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The effects of sugars and growth regulators on embryo- and callusogenesis in isolated ovules culture of beetroot, *Beta vulgaris* L.

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ABSTRACT

Haploid techniques have risen interest among researchers and breeders as they significantly reduce the time of creating pure lines in breeding programs, especially for biennial vegetable crops. The only way to produce doubled haploids used in representatives of the genus *Beta* is *in vitro* culture of non-pollinated ovules. This is a rather laborious method that requires manual labor to isolate beet ovules from flower buds, followed by their inoculation onto a culture medium. This method also has its drawback - the development of clones from the somatic tissues surrounding the embryo sac. At the same time, the yield of embryoids and subsequent regenerations is on average 12-14% of isolated ovules introduced into *in vitro* culture in the most responsive genotypes of sugar beet and 8% in beetroot. The selection of the optimal cultivation conditions for each genotype makes it possible to maximize the yield of regenerant plants in the culture of isolated ovules. This research is devoted to study the effect of various types of carbohydrates and growth regulators in the culture media on embryogenesis and callusogenesis in isolated ovules culture of five beetroot genotypes. We obtained embryoids and callus in isolated ovules culture of all studied genotypes using sucrose-based culture medium, while on a glucose- and fructose-based culture media or their combination, the number of callus and embryoids was much lower. Additionally, it has been shown that glucose has more negative effect on embryo- and callusogenesis than fructose. The addition of mannitol to the culture medium had a stimulatory effect on the ovules for one genotype only. The study of various combinations of phytohormones on 5 beetroot genotypes showed that gibberellic acid reduces the embryogenesis response of ovules in all genotypes.

Keywords: *Beta vulgaris* L., Callusogenesis, Gynogenesis, Beetroot, Doubled haploids, Embryogenesis.
Article type: Research Article.

INTRODUCTION

The current priority in vegetable breeding is the production of heterotic F1 hybrids based on the selection and crossing of inbred lines. The traditional method of creating inbred lines homozygous for genes that control economically valuable traits for biannual beet crop is self-pollination and selection during 4-6 generations, which requires 8-12 years (De La Fuente *et al.* 2013). Haploid techniques shorten this process up to 3-5 years (Zhuzhzhalova *et al.* 2020) and promote the manifestation of recessive alleles in haploids, hiding them in a heterozygous state in diploid plants, which facilitates the assessment and selection of plants with valuable traits (Doctrinal *et al.* 1989; Klimek-Chodacka & Baranski 2013). One of the most common techniques to produce doubled haploids of *Beta vulgaris* L. is the cultivation of isolated ovules (gynogenesis). Gynogenesis is simple technically but time-consuming method that requires manual isolation of each ovule under the stereomicroscope. It does not exclude the formation of donor plant clones from the somatic cells surrounding the embryo sac, which requires differentiation of homo- and heterozygote regenerant plants.

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Haploid formation is determined at the genetic level but proceeds under the effect of physiological and inducing factors, which directly affects the yield of regenerants of red and sugar beets (Baranski 1996). In addition, exogenous factors that affect the regeneration frequency of cultivated ovules are extremely important: composition of culture media, cold pretreatment of buds, temperature and cultivation conditions (Van Geyt *et al.* 1987; Lux *et al.* 1990; Gurel *et al.* 2000). The composition of phytohormones in the culture medium has a significant effect on the development of explants in ovule culture (Seman & Farago 1990; Gurel *et al.* 2000). EN Vasilchenko *et al.* (2017) reported that adding gibberellin (2 mg L^{-1}) to the culture medium for sugar beet ovules culture causes embryogenesis, while adding auxins (IBA) and cytokinins (6-BAP and kinetin) to gibberellin stimulate the growth of callus along with the embryoids. Wremerth, Levall (2003) recommend to add 0.5 mg L^{-1} 2,4-D and 0.3 mg L^{-1} 6-BAP for the media to induce embryo- or callusogenesis in sugar beet ovules at the first stage of cultivation. Sucrose is traditionally added to the culture medium as a source of carbohydrates. A high sucrose concentration of 80-100 g L^{-1} is recommended to use in the culture medium for sugar beet embryogenesis induction (Lux *et al.* 1990; Gurell *et al.* 2000; Wremerth & Levall 2003; Pazuki *et al.* 2018). Baranski (1996) used lower sucrose concentration of 60 g L^{-1} to induce beetroot gynogenesis. The aim is to study the effects of the carbohydrate and growth regulators of culture media on the rate of embryoids and callus formation of isolated non-pollinated beetroot ovules, *B. vulgaris* L.

MATERIALS AND METHODS

Growing conditions of donor plants

Genotypes of beetroot, *B. vulgaris* L., represented by F1 hybrids, Action and Kestrel, and F1, F2, F3 inbred lines of 2-3 generations from the collection of N.N. Timofeev Breeding Station LLC, were used to isolate ovules. Donor plants were grown in the field as well as in the greenhouse. Seeds were sown in open ground in early May according to two-line scheme 50 + 20 × 5. Nitrogen (N) Phosphorus (P) and Potassium (K) fertilizers were applied before sowing (15:15:15). Root crops were harvested and stored in October. In November, roots were transplanted into 10-liter pots to vernalize in greenhouse at +10-12 °C during the day and +5-6 °C at night. However, the air temperature was increased up to 15-20 °C, which stimulates growth and flowering. During flowering, no fertilizers or pesticides were applied.

Isolation and cultivation of ovules

To produce doubled haploids of beetroot by isolated ovules culture, Baranski-modified method of gynogenesis (1996) was used. For sterilization inflorescences were placed in 70% ethanol for 30 seconds, then in a 3% sodium hypochlorite with 2-3 drops of tween-20 for 10 minutes. Afterward, they were washed three times in sterile distilled water for 1, 5 and 10 minutes. Flower buds from the middle part of the primary and secondary inflorescences were collected. The ovules were removed from the buds using dissection needles and placed on MS culture medium supplemented with 7.5 g L^{-1} agar, 200 mg L^{-1} BAP, 500 mg L^{-1} IAA, and 60 g L^{-1} sucrose in Petri dishes 9 cm in diameter, 20 pieces per petri dish. Then they were incubated in complete darkness at 32 ± 0.1 °C until the appearance of embryoids and/or callus. The number of formed embryoids and callus was counted after 14 to 70 days of incubation, then they were transferred to a fresh MS culture medium containing 60 g L^{-1} sucrose, 7.5 g L^{-1} agar, 200 mg L^{-1} BAP, 500 mg L^{-1} IAA and kept in the climatic chamber at 24 ± 1 °C with a photoperiod of 16-hour day and 8-hour night. Regenerated plants were transferred to the same fresh medium.

Culture medium

The effect of various types of carbohydrates (sucrose, glucose, fructose) and mannitol in the culture medium on ovules (MS with the addition of 200 mg L^{-1} BAP, 500 mg L^{-1} IAA, 7.5 g L^{-1} agar) was studied in 3 genotypes: F1, F2, and F3. The following concentrations of sugars and mannitol were used in the experiment: sucrose 60 g L^{-1}, glucose 60 g L^{-1}, fructose 60 g L^{-1}, D-mannitol 60 g L^{-1}, as well as a combination of glucose 30 g L^{-1} and fructose 30 g L^{-1}. The standard for this experiment was MS medium supplemented with 60 g L^{-1} sucrose, 200 mg L^{-1} BAP, 500 mg L^{-1} IAA, and 7.5 g L^{-1} agar. Regenerated plants were cultured on the same culture medium. The effect of growth regulators in MS culture medium supplemented with 60 g L^{-1} sucrose 7.5 g L^{-1} agar was studied in 5 genotypes: Action F1, Kestrel F1, F1, F2, and F3. Four variants of phytohormone combinations were used: 1) IAA 0.5 mg L^{-1}, BAP 0.2 mg L^{-1} (control); 2) gibberellin 2 mg L^{-1}; 3) gibberellin 1 mg L^{-1}, IAA 0.5 mg L^{-1}, BAP 0.2 mg L^{-1}; 4) gibberellin 2 mg L^{-1}, IAA 0.5 mg L^{-1}, and BAP 0.2 mg L^{-1}.

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Statistical analysis

The SPSS 20.0 program was used to analyze the results by ANOVA test, this test shows the presence or absence of significant differences in the studied variables. Experimental data were processed on the basis of the methods of mathematical statistics. Analysis of variance and regression was carried out in MS Excel. The experiments were carried out in triplicate, one repetition corresponding to one Petri dish each with 20 isolated ovules. The significant differences of experimental variants were set at level of $P \leq 0.05$.

RESULTS

The effects of carbohydrate source

The effects of carbohydrates, sucrose, glucose, fructose and a combination of glucose, fructose, and mannitol in the culture medium on the rate of embryo- and callusogenesis by isolated ovules culture was studied using three genotypes of beetroot, *B. vulgaris* L. including F1, F2, F3. Sugar and mannitol were added to the medium at concentration of 60 g L^{-1}. The embryogenesis rate of each genotype was estimated (Table 1).

Table 1. Effects of sugars and mannitol in the medium on the embryo- and callus development in isolated ovules culture.

Genotype	Average number of morphogenic ovules per Petri dish (pcs).				
	Saccharose 60 g L^{-1} (control)	D-Mannitol 60 g L^{-1}	Glucose 60 g L^{-1} + fructose 60 g L^{-1}	Glucose 60 g L^{-1}	Fructose 60 g L^{-1}
F1	0.63 a	0.36 a	0.18 a	0.09 a	0.27 a
F2	0.75 a	0.00 b	0.10 b	0.00 b	0.14 b
F3	0.07 a	0.00 a	0.00 a	0.00 a	0.00 a

Note: lowercase letters *a, b, c* shows the difference among the variants in the row at P = 0.05.

Genotype F2 showed significant differences between the variants of the culture medium. The highest rate of embryogenesis was on the medium with 60 g L^{-1} sucrose. No significant differences were found among the rest of culture mediums. Genotypes F1 and F3 did not exhibit any significant differences among the variants. At the same time, a negative effect of glucose on embryogenesis can be noted when growing ovules on a medium with 60 g L^{-1} glucose, as well as in the combination of 30 g L^{-1} glucose and 30 g L^{-1} fructose. On glucose-containing media, all genotypes formed callus only, while other variants of the experiment showed the formation of both embryoids (Fig. 1) and callus (Fig. 2).

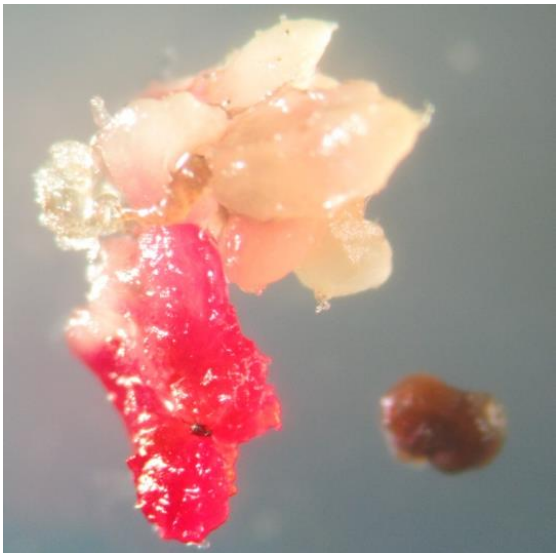

Fig. 1. Embryoids of beetroot in the culture of isolated ovules.

Fig. 2. Callus in the culture of isolated beetroot ovules.

Effects of growth regulators

Combinations of phytohormones in the culture medium were studied in 5 genotypes: F1, F2, F3, Action F1, and Kestrel F1 (Table 2). All the studied genotypes showed the development of embryoids during ovules culture on the culture medium with 0.5 mg L^{-1} IAA and 0.2 mg L^{-1} BAP. When they were cultured on the gibberellin-

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containing media, the rate of embryogenesis decreased, and callus developed from ovules. The worst variant of the culture medium was the one with gibberellin 2 mg L^{-1}, IAA 0.5 mg L^{-1}, and BAP 0.2 mg L^{-1}. This culture medium showed the development of callus only with the genotype F2.

Table 2. Effect of various combinations of phytohormones on the embryo- and callus development in isolated ovules culture.

Genotype	Average number of morphogenic ovules per Petri dish, pcs.			
	IAA 0.5 mg L^{-1}, BAP 0.2 m l-1 (control)	**Gibberellin 2 mg L^{-1}**	**Gibberellin 1 mg L^{-1}, IAA 0.5 mg L^{-1}, BAP 0.2 m L^{-1}**	**Gibberellin 2 mg L^{-1}, IAA 0.5 mg L^{-1}, BAP 0.2 m L^{1}**
F1	0.40 a	0.10 b	0.00 c	0.00 c
F2	0.90 a	0.20 a	0.40 a	0.20 a
F3	0.10 a	0.00 a	0.00 a	0.00 a
Action F1	1.00 a	0.00 a	0.66 a	0.00 a
Kestrel F1	1.66 a	0.00 b	0.00 b	0.00 b

Note: lowercase letters *a, b, c* shows the difference among the variants in the row at P = 0.05.

DISCUSSION

The overwhelming majority of researchers has been focused on the study of gynoginesis of sugar beet (D'Halluin & Keimer 1986; Van Geyt *et al.* 1987; Doctrinal *et al*. 1989; Seman & Farago 1990; Lux *et al*. 1990; Gurel *et al*. 2000; Wremerth & Levall 2003; Śliwińska *et al.* 2005; Tomaszewska-Sowa 2010; Vasilchenko *et al*. 2017; Pazuki *et al*. 2018; Zhuzhzhalova *et al.* 2020). There have been rare reports about the gynogenesis of beetroot (Baranski 1996), which show the influence of plant conditions, genotype, pretreatment, and some phytohormones on embryogenesis induction. Baranski (1996) used sucrose 60 g L^{-1} as a source of carbohydrates. Our experiment showed that the growing of ovules on the culture medium with sucrose as preferable source for all studied genotypes, leads to embryoids development predominantly, while using the media with monosaccharides and mannitol leads to callus development mainly. Vasilchenko *et al.* (2017), when obtaining doubled haploids of sugar beet, reported the positive effect of 2 mg L^{-1} gibberrelin on embryogenesis and subsequent development of regenerations. In our experiment, a combination of phytohormones, 0.5 mg L^{-1} IAA and 0.2 mg L^{-1} BAP was chosen as control, which caused the maximum response of ovules in Baranski's experiment (1996). Adding 1 and 2 mg L^{-1} gibberrelin to the culture medium in beetroot ovules caused the growth of non-morphogenic callus in addition to embryoids, however, its high concentration inhibited the development of ovules.

CONCLUSION

This paper evaluates the effects of carbohydrates (mono and disaccharides) and mannitol, as well as the effect of gibberellin on embryo- and callusogenesis by isolated ovules culture in beetroot. All the carbohydrates, as well as mannitol, exhibited supporting callus formation and embryogenesis, while the genotype-specificity of embryogenesis was noted. The rate of embryogenesis displayed an upward trend during the growing of ovules on a sucrose-containing medium. Growing of ovules on gibberellin-containing culture media negatively affected the development of embryoids in all studied beetroot genotypes. The best culture medium for the growing of beetroot ovules was the one which supplemented with 0.5 mg L^{-1} IAA, and 0.2 mg L^{-1} BAP.

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