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GENERATION OF *Rubus arcticus* L. HAPLOIDS THROUGH in vitro MICROSPORE CULTURE TECHNIQUE

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Abstract

Arctic bramble (*Rubus arcticus* L.) is a valuable small-fruit crop used as a plantation crop for a relatively short time. *R. arcticus* is a remontant donor in interspecific hybridization with *Rubus idaeus* L., though conditioning low yields to hybrids. So *R. arcticus* is primarily bred for yield enhancement; therefor, the acceleration of the breeding process is of great importance. This can be achieved using plants with a doubled haploid set of chromosomes. This paper is the first to describe the technique of production haploid plants of *R. arcticus* via in vitro microspore culture. In the experiments we used Finnish cultivars Pima and Mespi and Swedish cultivar Astra. To obtain donor explants, the method of forcing generative shoots was used throughout the year. Microspores were isolated from anthers with the use of manual homogenizer into a 1.5 ml micro test Eppendorf tube. The homogenate was added with 0.5 ml sterile water containing 30 g/l glucose, centrifuged at 4500 rpm, and the microspores were transferred with a microdoser to nutrient medium for morphogenesis initiation. To obtain microspores, the anthers were isolated from buds of 9 to 12 mm long 4-5 days before the flower bloomed. The concentration of the microspores in the suspension was about 40,000 per 0.5 ml sterile aqueous solution with glucose; for this, 60 anthers were crushed. To induce embryoidogenesis, we used the Murashige and Skoog (MS) plant growth medium supplemented with 0.50 to 2.00 mg/l growth regulator 6-benzylaminopurine (BA). After the appearance of embryoids, we used MS, 75 % MS, or 50 % MS growth media, and also the effect of carbohydrate sources, i.e. glucose, sucrose and maltose at a 20, 30 and 40 g/l dosage, was investigated. We have identified the following microspore development stages: tetrads, non-vacuolated microspore, strongly vacuolated microspore, three-cell pollen. It was found that MS nutrient media containing 1.5 mg/l BA provides for 23±3 embryoids on day 51±2 of culture. We have also found the effect of MS concentration and the source of carbohydrate nutrition on the growth of embryoids. The combination of 0.75 MS and 30 g/l glucose was the most effective leading to embryoid growth on day 12±2 and the appearance of leaflets on day 44±1. On day 40 of culture the embryoids reached 5±0.2 mm in length. The ploidy control of regenerant plants, by counting chromosomes and chloroplasts in the stomata guard cells, confirmed the haploid set of chromosomes ($n = 7$). These findings allow the use of the proposed technique to generate *R. arcticus* haploids which, after doubling the chromosome set, may be involved in breeding.

Keywords: *Rubus arcticus*, haploid, diploid, microspore culture, embryoid, morphogenesis, regenerant plant

Arctic bramble (*Rubus arcticus* L.) is a valuable berry plant. Despite the relatively short period of its use as a plantation crop, several highly productive

varieties were obtained. Arctic bramble is a donor of remontance and excellent taste traits in interspecific hybridization with *Rubus idaeus* L., however, the offspring inherits low productivity from arctic raspberry. That is why the *R. arcticus* is mainly bred for increased plant productivity. Therefore the acceleration of the selection and the use of haploids for breeding cross-pollinated plants are of particular importance [1-3]. The doubled haploid technology overcomes a number of breeding difficulties. Homozygous lines based on haploids can be obtained in 2-3 years [4]. At the same time, according to some researchers, the use of androcline regenerants is the only way to maintain the heterosis effect of a valuable hybrid line [5].

Obtaining haploid plants has a common experimental design plan, i.e. exposure to elevated and/or lower temperatures, chemical treatment of donor plants, change in the nutrient medium composition of growth regulators, in carbohydrate components, in anther and microspores age to better induce tissue culture and regeneration. To date, many papers describe methods for obtaining haploid plants of wheat [5, 6], cabbage [7-9], rapeseed [10], rice [11], carrots [12-14] but there are no works on production of haploid plants of *R. arcticus* L. in microspore culture.

There are a lot of factors influencing the activation of switching microspores from the gametophytic to the sporophytic pathway of development, for example, the types and concentrations of growth regulators [15-17] and carbohydrate nutrition [18-20].

Earlier, we have proposed a technique for *R. arcticus* clonal micropropagation [21]. To continue these studies, in the present work, we first obtained haploid regenerated *R. arcticus* plants in a microspore culture via optimizing the 6-benzylaminopurine concentration and carbohydrate nutrition to induce embryoidogenesis

Our goal was to develop a method for producing haploid plants of *Rubus arcticus* L. in microspore culture.

Materials and methods. The research was carried out in 2018 on Arctic bramble varieties Pima, Mespi from Finland and Astra from Sweden. Microspores were isolated from pollen, which were selected from buds of different ages and sizes.

To reduce contamination when introduced into in vitro culture and for year-round production of buds, plant forcing method was used [22]. Plants, after flowering was completed, were kept in pots for 30 days at 4 °C. After cold exposure, such plants again formed generative shoots.

The buds were sterilized 1 min in 70% aqueous solution of ethanol, then 15 min in 5% aqueous solution of sodium or potassium hypochlorite, and washed in sterile distilled water at least three times. The extracted anthers were collected in 1.5 ml Eppendorf microtubes, 60 anthers per each, and crushed with a manual homogenizer. Sterile water (0.5 ml, 30 g/l glucose) was added to the microtubes using a Proline 20-200 single-channel microdoser (Sartorius Proline, Finland). The tubes were centrifuged (Microspin FV-2400 minicentrifuge-vortex, SIA BioSan, Latvia) for 15 s at 4500 rpm. From each tube, a microspore suspension was transferred by microdoser into a 10 ml culture flask (a suspension from one tube was placed in one culture flask; approximately 80,000 microspores/ml. To isolate the anthers and microspores, 9-12 mm long buds were collected 4-5 days before blooming.

At each stage of in vitro microspore culture, the composition of the nutrient medium and the growth regulators were changed. To activate in vitro

morphogenesis, microspores were implanted on a Murashige-Skoog medium (MS) [23] supplemented with 100 mg/l mesinositol, 2 mg/l glycine, 0.5 mg/l thiamine, 0.5 mg/l pyridoxine, 30 g/l glucose and 5.0 g/l agar, pH of 5.8. As a growth regulator, 0.50 to 2.00 mg/l 6-benzylaminopurin (BA, AppliChem GmbH, Germany) was used. After the emergence of embryoids, MS medium (AppliChem GmbH, Germany) was used (full and reduced to 75% and 50% concentration of macro- and micronutrients according to the recipe) [23]. The influence of the carbohydrate source on the growth and development of embryoids was assessed using 20, 30 and 40 g/l glucose, sucrose and maltose.

Microspores, embryoids, and regenerants were grown under illumination of 1500 lux at a 16 h (day)/8 h (night) photoperiod and a temperature of about 25 °C. The 10 ml culture flasks were used for in vitro culture and during growth and development of embryoids, and 100 ml culture flasks for the regenerated plant growth.

Chromosomes were counted in root meristemic zone of the regenerated plants. The roots were placed in Carnoy's fixative [24] and allowed for 24 h, then washed for 20 min under running water, immersed in an enzyme mixture (0.3% pectinase, 0.3% macerase, 0.3% cellulase + citrate buffer) and incubated at of 37 °C for 2 h. Afterwards, the roots for 3 min were immersed in 60% acetic and crushed with a dissecting needle, then circled with a 3:1 fixative, shaken, washed in absolute alcohol, dried, stained with methylene blue, and washed in distilled water. Chromosomes were counted as per description [24] (Light Microscope Biomed-3, Biomed, Russia). To count the chloroplast number, 5 cuts from leaves of each plant, each cut of 5 mm in diameter, were used. The cuts were stained with iodine dye. In vitro growing regenerants were photographed (a Samsung NX-10 digital camera, Samsung, South Korea).

The arithmetic mean values (*M*) with standard error of the mean (±SEM) and coefficient of variation (*Cv*) are shown. The calculation of the confidence interval based on Student's *t*-distribution at a significance level of $p \leq 0.05$ was performed using Microsoft Excel 2010 statistical program.

Results. To start with, the number of microspores in the anthers was counted and the stages of microspore development were established depending on the bud length. Buds of 1.0 to 1.4 mm in length contained 70 to 86 anthers with 590 to 710 mature pollen grains per anther. We identified four main stages in microspore development (Fig. 1). Stage 1 is a tetrad (in buds from 4 to 5 mm long), stage 2 is an unvacuolated microspore with a central location of the nucleus (buds of 5 to 7 mm long), stage 3 is a strongly vacuolated microspore with a thickened wall, a large vacuole and a small nucleus located laterally (in buds of 7 to 8 mm long), and stage 4 is three-cell pollen (in buds longer than 8 mm) (see Fig. 1).

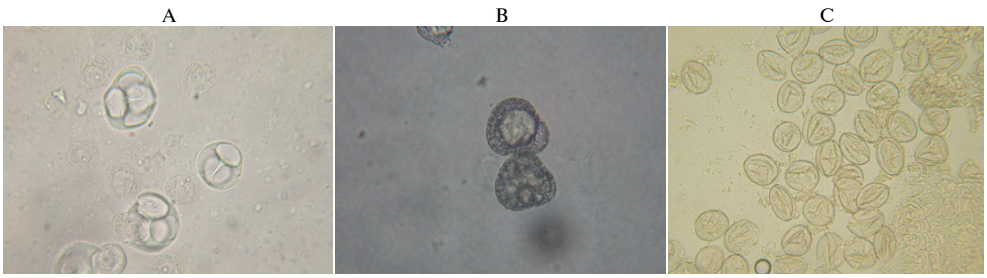

Fig. 1. Microspores of Arctic bramble (*Rubus arcticus* L.) Astra cultivar on Murashige-Skoog medium (30 g/l sucrose, 1.5 mg/l 6-benzylaminopurin, pH 5.8): A — tetrads, B — highly vacuolated microspore, C — mature pollen (magnification ×600, light microscope Biomed-3, Biomed, Russia).

1. Morphogenesis of microspores of different Arctic bramble (*Rubus arcticus* L.) cultivars on Murasige-Skoog medium at different concentration of 6-benzylaminopurine (BA)

Cultivar	BA, mg/l	Emergence of embryoids, days		Number of embryoids on day 60	
		M±SEM[1, 2]	*Cv*, %	*M*±SEM[1, 2]	*Cv*, %
Astra	0.50	63±2[a]	7.8	5±1[c]	11.2
	0.75	58±1[ab]	4.5	12±2[ac]	4.5
	1.00	55±1[bc]	10.0	14±2[ac]	6.3
	1.25	51±2[c]	9.2	23±3[b]	5.6
	1.50	51±3[bc]	7.8	20±1[b]	7.2
	1.75	51±3[bc]	8.2	9±2[c]	8.7
	2.00	0	0	0	0
Pima	0.50	65±3	2.6	4±1[a]	9.1
	0.75	54±2[a]	4.6	7±2[ab]	12.4
	1.00	53±2[a]	7.1	8±2[ab]	9.3
	1.25	53±1[a]	8.6	18±1	9.7
	1.50	54±2[a]	5.8	9±1[b]	6.5
	1.75	54±2[a]	4.3	8±2[ab]	7.7
	2.00	–	–	–	–
Mespi	0.50	63±2[a]	12.2	10±2[a]	5.4
	0.75	60±1[a]	12.1	13±2[ab]	6.5
	1.00	57±2[ab]	10.1	15±2[ab]	6.4
	1.25	53±2[b]	9.3	20±1	7.6
	1.50	52±1[b]	6.5	14±1[b]	3.8
	1.75	53±2[b]	3.4	14±2[b]	5.1
	2.00	63±2[a]	7.8	5±1[c]	11.2

N o t e. The sucrose concentration is 30 g/l, pH 5.8; 40,000 microspores per 0.5 ml. Each experiment was performed in a 3-fold repetition. Dashes mean the death of explants. 1 — a confidence interval based on Student's *t*-distribution at $p \le 0.05$; 2 — indicators in the column marked with the same letters (a, b, c) have no statistically significant differences at $p \le 0.05$ as per the Student's *t*-test.

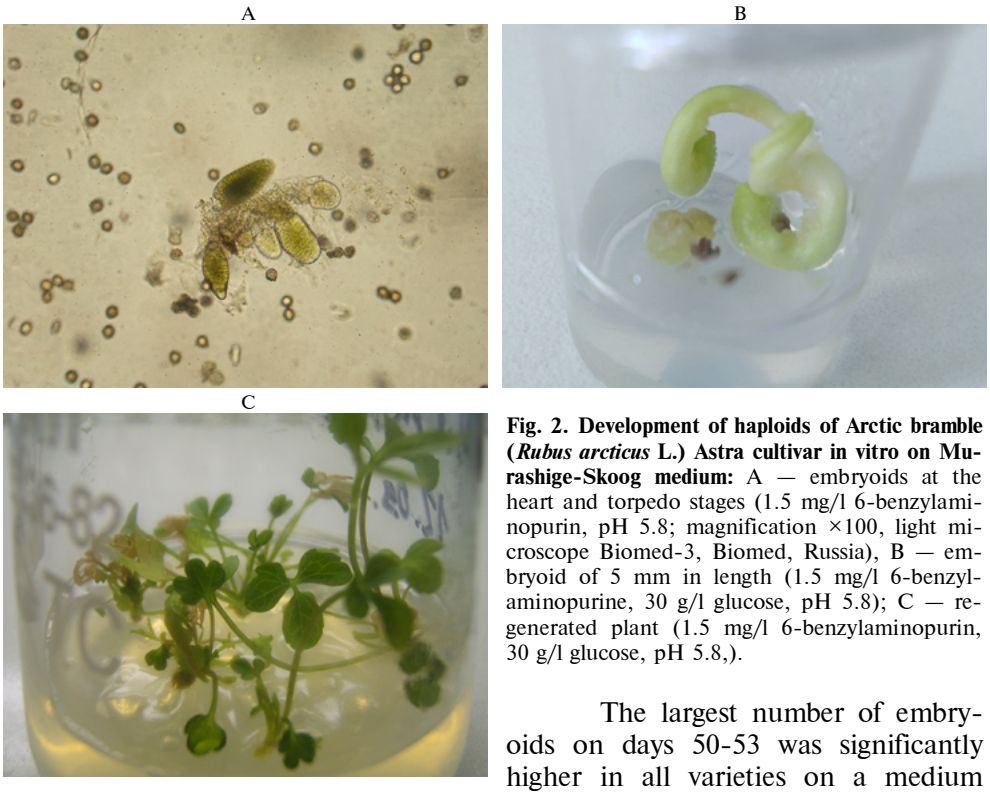

Fig. 2. Development of haploids of Arctic bramble (*Rubus arcticus* L.) Astra cultivar in vitro on Murashige-Skoog medium: A — embryoids at the heart and torpedo stages (1.5 mg/l 6-benzylaminopurin, pH 5.8; magnification ×100, light microscope Biomed-3, Biomed, Russia), B — embryoid of 5 mm in length (1.5 mg/l 6-benzylaminopurine, 30 g/l glucose, pH 5.8); C — regenerated plant (1.5 mg/l 6-benzylaminopurin, 30 g/l glucose, pH 5.8,).

The largest number of embryoids on days 50-53 was significantly higher in all varieties on a medium containing 1.5 mg/l BA (Table 1). Visually, embryoids were detected starting from the heart stage, when their size reached 0.5 mm. We did not reveal a reliable effect of the genotype on the morphogenesis of microspores in vitro. Though

we did not perform a special investigation of the influence of the stage of development of microspores on the morphogenetic activity, it is only necessary to note that the microspores that were placed on the nutrient medium were overwhelmingly at the stage of vacuolization.

In studies on the induction of embryogenesis and the production of secondary embryoids, various concentrations of BA, as a growth regulator, are actively used [25]. In our work, BA induced direct embryoidogenesis.

We also found out that more intensive growth processes occurred on a nutrient medium with a 75% macro- and microelements and 30 g/l glucose. In this variant, embryoid growth occurred on day 12, which was significantly different from other variants. On day 40, their size reached 5 mm (Fig. 2). However, the smallest death rate of embryoids was observed on a MS medium with a complete macro- and microelements (Table 2).

2. Growth of embryoids of Arctic bramble (*Rubus arcticus* L.) Astra cultivar in vitro on the Murashige-Skoog medium under different concentrations of macro- and microelements and carbohydrates

Micro- and macro-elements, %	Carbohydrate, g/l	Start of embryoid growth, days		Leaf emergence, days		Length in 40 days of growth, mm	
		$M\pm SEM^{1,2}$	*Cv*, %	$M\pm SEM^{1,2}$	*Cv*, %	$M\pm SEM^{1,2}$	*Cv*, %
100	Sucrose, 20	55±3	13.2	–	–	1.0±0.5^{a}	29.1
	Sucrose, 30	41±2^{a}	10.3	–	–	1.0±0.5^{a}	11.3
	Sucrose, 40	40±1^{a}	5.4	63±3^{a}	3.4	2.0±0.6^{ab}	7.1
	Maltose, 20	37±2^{a}	12.3	60±3^{a}	7.6	3.0±0.3^{b}	5.3
	Maltose, 30	19±1^{b}	5.4	49±2^{b}	2.1	3.0±0.5^{b}	5.6
	Maltose, 40	20±1^{b}	4.2	49±3^{b}	6.4	4.0±0.5^{b}	8.0
	Glucose, 20	25±3^{b}	14.1	49±4^{ab}	11.9	2.0±1.0^{ab}	14.6
	Glucose, 30	17±2^{b}	10.2	47±4^{ab}	9.5	3.0±0.3^{b}	7.5
	Glucose, 40	20±2^{b}	9.8	48±4^{b}	8.7	4.0±0.4^{b}	5.9
75	Sucrose, 20	–	–	–	–	–	–
	Sucrose, 30	43±3^{a}	6.8	60±3^{a}	13.2	3.0±0.3^{a}	11.2
	Sucrose, 40	40±2^{a}	8.2	60±4^{a}	12.5	3.0±0.3^{a}	8.4
	Maltose, 20	–	–	–	–	–	–
	Maltose, 30	21±2^{b}	5.5	46±2^{b}	5.6	4.0±0.4^{abc}	9.8
	Maltose, 40	20±2^{b}	4.3	46±2^{b}	8.1	5.0±0.5^{bc}	12.3
	Glucose, 20	25±3^{b}	11.2	46±3^{b}	10.3	4.0±0.3^{ab}	5.3
	Glucose, 30	12±2^{c}	5.2	44±1^{b}	7.0	5.0±0.2^{c}	7.9
	Glucose, 40	15±2^{c}	8.7	45±2^{b}	6.2	5.0±0.5^{bc}	9.2
50	Sucrose, 20	–	–	–	–	–	–
	Sucrose, 30	–	–	–	–	–	–
	Sucrose, 40	52±2^{a}	10.3	80±5^{a}	12.5	3.0±0.4^{a}	13.9
	Maltose, 20	–	–	–	–	–	–
	Maltose, 30	58±1	5.4	73±2^{a}	6.7	3.0±0.4^{a}	9.4
	Maltose, 40	50±2^{a}	7.9	70±1^{a}	8.3	3.0±0.5^{a}	6.7
	Glucose, 20	41±2^{b}	6.3	–	–	–	–
	Glucose, 30	39±2^{b}	7.5	–	–	–	–
	Glucose, 40	35±3^{b}	4.3	62±2	5.9	4.0±0.3^{a}	5.7

N o t e. The 6-benzylaminopurin concentration is 1.5 mg/l, pH 5.8. Each experiment was performed in a 3-fold repetition. Dashes mean the death of explants. 1 — a confidence interval based on Student's *t*-distribution at $p \le 0.05$; 2 — indicators in the column marked with the same letters (a, b, c) have no statistically significant differences at $p \le 0.05$ as per the Student's *t*-test.

Many authors note the positive effect of increased osmotic pressure, which is created at sucrose concentrations from 10 to 17%, on the emergence of embryoids [26-28], however, in our experiments, embryoidogenesis in *R. arcticus* actively occurred at a 3% concentration, and already at 4%, the rate of development decreased. This discrepancy in the results can be explained by the fact that we studied the transition from the embryoid stage to the regenerant plant, and not the stage of embryoid formation from the microspore.

The ploidy of regenerated plants was determined by counting the number of chromosomes and the number of chloroplasts in the stomata guard cells (Fig. 3). The number of chloroplasts in the stomata guard cells varied from 8 to 12 in

diploid plants, and from 2 to 6 in haploid plants. The possibility to use this indirect method to confirm ploidy has been proved in a number of works [29-31].

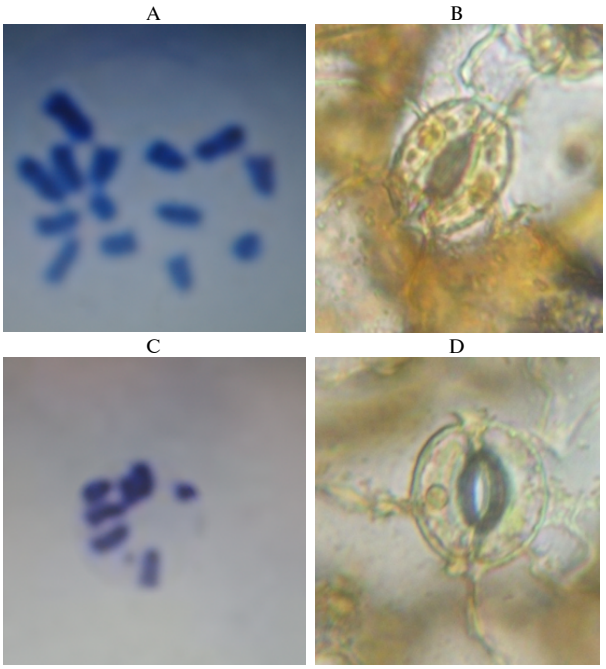

Fig. 3. Chromosomes of dividing cells in root meristem at the metaphase stage (A, C, magnification ×1000) **and chloroplasts of stomata guard cells** (B, D, magnification ×630) **in regenerants of Arctic bramble (*Rubus arcticus* L.) Astra cultivar:** A, B — the diploid ($2n$ = 14, 10 chloroplasts in the guard cells), C, D — the haploid (n = 7, 4 chloroplasts in the guard cells). Light microscope Biomed-3 (Biomed, Russia).

This investigation does not allow us to identify which particular stage of microspore development is better to produce haploid plants of Arctic raspberry, however, this is important for determining the factors that influence the switching of the development program of microspores from gametophytic to sporophytic [17, 19, 28, 32] . It can only be noted that most of the microspores were at the stage of vacuolization. One of the main factors of switching to the sporophytic pathway of development in the Arctic bramble microspores was the use of the growth regulator 6-benzylaminopurine and glucose as a carbohydrate source. The crucial point in obtaining haploid plants is not only the production of embryoids, but also the preservation of their viability and morphogenetic activity when transplanted to a fresh nutrient medium [33]. Out findings indicate that for a success, it is necessary to reduce the concentration of macro- and microelements in MS nutrient medium to 75% of their full amount, while a decrease to 50% leads to a decrease in viability.

Thus, to obtain haploid regenerants of *Rubus arcticus* L. varieties Pims, Mespi and Astra, it is advisable to use full Murashige-Skoog nutrient medium enriched with 1.5 mg/l 6-benzylaminopurine, which gives from 18±1 to 23±3 embryoids per 40,000 microspores. To regenerate plants from embryoids, one should use a 75% Murashige-Skoog medium supplemented with 30-40 g/l glucose and 1.5 mg/l 6-benzylaminopurin, which provides the shortest time of induction resulting in 5±0.2 mm embryoids by day 40. These findings and the developed technique can be used to create doubled haploids of other *R. arcticus* varieties for inclusion in breeding programs.

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