинства растений были миксоплоидные клетки. Семена дали только растения, имеющие клетки с плоидностью $2n = 6x$. Большинство фертильных растений содержало в листьях более чем 40 % гексаплоидных ($6x$) клеток. Доля гексаплоидных клеток и их распределение по плоидности зависели от генотипа материнского гибридного растения. Доля спонтанной диплоидизации также зависела от генотипа растения. В описываемом эксперименте колхицинирование не оказало достоверного влияния на выход фертильных растений. У одного гибридного растения обнаружена новая доминантная аллель, отсутствующая у обоих родителей, что указывает на возможное передвижение ретротранспозонов. Генетическое разнообразие растений-регенерантов, созданных методом культуры пыльников, вызвано как расщеплением родительских аллелей, так и сомаклональной изменчивостью.

Ключевые слова: культура пыльников; ДГ-линии; селекция пшеницы; плоидность; проточная цитометрия; iPBS.

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Wheat occupies the largest part of all cropland and continues to be the most important type of whole-grain food for humans all over the world. In Latvia, last years the main cereal crops are spring and winter wheat. Wheat breeding in the country continues since 1922. In Latvia, main wheat crop is common wheat *Triticum aestivum* L. ($2n = 6x = 42$) (Goncharov, 2002). The current breeding task is aimed to create new winter and spring wheat varieties adapted to conventional and organic management with grain quality corresponding to modern food industry requirements. For this purpose both traditional and biotechnology methods are applied. Different plant tissue culture methods are widely exploited to speed up breeding time of many crops and can be used during all breeding stages: for obtaining breeding source material, selection of best plants/lines and the fast genotype propagation. Involving doubled haploid (DH) lines in the breeding process is recognized as an appropriate approach especially in conditions of mid-size breeding institutions both to speed up time of obtaining of new varieties and decrease labour and technical resources. Advantages of using DH lines are well known: during a single generation not segregating lines homozygous in all loci are created what provides ability for true evaluate of breeding material in a rather short time. Additionally, novel important gene alleles could be quickly involved in new varieties. Usually, by involving DH technique creating of new varieties is shortening for 3–5, in some cases even more years (Grauda et al., 2010; Tadesse, 2012). In Latvia, new spring wheat variety 'Robijs' was created from a DH line, initiated by anther culture, only during 7 years (Grauda et al., 2009).

To produce DH lines such approaches, as hybridisation with haploproducer, as well as DH creating from pollen, anthers and ovarium *in vitro* cultures, are used. DH lines can be obtained by autopolyploidy or induced polyploidy as mitotic polyploidization, which is based on the doubling of chromosome set of somatic tissues of haploid plants (Ramsey, Schemske, 1998) and resulted in two (or more) pairs of homologous chromosomes. Production of DH plants by anthers culture is the most significant method used in the wheat breeding (Kasha, Maluszynski, 2003; Belchev et al., 2004; Grauda et al., 2009, 2010; El-Hennawy et al., 2011). Effectiveness of anther culture is found to be influenced by genotype of used hybrids, donor plant growth conditions, the developmental stage of microspores, spikes pre-treatment, and media components (Kasha, Maluszynski, 2003). Different investigations have been carried out to clarify mode of inheritance of microspore regeneration capacity in anther culture and *de novo* shoot organogenesis (Torp, Hansen, 2001; Xynias et al., 2001; Kasha, Maluszynski, 2003; Zamani et al., 2003; Jacquard et al., 2009; Ferrie, Caswell, 2010; Duclercq et al., 2011; El-Hennawy et al., 2011; Rubtsova et al., 2012) and to find out the ways to increase percentage of green plants-regenerants. Till now, the hybrid genotype is still the main limiting factor of androgenesis in tissue culture and therefore it is also the limiting factor for successful obtaining fertile plants-regenerants with stable diploid ($2n$) chromosome set as a starting plant of DH line.

Spontaneous doubling rate in wheat depends on the genotype and in different experiments varied from 0 to 68 % of the obtained plants-regenerants (Barnabás, 2003; Grauda et al., 2010, 2014; El-Hennawy et al., 2011). Colchicine is the most successful agent used for doubling of chromosome set. Colchicine inhibits spindle formation during mitosis and disturbs normal polar segregation of sister chromatids, what leads to doubling of chromosome number (Levan, 1938). Colchicine treatment in various concentrations was successfully applied during cultivation both anthers and plants-regenerants (Barnabás et al., 1991; Chen et al., 1994; Hansen, Andersen, 1998; Soriano et al., 2007). Nevertheless, the question about the most effective time and concentration of using the colchicine for anther culture method is still open. An important step to evaluate colchicine influence is determination of cell chromosome number. Use flow cytometry (FCM) makes the process of determination of ploidy of plants-regenerants quick and efficient. For this purpose DNA is bound with fluorescent dye and relative laser initiated fluorescence are recorded. The large number of cells can be evaluated in a very short time and therefore results are statistically significant and represent a whole population. Resulting relative fluorescence is calculated on data of measurement of fluorescence of 1000 cells as a minimum (Doležel et al., 1991; Galbraith, 2010). Genetic diversity of DH lines obtained from the same hybrid is usually associated with combinative variability based on parental plants genotypes.

Use of pollen or anther culture for DH production related to high level of cell stress during of different stages of *in vitro*: immature pollen pre-treatment, callus initiation and cultivation. As a result of cell stress during *in vitro* cultivation, so called somaclonal variation (Smith, Park, 2002; Khan et al., 2008) could be observed. Direct use of somaclonal variation in plant breeding is limited by a lot of factors: they are unpredictable, variation may be hereditary or epigenetic (Smith, Park, 2002). Moreover, somaclonal changes cannot be received for all traits, most of them are negative, new features are very rare. But on the other hand, somaclonal variation has benefits – changes may affect agronomically important characteristics, and finally, in *in vitro* conditions could be selected biotic and abiotic stress resistant genotypes. Frequency of somaclonal variation is related to genotype, type of explants, cultivation media, and time of cultivation *in vitro* (Jain et al., 1998).

Some authors (Kalendar, Schulman, 2006; Bairu et al., 2011) consider changes of activity and transposition of mobile genetic elements, including retrotransposons, to be the main cause of somaclonal variation. Retrotransposons are found in all plant genomes. In wheat retrotransposons compose till 68 % of whole genome (Li et al., 2004). Retrotransposons as repetitive sequences integrated between functional genes in the noncoding regions (Kubis et al., 1998; Bennetzen, 2000). Retrotransposons are source of spontaneous mutations, activity of retrotransposons associated with plant cell stress (Casacuberta, Santiago, 2003; Kalendar et al., 2010). After cell polyploidization transpositions are most common genetic alteration, hence retrotransposon-based methods are most effective for studying genetic diversity caused by somaclonal variation. Some authors (Kalendar, Schulman, 2006; Bairu et al., 2011) suggest that activity of transposons, including retrotransposons, is a base of somaclonal variation.

The goal of this study was evaluation of genetic diversity of wheat (*Triticum aestivum* L.) plants-regenerants produced by anther culture from hybrids involved in Latvian wheat breeding programme. By the reasons mentioned above a

Table 1. Hybrids used for obtaining plants-regenerants by anther culture

Hybrid designation	Cross combination	Type
A	Eminent/Uffo	Spring
B	Sarnuko//Bastian/Drifler	Spring
C	Uffo//Jasna/Jota	Spring
D	Bombona/Fasan	Spring
E	Compliment/Contact	Winter
F	CPBT W 143/Ada	Winter
G	Nic99-3009B/Raduga/Galahad	Winter
H	Schara/Nic04-4106B	Winter
I	Premjera/Nic04-3241A	Winter
J	Sepstra/Nic04-4106B	Winter
K	Fantazia/Olivin	Winter

universal retrotransposon based iPBS (inter primer binding sites) method was chosen for the study. The method developed by R. Kalendar et al. (2010) allows revealing the high level of genetic diversity and it is cost and labour effective. The method was successfully used for investigation of genetic diversity of several species (Kalendar et al., 2010; Smýkal et al., 2011; Grauda et al., 2014; Kalendar, Schulman, 2014; Kļaviņa et al., 2014).

Materials and methods

Plant material

Spring and winter common wheat *Triticum aestivum* L. F_1 and F_2 hybrids created at the Research Centre of the Institute of Agricultural Resources and Economics (former State Stende Cereals Breeding Institute, Latvia) were used for DH lines obtaining (Table 1). Donor plants were grown in greenhouse (+17 to +20 ºC at night, +25 to +30 ºC day, humidity ~70 %) or in room conditions (+20±2 ºC, humidity ~40 %, 16 hours photoperiod). Three weeks after sowing seedlings of winter genotypes were vernalised at +4 ºC for 8 weeks. Spikes of both wheat types were collected when most microspores were in the early or mid-uniculate stage. The developmental stage of microspores was determined by squashing in acetic carmine on a glass slide (Jacquard et al., 2003; Grauda et al., 2014).

Establishing of anther culture and obtaining plants-regenerants

Collected spikes were maintained at +4 °C for two weeks. Spikes were sterilised with 50 % commercial bleach (4 % of hypochlorite content) water solution for 17 minutes, and then rinsed 4 times with deionised and autoclaved water (Grauda et al., 2005). Anthers were separated from spikes and, in equivalent amounts, put on liquid induction medium AMC (Quinn, Keough, 2002) with 2.5 mg/l $CuSO_4 \times 5H_2O$ (Grauda et al., 2005). Petri dishes with anthers were maintained in a growth chamber at +29 ºC in dark conditions. After four weeks of cultivation obtained embryos about 2 mm in diameter were transferred onto regeneration medium 190-2 (Kunz et al., 2000). Embryos were cultivated under light (+24 ºC, 16 hours photoperiod, light intensity 3000 lx). When embryos started to develop green plantlets, they were transferred on rooting medium: MS (3 % sucrose, 0.6 % agar) with 1 g/ml activated charcoal. Plantlets were grown under light (+24 ºC, 16 hours photoperiod, light intensity 3000 lx).

After 2–4 weeks of cultivation on rooting medium the plantlets with developed leaves and roots were transferred into autoclaved mixture of soil and sand (1 : 1) and grown in a growing room (+20 ± 2 ºC, humidity ~40 %, 16 hours photoperiod). For the first days plantlets were covered with glass jars to maintain high level of humidity.

Colchicine treatment

For the doubling of chromosome set the plantlets with well-developed leaves and roots were chosen. Plants-regenerants obtained from each cross combination of spring wheat hybrids (Table 1, A–D) were split randomly into two equal groups – plants regenerants which were treated with colchicine, and another part which continued grown without colchicine treatment (control). For winter wheat control was established for two hybrid combinations (Table 1, E and F).

Roots of plants-regenerants were immersed in 0.2 % colchicine solution for 4 hours. Then the plants were rinsed with water, planted back into soil and cultivated in a growing room (+20 ± 2 ºC, humidity ~40 %, 16 hours photoperiod). Spring wheat plants-regenerants were grown in the mentioned conditions till maturity. Winter wheat plants-regenerants after 3 weeks of treatment by colchicine were transferred to a growth chamber (+1 to +7 °C, dim-light) for vernalisation. After 8 weeks of vernalisation survived plantlets were moved to a growing room (+20 ± 2 ºC, humidity ~40 %, 16 hours photoperiod) and grown till maturity.

Ploidy determination

In stage of tillering the second leaf from each plant-regenerant was collected for ploidy determination by flow cytometry. Collected plant leaves (approximately 0.5 cm^2) were prepared using CyStain PI absolute P reagent kit (Sysmex Partec GmbH., Germany) accordingly to the manufacturer protocol. Leaves were chopped by a razor blade in petri dishes, then added 500 µl of the extraction buffer solution per sample, and after 90 seconds were filtered by Partec 50 µm CellTrics

disposable filter. Samples were coloured with staining buffer solution with PI (propidium iodide) and RNAse in amount of 2.0 ml and incubated in dark conditions for 60 minutes. BD FACSJazz® cell sorter (BD Biosciences, USA) with flow cytometer function was used to determine ploidy of plant cells. The device was equipped with 100 µm nozzle and used phosphate-buffered saline (BD Pharmingen™ PBS, BD Biosciences, USA) as a sheath fluid. Cell nucleus counting events were triggered by forward-scattered signal. The excitation of the cell fluorescence was made by 488 nm Coherent Sapphire Solid State (blue) laser. The fluorescence emission was measured at 585 nm (bandwidth 29 nm). Before measurements, flow cytometer was calibrated using Sphero™ rainbow calibration particles (3.0–3.4 µm, BD Biosciences, USA) in phosphate buffered saline (PBS). The calibration was considered as successful if the coefficient of variance (CV) of the relative fluorescence of calibration particles did not exceed 3 %. Cell nucleus counts were gated by the intensity in fluorescence channel to include 95–99 % of target nucleus. The intensity of fluorescence was expressed in arbitrary units and analysed in logarithmic scale, used through the whole research. No less than 3×10^3 gated cell nucleus were analysed from each sample. Plants of wild wheat species *Triticum urartu* (2*x*) and *T. durum* (4*x*), and two common wheat (*T. aestivum*) varieties (6*x*) were used for establishing of relative fluorescence reference system.

Estimation of genetic diversity of plants-regenerants

Ten iPBS primers (Kalendar et al., 2010; Kalendar, Schulman, 2014) that showed high level of polymorphism of many organisms were tested on the preliminary sample set of wheat. Criteria for further analysing of diversity of plants- regenerants was the difference in loci produced by mother plants of used hybrids (varieties 'Olivin' and 'Fantasia'). Primers showing at least one different locus between mother plants were selected for analysing of all plant material for further studies. At first stage homogenity of mother plants on selected loci were tested, then selected primers were used for analysing of plants-regenerants. For genetic analysis DNA was extracted from dried leaves using NucleoSpin Plant II kit (Macherey-Nagel, mn-net.com) and standard protocol for plants. After homogenization of dried plant material lysis buffer PL2 was used (protocol part 2B), incubation time – 60 min. Extracted DNA was examined for quality test by 0.7 % agarose gel electrophoresis at 80 V for one hour, DNA quality also was detected by spectrophotometer. DNA amplification was performed in Gene Amp® PCR System 9700 thermocycler (Applied Biosystems) under the following conditions: denaturation 95 °C/3 min, then 30 cycles and three-steps PCR process: (denaturation at 95 °C/30 s; annealing at 50 °C/40 s; extension at 68 °C/60 s) and the final annealing temperature was 72 °C for 10 min. The concentration of DNA used in PCR reactions was 30–100 mcg/ml and *was quantified using* Eppendorf Biophotometer. The final concentration of reagents in PCR mixture was: 1xDream Taq Buffer (Fermentas), 200 µM dNTPs (Fermentas), 1 µM primer, 2.5 U per 25 µl *DreamTaq* polymerase (Fermentas) and 0.06 U per 25 µl *Pfu* DNA polymerase (Fermentas). PCR products were visualized by 1.7 % agarose gel electrophoresis with 25 × 10 gel track, in 1 X TAE buffer at 40 V for 17 hours. Gel was dyed for 40 minutes in deionized water with 20 µl/L 10 % water solution of ethidium bromide and rinsed for 10 minutes in deionized water. After visualization the agarose gel was documented with digital camera (Yılmaz et al., 2012).

Data processing

Generalized linear model (GLMs) was applied to detect factors that influence seed production. Logistic and Poisson distributions were used in the GLM analysis (Quinn, Keough, 2002). The best model was chosen based on Akaike's Information Criterion (AIC) values, and variance. The model with lowest AIC value was chosen. Groups of two hybrids offspring, which showed biggest difference in seed production (the greatest seed existence among the lines, and complete seed absence), were compared with t-test to determine the difference of percentage of hexaploid cells. Statistical calculations were made using R v. 3.2.3 and MS Excel software.

Results

Direct influence of cells ploidy on the formation of green plants-regenerants was not found. The loss of wheat plant-regenerants during transfer from *in vitro* culture to soil was about 10 %, and no effect of genotype or cell ploidy was observed. All evaluated wheat plants-regenerants have cells of different ploidy, including diploid ($2n = 6x$) cells (Fig. 1, *b*), nevertheless 60 % of those were mixoploids with wide spectrum of cells with different ploidy (from 2*x* till 6*x*) (Fig. 1, *c* and *d*).

Even plants-regenerants produced from the same hybrid combination had different proportions of cells with various ploidy, including different proportion of cells with 6*x*. This presented, in general, very high genetic diversity within and between offspring of different hybrids (Fig. 2). Only presence of 6*x* cells ensures the development of seeds, the plants- regenerants without hexaploid cells in all cases were sterile regardless of cell ploidy (2*x*, 3*x*, or 4*x*). The presence of 6*x* ploidy cells were found in all fertile plants-regenerants and ranged from 8 % till 85 %. On the other hand presence of 6*x* cells is not a guaranty of plant seed formation (Fig. 2, *a*, hybrid B). Several plants-regenerants (Fig. 2, F) had seeds at presence of even very small (less than 20 %) 6*x* cell percentage. Nevertheless, in the next generation offspring of such plants formed stable DH lines with cell ploidy $2n = 6x$. Usually fertile plants-regenerants had more than 40 % of hexaploid cells. As exception can be mentioned, that a plant-regenerant with proportion of cells with 6*x* ploidy more than 70 % (Fig. 2, *a*, hybrid A) did not produce seeds, although other regenerant from the same hybrid with only 32 % of 6*x* cells was fertile.

Comparison of spring and winter wheat types pointed out similar tendencies. All evaluated plants-regenerants of winter samples presented on Fig. 2 (hybrids E and F) were mixoploids before colchicine treatment and already contained cells with ploidy 6*x*, from them hybrid E produced only sterile plants-regenerants. Plants-regenerants of other winter wheat hybrids after colchicine treatment were partly fertile (Fig. 3, hybrids G–K), all of them also were mixoploid with different content of 6*x* cells before the treatment. The level of fertility depending of hybrid combination ranged in this set from 33 % (Fig. 3, hybrid H) till 64 % (Fig. 3, hybrid I).

The comparison with the control both in spring and winter wheat showed than colchicine treatment slightly enhancing percentage of hexaploid cells only for small part of plants-regenerants (data not shown). The influence of colchicine treatment on fertility of plants- regenerants was not observed.

Data analysis by generalized linear model (GLM) method allowed establishing factors that determine seed formation in plants-regenerants (Table 2). GLM models explaining presence of seeds and percentage of hexaploid cells in linkage with genotype and colchicine treatment. Percentage of hexaploid cells ($p < 0.001$) and genotype ($p < 0.01$) are significantly associated with seed formation. Statistically significant ($p < 0.001$) influence of genotype was observed in comparing of plants-regenerants obtained from genetically distant hybrids and presented different seed production frequencies. Percentages of hexaploid cells as factor explained the greatest data variance 34 %, showing also the lowest AIC value. The influence of colchicine treatment to increase of percentage of hexaploid cells was not significant ($p = 0.864$). Colchicine treatment did not enhance seed production of plants-regenerants.

Ten primers (Kalendar et al., 2010) were used for testing varieties 'Olivin' and 'Fantasia'. Among them three primers (2083, 2273, 2385) revealed differences between varieties in some loci. Primer 2083 showed difference in one locus. The presence of PCR-fragment (dominant allele) in 'Olivin' was observed, while 'Fantasia' is a carrier of recessive allele (lacking PCR fragment) in this locus (designated as locus 1). Primer 2273 revealed differences in two loci: locus 2 with dominant allele in 'Olivin' and recessive in 'Fantasia', and locus 3 with dominant allele in 'Fantasia' and recessive in 'Olivin'. Primer 2385 also produced two loci: locus 4 with dominant allele in 'Olivin' and recessive in 'Fantasia' and locus 5 with dominant allele in 'Fantasia' and recessive in 'Olivin'. All tested plants of both varieties were genetically homogenous.

Selected primers were applied for all obtained plants-regenerants from the hybrid 'Olivin'/'Fantasia'. Frequency of dominant alleles in plants-regenerants ranged from 66.0 % (primer 2385, locus 5) till 93.9 % (primer 2083, locus 1)

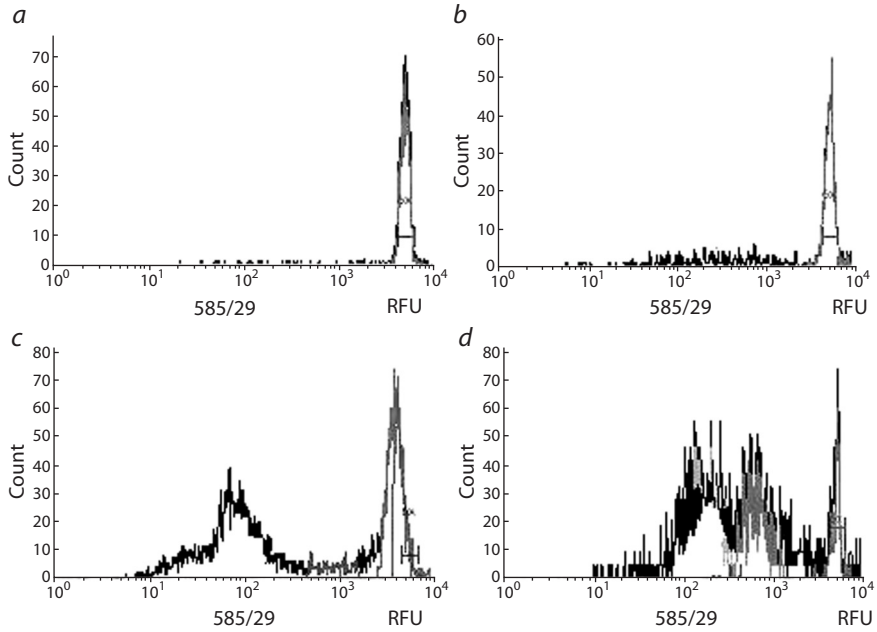

Fig. 1. Examples of flow cytometry results: fluorescence of cell nucleus depending on ploidy level.
a. Fluorescence of nucleus of 6*x* cells of variety 'Robijs' used as control. *b*. Fluorescence of cell nucleus of plants-regenerants with ploidy 2*n* = 6*x*. *c*. Fluorescence of cell nucleus of mixoploid plants-regenerants with leading cell ploidy 2*x* and 6*x*. *d*. Fluorescence of nucleus of mixoploid plants-regenerants with wide spectrum of cells with different ploidy: from 2*x* till 6*x*.

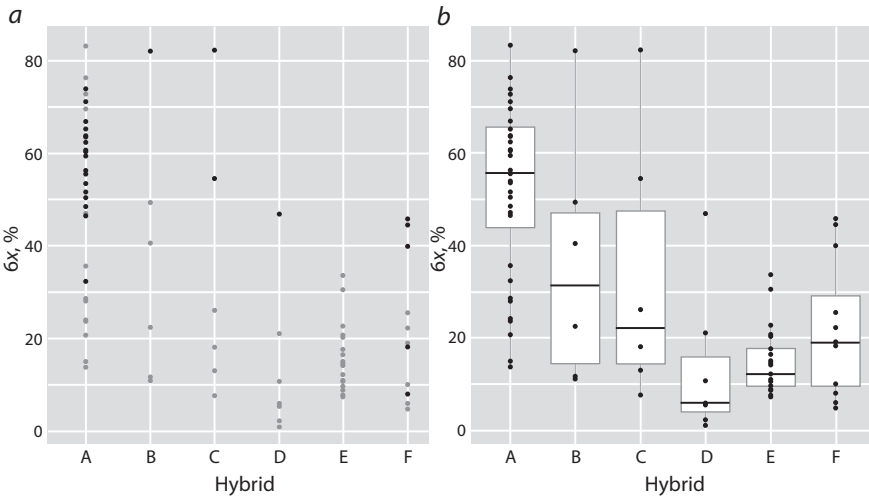

Fig. 2. Distribution of plants-regenerants obtained from genetically different F_1 hybrids (A–F) depending of percentage of 6*x* cells.
a. Distribution of fertile (black circles) and sterile (grey circles) regenerants. *b*. Total distribution of regenerants.

(Table 3). In one plant-regenerant (B1-2) *de novo* locus was identified by primer 2083 (Fig. 4), which was present neither in parents nor in other plants-regenerants.

Discussion

The anther culture is one of common methods for wheat DH obtaining. The method drawback is high genotype dependence in all crucial phases of microspore initiation and cultivation and growing of plants-regenerants. The improvement of mother plant cultivation, spikes pre-treatment and anther *in vitro* cultivation enhance the

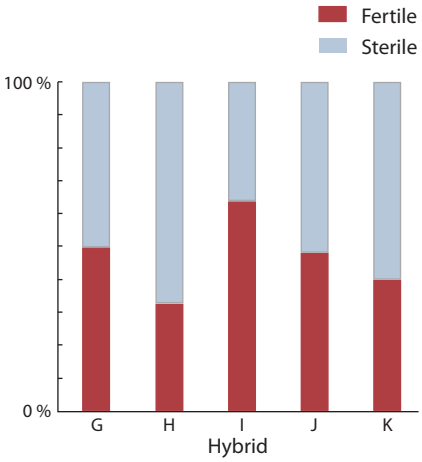

Fig. 3. Proportion of fertile and sterile plants-regenerants of different winter wheat F_1 hybrids (G–K).

frequency of microspore development till green plants-regenerants, but not always led to obtaining of large number of DH (Kunz et al., 2000; Tuvesson et al., 2003). Doubled haploid obtaining in *in vitro* system is related to high cell stress (Touraev et al., 1997) underlying genetic and epigenetic variation of plants-regenerants (somaclonal variation). In the anther culture each plant-regenerant is developed from one microspore (cell), which is subject to genetic changes induced by stress (Dhooghe et al., 2011). Probably, it also explains the high diversity of plants-regenerants by ploidy, including that of regenerants obtained from the same hybrid (Fig. 2). Flow cytometry gives possibility to examine the ploidy of several thousands (in this investigation 3×10^3) cells of each regenerated plant, therefore the obtained results are undeniable. Nevertheless, it is necessary to take into account that for ploidy analysis the leaves were used. In case of plant mixoploidy the percentage of 6*x* cells in leaves and in flower can be different. It is clear that seeds formed only plants-regenerants that have cells with 6*x* ploidy, majority of fertile plants-regenerants had more than 40 % of hexaploid cells. The percentage of 6*x* cells in plants-regenerants and diversity in cell ploidy demonstrated association with mother plant (hybrid) genotype. Percent of spontaneous diplodization of wheat plants-regenerants in anther culture also showed genotype (hybrid) dependence.

Table 2. Results of analysis by generalized linear model (GLM) of influence of different factors on producing of plants-regenerants

Related parameters	Variance, %	P-value	AIC
6*x* cells, % – presence of seeds	34.45	4.84e–07*	79.17
Genotype – presence of seeds	10.34	0.00133*	106.81
Colchicine treatment – 6*x* cells, %	–	0.864	127.13

* Significant relations.

Table 3. Frequency of dominant alleles in plants-regenerants of the hybrid Fantazia/Olivin

Primer	Locus designation	Number of tested individuals	Individuals with dominant allele, *n* (%)
2083	1	66	62 (93.9)
2273	2	75	56 (74.7)
2273	3	52	43 (82.7)
2385	4	48	44 (91.7)
2385	5	47	31 (66.0)

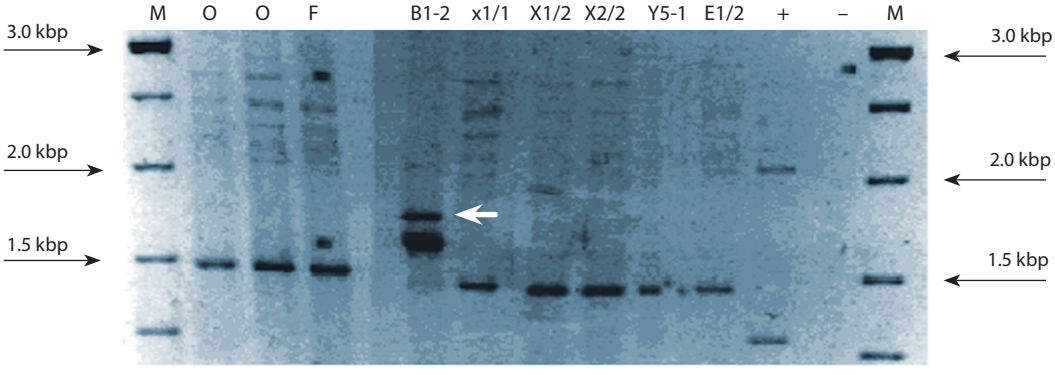

Fig. 4. PCR-products produced with the primer 2083.

M – weight marker; O – 'Olivin'; F – 'Fantasia'; B1-2 till E1/2 – plants-regenerants; „+/–„ – positive and negative controls. The new band, not presented in any of parents, is pointed by arrow.

The role of colchicine treatment in wheat DH lines production is still a question. For enhancing of DH producing and cell diplodization colchicine can be used as an additional stress factor in the medium of pre-treatment, in the induction medium and directly for the treatment of plants-regenerants (Pauk et al., 2003; Soriano, 2007). In our previous experiments about 10 % of wheat plants-regenerants spontaneous diplodization and producing seeds without colchicine treatment was evaluated. The use of colchicine treatment increased this rate, depending on the genotype, till 20–70 % (Grauda et al., 2014). However, in this study colchicine treatment had not significant positive influence on the increasing of diploid ($2n = 6x$) cell proportion and on fertility of plants-regenerants. It could be suggested that the method is not effective for genotypes without tendency to formation of diploid ($2n = 6x$) cells (self-diplodization).

Using *in vitro* methods, such as anther cultures, is related to development of novel structures, including calluses, what can have influence on the activity of retrotransposons. LTR retrotransposons are located mostly in uncoding regions of plant genome and cannot have direct effect on forming regeneration zones in gametic calluses and on development of plants-regenerants (Kalendar, Schulman, 2006). This assumption is confirmed by results of analysis of plants-regenerants using primer 2083: one of plants-regenerants (B1-2) contains new dominant allele in comparison with both parents what indicate a retrotransposon moving. However, this novelty has not interfered with the development of vital plant. In other cases, somaclonal variation induced by retrotransposons moving can affect different traits of plants. Those changes are unpredictable, they may be hereditary or epigenetic, and most of them are negative from agronomic point of view. For this reason DH lines formed by plants-regenerants often are unusable for obtaining new varieties (Pierik, 1997; Grauda et al., 2005). Certainly, the genetic diversity of the plants-regenerants obtained in anther culture is a combination of parent's allele segregation and somaclonal variation.

Use of all genotypes with different anther response for obtaining of large number of DH lines for breeding makes this method too expensive to be used for routine purposes. Another option is the use of anther culture only for responsive genotypes (Irikova et al., 2011; Murovec, Bohanec, 2012). In this case obtaining high number of DH lines from hybrids of unknown androgenesis response should be organized in two stages: the first – selection from breeding initial material hybrids responsive in anther culture, which produced embryos able to regenerate green plants, and, the second – producing of large number of DH lines from corresponding hybrids. Developing appropriate timeline for obtaining DH plants is option considerably decreasing expenses of DH lines production, especially for winter wheat, and, as well, give a possibility for evolution of some agronomic traits (for example disease resistance) already in the first cycle of creating DH lines.

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Conflicts of interest

The authors declare no conflicts of interest.

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