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INFLUENCE OF GENOTYPE AND CULTURE CONDITIONS ON *in vitro* GYNOGENESIS IN RED BEET (*Beta vulgaris* subsp. *vulgaris*)

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ABSTRACT

The process was examined or the effect of culture conditions on *in vitro* gynogenesis in red beet was analyzed, conditions were modified or optimized. A significant influence of the genotype on the gynogenesis process was demonstrated. Of the eight genotypes, 58.3% planted ovules regenerated embryo-like structures in breeding line 411, 2.1% in RA-10, RA-11, RA-12 breeding lines and 0.9% embryo-like structures in Opolski. For the gynogenesis induction, B5 medium containing 0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid was the most effective from all tested media. On this medium, the highest number of gynogenetic embryo-like structures was obtained. Most of the plants were regenerated on MS medium supplemented with 30 g L^{-1} sucrose, 0.2 mg L^{-1} 6-benzylaminopurine and 1 mg L^{-1} indole-3-acetic acid. Thirty nine percent of regenerated plants acclimatized. Cytometric evaluation of gynogenetic plants of four tested genotypes revealed that in three genotypes, 100% of tested plants were haploid. Plants showed diploid ploidy level in one genotype. Isoenzymatic analysis of gynogenetic plants demonstrated that 95% and 70% of examined populations were homozygotic for the phosphohexose isomerase isoenzyme and the aspartato aminotransferase isoenzyme, respectively. During the next generation sequencing, 93% of reads were successfully mapped, from which 83% to 85% were mapped in pairs. For 15% of pairs it was clear that obtained sequence was fully homozygous, the rest of the readings were not unambiguous, but similar to the sequence of a homozygous base pair system.

Keywords: gynogenesis, cytometry, isoenzymes, next generation sequencing

INTRODUCTION

Red beet is a common crop plant distributed throughout Asia Minor, the Mediterranean, and Europe. It is also known as an economically important plant. Due to the high content of biologically active substances, in particular betanin, red beet is classified as a nutraceutical food.

Currently, breeding of the new cultivar of crop plants is conducted with the use of traditional and biotechnological methods. Gynogenesis is one of the utilized methods, which allows researchers to obtain haploid plants and double haploid lines (DH) in a short period of time.

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Haploid plants became a valuable source for basic research such as genome mapping, genetic analyses, mutations, transformation, somatic hybridization, biochemical and physiological analyses, cytogenetic research, reference genome sequencing and genetic linkage analysis [Ferrie and Möllers 2011]. Most often, however, they are used in plant breeding programs. So far intensive research on production of haploid plants using gynogenesis were conducted mainly on sugar beet. First haploid plants of red beet were obtained by Hosemans and Bossoutrot in 1983 with the efficiency of 23 haploid plants produced from 10000 ovules [Hosemans and Bossoutrot 1983]. Subsequently, the successful induction of plant regeneration from unpollinated ovules was reported by Bossoutrot and Hosemans [1985]. Since then many researchers obtained embryos by gynogenesis in sugar beet, e.g. Gürel et al. [2000], Nagl et al. [2004], Tomaszewska-Sowa [2010], Aflaki et al. [2017], Pazuki et al. [2017]. For red beet, Barański [1996] obtained few haploid plants using gynogenesis. In 2021, two research teams confirmed the successful production of haploid red beet plants through *in vitro* gynogenesis [Zayachkovskaya et al. 2021, Kiszczak et al. 2021], and this was also confirmed by Kiszczak et al. [2023]. The process of induced gynogenesis is determined by numerous endogenous and exogenous factors such as genotype and the composition of induction and regeneration media. Genotypes vary greatly in their ability to form a gynogenetic embryo or plant regeneration [Gürel et al. 2000, Klimek-Chodacka and Barański 2013, Pazuki 2017]. Barański [1996] observed that ovules collected from donor plants with stable cultivar genotypes had a greater gynogenic ability than the ovules of hybrids or inbred lines. Other studies have confirmed genotypic differences in the efficiency of gynogenesis, but have not indicated that these differences are significant between stable varieties and inbred lines [Zayachkovskaya et al. 2021, Kiszczak et al. 2021]. In general, media based on N6 [Chu et al. 1975] and MS [Murashige and Skoog 1962] containing various growth regulator combinations were used to induce gynogenesis [Weich and Levall 2003, Aflaki et al. 2017, Pazuki et al. 2017]. Barański [1996] used N6 medium with the addition of IAA and 6-benzylaminopurine (BAP) to induce gynogenesis in red beet. However, after obtaining gynogenetic embryos Barański [1996] did not achieve direct conversion of sugar beet embryos into plants. Different authors obtained better results in androgenesis using IMB medium supplemented with thidiatabzuron (TDZ) [Zayachkovskaya et al. 2021] and B5 medium with the addition of IAA, BA and putrescine (Put.) [Kiszczak et al. 2021, Kiszczak et al. 2023].

Zayachkovskaya et al. [2021] obtained direct regeneration of callus in plants on MS medium containing BAP and GA_3, but the root system was weak, therefore passages of shoots were performed several times to medium without hormones. Kiszczak et al. [2021 and 2023] obtained plants with well-developed root system on MS medium supplemented with BA and IAA, however more shoots regenerated on medium with the addition of BA and Put. In the next stage, obtained shoots were rooted on ½ MS medium containing naphthylene-1-acetic acid (NAA) and Put.

Successful regeneration and adaptation are the most important stages in the whole procedure of deriving gynogenetic plants, but only ploidy level and homozygosity evaluation can confirm the obtaining of haploids or DH plants. The ploidy level of gynogenetic plants, can be confirmed by determination of the nuclear DNA content using flow cytometry [Bohanec 2013, de Oliveira et al. 2013, Keleş et al. 2016]. The above-mentioned authors have successfully used flow cytometry to determine the gametic origin of red beet [Zayachkovskaya et al. 2021, Kiszczak et al. 2021, 2023]. Another method for assessing the gynogenetic homozygosity of plants is the analysis of isoenzyme polymorphism. This approach enables the evaluation of differences in gene products at the protein level. Evaluation of isoenzyme polymorphism is commonly used to confirm the homozygosity of various plant species obtained in the gynogenesis process [Murovec and Bohanec 2012]. In case of red beet, authors have applied two isoenzymatic systems [Kiszczak et al. 2021, 2023].

According to Djedatin et al. [2017], next generation sequencing (NGS) is less expensive, more effective and quicker method for determining the homozygotic arrangement of alleles in the genome. NGS is known to be the most precise method that provides an immense amount of bioinformatic data. With advances of the NGS technology and DNA sequencing, it was

possible to use accurate genotyping as a tool for the genetic and evolutionary studies or in the process of accelerate the breeding processes [Song et al. 2016, Wang et al. 2016]. Polymorphism of the genome, including single nucleotide polymorphisms (SNPs), is determined by the NGS method [Kumar 2012, Gupta et al. 2017]. The spontaneous doubling of the genetic material often occurs in the gynogenesis process, which in case of the allelic forms of genes in tested isoenzymes, can cause difficulties for determination of the gametic origin of those plants. According to Djedatin et al. [2017], the most effective method for detection of the duplication of entire segments of the genome or even single genes is the NGS method. The above-mentioned method is very suitable for the isolation of homozygotic populations found in a transgenesis procedure [Passricha et al. 2016]. O'Malley et al. [2017] used results obtained from NGS for the isolation of homozygotic mutants from the population of *Arabidopsis thaliana*. Earlier in 2016, NGS sequencing, combined with Bulk Segregant Analysis, allowed researchers to accelerate the identification of causal mutations with a reference genome sequence in the sugar beet [Ries et al. 2016]. Szklarczyk et al. [2016] applied NGS as a supplementary method for the identification of mitochondrial DNA characteristics, which diversified the cytoplasmatic male sterile and male fertile forms of sugar beet. On the other hand, so far there is no information in the literature about the application of this method in the studies on the genome of red beet.

The aim of this study was to evaluate the influence of various factors on the gynogenesis process and haploid red beet plant regeneration. Different important factors for the gynogenesis process were under study, i.e. the induction medium, the genotype, media for gynogenesis induction and plant regeneration, acclimatization process. Ploidy of obtained plants was also evaluated and the usability of isoenzyme polymorphism analysis for the determination of homozygosity was tested. The correlation between the isoenzyme polymorphism and the analysis of the base pairs order in the genome of red beet on the basis of NGS were examined. The NGS analysis was also performed in order to obtain data that will be used in the databases. Thanks to the information included in the database, researchers will be able to design molecular markers and perform comparative transcriptomics. Knowing the nucleotide sequence of the genome or the transcriptome, it will be also possible to find single nucleotide mutations (SNPs) or simple sequence repeats (SSRs).

MATERIALS AND METHODS

Preparation of plant material

Roots of various red beet genotypes were provided by Breeding and Seed Company – Polan Sp. z o.o. in Cracow. The research was conducted in two vegetation seasons. In the first year, studies were conducted using breeding lines RA-10, RA-11, RA-12, RA-13, RA-14, 406, 411. In the second year of research, RA-5, 4/11, 5/11, 411 breeding lines were used. As a control, the roots of Opolski cultivar. Received roots of red beet plants with heterozygosity confirmed by breeding methods were placed in a substrate consisting of 1:3 (v/v) sand and soil and placed in a cold chamber at 4 °C for two-month vernalization. Then, roots were planted in plastic containers with a capacity of 20 L (two roots per container) in a growth chamber under controlled growth conditions at 18 °C during the day and 16 °C at night, with a 16/8 hour photoperiod.

General research plan

In the first stage of the study, the protocol for gynogenetic plant production was optimized for each red beet genotype. Initial research and then research on determining the composition of the medium that guarantees the formation of embryos were conducted on the Opolski cultivar (Fig. 1 A). In the following year, mainly optimization of the plant growth regulators (PGRs) composition was conducted on the 411 breeding line. At the stage of multiplication, the ploidy of obtained regenerated plants multiplication was analysed using flow cytometer. Shoots of breeding line No. 411 with cytometrically confirmed haploid number of chromosomes were placed on a solidified MS medium containing 5 g L^{-1} colchicine for 5 min [Pazuki et al. 2018] and then transferred onto MS media supplemented with 0.2 mg L^{-1} BAP and 1 mg L^{-1} IAA, on which roots have developed from shoots (Fig. 1 B, C). Plants with confirmed homozygosity were given to breeders, who included received plant material in their breeding programs (Fig. 1 D).

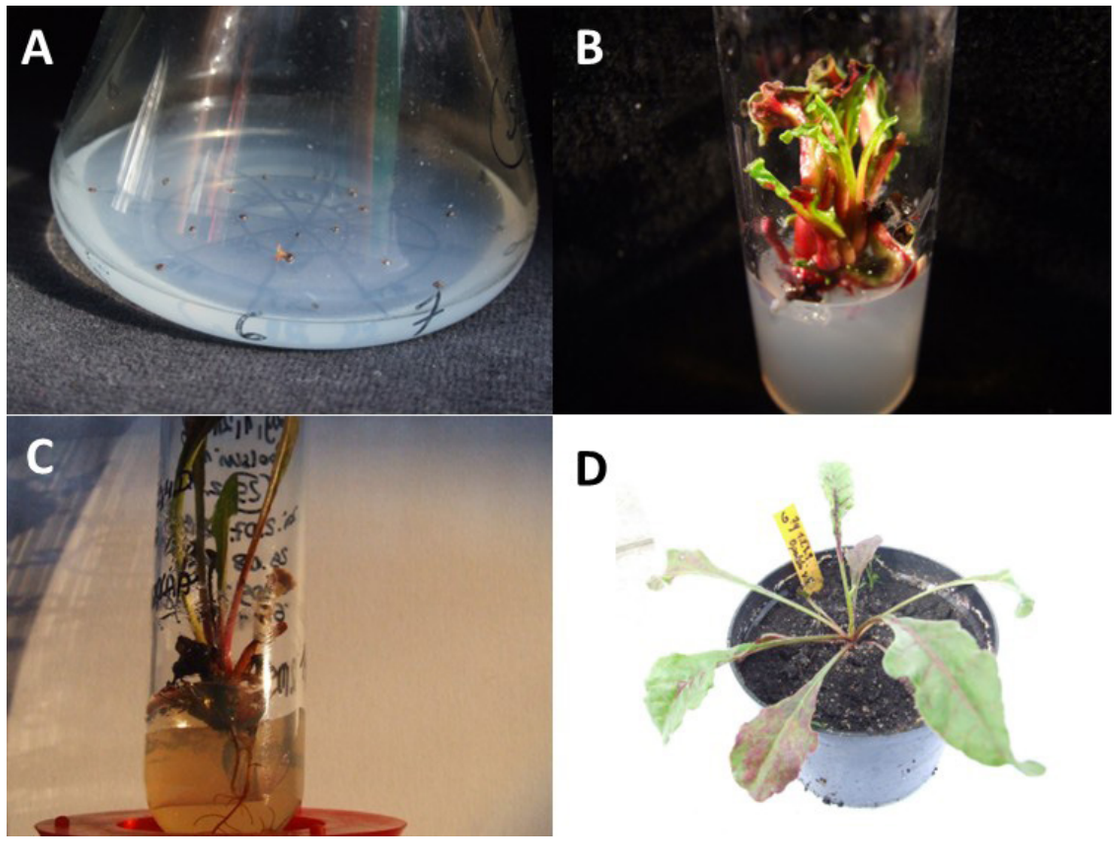

Fig. 1. The successive development stages of gynogenetic plants of red beet: A) gynogenetic embryo, B) regenerating plant, C) fully developed gynogenetic plant, D) acclimatized gynogenetic plants

Optimization of the protocol for gynogenetic plant production

Gynogenesis induction. Green, immature flower buds with unfolded petals of the cultivar and breeding lines were disinfected with 70% ethanol for 10 min and washed 2 times in sterile distilled water. Ovules were isolated from disinfected flower buds under a stereoscopic microscope. Using preparation needles, 24 ovules were placed in one Erlenmeyer flasks (100 mL) containing 30 mL of medium (media described below). All induction media were supplemented with 100 g L^{-1} sucrose and solidified with 6.5 g L^{-1} agar. The pH of all media was adjusted to 5.8 [Barański 1996]. The ovule cultures were kept at 27 °C at continuous light (24 hours a day) with photosynthetic photon flux density (PPFD) of 30 µmol m^{-2} s^{-1}. Formation of embryo-like structures (ELS) took place after 6–14 weeks. The efficiency of gynogenesis process was defined by the number of obtained ELS per 100 planted ovules (%).

Effect of genotype. In the first experiment, frequency of ELS formation was compared among all tested genotypes. Ovules were plated on the B5 [Gamborg et al. 1968] induction medium supplemented with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} IAA. This medium proved to be the most effective for inducing red beet gynogenesis in the preliminary studies conducted by the authors in the previous year.

Effect of medium composition. In the second experiment, the effect of medium composition on gynogenesis frequency was studied. Ovules of red beet Opolski were cultured on N6 media [Chu et al. 1975] or modified B5 (with the addition of 500 mg L^{-1} L-glutamine and 100 mg L^{-1} L-serine) supplemented with

0.1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) [Górecka et al. 2017] in the first variant or 0.2 mg L^{-1} BAP and 0.5 mg L^{-1} IAA in the second variant [Barański 1996, Górecka et al. 2017].

Plant regeneration. *Effect of the sucrose concentration*. The ELS were transferred to the media selected on the base of the preliminary studies, consisting of the N6 medium containing 0.2 BAP mg L^{-1}, B5 medium without hormones and MS medium supplemented with 1 mg L^{-1} TDZ with the addition of sucrose at concentrations of 10, 20, or 30 g L^{-1}.

All tested media were solidified with 6.5 g L^{-1} agar, pH adjusted to 5.6 [Ghosh et al. 2013]. ELS were cultured in a 30 mL tube containing 10 mL of medium, placed in a growth room and exposed to continuous light with Photosynthetic Photon Flux Density (PPFD) of 30 µmol m^{-2} s^{-1} [16 hours a day] at a temperature of 20 °C. Observations were made after six weeks of culture. One ELS was placed in each of the 10 tubes containing the tested media.

Effect of the PGR. In the next experiment, ELSs were transferred onto the MS regeneration medium containing 1 mg L^{-1} BAP with the addition of 30 g L^{-1} sucrose. Six weeks later, regenerating plants were placed on MS medium with BAP at a lower concentration of 0.2 mg L^{-1}, supplemented with various auxins, IAA or NAA each at the concentration of 1 mg L^{-1}. Observation of frequency and quality of regenerated plants was conducted after four weeks. At this stage, ploidy analysis was performed using a flow cytometer.

Acclimatization. Plants underwent the acclimatization process in order to conduct further studies on methods of chromosome doubling. Fully developed plants of red beet breeding line No. 411 were rinsed in distilled water after removing from tubes, dipped for a second in 2% Kaptan solution. Next seedlings in *ex vitro* conditions planted in multipots containing peat and sand medium (1:3, v/v), in high humidity conditions in a plastic tunnel localized in a growth chamber at a temperature of 20 °C during the day and 18 °C at night and the light intensity of 30 µmol m^{-2} s^{-1} for 16 hours. After 3–4 weeks, the plastic tunnel was gradually ventilated to reduce the humidity. In the fifth week, an observation of adapted plants was made. Plants that survived the acclimatization process were counted. In the final stage, adapted plants were transplanted to pots and cultured in the same growth chamber.

Ploidy evaluation. Flow cytometric analysis (FCM-DAPI) was performed on leaf samples taken from plant material. Samples of ca. 0.5 cm^2 of leaf blade were taken from the reference plants (donor diploid red beet cultivars) and regenerants. Plant tissue was ground in a Petri dish containing 0.5 mL of Partec buffer for nucleus isolation [Śliwińska 2008], with 1% polyvinylpyrrolidone (PVP-40), to which the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) was added (2 µg mL^{-1}). After adding 1 mL of isolation buffer, samples were filtered through a 30 µm filter and incubated at room temperature for 30–45 min in the dark. Fluorescence of nuclei was measured using CyFlow ploidy analyser (Partec, Germany) with software CyView (CyFlow PA, Partec), with UV-LED 365 nm. Sample measurements were performed for at least 1000 nuclei. The ploidy level was read on the histograms, expressed as the value of the position of the nuclear DNA fluorescence peak on the X axis. The external standard for determining the position of the DNA fluorescence peak on the X axis were the leaf samples of diploid reference plants. The position of the fluorescence peak for haploid plants should be within half of the value determined for diploid reference plants.

Homozygosity evaluation. Homozygosity was evaluated using isoenzymes and NGS for the selected obtained from 399, 426 and 521 plants of breeding line No. 411, which was produced using the optimized protocol developed in the first research stage.

Isoenzyme system. To assess homozygosity of 26 plants obtained in ovule cultures (genotype o. 411), two isoenzymes were analyzed: phosphoglucose isomerase (EC:5.3.1.9, PGI) and aspartato aminotransferase (EC 2.6.1.1, AAT) [Westphal and Wricke 1989, Kiszczak et al. 2011]. Electrophoresis was conducted on a 10% starch gel according to the Gottlieb [1973] method. Separation of enzymes was performed according to the Selander et al. [1971] protocol. Weeden and Gottlieb's [1980] method was used for visualization of polymorphism of tested isoenzymes.

RNA isolation. Three plants No. 399, 426 and 521 of breeding line No. 411, maintained in *in vitro* conditions have been used for this analysis. Total RNA used for the preparation of complementary DNA (cDNA) libraries (and further transcriptome analysis) was obtained from red beetroot plants grown *in vitro*. About 400 mg of leaves and stems were used for RNA iso-

lation from each one of three plant lines. Plant/fungi total RNA Purification kit (Norgen Biotek #25800) was used according to the procedure recommended by a manufacturer with a minor modification – added two extra washes of the column before elution of purified RNA. Eluted RNA was precipitated overnight at –20 °C after addition of 1/10 volume of 3 M sodium acetate, pH 5.2, and 2.5 volumes of cold (–20 °C) 99% ethanol. Purified RNA was pelleted by centrifugation (30 minutes at 14,000 rpm; 4 °C), washed twice with cold (–20 °C) 80% ethanol, dried at room temperature, resuspended in water. DNA was deigested using Turbo DNAse (Life Technologies kit #AM 1907) according to a standard procedure. RNA was precipitated, washed and resuspended finally in autoclaved MilliQ water (18.2 MΩ). Verification of the quality and concentration of the preparation was based on UV absorbance measurements in the range 140–220 nm (NanoDrop) and electrophoretic profile in a non-denaturing 2% agarose gel. Purified RNA was aliquoted and stored at –70 °C.

Sequencing of cDNA and construction of cDNA libraries. Preparations of total RNA have been sent to a commercial company Genomed S.A., (Warsaw, Poland). RiboZero cDNA libraries have been constructed there and sequenced on Illumina HiSeq platform d.

Bioinformatic analysis. Bioinformatic analysis was performed using CLC Bio Genomics Workbench software (QIAGEN (n.d.) https://digitalinsights.qiagen.com) and services like BLAST provided by NCBI. The raw reads have been trimmed and filtered for quality, then mapped to a reference transcriptome of sugar beet (*Beta vulgaris*) published by Dohm et al. [2014]. The reference consisted of 29,088 contigs of average length 1,526 nts.

Statistical analyses. A flask containing 48 ovules was treated as a repetition in conducted experiments. The number of repetitions varied in a particular experiment and was dependent on the availability of plant material. Obtained data were analyzed using ANOVA/MANOVA multivariate models and non-parametric analyses such as the Kruskal and Wallis [1952], at an adopted level of significance of $\alpha = 0.05$. Statistical analyses were performed using Statistica 8.0 software package for Windows (StatSoft Inc. Tulsa, USA).

RESULTS

Gynogensis induction

The highest percentage of gynogenetic ELS/100 ovules (58.3) was obtained in red beet breeding line No. 411 and the lowest in Opolski (0.9 ELS/100) (Table 1). No embryos were obtained in three breeding lines (RA-13, RA-14 and 406). The most effective medium for gynogenesis induction in red beet was B5 medium supplemented with 0.1 mg L^{-1} 2,4-D (Table 2). On this medium, 2.5 out of 100 planted ovules formed ELSs. More than twice less of these

Table 1. The influence of the genotype on the gynogenesis induction in ovule *in vitro* culture of red beet

Genotype	Number of cultured ovules	Number of obtained ELS	Number of responding ovules	Number of ELS per 100 ovules
RA-10	48	1	1	2.1b*
RA-11	48	1	1	2.1b
RA-12	48	1	1	2.1b
RA-13	24	0	0	0
RA-14	47	0	0	0
406	96	0	0	0
411	24	14	1	58.3a
Opolski	216	2	2	0.9b

* Combinations located in the same homogeneous group (with the same letter) do not differ statistically at a significance level of $\alpha = 0.05$. Kruskal-Wallis test.

Table 2. Effect of the medium on the gynogenesis efficiency in ovule cultures of red beet Opolski cultivar

Medium	Number		
	cultured ovules	ELS	ELS per 100 plated ovules
B_5 + 2,4D	80	2	2.5a*
B_5 + BA, IAA	216	2	0.9a
N_6 + 2,4D	128	2	1.6a
N_6 + BA, IAA	54	0	0.0a

* Combinations located in the same homogeneous group (with the same letter) do not differ statistically at a significance level of $\alpha = 0.05$. Kruskal-Wallis test.

structures were obtained on the same medium containing BA and IAA. Whereas, on N6 medium, in the presence of 2,4-D, 1.6 ELSs per 100 ovules were produced. No ELSs were formed on N6 medium containing BAP and IAA.

Plant regeneration

Regenerated shoots of various quality and/or callus formation were obtained after transferring gynogenetic ELSs with a different frequency depending on regeneration media (MS, N6 and B5) and sucrose concentration (10, 20 and 30 g L^{-1}) – Table 3. The highest number of shoots was obtained on MS medium containing 30 g L^{-1} sucrose, that is 2,88 average per 1 ELSs, also the highest number of callus (2,89 per 1 ELSs) was observed on MS medium. When this medium contained lower amount of sugar (10 and 20 g L^{-1}), approximately twice less shoots were obtained. No shoots developed from gynogenetic embryos on B5 medium; however, a small amount of callus formation was observed.

On the most effective regeneration medium (MS supplemented with 30 g L^{-1} sucrose) the effect of growth regulators (BAP in combination with IAA or NAA) on shoot development of red beet breeding line No. 411 was examined. Obtained results indicate that whole plants with a well-developed root system can be obtained on media supplemented with both types of auxin combined with BAP (Table 4). However, the higher number of well-developed plants was obtained on medium containing 0.2 mg L^{-1} BA and 1 mg L^{-1} IAA. All regenerated plants (18) of this cultivar were planted *ex vitro* and 39% survived the acclimatization process.

Ploidy evaluation

All tested plants of the Opolski red beet, as well as the 411 and 5/11 breeding lines contained the amount of DNA in the cell nuclei corresponding to the haploid number of chromosomes (Table 5, Fig. 2). Plants RA-5 breeding line consisted of DNA equivalent to a diploid number of chromosomes.

Homozygosity – isoenzyme analysis

Homozygosity analysis of gynogenetic plants from breeding line No. 411 showed that in case of PGI isoenzyme, 95% of examined plants were homozygotes and 5% were heterozygotes. Regarding the indole-3-acetic acid (AAT) isoenzyme, 70% of these plants were homozygous, 23% heterozygous and for the remaining 7%, due to the illegible bands polymorphism, we were not able to confirm their homozygosity.

Homozygosity – NGS

Ninety three percent of reads were successfully mapped for each from three tested genotypes, from which 83% to 85% was mapped in pairs. For the set of 29,088 reference transcripts with a total length of 44,686,800 nucleotides, the following fragments were mapped respectively: 16,530,673 fragments (total length of mapped fragments: 1,645,771,149 nts) for sample No. 399, 56,121,204 fragments (5,398,960,791 nts) for sample No. 426, 39,111,596 fragments (3,901,891,001 nts) for sample No. 521. The number of sugar beet transcripts, to which reads obtained for the samples of red beet were mapped (with the applied mapping parameters: 60%, 80%), is

Table 3. The effect of three sucrose concentrations (10, 20, 30 g L^{-3}) in three media (MS, N6, B5) on the regeneration of shoots from ELS formed by gynogenesis in red beet Opolski cultivar

Medium/sucrose concentration g L^{-3}	Number of cultures ELS	Multiplication – the average per 1 embryo			
		shoots without root			callus
		long more than 0.5 cm	long less than 0.5 cm	total number	
MS-10	62	0.40b*	0.83b*	1.23b*	2.78ab*
MS-20	58	0.30b	0.88b	1.18b	2.89a
MS-30	61	0.77a	2.11a	2.88a	2.56ab
N6-10	60	0.22b	0.11bc	0.33cd	1.11b
N6-20	63	0.11b	0.78b	0.89bc	1.44b
N6-30	59	0.00b	0.00c	0.00d	2.00ab
B5-10	61	0.00b	0.00c	0.00d	1.00b
B5-20	58	0.00b	0.00c	0.00d	1.00b
B5-30	58	0.00b	0.00c	0.00d	1.00b

* Combinations located in the same homogeneous group (with the same letter) do not differ statistically at a significance level of α = 0.05. Kruskal-Wallis test.

Table 4. The effect of PGR (BA 0.2 mg L^{-1}, IAA 1 mg L^{-1}, NAA 1 mg L^{-1}) on the number of obtained regenerants from gynogenetic embryos of red beet on MS medium (breading line 411) – the average per 1 embryo

Medium	Number of cultures ELS	Frequency of plant regeneration		
		shoots		without regeneration
		with root	without root	
BA, IAA	63	0.25a*	1.31a	0.02a
BA, NAA	51	0.04b	1.68a	0.06a

* Combinations located in the same homogeneous group (with the same letter) do not differ statistically at a significance level of α = 0.05. Kruskal-Wallis test.

presented in Table 6. A list of observed variants was made for every tested plant (in a simplified form: the differences in sequence in comparison with the reference transcripts).

Bioinformatic analysis

The total number of 100 nt paired reads obtained for three analyzed plants: No. 399, 426 and 521, was 17,755,074, 57,828,394 and 41,841,448, respectively.

The percentage of reads mapped successfully was 93.10%, 93.59%, and 93.48% of their total number for three samples. From 83.03% to 84.98% reads were mapped in pairs, with the observed distance in pairs from 83–334 nt, which increased their effective length. Library reads were mapped (separately for every sample) based on the sequence of 29,088 transcripts read for sugar beet [Dohm et al. 2014].

During analysis, the possibility of the occurrence of sequencing errors was taken into consideration, therefore an advanced software with complex algorithms was used for the elimination or reduction of those errors. The possibility of the presence of several copies of the same genes was also considered. In the course of analysis, 172,710 potential variants diversifying transcriptome of sugar beet and tested breeding lines of red beet were identified, which in conclusion

Table 5. Ploidy evaluation of gynogenetic plant material conducted during the multiplication of red beet plants

Genotype	Number of rosettes	Ploidy			
		1x		2x	
		number	%	number	%
Opolski	18	18	100	0	0
411	24	24	100	0	0
RA 5	18	0	0	18	100
5/11	2	2	100	0	0

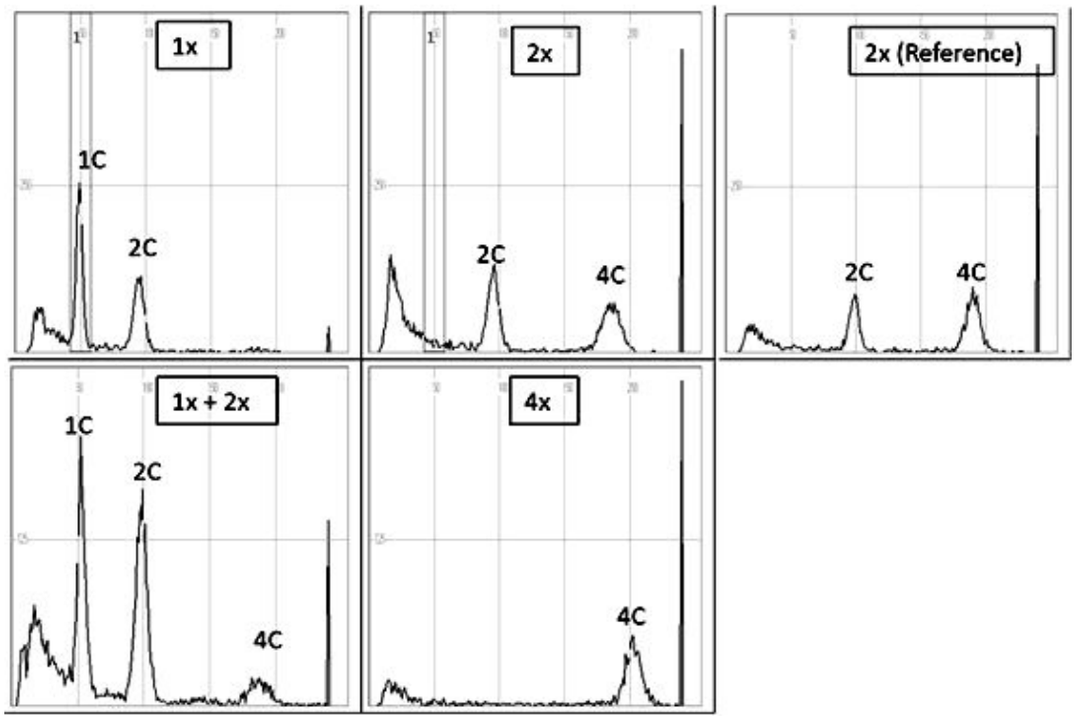

Fig. 2. Sample histograms of cytometric analysis of haploid (1x), diploid (2x), mixoploid plants with a haploid and diploid genomes (1x + 2x), tetraploid (4x) plant of the red beet line 3/2010 and diploid reference plant (donor plant Czerwona Kula)

gave 86,355 potential sites of difference. For more than 95% of those spots, the heterozygosity of tested plants was not specified (399, 426 and 521). The remainder of the sequence was fully homozygotic. Approximately 20,000 identical single positions were examined within the reference transcriptome. During the analysis, all chromosomes nine were identified by at least 400 transcriptomes (genes or their fragments). For each chromosome of tested breeding line of red beet at least 4,000 variants (SNV, MSV or ins/del) were analyzed for the homozygosity (Table 7).

DISSCUSION

In red beet, a significant influence of genotype on the efficiency of gametic embryogenesis was confirmed. Barański [1996] observed gynogenesis in all tested red beet cultivars, but the frequency of embryo

Table 6. The number of transcripts, on which the sequence reads were mapped for the tested red beet breeding line no 411. The total number of reference transcripts: 29,088

Plant individuals	The number of reads mapped on transcripts		
	>0	>10	>100
399	23,310	19,644	13,291
426	24,930	22,532	17,703
521	25,128	22,853	18,148

Table 7. The number and the character (heterozygosity/homozygosity) of the discovered sequence variants observed in the transcriptome of three plants line no 411 of red beet at various limits of the number of single sequence reads in the place of the occurrence of tested variant

Plant individuals	The number of all nucleotide/nucleotides reads including the variant		
	>0	>20	>200
	hetero*/in total	hetero*/in total	hetero*/in total
399	8,392/88,306	3,747/45,483	330/4,377
426	18,333 /163,238	12,870/111,444	701/17,318
521	18,750 /172,710	12,446/113,927	985/13,836

* The numbers presented in the table as a „hetero" are referring to the number of possible variants. The amount of places of their occurrence in the analyzed transcripts of red beet was at least two times lower.

formation was dependent on genotype and ranged from 0-2.86%.

In 2021, Zayachkovskaya et al. obtained a higher induction factor dependent on the genotype, up to 25% of induced ovules. The highest gynogenesis efficiency of 33% was obtained by Kiszczak et al. [2023]. In their studies, the number of obtained gynogenetic embryos was dependent on the genotype. In presented studies, we also confirmed that the efficiency of gynogenesis depends on the genotype. We obtained embryos in several genotypes. In the breeding line, we found the presence of embryos in over 58.3% ovules, but e.g. in the RA-13 line, no gynogenetic sources were observed.

Medium composition is one of the most important factors in the induction of haploids either in the process of androgenesis or gynogenesis. Barański [1996] noted that the use of N6 medium supplemented with 0.5 mg L^{-1} IAA and 0.2 mg L^{-1} BA was the most effective in red beet embryo formation from ovules. We did not receive any gynogenetic embryos on this medium, while the highest number of embryos was received on B5 medium with the addition of 2,4-D. The above-mentioned auxin has the best ability to induce cell divisions and callus differentiation [Zheng et al. 1999] and its usability for inducing gynogenesis process in plants was confirmed by various authors [Rekha et al. 2013, Alan et al. 2016]. In the studies presented by Kiszczak et al. 2023, this auxin added to B5 medium did not cause a significant increase in the number of gynogenetic embryos, but increased on the N6 medium. However, in these experiments, authors obtained most of embryos on cultures conducted on the media with the addition of polyamines. In our study, in the presence of 2,4-D, considerably fewer embryos were obtained on N6 medium compared to B5 medium.

Plant regeneration is the next very important stage in the process of obtaining DH plants via gametic embryogenesis [Górecka et al. 2009, Kiszczak et al. 2015]. Medium is one of the main factors affecting the efficiency of plant regeneration in this process [Se-

gui-Simarro and Nuez 2008, Wędzony et al. 2009]. In 2017, Pazuki carried out the regeneration process in one stage using MS medium, but with the addition of BAP, that resulted in 18.98% of plants. Direct germination and formation of microrosettes occurred when the embryoid was placed on regenerating MS medium with the addition of 1 mg L^{-1} BAP and 0.1 mg L^{-1} GA_3 [Zayachkovskaya et al. 2021]. However, in these experiments, the shoots did not develop or developed roots poorly, therefore additional passages on the hormone-free MS medium were performed. Other researchers obtained direct regeneration into plants red beet on MS medium supplemented with 0.2 mg L^{-1} BA and 1 mg L^{-1} IAA, but this method was inefficient [Kiszczak et al. 2023]. Therefore, regeneration from callus was conducted in two stages. Authors regenerated shoots on MS medium supplemented with BA and 0.5 mg L^{-1} putrescine, afterwards rhizogenesis was conducted on MS medium containing ½ MS macronutrients and supplemented with NAA at the concentrations of 1 or 3 mg L^{-1} and Put at 0.5 or 160 mg L^{-1}. In our studies, higher numbers of fully developed plants (reaching 10%) were obtained on MS medium, compared to N6 and B5 media. This confirms that MS-based media are the most suitable for regeneration of plants from gynogenetic embryos in red beet. The authors applied the standard of 30 g L^{-1} of sucrose. The increased presence of callus in our studies was due to the application of 0.2 mg L^{-1} BAP and 1 mg L^{-1} NAA for the regeneration of red beet embryos. Similar results were demonstrated earlier by Gürel et al. [2000]. Authors applied the same combination of two growth regulators in the concentration of 1 mg L^{-1} BAP and 0.5 mg L^{-1} NAA. They observed a higher amount of formed callus comparing to other media used for the regeneration in *in vitro* cultures of sugar beet.

In presented studies, only haploids underwent the acclimatization process, which in general are characterized by lower vigor [Murovec and Bohanec 2012]. In case of sugar beet, Gośka et al. [2004] selected only diploid gynogenetic plants for the acclimatization, which allowed approximately 95% of plants to adapt to *ex vitro* conditions. In 2010, Tomaszewska-Sowa acclimatized almost 80% of gynogenetic plants of sugar beet. Some authors are emphasizing the special significance of the root system for the efficiency of acclimatization [Salvi et al. 2002]. Our observations of the acclimatization process of carrot androgenetic plants [Kiszczak et al. 2018] and current studies on the gynogenetic red beet regenerants confirm this thesis. It is most likely that one of the reasons a low percentage of plants in our experiments adapted was the very poor root system of haploid plants.

Our research has shown that the tendency to spontaneously double the chromosome number was strongly dependent on the genotype. All the gynogenetic plants (18 pcs.) of Opolski red beet and two breeding lines RA 5, 5/11 were haploids, whereas in one breeding line 4/11 and 411 all 24 pcs. gynogenetic plants were diploids. The emergence of breeding line with a doubled set of chromosomes is probably related to the occurrence of the phenomenon of endoreduplication [Joubes and Chevalier 2000]. Strong DNA endoreduplication was also observed during flow cytometry analysis in our study. Lukaszewska et al. [2011] observed this phenomenon in *in vitro* cultures of sugar beet. Authors showed that the application of medium with NAA at a concentration of 1 mg L^{-1}, the same concentration as used in our experiments, intensified the process of endoreduplication. These observations indicate that doubling the chromosome number may be associated with tendency for a given genotype to endoreduplication.

During the homozygosity analysis with the use of two isoenzymatic systems, PGI and AAT, the polymorphism that allowed recognition of homozygote from heterozygote was obtained for the PGI isoenzymatic system. Sabir et al. [1992] showed the usability of this isoenzymatic system for the analysis of the somaclonal variation frequency in plant material of sugar beet and chard, propagated *in vitro*. Authors also observed polymorphism in the PGI system, whereas the AAT system did not generate any variations in the bands. Ludina and Levites [2003] assigned the absence of the polymorphism for the malate dehydrogenase isoenzyme in the studies on the population of sugar beet to the not-allelic character of isoenzymes located in various cellular organelles. This finding indicates that genes of an isoenzyme, such as AAT, can be inherited with deviation from standard Mendel's law. The appearance of a heterozygotic pattern of bands for both isoenzymes in tested population, may be due to the reasons explained above, also described by Levites et al. [2005]. In conducted studies, authors demonstrated

that spontaneous polyploidization caused by their prolonged culturing occurs in the haploid tissues of sugar beet under *in vitro* conditions. According to their results, the emergence of heterozygotes in polymorphic populations regarding the isocitrate dehydrogenase and 6-phosphogluconate dehydrogenase isoenzymes in combination with the simultaneous homozygotic profile for the other isoenzyme in the same plants indicate the occurrence of spontaneous polyploidization.

Evaluation of the homozygosity of three red beet plants [399, 426 and 521 breeding lines] was performed on the basis of the transcriptome analysis [read with the use of the high-throughput sequencing and NGS] in terms of the occurrence of different variants of nucleotide sequences [SNV, MNV, ins/del]. Results presented in Table 1 indicate that even in consideration of only reference transcripts, for which at least 100 mapped reads were obtained [approximately 100 nt each], conducted analysis included from 45% to 62% of potential red beet genes. High percentage [93%] of mapped reads, when adding the reads mapped in pairs with the distance in line with the expectations, indicates the high reliability of obtained results. Lower percentage of mapped reads was obtained by various researchers in other plant species, for example Wang et al. [2016] achieved 70% of mapped reads in corn. Obtained results are considered to be significant only when 95% of genes are mapped in comparison with cDNA databases [Claros et al. 2012]. Analysis was performed on the transcripts originated from all red beet chromosomes, which allowed for the detection of potential aberrations during the chromosome duplication. The occurrence of well documented (over 200 single reads) cases of simultaneous presence of two variants (heterozygosity) was discovered on every tested chromosome. However, the overwhelming part of the genome had a homozygotic character (Table 2). The appearance of false segmental duplications in the assemblies, which occurs when heterozygous sequences from two haplotypes are assembled into separate contigs and are scaffolded adjacent to each other rather than being merged, this is the main problem during the analysis of the material derived from a heterozygotic plant [Kelley and Salzberg 2010]. This can also be referred to as the process of spontaneous doubling of chromosomes that occur while obtaining plants through gynogenesis. Therefore, part of the 200 single reads may be incorrectly categorized, which in reality leads to the appearance of a greater number of homozygotic variants. It should be emphasized that applied method was considerably more sensitive to the detection of differentiation variants (heterozygosity) in the tested genomes in comparison to the classic methods.

CONCLUSION

The influence of individual factors on the gynogenesis process was determined in the presented studies and their optimal range for obtaining the highest number of doubled haploids of red beet. A significant influence of genotype on the efficiency of red beet ovule cultures was confirmed. In conducted experiment the most effective medium for gynogenesis induction proved to be the B5 medium containing 0.1 mg L^{-1} 2,4-D. Based on results obtained after analyzing the influence of various media base it was shown that MS containing 30 g L^{-1} sucrose was the best medium. It was also proven that the gynogenesis process was the most effective on the media with the addition of 0.2 mg L^{-1} BA and 1 mg L^{-1} IAA. The study of the nuclear DNA content at the stage of multiplication of the genotype 411 and after acclimatization of the genotype 5/11 showed that they are haploids. The analysis of polymorphism of two isozymes PGI and AAT demonstrated that the majority of the regenerants displayed the homozygous band pattern. To confirm obtained results, next generation sequencing was performed, within which 3 tested genotypes were mapped using sugar beet transcripts. Subsequently, bioinformatic analyzes were performed based on the obtained transcripts. During the analysis, all chromosomes (nine) were identified by at least 400 transcriptomes (genes or their fragments). For each chromosome of tested breeding line of red beet at least 4,000 variants (SNV, MSV or ins/del) were analyzed for the homozygosity.

Significance statement

Optimal parameters of each factor were defined for obtaining haploid plants of red beet in gynogenesis process. Ploidy analysis confirmed the presence of haploid variants among the obtained multiplications.

The gametic origin of these plants was confirmed after obtaining homozygotic polymorphism systems

of PGI and AAT isoenzymes during the study of regenerants. For the first time, mapping of regenerants of the three studied genotypes to sugar beet transcripts was carried out and a picture was obtained confirming that the predominant part of the genome was homozygous. Application of different evaluation methods confirmed the gametic origin of regenerants obtained by gynogenesis in beet ovule cultures.

AUTHORS' CONTRIBUTIONS

Waldemar Kiszczak, Maria Burian, Tadeusz Malinowski, Małgorzata Podwyszyńska, Krystyna Górecka contributed to the study conception and design. Material preparation, data collection and analysis were performed by Waldemar Kiszczak, Krystyna Górecka and Maria Burian. The first draft of the manuscript was written by Waldemar Kiszczak, Krystyna Górecka and Małgorzata Podwyszyńska and they authors and Marcin Domaciuk read and approved the final manuscript.

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