GENETIC AND PHYSIOLOGICAL ASPECTS IN ANDROGENESIS OF BARLEY

Summary of doctoral dissertation
Biomedical sciences, agronomy (06B)
This doctoral dissertation was prepared at the Lithuanian Institute of Agriculture during 2000-2004. Part of the work was carried out at the Laboratory of Plant Physiology and Biology of Champagne-Ardenne University in Reims, France.

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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 crops. Growing in suitable soils and sufficiently fertilized, barley can produce a grain yield higher than that of oats. In the development of self-pollinating plant cultivars, the most important and time-consuming period is from crossing paternal forms to production of pure lines. Methods of modern biotechnology allow to accelerate the process of breeding and haploid production is one of the most widely used biotechnological methods in cereal breeding. Haploid material makes creation of genetic variety, especially identification and stabilization, easier [Oury et al., 1993].

The androgenetic process is usually used for producing doubled haploid lines from microspores normally destined to produce mature pollen grains. In barley, a high number of albino plants are regenerated, limiting the exploitation of this technique. 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 of more than 200 cultivars of plants have been obtained from anthers and microspores by the method of androgenesis [Dewey, 1984; Oury et al., 1993; Caredda and Clement, 1999]. Many new cultivars of barley (more than 50) and wheat have been created using this method. The method is successfully used in rice, oats, rye, and wheat-rye breeding. The DH method is widely used in the programmes of industrial crops (oilseed rape, tobacco and sugar beet).

Haploid production system is used both in fundamental genetics and in practical plant breeding. At haploid level, each gene is hemizygotic. After the doubling of chromosomes, each chromosome has an identical copy and each gene becomes homozygotic. The doubling of haploids of self-pollinating plants means that a plant breeder can get the fixing of small parts in the early stage, and this makes it possible to estimate the first breeding material quickly and exactly.

In self-pollinating plants (barley and wheat), anther culture is used for F1 hybrids that have been produced after crossing of two lines with desirable traits that are aimed to be transferred into the homozygotic state in the first generation. This method accelerates breeding process by 3-5 years [Manninen, 1997; Sarrafi and Ghaemi, 1997].

Success of anther culture method depends on plant growing conditions, plant genotype, and choice of medium [Caredda et al., 2000]. It is very important to find such genotypes whose anthers form morphogenetically active structures. Most scientists using the method of anther culture report that morphogenetic potential of callus and embryoids is genetically predetermined [Andersen et al., 1987; Maxim, 1995; Sarrafi and Ghaemi, 1997; Caredda and Clement, 1999].

Aim of the study

The aims of the study were:
(i) To optimise spring barley double haploid (DH) production in anther culture in order to increase the yield of green regenerants;
(ii) To compare the dynamics of chlorophyll and the changes in total RNA in connection with the problem of albinism in anther culture.

Tasks of the study

1) To estimate the effect of the donor-plant genotype and its growing conditions on the formation of green regenerants;
2) To evaluate the efficiency of anthers in relation to plant tiller position in the tillering sequence;
3) To choose the most effective way of the anther cold pre-treatment at 4°C;
4) To investigate the effect of micro salts on anther productivity;
5) To estimate the androgenic potential of Lithuanian cultivars of spring barley;
6) To investigate the dynamics of chlorophylls $a$ and $b$ in etiolated and androgenic plants;
7) To evaluate the changes in total RNA migration profiles of barley at different stages of anther culture.
The novelty of the work
A number of aspects of the work are novel, and have been developed or used for the first time:

1) The importance of the position of tillers in a plant for the efficiency of microspore culture and green plant regeneration has been revealed. The best results were obtained taking anthers from the tillers of the second tillering.

2) The optimal concentration of copper sulphate for the yield increase of green regenerants in anther culture has been identified.

3) The androgenic potential of Lithuanian cultivars has been estimated. Cvs. ‘Aidas’, ‘Alsa’, ‘Auksiniai’ and ‘Aura’ were found to form green regenerants from androgenic structures in anther culture.

4) The dynamics of quantities of \( a \) and \( b \) chlorophylls and their relations within etiolated and androgenic plants of the contrasting cultivars of ‘Igri’ and ‘Cork’ have been compared.

5) The changes in total RNA profiles at different stages of anther culture has been observed. It has been found that the total RNA profile of the anthers of cv. ‘Cork’ changes during the pre-treatment. The changes have been recorded in embryoids and albino regenerants of cv. ‘Cork’ and ‘Igri’.

Practical relevance
The protocol of anther culture method allowing to increase production of homozygotic lines of spring barley has been developed. The greater efficiency of this method will allow to use anther culture in barley breeding programmes more widely and will accelerate development of new barley cultivars.

Approval
The work results have been published in three scientific articles and presented in three scientific conferences. Two of them have been published in ‘Agriculture. Scientific articles’ journal, which is included in the list of Lithuanian scientific publications approved by the Lithuanian Council of Science. The third article has been published in the edition of Lithuanian science not included in the above-mentioned list.

Volume and structure of the work
The doctoral dissertation is written in Lithuanian. It consists of six parts: introduction, research overview, methods of the work, results of the work, conclusions, references and four annexes: recommendations for breeding, list of scientific publications relevant to the research topic acknowledgements and appendix. The dissertation comprises 93 pages, including 16 tables and 31 pictures.

MATERIALS AND METHODS

Experimental subject and general methods
The research was carried out at the Laboratory of Genetics and Physiology of the Lithuanian Institute of Agriculture (Akademija) in 2000-2004. The estimation of anther efficiency according to the sequence of plant tillering, the identification of copper sulphate concentration for the anther efficiency, the dynamics of quantities of \( a \) and \( b \) chlorophylls, and the analyses of total RNA profiles were carried out at the Laboratory of Plant Biology and Physiology of Champagne-Ardenne University in Reims, France.

The barley F$_1$ hybrids were obtained by crossing using “twirl” method [Guliaev and Gujov, 1978]. The obtained hybrids were sown in the greenhouse in November – December; part of F$_1$ seeds were sown for reproduction, and the other part as a donor material for production of DH lines using anther culture.

The donor material was grown in the greenhouse in winter (in November – February) under controlled conditions (photoperiod: 16/8 h, light intensity: 18000-20000 Lx, temperature: 10-16±2°C).

Spring barley was planted in vegetative pots, four plants per pot; pot volume was 7 l, and it contained about 6 kg of soil. The soil was prepared using 2/3 of composted soil and 1/3 of peat mixture. During plant growth fertilisation and spray applications against diseases and pests were minimal so as not to influence the development of microspores [Caredda and Clement, 1999].

The spikes were collected at uninucleate stage of microspores in anthers [Szarejko, 1996]. After testing of anthers suitability for the culture they were prepared for low-temperature pre-treatment. Manipulations were carried out using two different methods developed by the researchers I. Szarejko and S. Caredda [Szarejko, 1996; Caredda, 2000].

According to Szarejko’s method, the tillers were sterilised by spraying with ethanol of 70° after having primarily removed leaves from the tillers. The ears were dissected from the sterile tillers, and the awns were removed from the ears. After estimating of microspore development stage by microscope, the ears were transferred into a sterile Petri dish, three ears per dish with a small dish inside containing sterile water to keep humidity. Each dish was sealed with parafilm to avoid dehydration and to maintain viability of microspores during the pre-treatment. The Petri dishes were pre-treated in the dark for 21-28 days (4±1°C) after wrapping them into aluminium foil.

Using the method developed by Caredda, after estimating microspores development stage by microscope, the ears and anthers were sterilised using ethanol of 70°. The anthers were planted into Petri dishes of 5 cm diameter, 30 anthers per dish. They were pre-treated at 4°C in the dark at 80% of 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 was wrapped in aluminium foil.

After pre-treatment, anthers were placed onto the culture medium without rinsing. The anthers were carefully dissected from ears using pincers and transferred on the agarized medium [Szarejko, 1996]. The anthers removed from the mannitol solution were transferred on the anther culture medium [Caredda, 2000]. 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 beginning of the structures (callus and/or embryoids) formation.

After 20-30 days from the beginning of anther culture 1-2 mm callus or embryoids were formed. Subsequently they were transferred on the regeneration medium in the Petri dish. After 2 weeks, green plants were transferred into culture tubes containing 5 ml of regeneration medium and were allowed to grow for 4 weeks in the culture chamber.

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

The analysis of the chromosome number of green regenerants was carried out using a flow - cytometer Partec Analyser (PA) [Dolezel et al., 1994].

The selected haploids were colchicines-treated by 0.1% colchicine solution [Szarejko, 1996]. After colchicines treatment the plants were transferred into vegetative pots containing a mixture of soil/peat (2/3 of compost soil and 1/3 of peat) and placed in the greenhouse for 2-4 weeks.
DH plants were grown in the greenhouse until the full maturity of seed. The collected DH line seeds (1 green plant per 1 DH line) were passed on to the barley breeder Dr. Algė Leistrumaitė from the Cereal Breeding Department for further testing.

Details of the experimental methods

Comparison of growth conditions for donor material
The experiments were carried out at the laboratory of Genetics and Physiology of the Lithuanian Institute of Agriculture in 2003 using Caredda’s method [Caredda, 2000]. The research material was the breeding F1 lines of spring barley (catalogue No.8556, No.8615, No.8620, No.8650 and No.8659).

The donor material was grown:
(1) In the greenhouse under controlled conditions, at relatively low day/night temperatures of 8-15°C/8-10°C, at light intensity of 100 µM photons m⁻² s⁻¹, and photoperiod of 16 h; the plants were not fertilised, and no spray applications against diseases and pests were used. The donor material was sown in the pots on the 15th of October 2002, and collection of ears for the anther culture was started on the 23rd of January 2003.

(2) In the field under natural conditions. The breeding lines of barley were grown in the nine-field rotation field of the Plant Breeding Department. In spring when the soil had dried, it was harrowed, 60 kg ha⁻¹ of nitrogen was applied and then the soil was cultivated and harrowed again. The spring sowing was started at the end of April. Collection of the first ears for the anther culture was begun on the 16-17th of June 2003.

The number of responding anthers, embryoids and green/albino plants were recorded.

Estimation of anther efficiency according to the tiller position in the tillering sequence
The experiment was carried out at the Laboratory of Plant Biology and Physiology at Reims University (France) in 2002. The investigation material was spring barley ‘Cork’, which was used as a genotype producing a high percentage of albino plants in anther culture and winter barley ‘Igri’ producing high percentage of green plants. The experiment was carried out using the anther culture method of Caredda [Caredda, 2000].

The anthers were taken from (1) the ears of the main tillers, (2) the ears of the tillers of the first tillering, (3) the ears of the tillers of the second tillering, (4) the ears of the tillers of the third tillering and (5) the ears of the tillers of the fourth tillering.

The number of responding anthers, embryoids and green/albino plants was recorded.

Optimisation of pre-treatment method
The experiment was carried out at the Laboratory of Genetics and Physiology of the Lithuanian Institute of Agriculture in 2001 and 2003 using Szarejko [Szarejko, 1996] and Caredda [Caredda, 2000] methods. The material was the breeding F1 lines of spring barley (catalogue No.8317, No.8331, No.8332 and No.8341).

(1) Ears were dissected from sterile tillers, and awns were removed from the ears and were placed in Petri dishes (according to Szarejko). After the estimating of the developmental stage of microspores by microscope, the ears were placed into a sterile Petri dish with a small dish containing sterile water to prevent dehydration. Each dish was sealed with parafilm to prevent dehydration of ears and to maintain viability of microspores during the pre-treatment period. The Petri dishes were pre-treated at 4±1°C in the dark for 21-28 days wrapped in aluminium foil;

(2) The tillers were kept in situ (according to Szarejko) in Knop’s solution (Ca(NO₃)₂·4H₂O 1.43 g l⁻¹, KH₂PO₄ 0.25 g l⁻¹, MgSO₄·7H₂O 0.25 g l⁻¹, KCl 0.125 g l⁻¹, FeCl₆·6H₂O 0.0125 g l⁻¹) and pre-treated at 4±1°C in the dark for 21-28 days;

(3) The collected ears were sterilised by ethanol 70%. The anthers were dissected and were placed in Petri dishes 5 cm in diameter with mannitol (according to Caredda). Mannitol concentration 62 g l⁻¹, 30 anthers per pot, osmotic pressure of 180 mosm l⁻¹. Each dish was sealed
with parafilm to prevent spillage of the solution. The Petri dishes were wrapped into aluminium foil and kept at 4±1°C temperature in the dark for 3-4 days.

The number of responding anthers, embryoids and green/albino plants was recorded.

The effect of microelements on the green regenerants formation in anther culture.
The experiment was carried out at the Laboratory of Genetics and Physiology of the Lithuanian Institute of Agriculture in 2003, using Caredda’s method [Caredda, 2000]. The experimental material was the breeding F1 lines of spring barley (catalogue No.8331, No.8332 and No. 8341) and winter barley ‘Igri’ producing green planlets in high percentage (control).

The anthers were transferred into Petri dishes of 5 cm diameter with mannitol (62 g l⁻¹) and the solution of 10 µM, 30 anthers per dish: (1) without microsalts (control); (2) MgSO₄·7H₂O, (3) MnSO₄·H₂O, (4) FeSO₄·7H₂O, (5) CuSO₄·5H₂O and (6) ZnSO₄·7H₂O. After removal from the mannitol solution, the anthers were transferred on the anther culture medium with the solution of 10 µM: (1) without microsalts (control); (2) MgSO₄·7H₂O, (3) MnSO₄·H₂O, (4) FeSO₄·7H₂O, (5) CuSO₄·5H₂O and (6) ZnSO₄·7H₂O. The control anther culture medium contained: NH₄NO₃ 0.166 g l⁻¹, KNO₃ 1.9 g l⁻¹, KH₂PO₄ 0.17 g l⁻¹, K₂B₂O₃ 6.2 mg l⁻¹, KI 0.83 mg l⁻¹ and Na₂MoO₄·2H₂O 0.25 mg l⁻¹. After 20-30 days from the beginning of anther culture, the formed embryoids were transferred on the regeneration medium [Caredda, 2000] into the Petri dishes.

The number of responding anthers, embryoids and green/albino plants was recorded.

The effect of copper sulphate on anther culture efficiency
The experiment was carried out at the Laboratory of Plant Biology and Physiology of Reims University (France) in 2002. The research material was spring barley ‘Cork’ used as a genotype in anther culture producing high number of albino plants and winter barley ‘Igri’ producing high number of green planlets. The experiment was carried out according to Caredda’s anther culture method [Caredda, 2000].

The anthers were pre-treated as follows: 30 anthers were placed per dish with mannitol (62.0 g l⁻¹) at different concentrations of copper: (1) 0, (2) 10 µM, (3) 20 µM, (4) 30 µM, (5) 40 µM, (6) 50 µM, (7) 60 µM, (8) 70 µM and (9) 80 µM, the Petri dishes were kept at 4±1°C temperature in the dark for 3-4 days wrapped in aluminium foil. After pre-treatment, the anthers were transferred on the anther culture medium with different concentrations of copper sulphate: (1) 0, (2) 10 µM, (3) 20 µM, (4) 30 µM, (5) 40 µM, (6) 50 µM, (7) 60 µM, (8) 70 µM and (9) 80 µM.

The number of responding anthers cultivated in the light and in the dark, as well as the number of embryoids and green structures (embryoids and green regenerants) formed were recorded.

Testing of auxins in the regeneration medium
The experiment was carried out at the Laboratory of Genetics and Physiology of the Lithuanian Institute of Agriculture in 2001, employing Szarejko’s method [Szarejko, 1996]. The research material was the breeding F1 line of spring barley (catalogue No.8332, No.8337 and No.8390).

The new physiological analogues TA-12 and TA-14 of IAA α-naphthylacetic acid were obtained from the Laboratory of Plant physiology of the Institute of Botany (Habil. Dr. L. Navickienė). According to Szarejko’s method, the formed callus was transferred onto the regeneration medium with different growth regulators: (1) 0.5 mg l⁻¹ IAA, (2) 0.2 mg l⁻¹ TA-12 and (3) 0.2 mg l⁻¹ TA-14. One half of callus was grown (1) in culture tubes of 1.5 cm diameter, and the other half (2) in Petri dishes of 9.0 cm diameter with different auxins.

After 30 days cultivation, the development of plants and callus was recorded.

Estimation of the androgenic potential of Lithuanian spring barley cultivars
The experiments were carried out at the Laboratory of Genetics and Physiology of the Lithuanian Institute of Agriculture at the Department of Cereal Breeding.

The experimental material:


The androgenic potential of spring barley cultivars in anther culture was estimated according to the yield of green regenerants and other indicators of morphogenetic potential.

Analysis of the content of chlorophylls a and b

The study was carried out at the Laboratory of Plant Biology and Physiology of Champagne Ardenne University in Reims, France in 2002. The experimental material: spring barley ‘Cork’ used as a genotype producing albino plants in anther culture at a high percentage, and winter barley ‘Igri’ producing green regenerants at a high percentage [Wojnarowiez, 2002]. The androgenic plants were obtained according to Caredda’s anther culture method [Caredda, 2000]. The etiolated plants were grown at 20±2°C temperature in the dark for 8 days [Kouril et al., 1999]. The test samples were taken after 8 days etiolation.

The content of chlorophylls a and b was tested in (1) androgenic and (2) etiolated plants. The content of pigments in the etiolated plants was tested every 24 hours up to the 10th day. In the androgenic plants (green (1) and albino (2) regenerants) the content of pigments was measured after 18 days regeneration, taking samples every two days up to 30th day of regeneration (3 replicates). The measurements were carried out by a spectrophotometer ‘Novaspec® II’ at 663 nm, 647 nm and 470 nm of the wavelength.

The content of chlorophylls was calculated:

The concentration of chlorophyll a (mg l⁻¹): \( C_a = 12.25 \times D_{663\text{ nm}} - 2.79 \times D_{647\text{ nm}} \)

The concentration of chlorophyll b (mg l⁻¹): \( C_b = 21.5 \times D_{647\text{ nm}} - 5.1 \times D_{663\text{ nm}} \)

The ratio of chlorophyll a to chlorophyll b: \( C_a / C_b \)

Extraction of total RNA

The research was carried out at the Laboratory of Plant Biology and Physiology of Champagne Ardenne University in Reims, France in 2002. The research material was spring barley ‘Cork’ and winter barley ‘Igri’. RNA was extracted from: (1) the leaves of donor plant, (2) the anthers of donor plant at uninucleate stage of microspores, (3) the anthers of donor plants after 3-4 days pre-treatment, (4) the embryoid structures of 28 days cultivation, (5) the green leaves of regenerants and (6) the leaves of albino regenerants. The green and albino regenerants were obtained using Caredda’s method of anther culture [Caredda, 2000].

100 mg of leaves and embryoids and 50 mg of anthers were used for each extraction. The total RNA was extracted using recommended methods and the set of Qiagen company (Cat. No. 74904).

Statistical data analysis

The research data were processed using statistical analysis (unifactorial, two-factorial and three-factorial experiments) for quantitative and qualitative parameters and the set of statistical data analysis software “SELEKCIJA” (author Dr. P. Tarakanovas). The experiments included many variants, therefore we used the multiple Duncan criterion. The essence of this method is sorting the average of variants according to their value, thus obtaining a progressively increasing data line. The data of the experiments were treated according to this criterion using the software “ANOVA” [Tarakanovas and Raudonius, 2003].

Theoretical lines of regression give the results of chlorophylls. The regression determines how much on average the value of X changes when changing the value of Y, and vice versa. Linear regression equations \( y(x) = a + bx \) and determination factors (R²) have been calculated using the software “STAT” [Tarakanovas and Raudonius, 2003].
RESULTS

The effect of donor material growth conditions on the efficiency of androgenesis

The process of androgenesis in barley has three stages: 1) induction – the usual development of gametophyte is blocked and an alternative sporophyte programme is induced; 2) cultivation – the microspores produce callus or embryoid structures; 3) regeneration – haploid plants are regenerated from androgenic embryoids or callus.

A great obstacle in barley anther culture is a distinct manifestation of albinism. The chloroplasts of microspores lose their inner membrane, are filled with lipids and globulins, and chlorophyll $a$ is not synthesized from protochlorophyllid $a$ [Manninen, 1997]. The DNA of microspore chloroplasts is damaged at the early stage of microspores development. The efficiency of the anther culture method is largely dependent on the plant genotype and on cultivation conditions [Cistue et al., 1995; Sarrafi and Ghaemi, 1997; Jacquard et al., 2003]. 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 the wheat anther culture [Andersen et al., 1987].

The efficiency of anthers was evaluated and compared for two growth conditions: (i) the donor material was grown in the field conditions; (ii) in the greenhouse conditions. The experiment was carried out with 5 selected F$_1$ lines.

The data of the experiment show that the donor material growth conditions affect the efficiency of androgenesis in anther culture. The most responding anthers were obtained from the breeding F1 line No.8650 (49.3%) when the plants were grown in the greenhouse under controlled conditions. After isolating the anthers from the barley grown in the greenhouse, the embryoids were formed by approximately 24.8% of anthers, and after isolating the anthers from the plants grown in the field conditions, the formation of embryoids was as low as 4.1%. In May 2003, there was less precipitation compared with the average amount of precipitation for May, and this might have determined the lower efficiency of barley anthers obtained from the ears grown in the experimental field.

The yield of green regenerants depended on the donor plant growth conditions. High induction of embryoids does not assure a sufficiently high yield of green plants. The averaged data show that the anthers of the plants grown in the greenhouse always produce green plants, while two genotypes taken from the field did not produce green plants at all, and the other two genotypes taken from the greenhouse regenerated at lower frequency (Fig. 1).

![Graph showing the effect of donor material growth conditions on the formation of green regenerants](image)

**Fig 1.** The effect of donor material growth conditions on the formation of green regenerants

One of the most important factors determining the yield of green regenerants in anther culture is genetic predetermination of plant. For example, F$_1$ hybrid No.8620 of spring barley regenerated as high as 32.5% of green plants.
The breeding F1 line No.8556 produced green plants at a quite high rate (27.5%). Komatsuda evaluated that shd1 gene which is on the second chromosome of barley affects the formation of green plants from embryoids by 65.0% [Beck et al., 2000], therefore the main factor affecting the formation of green regenerants in anther culture is the genetic predetermination of a donor plant.

The plants grown in the greenhouse derived substantially more embryoids and regenerated more green plants compared with the donor plants grown in the field conditions (in natural conditions). It is clear that the comparison of the variants ‘In the experimental field’ and ‘In the greenhouse’ includes many components: temperature, light, humidity and other factors. Our experiment provided a valuable comparison and our data have shown that anther culture is more effective for donor plants grown in the greenhouse. This determined the course of our further investigations, and only the plants grown in the greenhouse were used for optimisation of the anther culture method.

In conclusion, the anthers that were isolated from the barley grown in the greenhouse on average produced more green regenerants (8.4 green regenerated plants per 100 responding anthers) than the anthers obtained from the barley grown in the field conditions (6.7 green regenerated plants per 100 responding anthers).

Efficiency of anther culture as affected by the tiller position in the tillering sequence

During the previous experiments, attention was paid to the fact that efficiency of anthers isolated from the same donor plant is variable. It was therefore decided to study whether the tiller position (main or secondary) had any influence on the efficiency of the anthers. For this purpose, the tillers of ‘Cork’ and ‘Igri’ were marked, and the anthers from the ears were taken from the tillers according to the tillering sequence.

The experimental results show that the anthers isolated from the different tillers produced the embryoids at different rate. Cv. ‘Cork’ was noted for the highest efficiency of anthers (17.0%) when the anthers were collected from the ears of the tillers of the first tillering. The microspores of ‘Igri’ were found to produce embryoids quite efficiently (16.7%) when the anthers were collected from the tillers of the second tillering.

The experimental evidence suggests that the anther efficiency of ‘Igri’ and ‘Cork’ tended to be different according to the formation of green plants from the tillers of different position in the tillering sequence (Fig. 2).

**Fig. 2.** The effect of tiller position in the tillering sequence on the formation of green regenerants in anther culture (0- main tillers; 1- tillers of the first tillering; 2-tillers of the second tillering; 3- tillers of the third tillering; 4-tillers of the forth tillering)
‘Igri’ produced green plants from the anthers from the main tillers at a fairly high rate; while for ‘Cork’ the anthers from the secondary tillers were more efficient. This tendency was revealed while comparing the efficiency of the anthers of ‘Cork’ according to the tiller position, which is very important. For the first time it was shown by experiment that albino regenerants are formed at high percentage from the main tillers, whereas the anthers of the secondary tillers produced a high number of green plants.

We were the first to discover the importance of tiller position for the efficiency of anther culture and to show that when the anthers of main tillers produce albino regenerants, it is possible to improve the yield of green regenerants using the anthers of secondary tillers.

These results will be published in the journal “Plant Cell Reports” (ISSN: 0721-7714) in collaboration with the colleagues at the Laboratory of Biology and Physiology of Champagne Ardenne University in Reims, France.

In conclusion, the anthers isolated from the ears from the tillers of the second tillering on average produce more green regenerants (54.6%) than the anthers isolated from the ears from the main tillers (40.6%). The microspores in the anthers of the tillers form the fourth tillering were sterile.

**Pre-treatment of the anthers by of temperature (4°C) stress in different ways**

Treatment of the anthers by different stress factors before planting them in vitro has a positive effect on morphogenic structure formation in anther culture. The mechanism that predetermines higher rate of structure formation in anther culture under stress affects has not been widely studied yet. Low positive temperatures are thought to change ageing of the cells in the anther, and during degradation the tapetum of the anther wall stimulates a higher number of microspores to be developed in sporophytic way.

It has been demonstrated in the numerous studies that it is necessary to keep anthers at low temperature before transferring them into the culture [Oury et al., 1993; Hossini-Salekdeh and Abd-Mishani, 1998].

Four breeding F1 lines were used in the experiment aiming to investigate how the productivity of the anthers in vitro depends on the way of pre-treatment. The anthers were pre-treated at 4°C: (1) ears kept in Petri dishes for 21-28 days, (2) tillers kept in situ for 21-28 days and (3) anthers kept directly in Petri dishes with mannitol for 3-4 days.

The number of responding anthers and formation of androgenic structures varied depending on pre-treatment method and also these parameters were genotype dependant. The breeding F1 line No.8317 responded best (24.0%) when the ears were pre-treated in Petri dishes with mannitol. Mannitol \( \text{C}_6\text{H}_{14}\text{O}_6 \) increases osmotic pressure of solution, which leads to dehydration of anthers. Affected by mannitol under the osmotic pressure the microspores spread out on the nutrient medium [Caredda et al., 2000; Kasha et al., 2001], therefore this method provides significantly higher yield of responding anthers. The lowest number of responding anthers was recorded in the breeding F1 line No.8331 (0.5%).

Our results demonstrated (Fig. 3) an average formation of green regenerants at the rate of 3.2% when anthers were pre-treated in Petri dishes with mannitol. The pre-treatment by Szarejko method allowed an average production of green regenerants from the anthers at the rate of 2.2%. Applying pre-treatment (1) callus induction was higher than embryoid induction applying pre-treatment (3), however the regeneration from embryos was more efficient. On average the highest number of green regenerants obtained was at the rate of 3.3% for No.8341. The breeding F1 lines No.8332 and No.8331 also produce high percentage of green plants (2.1% and 2.0%).

The genotype effect was identified in relation to different pre-treatment method applied for the anthers. Three breeding F1, lines No.8317, No.8331 and No.8332, produced similar number of green plants when the ears were pre-treated in Petri dishes for 21-28 days and in Petri dishes with mannitol for 3-4 days. Line No.8341 was superior for green plant regeneration when anthers were pre-treated in tillers in situ and directly in Petri dishes with mannitol, however pre-treatment method (1) was exceptionally inefficient for this genotype. Other authors also suggest that the genotypes are
different in their response to pre-treatment and to different combinations of stress [Hossini-Salekdeh and Abd-Mishani, 1998; Manzyuk and Belinskaya, 2000]. Better results are obtained when anthers are dissected from ears and pre-treated in Petri dishes than when they are kept in tillers *in situ* [Sibi and Fakiri, 2000].

![LSD_{0.01}=5.02](image)

**Fig. 3.** The effect of pre-treatment on the formation of green regenerants

In conclusion, the comparison of the different pre-treatment methods has demonstrated that the most efficient was Caredda method when anthers were kept at 4°C in mannitol for 3-4 days. The yield of green regenerants was 1.5 times higher (average data for four breeding lines) than when anthers were kept in tillers *in situ* and in Petri dishes according to Szarejko.

### The effect of microelements on formation of green regenerants

The microelements affect anther culture efficiency, embryoid formation, and plant formation. Number of different authors studied and identified an effect of five microsalts used in anther culture: magnesium sulphate (MgSO₄), manganese sulphate (MnSO₄), iron sulphate (FeSO₄), copper sulphate (CuSO₄), and zinc sulphate (ZnSO₄) [Castillo and Cistue, 1993; Luckett and Smithard, 1995; Caredda and Clement, 1999]. The microelements are active in different biochemical processes of plant: magnesium (Mg²⁺) in chlorophyll biosynthesis, manganese (Mn²⁺) in photosynthesis, iron (Fe²⁺) in the fixation of nitrogen and the process of photosynthesis, copper (Cu²⁺) in photosynthesis and seed formation, and zinc (Zn²⁺) affects activity of enzymes and it is a metabolite in the pathway of auxins [Marschner, 1995].

Cv. ‘Igri’ was used as a control in this experiment. This genotype forms the embryoids in anther culture which regenerate green plants at a high percentage [Jacquard et al., 2003]. Therefore, it was useful to included ‘Igri’ in our experiment on microsalts and to have it as reference to the results of breeding F1 lines No.8331, No.8332 and No.8341. We tested five microsalts (MgSO₄, MnSO₄, FeSO₄, CuSO₄ and ZnSO₄) at a concentration of 10 µM. The content of the anther culture medium was as following: NH₄NO₃ 0.166 g l⁻¹, KNO₃ 1.9 g l⁻¹, KH₂PO₄ 0.17 g l⁻¹, K₂BO₃ 6.2 mg l⁻¹, KI 0.83 mg l⁻¹ and Na₂MoO₄·2H₂O 0.25 mg l⁻¹.

Our results demonstrated that barley genotype predetermines the rate of responding anthers. Cv. ‘Igri’ was superior for the anther induction (43.0%) using copper sulphate in anther culture media. The anthers of the genotype No.8332 responded positively to magnesium sulphate (30.0%). Mg SO₄, Fe SO₄ and CuSO₄ demonstrated positive effect in anther response in comparison to the control.

Our data show the highest anther culture efficiency (18.1%) using 10 µM of copper sulphate (Fig. 4) in the medium. The anthers formed embryoids sufficiently on the media with magnesium sulphate (13.2%) and iron sulphate (12.5%). Green plants also were regenerated at a high rate of
15.8% using 10 µM concentration of copper sulphate. Zinc sulphate at 10 µM had negative effect on anther induction, i.e. green regenerants were not produced from embryoids.

Control cv. ‘Igri’ responded well in the anther culture (32.7%) without any supplement of tested microsalts in the culture media. ‘Igri’ anthers perform readily when used in the anther culture and this genotype produce high number of embryos and green plants stably and easily. This cultivar has a high androgenic potential which allows the formation of embryos from the microspores without any addition of microsalts in the medium, while barley hybrid lines have low androgenic potential.

The positive effect of microsalts could be related to some advantage such as better viability of the microspores after temperature stress, and this could also increases the regeneration rate of green plants. The effect of microsalts was discriminated within the first week after transferring the anthers onto the anther culture medium. Our results show that copper sulphate (CuSO$_4$) had the strongest positive effect on the formation of green plants. This could related in such a way that Cu$^{2+}$ takes part in the stabilization of chlorophyll and subsequently this reduces the yield of albino regenerants.

In conclusion, data our experiment reveals that microelements are useful in the anther culture of barley added in media of induction and anther culture, they lead to better survival of microspores during the entire androgenesis. Our results suggest that copper sulphate (CuSO$_4$) has the greatest positive effect on the formation of green regenerants (15.8%) in comparison to other microsalts tested.

**The optimisation of the concentration of copper sulphate**

Cu$^{2+}$ is a necessary component in plant. In absence of copper plants die, and at the condition of Cu$^{2+}$ deficiency they appear to be damaged. The majority of enzymes include copper which increases their activity, stimulates photosynthesis, and it also takes part in the processes of oxidation and in nitrogen metabolism. Copper is necessary for the synthesis of vitamin B$_1$, chlorophylls and albumen. In our work it was important to determine the optimal concentration of copper (Cu$^{2+}$) for the formation of green regenerants in the anther culture.

Two contrast cultivars were used in the anther culture: spring barley ‘Cork’ that is used as a genotype producing albino plants at a high rate and winter barley ‘Igri’ as a genotype producing green regenerants at a high rate [Wojnarowiez, 2002]. The anthers were cultivated in the dark for 4 weeks and in the light for 2 weeks. Further, they were kept in the light without transferring onto regeneration medium. The response of anthers was determined: (i) kept in the dark, (ii) kept in the light, and (iii) total response in the dark and in the light. The number of embryos per 100
responding anthers was calculated. The number of green structures accounts for green regenerants and green embryoids in total.

Our results show that the concentration of CuSO₄ at 20 µM affects the formation of green structures from the anthers of ‘Cork’ (Fig. 5a). The percentage of green structures increased 10 times in comparison to the no copper sulphate supplement in the medium of anther culture. From the data estimated on the anther response and green structure formation for the anthers of ‘Cork’ it is suggested that 10 µM concentration copper sulphate is the most efficient.

Cv. ‘Igri’ was superior for anther induction (99.6%) using the concentration of copper sulphate at 10 µM in the media of induction and anther culture (Fig. 5b).

![Graph a) and b) showing the effect of copper sulphate (CuSO₄·5H₂O) concentration on the efficiency of anthers culture and formation of green structures in ‘Cork’ (a) and ‘Igri’ (b).]

Nonetheless, the anthers of cv. ‘Igri’ have formed embryoids without using CuSO₄ and using the concentrations of copper sulphate at 20 µM, 30 µM, 40 µM, 50 µM, 60 µM and 70 µM. However, green structures in ‘Igri’ were better formed at 10 µM concentration of copper sulphate. In the future, the effect of copper sulphate on the formation of green plants should be investigated in much more detail, i.e. using the method of RNA Differential display (Brzostowicz et al., 2000; Colomenares et al., 2000).

The data of this experiment suggest that the concentration of copper sulphate (CuSO₄) at 10 µM is the most efficient for the formation of green plants. We also came to the conclusion that for the genotypes with a high rate of formation of green regenerants it is possible to omit the transferring of green plants onto the regeneration medium. Plants from the anther culture medium can be transferred directly into the vegetative pots with the mixture of soil. This will allow saving of the time and reagents.
In conclusion, we suggest an optimal concentration of copper sulphate at 10 µM in the media of induction and anther culture which increases the yield of green regenerants by 14.0%.

The comparison of the effect of auxins on regeneration
The phytohormones affect growth and division of the cells, process of adaptation and ageing, transportation of substances, synthesis and many other vital processes in plants. Auxins induce the synthesis of ethylene and affect the amount of cytokinines in plants [Ouedrago et al., 1998]. It was found in oilseed rape that two classes of phytohormons induce and stimulate the growth of callus and organogenesis: auxins NAA, 2,4-D and IAA, and cytokinines BAP and kinetin. The synthetic physiological analogues of α-naphtylacetic acid, TA-12 and TA-14, in the combination with the cytokinines stimulate the growth and rizogenesis in the cells of oilseed rape callus, and TA-14 used separately stimulate the formation of shoots. These synthetic auxins for oilseed rape are more efficient than 2,4-D, NAA and IAA [Mackevičius et al., 1999].

The regeneration medium was tested using auxins: IAA, TA-12 and TA-14. Three breeding F1 lines were used to investigate efficiency of anthers in vitro as affected by the conditions of callus cultivation. During the experiment, callus was cultivated: (i) in Petri dishes and (ii) in culture tubes. These experiments demonstrated that response to TA-12 and TA-14 depended on the barley genotype and the anther cultivation method. The highest callusogenesis rate in the anther culture was identified for breeding line No.8332 when callus was pre-treated in culture tubes with 0.5 mg l⁻¹ IAA in the regeneration medium. High callusogenesis rate of 68.6% was found for the breeding F1 line No.8390 grown in Petri dishes with 0.2 mg l⁻¹ TA-1. The highest regeneration percent was in the callus of the breeding line No.8390 (5.1%) when callus was allowed to grow in Petri dishes with 0.5 mg l⁻¹ IAA in the regeneration medium. In total, the regeneration potential of the callus of breeding F1 lines in this experiment was low. For example, the callus that was induced from the breeding line No.8332 formed only 1.2% of albino regenerants in Petri dishes with 0.5 mg l⁻¹ IAA in the medium.

The shoot regeneration using TA-12 and TA-14 was not as intensive as using IAA. Only albino regenerants were regenerated. It was identified that using TA-12 and TA-14 the organogenesis in callus is induced at a lower rate in comparison to using IAA. It suggests that the level of endogenic phytohormones was predetermined by plant genotype, so in this way that effect of egzogenic hormones for callusogenesis and organogenesis process was not clearly identified. It is known that synthetic analogues TA-12 and TA-14 are effective for increase of both green and dry mass in field conditions. They affect the growth of vegetative organs and have positive effect on the formation of generative organs as well [Mackevičius et al., 1999].

The microclimatic conditions of callus cultivation were important for callus organogenesis. Using culture tubes (Ø 1.5 cm) plant regeneration was better than cultivating callus in Petri dishes (Ø 9.0 cm). Some authors point out that there must be optimal microclimate in vitro culture to be able to establish a high regeneration rate [Wang et al., 1993; Jacquard et al., 2003]. The larger volume is more suitable for callus organogenesis.

We conclude that physiological auxin analogues of α-naphtylacetic acid, TA-12 and TA-14, have no positive effect in barley anther culture for callusogenesis and regeneration in comparison to 3-indolilacetic acid (IAA). Regeneration from callus is better in Petri dishes (Ø 9.0 cm) than culture tubes (Ø 1.5 cm).

Evaluation of androgenic potential of Lithuanian spring barley cultivars
Experiments were carried out in 2001 by Szarjeko method [Szarjeko,1996] and in 2004 by using more recent method of Carreda [Carreda, 2000].

In 2001 barley cultivars varied significantly according to their response in the anther culture. The highest induction response in anther culture was determined for ‘Aura’ (6.0%). Anthers of cv. ‘Ūla’ produced the highest number of callus per anther (800.0 callus per 100 responding anthers), similar results were for ‘Aidas’ (600.0 callus per 100 responding anthers) (Fig. 6 a). ‘Džiugiai’ and
‘Auksiniai 3’ showed low callus formation in the anther culture (100.0 and 107.1 callus per 100 responding anthers, respectively). In cv. ‘Auksiniai 3’ anther response was as low as 4.7%, and callus was formed at a low rate only of 107.1 per 100 callus per responding anthers. In cv. ‘Ūla’ the frequency of responding anthers was low (1.7%), however it was superior for the number of callus per responding anthers, 800.0 callus per 100 responding anthers.

The highest callusogenesis rate was identified for cv. ‘Aura’, i.e. 116.7 regenerants per 100 responding anthers. Cv. ‘Alsa’ was superior for callus formation (420.0 callus per 100 responding anthers), however this callus had no morphogenetic potential and no regenerants were produced in the anther culture. Cv. ‘Aura’ was the only cultivar which performed successfully for green regenerant formation in anther culture using Szarjeko method [Szarjeko, 1996] (Fig 6c).

![Fig 6. Formation of spring barley regenerants in the anther culture of Lithuanian cultivars: a) EPSk, callus per 100 responding anthers; b) EPSe, embryoids per 100 responding anthers; c) Rgk, green regenerants (developed from callus) per 100 responding anthers; d) Rge, green regenerants (developed from embryoids) per 100 responding anthers](image)

Better results were achieved in terms of green regenerant production in 2004 using Carreda method. Cv. ‘Alsa’ was found to be superior for the rate of responding anthers (22.7%). In this experiment embryoids were formed in the anther culture for all 10 spring barley cultivars. 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) (Fig. 6b). The lowest rate of embryoid formation was identified for ‘Auksiniai’ (162.0 embryoids per 100 responding anthers). 5.3% of ‘Auksiniai’ anthers were productive, but only 162.5 embryoids were formed per
100 responding anthers. The lowest rate of responding anthers was in ‘Luokė’ (0.3%), however significantly high number of 200.0 embryoidogenic structures per 100 responding anthers was identified in this case. These results suggest that anther culture response is predetermined by the genotype. Lithuanian cultivars show high variation in terms of anther culture response and some of them perform quite readily, however most cultivars (about 70%) are found to be difficult.

Cv. ‘Alsa’ was superior for embryoidogenesis in anther culture using Carreda method, i.e. 21 regenerants were formed from 239.0 embryoids. High formation rate of embryoids was determined for ‘Auksiniai II’ and ‘Luoke’ (200.0 embryoids per 100 responding anthers), but no regeneration from these structures was induced. The embryoids of cv. ‘Aura’ produced the highest number of albino regenerants (40.0 regenerants per 100 responding anthers). For ‘Auksiniai 3’, ‘Gintariniai’ and ‘Ūla’ only albino regenerants were formed (10.0, 33.3 and 33.3 per 100 responding anthers, respectively).

The regeneration potential of Lithuanian spring barley cultivars by direct embryoidogenesis in the anther culture was evaluated in this experiment. Green regenerants were developed from the embryoids of ‘Aidas’, ‘Alsa’ and ‘Auksiniai’ using Carreda method (Fig. 6d). Cv. ‘Alsa’ was determined to have the highest androgenic potential, while for cv. ‘Auksiniai 3’ the highest rate of embryoid formation was identified. The highest number of albino regenerants was identified for Cv. ‘Aura’.

Our results confirm that induction response in anther culture, formation of embryoids, regeneration potential and the ratio of green regenerants to albino are controlled genetically as it was discussed in literature [Maxim, 1995; Caredda and Clement, 1999; Beck FX et al., 2000; Caredda et al., 2000].

Study of chlorophyll $a$ and $b$ content in relation to albino formation in anther culture

Changes in the content of chlorophylls $a$ and $b$ could be related with albino phenomenon in the anther culture. Two standard cultivars of barley were used in this experiment: cv. ‘Cork’ was used as a genotype producing a high number of albino plants in anther culture and winter barley cv. ‘Igri’ as a genotype producing high number of green plants [Wojnarowiez, 2002]. The content of chlorophylls $a$ and $b$ and their ratios in etiolated and androgenic plants were determined.

Results on control plants (plants were not etiolated prior to experiment) grown under standard light/dark regime have shown that the content of chlorophylls in dynamics increases in ‘Igri’ more intensively than in ‘Cork’ during ten days (Fig. 7a). In ‘Igri’ the content of chlorophylls increased by 0.926 µg g$^{-1}$ per day, and in ‘Cork’ the content of chlorophylls increased by 0.531 µg g$^{-1}$ per day.

It was identified in dynamics for etiolated plants after transferring them for 10 days to the light that the content of chlorophylls in plants of ‘Igri’ had increased by 1.384 µg g$^{-1}$ per day, and in the etiolated plants of ‘Cork’ – by 1.221 µg g$^{-1}$ per day (Fig. 7b). Chlorophyll $a$ was detected in one minute after the etiolated plants were transferred to the light. This suggests that plants are likely to synthesize protochlorophyllid $a$ in the dark which further, at light, is transformed into chlorophyll $a$. Results showed that in the etiolated plants after transferring them for 10 days to the light the rate of recovery of photosynthesis in ‘Igri’ was more intensive than in ‘Cork’ plants.
The content of chlorophyll \(a\) in green plants is twice as that as of chlorophyll \(b\) [He et al, 1999]. In our experiment we estimated ratios of chlorophylls \(a\) and \(b\) in dynamics within ten days. In non-etiolated plants the ratio of chlorophylls decreased by 0.112 times in ‘Igri’ and by 0.060 times in ‘Cork’ (Fig. 8a).

Theoretically, the optimal \(a/b\) chlorophyll ratio is 3/1 [Lichtenthaler, 1987]. The non-etiolated ‘Cork’ plants reached the optimal 3/1 ratio in shorter time than ‘Igri’ plants.

The etiolated plants were transferred for 10 days to the light and \(a/b\) chlorophyll ratios of the etiolated plants were estimated in dynamics of recovery. This \(a/b\) chlorophyll ratio decreased by 0.054 times per day in ‘Igri’ and by 0.105 times per day in ‘Cork’ (Fig. 8b).

The etiolated plants grown in the dark had no chlorophyll and were white and yellow. These plants appear to be very weak, their leaves and tillers did not develop properly. The recovery of \(a\) and \(b\) chlorophylls started in one minute after transferring them to the light.
Fig. 8. Changes of chlorophyll a/b ratio in ‘Cork’ and ‘Igri’ every 24 hours up to the 10 days: (a) control seedlings grown under standard light/dark regime ($Y_{Ic}=-0.112x+2.37$, $R^2=0.62$, $Y_{Cc}=-0.060x+2.34$, $R^2=0.14$) and (b) etiolated seedlings transferred to standard light/dark regime ($Y_{Ie}=-0.054x+2.65$, $R^2=0.12$, $Y_{Ce}=-0.105x+3.20$, $R^2=0.25$). The Chl a/b ratios were measured by spectrophotometric [Lichtenthaler, 1987; Kouril et al., 1999] method in 80% acetone are room temperature.

The content of chlorophylls a and b in the green regenerants increased in dynamics of 30 days (Fig. 9a). The increase of chlorophyll in green ‘Igri’ regenerants was determined by 87% by the time from the start of regeneration and only by 13% by other uncontrolled factors. The dynamics of chlorophyll increase in green regenerants of ‘Cork’ was affected in similar way, i.e. the content of chlorophylls was determined by the time by 97%, and by other uncontrolled factors only by 3%.

Green ‘Igri’ regenerants had higher content of chlorophyll than green ‘Cork’ regenerants after 30 days regeneration and growth in the light. This suggests that the efficiency of photosynthesis in the regenerants of ‘Igri’ is more efficient by 50% than in the green regenerants of ‘Cork’.

The albino regenerants do not synthesise chlorophylls because of genetic disorders. Protochlorophylls a and b as were found in the albino regenerants, but not chlorophylls a and b. Changes in the ratio in dynamics showed increase by 0.084 times for regenerants of ‘Igri’ for every two days during 30 days, and by 0.117 regenerants of ‘Cork’.

In conclusion, the content of chlorophylls in the etiolated and not etiolated plants during ten days increased at a higher rate in ‘Igri’ than ‘Cork’. This suggests that photosynthesis recovers more efficiently in the etiolated ‘Igri’ plants than in ‘Cork’ plants.

The content of chlorophylls a and b in the green regenerants of ‘Igri’ increased significantly higher than in the green regenerants of ‘Cork’. This could be the reason of higher efficiency of photosynthesis in the green regenerants of ‘Igri’ than of ‘Cork’. Photosynthesis was found to be blocked in all albino regenerants both of ‘Igri’ and of ‘Cork’.
Changes in the profiles of total RNA at the different stages of anther culture

Total RNA was extracted from ‘Igri’ and ‘Cork’: (1) donor plant leaves, (2) anthers with the microspores at the uninucleate stage, (3) anthers after pre-treatment, (4) embryos cultivated for 28 days, (5) leaves of green and (6) albino regenerants. The aim of the experiment was to compare the profiles of total RNA of contrast cultivars in the plants and in the anther culture.

Results have demonstrated that ‘Igri’ leaves, anthers at vacuolated microspores stage, anthers after the pre-treatment, embryos and green regenerants have identical RNA profiles with characteristic RNA bands within the range of 1.4 - 0.6 Kb (Fig. 10a). This profile comprises four specific RNA fragments, 1.4, 0.8, 0.75 and 0.6 Kb in size. Albino regenerants of ‘Igri’ were missing the fragments of 0.75 Kb and 0.6 Kb in comparison to specific RNA profile of donor material.

Cv. ‘Cork’ leaves and anthers with microspores at the uninucleate stage and green regenerants had identical RNA profiles with characteristic RNA bands within the range of 1.4 – 0.6 Kb (Fig. 10b). Four specific RNA fragments are discriminated of 1.4, 0.8, 0.75 and 0.6 Kb in size. After pre-treatment the anthers have lost two characteristic fragments of about 0.75 Kb and 0.6 Kb. Same fragments were missing in RNA of the embryos after 28 days cultivation and in RNA of albino regenerants.

Cv. ‘Cork’ is used in the anther culture as a genotype forming high number of albinos. RNA analysis provided as with an evidence of changes in the fractional composition of RNA. These changes occur in the anthers after the pre-treatment, and they resemble the ones discriminated in the embryos and albino regenerants.
CONCLUSIONS

1) The specific response of hybrid lines of barley F₁ to changes in the conditions of anther culture and their different ability to regenerate green viable regenerants shows that these characteristics are determined by genetic factors.

2) Results on five hybrid lines of barley suggest that anthers isolated from the plants grown in the greenhouse are likely to form a higher percentage of green regenerants (8.4 green regenerants per 100 responding anthers) compared to the anthers taken from the plants grown under the field conditions (6.7 green regenerants per 100 responding anthers). The breeding line F₁ No.8620 does not follow this regularity.

3) The study on contrasting cvs. ‘Cork’ (low number of green regenerants) and ‘Igri’ (high number of green regenerants), shows that the anthers taken from the tillers of the second tillering form a higher number of green regenerants (54.6% from all regenerants) compared to the anthers taken from the tillers of the main (40.6%) and the first (37.6%) tillering. This effect is especially strong for cv. ‘Cork’ (27.3%, 0.0% and 7.8%, respectively). The anthers from the third tillering are inefficient in the anther culture because of the high production of albino regenerants (93.8%).

4) The study on four hybrid lines of barley indicates that pre-treatment of anthers in mannitol solution (according to Caredda’s method) enables 1.5 times higher green regenerant production compared to ear pre-treatment in Petri dishes and tiller pre-treatment in situ (according to Szarejko’s method). However, the in situ method was effective for hybrid line No.8341.

5) Comparison of the effect of five micro salts (MgSO₄, MnSO₄, FeSO₄, CuSO₄ and ZnSO₄) on regeneration revealed that 10µM CuSO₄ addition into induction medium increased the yield of green regenerants by on average 14.0–15.8%, except for F₁ hybrid line No.8332, where a greater positive effect was identified by addition of MnSO₄.

6) The use of IAA analogues, TA-12 and TA-14, in callus regeneration medium had no positive effect on the yield of green regenerants in barley F₁ hybrids compared with the use of IAA.

7) Comparison of Lithuanian barley cultivars according to the yield of green regenerants suggests that the highest percentage of green regenerants is produced while cultivating anthers by the modified method of Caredda. The study on 10 cultivars using his method showed that green regenerants can be obtained from the embryos of cvs. ‘Alsa’, ‘Aidas’ and ‘Aukšniai’. For cv. ‘Aura’ green regenerants can be obtained only from the callus induced in the anther culture by Szarejko’s method.
8) Research on chlorophyll content dynamics showed that the androgenic plants of cv. ‘Igri’ accumulated a higher content of chlorophyll compared to the regenerants of cv. ‘Cork’. This deficiency of chlorophyll in regenerants may be directly related to a high number of albinos in the anther culture of cv. ‘Cork’.

9) Comparison of the fractional composition of total RNA of cvs. ‘Igri’ and ‘Cork’ at different stages of anther culture showed some differences in RNA profiles for the anthers after the pre-treatment and embryoids. This suggests that the loss of certain RNA fractions during cultivation of anthers of cv. ‘Cork’ may be related to the high manifestation of albinism.

LIST OF PUBLICATIONS

Articles:

Conference thesis:

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**Short profile of the author of the thesis**

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