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Androgenesis in anther culture of Lithuanian spring barley cultivars

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The method of anther culture was used for the production of doubled haploids in Lithuanian spring barley cultivars. Two methods, (i) regeneration from callus (Szarjeko's method) and (ii) direct regeneration from embryoids (Caredda's method) were applied to determine the androgenic potential according to the green regenerant yield and other morphogenetic factors.

Green double haploid regenerants were obtained in four Lithuanian spring barley cultivars ('Aura', 'Aidas', 'Alsa' and 'Auksiniai') out of 10 studied. 'Aura' was the best for regenerant formation rate from callus by Szarjeko's method. Applying Caredda's method, green regenerants were obtained from embryoids in cvs. 'Aidas', 'Alsa' and 'Auksiniai'. Cv. 'Ūla' showed the highest callus formation rate at the level of 800.0 calli per 100 responding anthers. The highest rate of embryoid formation was determined in cv. 'Auksiniai 3' (580.0 embryoids per 100 responding anthers).

Key words: spring barley, anther culture, callus, embryoids, doubled haploids

INTRODUCTION

Spring barley is the most widely grown spring cereal in Lithuania. According to the data of the Lithuanian Department of Statistics, in 2003 cereals accounted for 65.7% of the total area under the crops. Growing in suitable soils and sufficiently fertilized, barley can produce a grain yield higher oats.

Methods of modern biotechnology allow to accelerate the process of breeding, and haploid production is one of the most widely used biotechnological methods in breeding of self-pollinating cereal crops. Anther culture is used for barley F_1 hybrids produced after crossing two lines with desirable traits. DH production is aimed for gene transfer into the homozygotic state in the first generation. Recessive mutations, important recombinations and other genomic changes can be found in double haploid (DH) more easily. DH can be used for genetic analysis, gene mapping and gene engineering. DH material makes easier identification and stabilization of genetic variation [1]. This method accelerates breeding by 3–5 years [2, 3].

Success in the anther culture method depends on plant growth conditions, plant genotype, and growth medium [4]. Most scientists using the method of anther culture report that the morphogenetic potential of callus and embryoids is genetically predetermined [5–7]. In barley, a high number of albino plants are regenerated, limiting the exploitation of this technique.

The microspore enters the androgenesis following two patways: (1) the microspore develops into a haploid callus from which haploid plants can be regenerated (according to Szarejko's method) and (2) the microspore directly develops into a haploid embryo which further regenerates into a haploid plant (according to Caredda's method). It is important to find such genotypes whose anthers form morphogenetically active structures. Applying Szarejko's and Caredda's methods, we evaluated the anther culture potential for DH production in ten Lithuanian spring barley varieties.

MATERIALS AND METHODS

Plant material

The research was carried out at the Laboratory of Genetics and Physiology of the Lithuanian Institute of Agriculture.

Haploid production was investigated:

(i) using Szarejko's method [8] in cvs.'Aidas', 'Alsa', 'Auksiniai', 'Auksiniai 2', 'Auksiniai 3', 'Aura', 'Džiugiai' and 'Ūla';

(ii) using Caredda's method [4] in cvs. 'Aidas', 'Alsa', 'Auksiniai', 'Auksiniai 2', 'Auksiniai 3', 'Aura', 'Džiugiai', 'Gintariniai', 'Luokė' and 'Ūla'.

Seeds were germinated on humidified filter paper in Petri dishes for four days at room temperature and ambiant light. Seedlings were planted in 20 cm diameter pots containing a mixture of peat moss and soil (1:1). Plants were grown in the greenhouse at 25 °C for a week under a 16 h photoperiod (18.000–20.000 lx) at an approximately 80% relative humidity. Natural light was supplemented from September to April with artificial sodium lighting (400 Watts Sodiclaude) to maintain a photon flux density of 300–350 μE /m^2/s^1 at the soil surface.

Stress such as pesticide treatment, water deficiency or temperature fluctuation was avoided during plant growth.

Callus induction in anther culture (Szarejko's method)
Spikes containing microspores at uninucleate stage were removed from leaf sheaths and placed into two-compartment Petri dishes, with a few drops of sterile water in the first compartement. Petri dishes were sealed with parafilm, wrapped in foil and stored in the refrigerator or a cold room at 4–5 °C for 21–28 days.

After pretreatment, anthers were cultured on the Szarejko (1996) medium composed of macro-element salts including KNO_3 (2.6 g l^{-1}), NH_4NO_3 (0.200 g l^{-1}), $(NH_4)_2SO_4$ (0.400 g l^{-1}), $NaH_2PO_4 \cdot H_2O$ (0.150 g l^{-1}), KH_2PO_4 (0.170 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (0.600 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.300 g l^{-1}); micro-element salts including KI (0.800 mg l^{-1}), $MnSO_4 \cdot H_2O$ (5.0 mg l^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.250 mg l^{-1}), $CoCl_2 \cdot 6H_2O$ (0.025 mg l^{-1}), H_3BO_3 (5,0 mg l^{-1}), $ZnSO_4 \cdot 7H_2O$ (2,0 mg l^{-1}), $CuSO_4 \cdot 5H_2O$ (0,025 mg l^{-1}), Fe-Na-EDTA (40 mg l^{-1}); and other inorganic salts: $AgNO_3$ (10 mg l^{-1}) and $KHCO_3$ (50 mg l^{-1}). This medium was supplemented with 0.3 g l^{-1} casein hydrolysate and 60 g l^{-1} maltose as a carbohydrate source. The pH was adjusted to 5.6 before the addition of agarose (6 g l^{-1}). After autoclaving, filter-sterilized vitamins, including myo-inositol (2.0 g l^{-1}), thiamine-HCl (1.0 mg l^{-1}), nicotinic acid (0.5 mg l^{-1}), ascorbic acid (1.0 mg l^{-1}), pyridoxine-HCl (0.5 mg l^{-1}), and growth hormones including IAA (2 mg l^{-1}) and BAP (1 mg l^{-1}) were added to the medium. Thirty anthers were placed per 5 cm Petri dishes. Dishes were sealed with parafilm and maintained in the culture chamber at 26 ± 2 °C, with 85% relative humidity, in the dark for 21–28 days.

Embryoid formation in anther culture (Carreda's method)
Using the method developed by Caredda, after estimating the microspore development stage with a microscope, the ears and anthers were sterilised using 70° ethanol. The anthers were placed into Petri dishes 5 cm in diameter, 30 anthers per dish. They were pre-treated at 4 °C in the dark at a 80% relative humidity and for 3–4 days in mannitol (62.0 g l^{-1}) providing an osmotic pressure of 180 mosm l^{-1}. Each dish was sealed with parafilm to prevent spillage of solution, and wrapped in aluminium foil.

The anthers removed from the mannitol solution were transferred on the anther culture medium [4]. The anthers were allowed to grow in the thermostat at a constant temperature of 26 ± 2 °C in the dark for 2–4 weeks, monitoring the initiation of embryoid formation.

Plant regeneration
When microspore-derived embryos measured approximately 1–2 mm, responding anthers were collected and transferred onto a Szarejko [8] or Caredda [6] regeneration medium. The differences between the regeneration and the culture medium corresponded to the replacement of maltose by sucrose, the replacement of agarose by agar and the lower concentrations of plant growth regulators (0,4 mg/l auxin and cytokinin). The Petri dishes were maintained in the culture chamber at 26 ± 2 °C and 85% relative humidity with a 16 h photoperiod at 18.000–20.000 lx. After two weeks on the regeneration medium, green and albino plants were counted.

When the green regenerants reached the length of approximately 5–7 cm in coleoptiles and 1–2 cm in roots and 1–2 green leaves, they were removed from the culture tubes using pincers and transferred into pots containing a sand / turf / soil mixture (1/1/1). The covered pots were kept in a climate chamber or in the greenhouse under controlled plant growth conditions (the photoperiod 16/8 h, light intensity 18000–20000 lx, temperature 14–16 ± 2 °C).

Data statistics
At least 300 anthers from 12 different spikes were used for each test. Data were processed using statistical analysis for quantitative and qualitative parameters and the SELEKCIJA set of statistical data analysis software (author P. Tarakanovas).

RESULTS

DH regeneration from callus
Barley cultivars varied significantly according to their response in the anther culture. The highest callus induction was determined for 'Aura' (6.0% of responding anthers) (Table 1). Anthers of cv. 'Ūla' produced the highest number of calli per anther (800.0 calli per 100 responding anthers); the results similar were for 'Aidas' (600.0 calli per 100 responding anthers). 'Džiugiai' and 'Auksiniai 3' showed low callus formation in the anther culture (100.0 and 107.1 calli per 100 responding anthers, respectively). In cv. 'Auksiniai 3' anther response was as low as 4.7%, and callus was formed at a low

Table 1. **Formation of spring barley regenerants from callus in anther culture of Lithuanian cultivars (Szareiko's method)**

Cultivars	RA (%)	CA/RA	RP/RA	GR/RA
Aidas	2.7	600.0	87.5	0.0
Alsa	5.0	420.0	0.0	0.0
Auksiniai	1.0	166.7	33.3	0.0
Auksiniai 2	0.0	0.0	0.0	0.0
Auksiniai 3	4.7	107.1	57.1	0.0
Aura	6.0	366.7	116.7	16.7
Džiugiai	0.7	100.0	50.0	0.0
Ūla	1.7	800.0	20.0	0.0
$LSD_{0.01}$	0.87	98.42	35.82	3.82

RA, responding anthers; CA/RA, callus per 100 responding anthers; RP/RA, regenerated plantlets per 100 responding anthers; GR/RA, green regenerants per 100 responding anthers.

rate only of 107.1 calli per 100 responding anthers. In cv. 'Ūla' the frequency of responding anthers was low (1.7%), however, it was superior by the number of calli per responding anthers (800.0 per 100).

The regenerants were recorded in six varieties out of the eight studied. However, most of the regenerants from calli were albino type plants. Cv.'Alsa' was superior for callus formation (420.0 calli per 100 responding anthers), however, this callus had no morphogenetic potential and no regenerants were produced. The highest plant regeneration rate was identified for cv. 'Aura' – 116.7 regenerants per 100 responding anthers. Cv. 'Aura' was the only cultivar which performed successfully for green DH regenerant formation in anther culture using Szarjeko's method [8].

DH regeneration from embryoids

The regeneration potential of ten Lithuanian spring barley cultivars by direct embryoidogenesis in the anther culture was evaluated in this experiment using Carreda's method. Embryoids were formed in the anther culture for all ten spring barley cultivars (Table 2, Figure). Cv. 'Alsa' was found to be superior by the rate of responding anthers (22.7%). The lowest rate of responding anthers was in 'Luokė' (0.3%), however, a significantly higher number of 200.0 embryoidogenic structures per 100 responding anthers was identified in this case. The highest rate of embryoid formation was identified for 'Auksiniai 3' (580.0 embryoids per 100 responding anthers) and 'Aura' (540.0 embryoids per 100 responding anthers). 5.3% of 'Auksiniai' anthers were productive, but only 162.5 embryoids were formed per 100 responding anthers. Using Carreda's method, green regenerants were developed from the embryoids in three cultivars ('Aidas', 'Alsa' and 'Auksiniai'). 'Auksiniai 3', 'Aura', 'Gintariniai' and 'Ūla' formed only albino regenerants (10.0, 40.0, 33.3 and 33.3 per 100 responding anthers, respectivley). These results suggest that anther culture response is predetermined by the genotype. Lithuanian cultivars show a high variation in terms of anther culture response and some of them perform quite readily, however, most cultivars (about 70%) have been found difficult to perform.

Table 2. **Formation of spring barley regenerants from embryoids in anther culture of Lithuanian cultivars (Carreda's method)**

Cultivars	RA (%)	EM/RA	RP/RA	GR/RA
Aidas	3.3	270.0	10.0	10.0
Alsa	22.7	351.5	30.9	4.4
Auksiniai	5.3	162.5	18.8	6.3
Auksiniai 2	1.0	200.0	0.0	0.0
Auksiniai 3	3.3	580.0	10.0	0.0
Aura	1.7	540.0	40.0	0.0
Džiugiai	1.3	175.0	0.0	0.0
Gintariniai	1.0	266.7	33.3	0.0
Luokė	0.3	200.0	0.0	0.0
Ūla	4.0	250.0	33.3	0.0
$LSD_{0.01}$	0.31	62.31	5.41	0.52

RA, responding anthers; EM/RA, embryoids per 100 responding anthers; RP/RA, regenerated plantlets per 100 responding anthers; GR/RA, green regenerants per 100 responding anthers.

DISCUSSION

A great obstacle in barley anther culture is a distinct manifestation of albinism. The reason for this is that the chloroplasts of microspores lose their inner membrane and become filled with lipids and globulins, and chlorophyll *a* is not synthesized from protochlorophyllid *a* [2]. The DNA of microspore chloroplasts is damaged at the early stage of microspore development. The efficiency of the anther culture method is largely dependent on plant genotype and cultivation conditions [3, 6]. Andersen has found that the genetic nature of donor plant affects the formation of embryoids by 20–40% and formation of green regenerates by 50–80% in wheat anther culture [9].

Our results confirm that induction response in anther culture, embryoid formation, regeneration potential and the ratio of green regenerants to albino are controlled genetically as reported in literature [1, 6, 10]. Komatsuda has evaluated that the *shd1* gene which is on the second chromosome of barley affects the formation of green plants from embryoids by 65.0% [11], therefore the main factor affecting the formation of green regenerants in anther culture is the genetic predetermination of a donor plant.

The androgenic potential of Lithuanian spring barley cultivars in anther culture has been determined as follows: cv. 'Aura' has the best regeneration rate from callus using Szarjeko's method; by Caredda's method

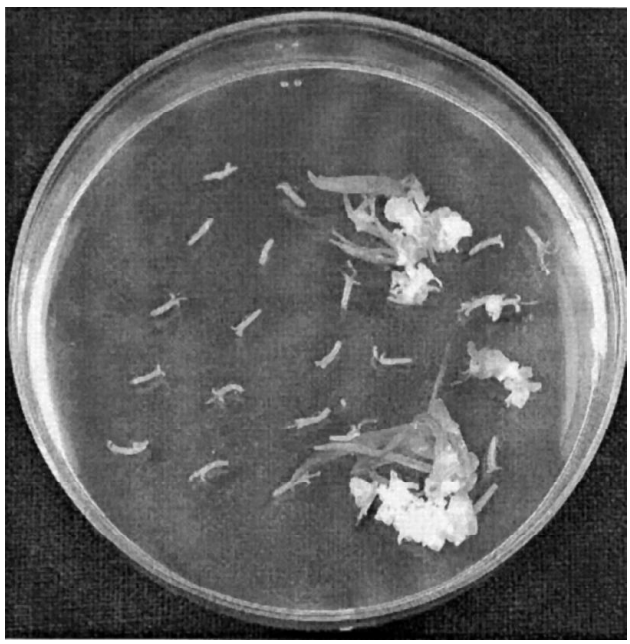

Figure. Direct green plant regeneration in barley anther culture embryoidogenesis from microspores with globular stage embryoid (Carreda's method)

green regenerants from embryoids have been produced in cvs. 'Aidas', 'Alsa' and 'Auksiniai'.

A comparison of Lithuanian barley cultivars according to the yield of green regenerants suggests that a higher percentage of green regenerants is produced while cultivating anthers by the modified method of Caredda. A study on ten cultivars using his method has shown that green regenerants can be obtained from the embryoids of cvs. 'Alsa', 'Aidas' and 'Auksiniai'. Cv. 'Aura' is exceptional as regards green regenerants obtained from the callus induced in the anther culture by Szarejko's method.

These results show that the ability to produce green androgenic plants is dependent upon the genotype and suggest that deeper genetic studies have to be undertaken in order to characterize this parameter.

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LIETUVIŠKŲ VASARINIŲ MIEŽIŲ DULKINIŲ KULTŪROS TYRIMŲ REZULTATAI

Santrauka

Vasarinių miežių dvigubų haploidų (DH) gavimas dulkinių kultūros metodu atliktas 2001 m. ir 2004 m. Lietuvos žemdirbystės instituto Genetikos ir fiziologijos laboratorijoje (Akademija). Bandymo metu įvertintas vasarinių miežių veislių androgeninis potencialas dulkinių kultūroje pagal žalių regenerantų išeigą ir kitus morfogenetinių potencialo rodiklius.

Tiriant vasarinių miežių lietuviškų veislių androgeninį potencialą nustatyta, kad daugiausia kaliaus suformavo veislės „Ūla" (800 kaliaus 100-ui produktyvių dulkinių) dulkinės ir daugiausia embrioidų – veislės „Auksiniai 3" (580 embrioidų 100-ui produktyvių dulkinių) augalai. Palyginus lietuviškas miežių veisles pagal žalių regenerantų išeigą nustatyta, kad daugiau žalių regenerantų gaunama dulkines kultivuojant pagal Caredda metodą – ištyrus 10 veislių, žali regenerantai buvo gauti iš veislių „Aidas", „Alsa" ir „Auksiniai" embrioidų. Szarejko metodu žali regenerantai gauti tik iš veislės „Aura" dulkinių kaliaus.