Development of embryoids by microspore and anther cultures of red beet (*Beta vulgaris* L. subsp. *vulgaris)*

Powstawanie zarodków w kulturach mikrospor i pylników buraka czerwonego (*Beta vulgaris* L. subsp. *vulgaris)*

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Abstract

So far there is no information about receiving red beet androgenic embryos by androgenesis. Several factors were tested which affected this process: starch accumulation in microspores, correlation between bud length and microsporogenesis course, induction and regeneration medium composition. Ploidy level of obtained regenerants were evaluated. Treating anthers with α-amylase or watering donor plants with gibberellin increased number of obtained androgenic embryos. The highest percentage (80%) of microspores at uninuclear stage appeared in buds with 1.3-1.5 mm. The B_5 medium with 100 g·L^{-1} sucrose and 0.1 mg·L^{-1} 2,4-D (2, 4-dichlorophenoxyacetic acid) proved to be better for inducing androgenesis than MS medium supplemented with 0.2 mg·L^{-1} BAP (6-benzylaminopurine) and 0.5 mg·L^{-1} IAA (indole-3-acetic acid). First androgenic embryos were placed on B_5 medium without plant growth regulators and then on MS medium containing 0.2 mg·L^{-1} BAP and 1 mg·L^{-1} NAA (α-naphthaleneacetic acid). Androgenic embryos died on B_5 regeneration medium without plant growth regulators. On MS medium first shoots and callus with and without roots were obtained. Rosettes withered during following passages whereas callus tissue developed further. The quantity of DNA in this tissue equivalent to 4X chromosomes.

Keywords: androgenesis, anther culture, isolated microspore, microsporogenesis, ploidy, red beet

Streszczenie

Do tej pory nie udało się uzyskać zarodków na drodze androgenezy gametycznej u buraka ćwikłowego. W doświadczeniach przebadano kilka czynników wpływających na ten proces: nagromadzenie skrobi w mikrosporach, korelację długości pylnika do przebiegu mikrosporogenezy, skład pożywki do indukcji i regeneracji. Oceniono ploidalność uzyskanych regeneratów. Traktowanie pylników α-amylazą oraz podlewanie roślin donorowych gibereliną wpłynęło na wzrost liczby uzyskanych pylników. Badając przebieg mikrosporogenezy stwierdzono, że najwięcej mikrospor w fazie jednojądrowej znajdowało się w pąkach o długości 1.3-1.5 mm. Pożywka B_5 zawierająca 100 g·L^{-1} sacharozy oraz 0.1 mg·L^{-1} 2,4-D (kwas 2,4-dichlorofenoksyoctowy) okazała się korzystniejsza do indukcji androgenezy u buraka czerwonego w stosunku do pożywki MS wzbogaconej 0.2 mg·L^{-1} BAP (6-benzyloaminopuryna) oraz 0.5 mg·L^{-1} IAA (kwas indolilooctowy). Uzyskane zarodki androgenetyczne umieszczono na pożywkach regeneracyjnych: B_5 bez dodatku substancji wzrostowych oraz na pożywce MS zawierającej 0.2 mg·L^{-1} BAP i 1 mg·L^{-1} NAA (kwas α-naftylooctowy). Na pożywce B_5 zarodki zamarły. Natomiast pierwsze pędy oraz kalus z korzeniami lub bez zregenerowano na pożywce MS. Pędy po następnym pasażu zamarły. Badanie cytometryczne wykazało, że zregenerowana tkanka kalusowa była tetrapliodalna.

Słowa kluczowe: androgeneza, burak czerwony, izolowane mikrospory, kultury pylnikowe, mikrosporogeneza, ploidalność

Introduction

The best for industrial purposes are hybrid beet varieties characterized by high yields and size uniformity. Obtaining homozygous parental lines for hybrid breeding by traditional methods is time-consuming and difficult because of the occurrence of inbreeding depression. Application of *in vitro* androgenesis allows to shorten the period required to obtain new varieties (Bajaj, 1990). There are a lot of reports about the induction of androgenic androgenic embryos in suger beet with the use of gynogenesis. Previously, homozygous plants of red beet were successfully obtained only by gynogenesis (Barański, 1996). Therefore, it was decided to try to derive homozygous plants of red beet by using androgenesis despite the fact that there was no information in the world literature about obtaining androgenic androgenic embryos and plants of this species with the use of androgenesis. The aim, therefore, was to determine the external indicators of microspore development stages optimal for androgenesis induction, medium composition, plant regeneration and ploidy evaluation.

Materials and methods

In order to implement the experiments material for anther and isolated microspore cultures consisted of flower buds of the commercial varieties ‘Opolski’, ‘Karmazyn’,

and ‘Czerwona Kula’, and plant breeding line. Donor plants were grown in a growth chamber at temperature recommended by Tsao and Lo (2006) +18 °C during the day (16-hour) and +16 °C at night (8-hour).

The course of microsporogenesis was studied in “Czerwona Kula” cultivar. Buds with not parted petals were collected in three size ranges: 0.9-1.2 mm, 1.3-1.5 mm, and 1.6-2.0 mm. Attempts were made to eliminate the accumulation of starch grains in microspores, which prevented observations of their internal structure. Buds taken from donor plants were soaked for 4 hours in human alfa-amylase (from barley,type II-B, Sigma-Aldrich, 200 $mg \cdot L^{-1}$ in distilled water) in an incubator, in the dark, at +36 °C. Then buds were crushed, stained with acetocarmine and left in the light for 1-2 hours and subsequently examined under a light microscope with immersion at a magnification of 1000x. During the examination the following microspore developmental stages were distinguished: tetrad, uninuclear, binuclear and unidentified (Figure 1 C, D, E).

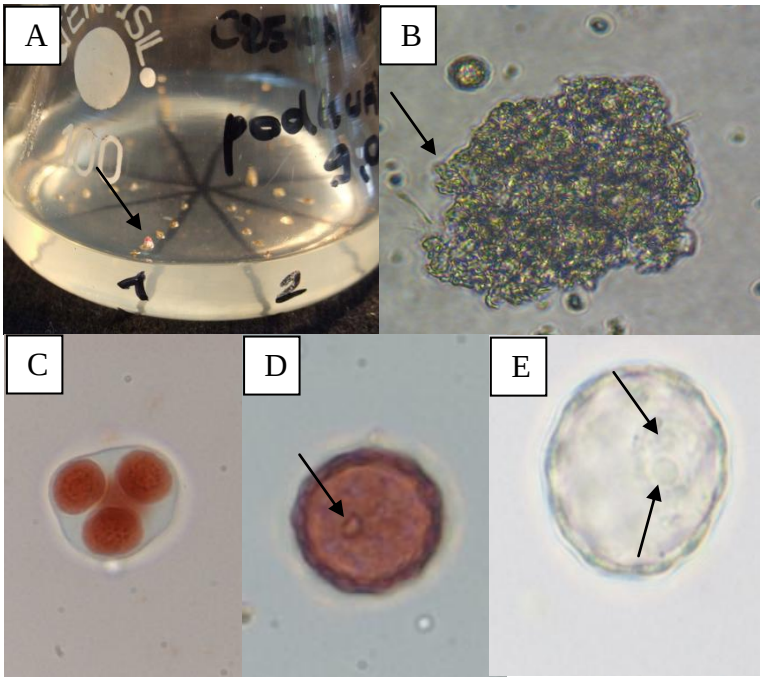

Figure 1. A – anther culture of var. Czerwona Kula, 4 week of culture, B – multicellular structures in isolated microspore culture, C - tetrad of microspores, D - microspore in uninuclear stage, E - microspore in early binuclear stage

Rycina 1. A – zarodek w kulturach pylnikowych (stadium globularne), B – wielokomórkowe globularne i embrioidalne struktury w kulturach izolowanych mikrospor, C – tetrada mikrospor, D – mikrospory w stadium jednojądrowym, E – mikrospory we wczesnym stadium dwujądrowym

To eliminate the numerous starch grains from microspores which hinders the induction of androgenesis, two methods were used on 3 varieties and one

commercial line: watering donor plants with a solution of gibberellin (GA_3) and alfa-amylase treatment of anthers. A solution of GA_3 in a concentration 50 mg·L^{-1} H_2O and volume of 250 ml was applied twice a week with per bucket from the time of planting. In the second method anthers isolated from flower buds were placed on sterile filter papers moistened with a sterile solution of α-amylase at 3 mg in 80 ml H_2O (from barley malt, type VIII-A, Sigma-Aldrich) **,** for 2 minutes and then transferred onto the medium for inducing androgenesis. The control for this experiment were anthers not treated with α-amylase collected from plants grown in a growth chamber and regularly watered.

For sterilization of flower buds for anther cultures, 70% ethanol for 2 minutes or 70% ethanol for 2 minutes followed by 0.1% $HgCl_2$ + 0.1% Tween 20 for 8 minutes were used and rinsing three times in sterile distilled water. Anthers and microspores were isolated from sterilized flower buds according to the procedures used for carrot (Górecka et al., 2005; Górecka et al., 2010).

To establish anther cultures, two size ranges of buds were used (0.9-1.2 mm and 1.3-1.5 mm). Anthers in sterile conditions were placed in flasks with medium. To induce androgenesis in anther cultures were used: B_5 media (Gamborg et al., 1968) with different sucrose content (140 g·L^{-1} and 100 g·L^{-1}) supplemented with 0.1 mg·L^{-1} 2,4-D; MS media (Murashige and Skoog 1962) with a sucrose content of 100 g·L^{-1}, supplemented with 0.1 mg·L^{-1} 2,4-D, 0.2 mg·L^{-1} BAP and 0.5 mg·L^{-1} IAA, both set with 6.5 g·L^{-1} agar, pH 5.8. Anther cultures were kept at a temperature of +35 °C for 24 h and then were carried out in the darkness at +24 °C (Nagl et al., 2004). To obtain a larger number of androgenic embryos in the following experiment anthers of “Czerwona Kula” variety were laid out on medium.

For sterilization of microspore cultures 70% ethanol was also used, but the duration of treatment was 10 minutes. In the second method for microspore cultures, colloidal silver (non ionic, 50 ppm, Nano Tech) at 2 ml·L^{-1} was added to the medium. In each treatment, the plant material was rinsed twice with sterile distilled water. Inflorescences of “Czerwona Kula” variety with undeveloped buds were homogenized in liquid medium and then centrifuged.

Obtained suspension was brought to microspore concentration of 120 thousand/ml in medium and pour into 24 Petri dishes. For this purpose following media were used: B_5 medium containing 100 g·L^{-1} sucrose and 0.1 mg·L^{-1} 2,4-D, pH 5.8, and a medium supplemented with β-amylase (from barley,type II-B, Sigma-Aldrich) at 70 mg·L^{-1}. Isolated microspore cultures were conducted in the darkness at a constant temperature of +27 °C applied by Górecka et al. (2010) to carrot isolated microspores cultures.

After about 4 weeks of anther and microspore culture, the emerging regular androgenic embryos as well as embryoidal (in globular, heart-like stage of development) structures were counted (Figure 1 A, B). Androgenesis efficiency was calculated as the number of embryoids obtained per 100 cultured anthers in anther culture or 1 embryo per one petri dish. Obtained embryoids were transferred onto regeneration media: B_5 without plant growth regulators and MS containing 0.2 mg·L^{-1} BAP and 1 mg·L^{-1} NAA. After 4-6 weeks, a passage was performed onto a medium with the same composition.

Obtained regenerants underwent ploidy examination with the use of flow cytometer. Samples were prepared in accordance with the recommendations of Galbraith (1984) with slight modifications. Approx. 20 mg of young leaves were finely divided with a razor blade in a Petri dish in the presence of 2 ml buffer for cell lysis CyStain® DNA and with 0.1% PVP-40. After the addition of fluorochrome DAPI – 4',6-diamidino-2-phenylindole, in the amount of 0.1 mg in 1 ml, the suspension was filtered through a Partec CellTrics® nylon filter with 50 µm pores (Partec GmbH, Münster, Germany). Samples prepared in this way were analyzed using a Partec CA-II flow cytometer (Partec GmbH, Münster, Germany).

Obtained data were analyzed using multivariate statystic ANOVA/MANOVA non-parametric analyses such as the par 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). A flask containing 48 anthers or a Petri dish with the microspore concentration of 120 pieces per ml of medium was counted as a repetition. The number of repetitions for each experiment was different and depended on the number of buds produced by donor plants. The exact numbers of used anthers have been given in the Table 1.

Results and discussion

In this study, androgenic embryos were successfully obtained in anther cultures of red beet in Czerwona Kula, Opolski varieties and 1 breeding line. In isolated microspore cultures, 4 multicellular structures were formed in 3 Petri dishes from 24 established dishes from Opolski variety. This is the first information on the subject of obtaining androgenetic androgenic embryos of this plant because, as already stated in the introduction, there is no information on this topic in the available literature.

Embryo induction in plant anther cultures requires optimal parameters of factors indicating the changes in development trail of gametic cell, transferred their development from gametic to sporophytes orientation. Many factors are responsible for successful anther culture (Silva, 2012). The nuclear stage of microspore is a key factor for embryogenic response and the mid-uninucleate stage to the early binucleate stage is the responsive stage in most cases (Don Palmer and Keller, 2005).

Many authors demonstrated that the length of anthers is good indicator of microspore development stage course. During the stages of microsporogenesis observed in red beet buds not treated with amylase, it was noted that 80% of microspores in tetrad stage occurred in the 0.9-1.2 mm range of bud length. In the other ranges of bud length, starch grains hampered the observation of microspore development phases.

After application of human α-amylase solution, in which starch grains were disintegrated, it was found that in buds with a length of 0.9-1.2 mm, most of microspores (80%) were at tetrad stage. In buds 1.3-1.5 mm long, those at the uninuclear stage accounted for more than 80%, and the binuclear ones for 15%. Uninuclear microspores also predominated in buds longer than 1.5 mm (58%). Based on the results, it was ascertained that bud length was correlated with the developmental stage of microspores, and was therefore a good indicator. For many

plants, the uninuclear stage is the most suitable for stimulating the development of androgenetic androgenic embryos (Górecka et al., 2009).

A strong accumulation of starch grains was found in microspores, which prevented from establishing polarity necessary for determining the site of sporoderm rupture and the orientation of the androgenic embryo body axis (Dubas et al., 2010).

Asatsuma et al. (2005) found that α-amylase plays an important role in the breakdown of starch in leaves of rice. In this study, the use of α-amylase caused the disintegration of starch grains in microspores cultured on induction medium, which resulted in embryo formation in anther cultures of 'Opolski' cultivar and X breeding line.

This was in contrast to isolated microspore cultures, where α-amylase did not induce embryo formation. Munekata and Kato (1957) showed that GA_3 markedly stimulated the activity of α-amylase. Therefore, treating donor plants with GA_3 caused an improvement in embryo formation in anther cultures of 'Czerwona Kula' cultivar, while from anthers of 'Karmazyn' cultivar, despite the application of different methods of eliminating starch grains from microspores, no androgenic embryos were obtained (Table 1).

Table 1. Effect of the methods used to eliminate starch grains in microspores on the induction of embryoids in anther cultures of red beet

Tabela 1. Wpływ różnych metod usuwania ziaren skrobi z mikrospor na powstawanie zarodków w kulturach pylnikowych buraka czerwonego

Genotype	Treatment	Number of laid out anthers	Number of embryoids	Number of embryoids per 100 laid out anthers
'Opolski'	control	642	1	0.2 b[a]
	α-amylase (anthers)	202	1	0.5 a
'Karmazyn'	control	724	0	0.0 d
	α-amylase (anthers)	160	0	0.0 d
'Czerwona Kula'	control	518	0	0.0 d
	α-amylase (anthers)	818	0	0.0 d
	watered with gibberellin	777	7	0.6 a
'Line X'	control	424	0	0.0 d
	α-amylase (anthers)	842	1	0.1 c
	watered with gibberellins	756	0	0.0 d

[a] Combinations within the same homogenous group (having the same letter) do not differ significantly from each other at a significance level of α = 0.05. Wilcoxon signed-rank test.

[a]Kombinacje znajdujące się w tej samej grupie jednorodnej (oznaczone tą samą literą) nie różnią się statystycznie istotnie między sobą przy poziomie istotności α=0.05. Test Wilcoxona.

Effective sterilization of plant material introduced to *in vitro* culture should be characterized by full elimination of infection factors (contaminations) from material and the lack of negative effects for plant material. In conducted experiment more effective in sterilizing flower buds for anther cultures proved to be the method of treating with 70% ethanol for 2 minutes, then with 0.1% $HgCl_2$ + 0.1% Tween 20 for 8 minutes, followed by rinsing three times in sterile distilled water. By contrast, while establishing isolated microspore cultures, the most effective proved to be sterilization of flower buds with 70% ethanol for 10 minutes, followed by rinsing twice in sterile distilled water and addition of colloidal silver to the medium. Soltanloo et al. (2010) emphasize the need to select the most appropriate concentration of colloidal silver so that it fulfills the dual role of a sterilizing agent and an agent stimulating the growth of cultures.

In the studies of androgenesis in vegetable crops, extensive experiments were conducted in order to develop the right medium for inducing this process. In the case of head cabbage, a significant improvement was obtained in the effectiveness of embryogenesis in anther cultures by increasing the concentration of sucrose to 140 $g \cdot L^{-1}$ (Górecka, 1998). Metwally et al. (1998) were using sucrose at 30 to 150 $g \cdot L^{-1}$ in a medium for inducing androgenesis in *Cucurbita pepo* and obtained the best results when the concentration of sucrose was 150 $g \cdot L^{-1}$. In red beet, increased sucrose concentrations did not produce positive effects. In the present study, in red beet anther culture of “Czerwona Kula” cultivar increased sucrose concentrations did not produce positive effects. Embryoids were obtained on B_5 medium supplemented with 100 $g \cdot L^{-1}$ sucrose and 0.1 $mg \cdot L^{-1}$ 2,4-D. The number of 384 anthers were placed on this medium, on which 40 embryoids in globular and heart-like stage of development arised. No embryoids were formed on 432 anthers laid out on MS-based media. Eight androgenic embryos transferred onto B_5 regeneration medium without plant growth regulators died and that is why this medium was not used again. Other thirty two androgenic embryos regenerated on MS medium containing 0.2 $mg \cdot L^{-1}$ BAP and 1 $mg \cdot L^{-1}$ NAA. On this medium 3 small rosettes without root system were received, also 3 callus clumps with roots and 18 clumps without roots. Other androgenic embryos died (Table 2). During following passages all rosettes turned black and died, only callus multiplication survived originating from 1 embryo. Gurel and Gurel (1998) in their experiments showed that application of medium supplemented with 1 $mg \cdot L^{-1}$ BAP and 0.2 $mg \cdot L^{-1}$ NAA caused a significant increase in the number of forming callus, which is the basis for morphogenesis of *in vitro* shoots and roots in suger beet ovary cultures. In conducted studies small rosettes were regenerated, which subsequently blacken and died. This problem occurred during studies conducted by Klimek-Chodecka and Barański (2013). Authors noticed genotype relation in respect of this feature in DH plants derived from suger beet gynogenesis.

Table 2. Red beet anther culture of “Czerwona Kula”

Tabela 2. Kultury pylnikowe buraka czerwonego, odmiana “Czerwona Kula”

Stages of androgenesis	Number of laid out anthers and obtained different category of explants	
	B_5 induction medium	MS induction medium
Anthers laid out (Cultured anthers)	384	432
Obtained embryoids	40	0
Died embryoids	8	0
Embryoids passaged on MS regeneration medium	32	0
Died embryoids after passage	8	0
Regenerated rosettes without roots	3	0
Regenerated callus clumps with roots	3	0
Regenerated clumps without roots	18	0
Died rosettes without roots (next pass.)	3	0
Died callus clumps with roots (next pass.)	18	0
Survived clumps without roots	1	0

Ploidy analysis of obtained callus revealed 4x chromosome number, which indicated that endoreduplication occurred repeatedly in this tissue. Śliwińska and Łukaszewska (2005) discovered occurrence of nuclear DNA reflecting the 8x number of chromosomes in forming roots and hypocotyl during germination of suger beet seeds in conducted studies on ploidy changes. This may suggest that callus obtained in this experiment differentiate into shoots and root cells. To summarise described experiments it can be concluded that the best effects of androgenesis in red beet was obtained when donor plants were watered with gibberellins. For plant material sterilization the method of treating with 70% ethanol for 2 minutes was used, then with 0.1% $HgCl_2$ + 0.1% Tween 20 for 8 minutes, followed by rinsing three times in sterile distilled water.

The best medium for induction of androgenesis in anther cultures was B_5 medium supplemented with 100 $g \cdot L^{-1}$ sucrose and 0.1 $mg \cdot L^{-1}$ 2,4-D. At this stage of this studies on MS medium containing 0.2 $mg \cdot L^{-1}$ BAP and 1 $mg \cdot L^{-1}$ NAA regeneration was obtained but in the following passages received callus died. Although this callus

allowed to perform ploidy evaluation which revealed that explants had 4x chromosome number. These studies give the opportunity of developing this method in red beet.

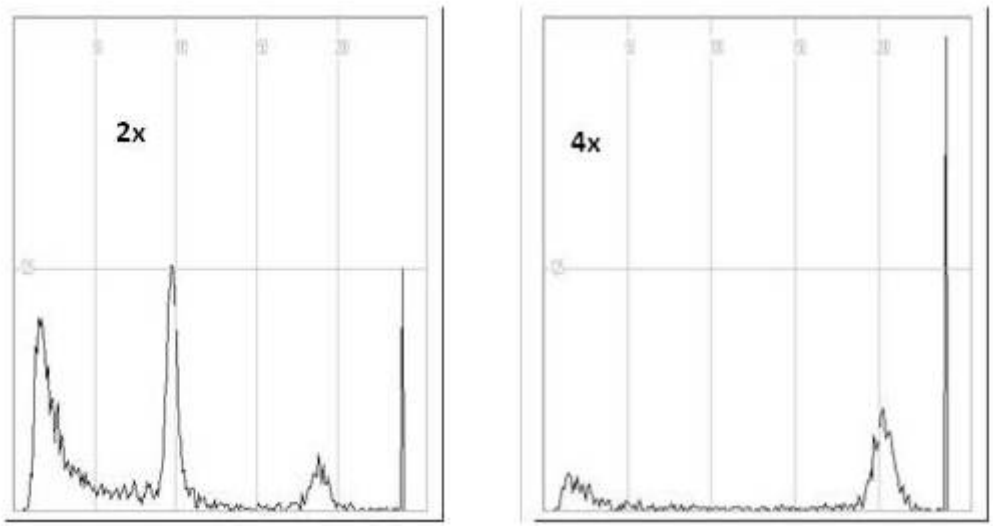

Figure 2. Histograms of flow cytometric ploidy level analysis of red beet, Czerwona Kula cultivars : 2x – control plant, 4x - androgenetic plant

Rycina 2. Histogramy z cytometrycznej analizy ploidalności buraka czerwonego odmiany Czerwona Kula : 2x – roślina kontrolna, 4x – roślina androgenetyczna

Conclusions

1. The most effective method of elimination of starch grains that limits androgenesis was preculture of anthers in aqueous α-amylase solution.
2. Optimal bud lenght for androgenesis induction in tested variety was the lenght in the range of 1.3 to 1.5 mm.
3. The B_5 medium with 100 $g \cdot L^{-1}$ sucrose and 0.1 $mg \cdot L^{-1}$ 2,4-D proved to be better for inducing androgenesis.
4. Callus regeneration occurs on MS medium containing 0.2 $mg \cdot L^{-1}$ BAP and 1 $mg \cdot L^{-1}$ NAA.

Acknowledgements

Research funded by Polish Ministry of Agriculture and Rural Development, task No 65 entitled “ Receiving homozygous red beet plants with the use of gametic embryogenesis.”

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