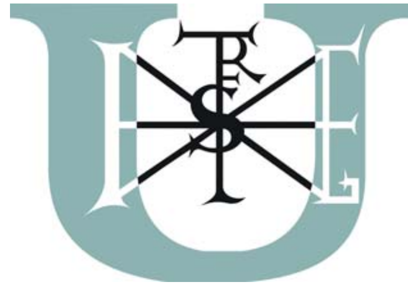

SZENT ISTVÁN UNIVERSITY

IN VITRO SELECTION OF MICROSPORES TO ENHANCE THE OXIDATIVE STRESS TOLERANCE OF MAIZE

Ph.D Thesis

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1. BACKGROUND AND OBJECTIVES

In addition to the challenge represented by the extreme weather conditions experienced nowadays (sudden warming up early in the year, heat waves in May, dry or excessively wet summers, the late arrival of spring, long periods of hot weather) and by the increasing cost of raw materials, plant breeders must also be prepared to develop new breeding material or hybrids with great ecological plasticity and resistance to abiotic (e.g. cold, drought, etc.) and biotic (pathogens) stress factors within a short space of time.

Numerous methods are now available to plant breeders to optimise the gene composition and gene functioning of the breeding materials, including both conventional methods (selection based on phenotype or molecular markers) and up-to-date gene technological and biotechnological breeding methods.

Conventional breeding is very time-consuming, a problem that can be overcome by using biotechnological methods for the effective improvement of the general adaptability of hybrids.

The development of genetically modified plants could be a useful technique for the targeted alteration of plant stress tolerance, but at present the registration and acceptance of such organisms has not yet been clarified in the European Union.

Another way of enhancing the stress tolerance of plants is *in vitro* selection in tissue culture. The advantages of this technique have been confirmed in numerous experiments on potato and tobacco. Plants selected from tissue cultures of somatic origin after treatment with stress-inducing chemicals proved to be more tolerant of cold and certain pathogen infections than the control plants. This method is primarily suited to dicotyledonous plants. It is difficult to develop fertile plants of monocots from somatic cells. The advantage of plant regeneration based on haploid induction is that the breeding time can be reduced by 6–8 years, as homozygous progeny are already produced in the first doubled haploid (DH) generation. This is of particular importance in the breeding of maize (Heszky, 2003). In recent years an anther culture system suitable for routine application has been elaborated in the Plant Cell Biology Department of the Agricultural Institute (Centre for Agricultural Research, Hungarian Academy of Sciences), making the *in vitro* selection of haploid tissue cultures possible.

Against this background, the aims of the Ph.D. work can be summarised as follows:

- The use of *in vitro* microspore selection to develop homozygous DH maize lines resistant to oxidative stress induced using compounds generating reactive oxygen species: paraquat (Pq), menadione (MD), methionine+riboflavin (MR) and *tert*-butyl

hydroperoxide (t-BHP). Analysis of the effect of these selection agents on microspore development using cytological and histological methods.

- Testing of the selected plants to determine whether they really have greater tolerance to oxidative stress than the control plants. For this purpose, the analysis of the physiological, biochemical and agronomic traits of the selected plants.
- If a selection method is successfully elaborated, the selection and testing of F_1 hybrids with important agronomic traits and the analysis of chilling tolerance at emergence.

2. MATERIALS AND METHODS

2.1. Development of plants of microspore origin and their *in vitro* selection

2.1.1. Donor plants

The basic material for the experiments was an F_1 maize hybrid of Chinese origin (A18) with good haploid induction ability, the parents of which were exotic DH lines arising from anther culture. After the model experiment, the F_1 hybrids H1, H2 and H3 were tested in the selection experiments.

The seeds of the donor plants were germinated at 26°C in a Conviron TCL germination cabinet, after which the seedlings were planted into 20-litre buckets and grown in a Conviron GB-48 growth chamber at a day-night temperature of 18/15°C, with 16 hours of photosynthetically active radiation (PAR) at 200 µmol m^{-2} s^{-1} and 80% relative humidity. The collection of the tassels required for anther culture was begun before tasselling, approx. 75±4 days after germination. Most of the anthers contained microspores in the mid-uninuclear stage, as confirmed by carmine acetic acid staining (Alexander, 1969). The young tassels were wrapped in aluminium foil and kept in the dark for seven days at 7°C.

2.1.2. Anther culture and plant regeneration

The anther culture was performed using the protocol developed in the Cell Biology Department (Barnabás, 2003). Selected tassel branches were sterilised and then rinsed three times in sterile water. The nutrient media were sterilised by autoclaving at 121°C for 20 min. The anthers were placed on modified Yu Pei medium (Genovesi and Collins, 1982; Barnabás, 2003) under aseptic conditions and then incubated in the dark at 29°C at 80% relative humidity for about a month. The embryo- or callus-like structures induced were counted and then placed on modified N_6 regeneration medium (Chu, 1978; Barnabás, 2003), where they were cultured at 26°C with 16 h illumination at a light intensity 50 µmol m^{-2} s^{-1} PAR until the

root and shoot length reached 0.5 cm. The plants were then transferred to hormone-free medium containing 2% sugar in 0.2 l glass container and grown until they were strong enough to be planted out into 5 cm diameter jiffy pellets (Jiffy Products International AS Ltd.). They were covered with plastic film and gradually adapted to the 70% relative humidity in the growth chambers by removing the film for a short period several times a day over a period of approx. 3 weeks. After this adaptation period they were grown in the growth chamber using the "BK" programme designed for maize (Tischner et al., 1997). Observations were made on the development and flowering of the plants, which were self-pollinated.

2.1.3. *In vitro* selection

For the purposes of *in vitro* selection, various compounds generating reactive oxygen species were added to the induction and regeneration media in several concentrations (Ambrus et al., 2006). These were as follows:

- paraquat (methyl viologen, Sigma M-2254) at concentrations of 0.5, 1, 5 and 10 μM (Pq),
- L-methionine (Reanal) combined with the same quantity of riboflavin (Sigma R-4500) at concentrations of 1, 10, 50 and 100 μM (MR),
- menadione (Sigma M-5625) at concentrations of 10, 50, 100 and 1000 μM (MD), and
- *tert*-butyl hydroperoxide (Merck S2676045) at concentrations of 100, 1000 and 10,000 μM (*t*-BHP).

For each treatment 5000–8000 anthers were placed on the nutrient medium.

2.1.4. Cytological and histological analyses

The cytological and histological analyses were performed on microspores and on embryo- and callus-like structures (MDS) arising from 7- and 30-day anther cultures, respectively.

The viability of the microspores was tested by means of fluorescein diacetate (FDA, Serva 21575) staining (Widholm, 1972). The analysis of the cell nuclei and of embryo development involved staining with 4',6-diamidino-2-phenylindole (DAPI) (Vergne et al., 1978).

Structures originating from 30-day anther cultures were embedded in synthetic resin for the histological analysis (Spurr, 1969). Semi-thin (1.0 μm) sections were cut with a Reichert-Jung Ultracut E microtome (Leica Mikrosysteme, Bensheim, Germany). The sections were

stained with 0.5% toluidine blue solution prior to the light microscope analysis (Sárkány and Szalai, 1957).

2.2. Development of DH_1 progeny plants; physiological and biochemical testing

2.2.1. Raising of DH_1 progeny

The DH_1 plants required for physiological and biochemical analysis were raised in a growth chamber as described above. Tests were made on fully developed leaves not yet exhibiting any signs of aging, and were terminated before flowering.

2.2.2. Plant treatment and the determination of oxidative stress tolerance

The oxidative stress tolerance of the plants was examined in flotation experiments. Leaf discs 1.3 cm in diameter were cut using a cork borer and floated in Tris-HCl solution (50 mM, pH 7.6) containing 50 μM Pq, using 1 ml/leaf disc. The control solution did not contain Pq. After infiltration with the control or Pq-containing solution, the leaf discs were floated for 4 or 24 h with 400 μmol m^{-2} s^{-1} PAR illumination (Darkó et al., 2009). The leaf discs were then used for physiological and biochemical analyses and measurements.

The oxidative stress tolerance of the plants was tested using the following techniques:

- **Measurement of chlorophyll *a* fluorescence induction**: after floating the leaf discs for 4 h, measurements were made with a PAM 2000 fluorimeter (Walz, Effeltrich, Germany). The optimum quantum efficiency of photochemical system II (PS II), F_v/F_m, was calculated from the measured parameters using the equation $F_v/F_m = (F_m - F_0)/F_m$ (van Kooten and Snel, 1990).
- **Determination of chlorophyll (*a*+*b*) content**: after floating the leaf discs for 24 h, analysis was made at wavelengths of 646.8, 663.2 and 750.0 nm using a Cary-100 UV-Vis spectrophotometer. The chlorophyll (*a*+*b*) content was determined from the results in terms of μg/g fresh weight (Lichtentaler, 1987).
- **Determination of ion leakage**: after floating the leaf discs for 24 h, the electrical conductance of the solution was recorded using an Automatic Seed Analyser (ASA610, Agro Sciences, USA) (Darkó et al., 2009).
- **Measurement of antioxidant enzyme activity**: after floating the leaf discs for 4 h, the enzyme activity was measured using a Cary-100 UV-Vis spectrophotometer (Varian, Mulgrave, Australia). The activity of the SOD enzyme (EC 1.15.1.1.) was measured using a modification of the method of Paoletti and Mocali (1990), that of

GR (EC 1.6.4.2.) according to Smith et al. (1988), that of GST (EC 2.5.1.18.) as reported by Habig et al. (1974), that of APX (EC 1.11.1.11.) according to Nakano and Asada (1987) and that of CAT (EC 1.11.1.6.) as described by Aebig (1983).

- ***In situ* detection of toxic oxygen species**: after flotation for 24 h, the leaf discs were stained with nitrotetrazolium blue (NBT, Sigma 6876) for the detection of superoxide radicals (Fryer et al., 2002) and with 3,3'-diaminobenzidine (DAB, Sigma D-8001) to monitor the accumulation of H_2O_2 (Thordal-Christensen et al., 1997).

2.2.3. Calculation of resistance factors

Resistance factors were calculated from the results of the physiological measurements in order to make it easier to evaluate the data and to compare the DH lines.

The resistance factor of the chlorophyll fluorescence induction parameter was determined using the following equation:

$$Rf_{Fv/Fm} = P_x[(Fv/Fm)_{Pq}/(Fv/Fm)_B]/DH[(Fv/Fm)_{Pq}/(Fv/Fm)_B]$$

where Fv/Fm = the optimum quantum efficiency of PS II,

DH = non-selected DH lines,

P_x = genotypes selected using Pq ($P_{1–15}$),

B = values measured in buffer in the flotation experiment,

Pq = values measured in solution containing Pq.

The resistance factor calculated on the basis of the chlorophyll content was determined using the following equation:

$$Rf_{Chl} = DH(Chl_B–Chl_{Pq})/P_x(Chl_B–Chl_{Pq})$$

where Chl = the chlorophyll (*a*+*b*) content of the leaf discs.

For other legends, see above.

The resistance factor calculated from the results of ion leakage measurements was determined using the equation:

$$Rf_{Cond} = DH(Cond_{Pq}–Cond_B)/P_x(Cond_{Pq}–Cond_B)$$

where Cond = the electrical conductivity of the solution in which the leaf discs were placed.

For other legends, see above.

2.2.4. Chilling tolerance tests at emergence

The 'cold test' of chilling tolerance at emergence was performed as described by Herczegh (1978), Marton (1992) and Marton and Kőszegi (1997). The kernels were germinated at two temperatures: at 22°C and in the cold, at 8°C. The time required for

germination and the germination % were recorded at both temperatures and the germination index (GI) of each line, i.e. the ratio of the maximum emergence % to the number of days to germination, and the chilling tolerance factor, calculated as $Px(GI_{T8°C}/GI_{T22°C})/DH(GI_{T8°C}/GI_{T22°C})$, were calculated from the results.

2.2.5. Field tests

The selected lines were also tested in the field in order to observe the effect of selection on the agronomic traits of the selected genotypes. In the course of the field tests a record was made of the germination percentage, the plant height, 50% tasselling and silking (expressed as the number of days from sowing to flowering), the fertilisation percentage (ratio of actual to potential fertilisation) and the kernel number.

2.2.6. Statistical evaluation

In most cases the data were evaluated (mean, deviation) using the Excel (2007) program. Differences were considered to be significant if there was no overlapping between the deviations. Where necessary, analysis of variance (indicated by * and **) and Tukey's post-hoc test (Statistica, 6.1. Statsoft) were applied for the statistical evaluation of the data.

3. RESULTS

3.1. Effect of selection agents on microspore development; *in vitro* microspore selection

Based on the viability determined by means of FDA staining, it could be seen that all the selection agents caused a significant reduction in the number of microspores and microspore-derived structures (MDS) obtained in both the 7- and 30-day anther cultures compared with the control anther cultures containing no selection agents.

All the selection agents were found to cause a significant, concentration-dependent reduction both in the responsiveness of the anthers and in the number of microspore-derived structures compared with the control.

It was also noted that the selection agents influenced the time when MDS appeared. The majority of MDS appeared in the 3rd week of incubation in the control cultures, while this was delayed to the 4th week after treatment with Pq, MR or *t*-BHP and until the 6th week in the case of MD treatment.

Some of the ROS compounds also exerted an effect on the type of MDS formed. In the control cultures the ratio of embryo-like and callus-like structures averaged around 30:70,

while the ratio of callus-like structures significantly increased to 82% in response to *t*-BHP and that of embryo-like structures to 48% after treatment with MD.

The results of the cytological and histological analyses confirmed earlier findings demonstrating that the selection agents retarded MDS development. DAPI staining revealed chromosome condensation, degenerated nuclei and cell degradation in 7-day cultures, while cell death and amorphous, abnormal embryos and calli were observed in the 30-day cultures. Tissue differentiation could be detected on semi-thin sections of the structures arising from 30-day cultures even in the control, while heterogeneous cell populations were observed in response to Pq, the accumulation of lipid droplets after MR treatment, and enlarged cell nuclei and heavily stained patches in the case of MD. Treatment with *t*-BHP resulted in numerous large cell organelles, the membranes of which were heavily stained by toluidine blue.

The selection agents reduced the regeneration % and the number of fertile plants compared with the control cultures.

The number of fertile plants regenerated from each hybrid after treatment with the individual selection agents is presented in Table 1.

Table 1: Number of fertile plants obtained as the result of *in vitro* selection

Hybrids	Control	Pq		MR	*t*-BHP	
		0.5 µM	1 µM	10 µM	100 µM	1000 µM
A18	**28**	**10**	**5**	**10**	**8**	**2**
H1	**15**	**9**	**7**	**1**	**2**	**0**
H2	**7**	**4**	**3**	**4**	**6**	**0**
H3	**10**	**4**	**2**	**2**	**2**	**1**

3.2. Physiological, biochemical and field analysis of the DH_1 progeny generation of plants selected on medium containing Pq

The determination of the chlorophyll *a* fluorescence induction parameter revealed nine genotypes selected from the A18 hybrid, nine from H1, two from H2 and two from H3 which exhibited a significantly smaller reduction in photosynthetic activity compared to the relevant non-selected genotype in response to Pq.

The chlorophyll (*a*+*b*) content was only determined for the hybrid A18. It was found that, while Pq treatment reduced the chlorophyll content by around 25% in non-selected DH lines and in the hybrid, this figure was only ~5% for eight selected lines.

The determination of ion leakage in response to Pq treatment revealed six lines for hybrid A18, nine for H1, two for H2 and two for H3 where the electrical conductivity

decreased to a significantly smaller extent than for the non-selected DH lines and the initial hybrids.

The accumulation of toxic oxygen species in response to Pq was also monitored by means of *in situ* staining in the case of hybrid A18. The results showed that staining was less intense in the selected lines, indicating that fewer toxic oxygen species were accumulated in response to Pq in these lines than in the non-selected DH lines and in the initial hybrid.

The activity of antioxidant enzymes was analysed for the hybrid A18. For all the antioxidant enzymes tested it could be said that four of the selected lines exhibited greater antioxidant capacity than the non-selected DH lines or the initial hybrid, and that these also responded with greater activity to Pq treatment.

As regards the resistance factors, the results indicated that six lines from the A18 hybrid had significantly higher values for all three traits for which resistance factors were calculated. Significantly higher resistance factors were recorded for seven lines from hybrid H1, two from H2 and two from H3 than for the non-selected DH lines or the initial hybrids for both traits examined.

With respect to the chilling tolerance of the selected lines at germination, it was found that the germination % decreased and the time required for germination increased in response to cold treatment. When resistance factors were calculated for chilling tolerance, higher values than those of non-selected DH lines or the initial hybrids were recorded for three lines from the A18 hybrid, 13 from H1, five from H2 and five from H3.

In summary, a total of 13 selected genotypes, three from hybrid A18, six from H1, two from H2 and two from H3, proved to have better resistance factors in terms of both Pq-induced oxidative stress and chilling stress at germination than the non-selected DH lines or the initial hybrids.

Based on the field tests it was established that selection did not cause a deterioration in the major agronomic parameters tested, while a substantial improvement was achieved in terms of flowering time, which could be important from the breeding point of view.

3.3. Novel scientific results:

- Chemicals inducing oxidative stress were used for the first time in *in vitro* microspore cultures, and the effect of these compounds on the development of maize microspores and on the fine structure of the differentiated microspore derived embryos or calli was described.
- Fertile DH maize plants were produced for the first time by means of *in vitro* microspore selection involving chemicals inducing oxidative stress, and the DH_1 progeny generation of these plants was developed.
- Physiological and biochemical tests were used to demonstrate that many DH plants selected using Pq had greater tolerance to Pq, which generates toxic oxygen species, than the control plants.
- It was proved that the oxidative stress tolerance developed in the course of *in vitro* microspore selection is inherited.

4. DISCUSSION (CONCLUSIONS AND RECOMMENDATIONS)

4.1. Effect of selection agents on microspore development and plant regeneration

The selection agents applied were found to retard cell division processes in cultured microspores compared to the untreated control. The most drastic effect was exerted by MD (Ambrus et al., 2005), in agreement with other authors who reported the influence of MD on cell division processes (Thor et al., 1982; Prasad et al., 1994; Reichheld et al., 1999). This was confirmed by the results of histological analysis. The majority of cell nuclei in structures differentiated from microspores exhibited morphological abnormalities (cell nucleus fragmentation, lysis). All the treatments caused substantial lipid accumulation in the cells of the structures formed from the microspores. It is generally considered that this can be attributed to the intense stress effects to which the cells are exposed.

In control cultures without selection agents the ratio of embryo-like to callus-like structures was in agreement with data from the literature (Kovács et al., 1999), but there was a significant increase in the ratio of embryoids after MD treatment, while the ratio of callus-like structures rose in response to *t*-BHP treatment. It appears likely that MD increases the number of symmetrical divisions through its effect on the cytoskeleton; data in the literature indicate that embryoids are typically formed following symmetrical cell division.

Previous studies revealed that the effects reported by other authors for the applied selection agents were also manifested in the cells of structures developed from *in vitro*-cultured microspores (lipid accumulation, abnormal cell nuclei). As microspores contain no chloroplasts, the destructive effect exerted on the photosynthetic apparatus could not be examined at the structural level in this work. It can thus be assumed that in microspores, too, the secondary effect of Pq is manifested, i.e. mitochondrial complexes I and II act as electron donors for the formation of superoxide radicals (Cochemé and Murphy, 2008). Further research will be required, however, to confirm this. Presumably structures that died did not possess a satisfactory detoxification mechanism. Sufficiently detailed observations could not be made due the difficulties encountered during ultrastructural analysis, so the work needs to be continued in order to achieve a more complete picture.

Fertile plants were successfully obtained from the 5000–8000 anthers cultured for each concentration of each selection agent, but the number achieved was very low in the case of MD treatment. When evaluating the results in terms of fertility, however, it should be noted that plant regeneration is one of the most difficult parts of DH development. Even when plants were successfully raised, proterandry or protogyny were often observed, in other words, either the female or male flowering was delayed to such an extent that self-fertilisation became impossible. In order to improve the method, it is recommended that such problems should be avoided by early colchicine treatment, as data in the literature suggest that a short period of treatment with a low concentration of colchicine at the beginning of induction overcame flowering problems (Barnabás et al., 1999; Kovács et al., 1999).

The low number of plants developed after treatment with MD was unexpected. As this selection agent was found to increase the ratio of MDS in the cultures, it was expected that a larger number of plants would be successfully regenerated. Due to the effect of MD on the regulation of the cell cycle, the embryos formed were not functional and did not germinate after being transferred to regeneration medium. The influence of MD on symmetrical cell division is interesting and would be worth further cytological and molecular biological analysis in both maize and other cereals.

In the light of the results the question also arises of whether mutations may be induced during the selection process. Considering the number of F_2 microspores exposed to treatment (approx. 10 million/treatment) this is conceivable, but it appears more likely that the broad selection basis provided by the variability arising from genetic segregation could be the explanation for the success of selection for oxidative stress tolerance. This is backed up by the fact that experiments performed as a continuation of the work discussed in the thesis have

shown that this enhanced level of oxidative stress tolerance is stably inherited in later DH generations ($DH_{1–5}$).

4.2. Physiological and biochemical analysis of the DH_1 progeny generation of *in vitro*-selected lines

In order to determine whether the DH_1 progeny of Pq-selected lines really possessed greater oxidative stress tolerance than the control genotype, various physiological and biochemical tests were performed.

Six of the lines selected using Pq from the A18 hybrid had significantly higher resistance factors than the control genotype for the traits tested (Fv/Fm, electrical conductance, chlorophyll content), indicating that in these genotypes the Pq-induced accumulation of toxic oxygen radicals was not as intense as in the control plants, as also shown by means of *in situ* staining. Pq-selected leaves accumulated fewer toxic oxygen species in response to Pq treatment, so the leaves exhibited less staining.

It was also found that lines originating from medium containing a higher concentration of Pq DH_1 generation had the highest resistance factor. This means that the greater the selection pressure (below the lethal concentration), the greater the level of tolerance achieved.

The analysis of certain antioxidant enzymes revealed that the activity of numerous antioxidant enzymes was higher in the selected lines, both in the control and under Pq-induced oxidative stress conditions. Nevertheless, the individual lines were found to have diverse levels of antioxidant capacity, which could have been the consequence of selecting microspores with different combinations of gene. It was also established that genotypes with high resistance factors exhibited greater basic and Pq-induced activity for a number of enzymes. This could represent an advantage for complex protection against a variety of ROS.

The chilling tolerance index at germination was determined for the selected lines. In Hungary low temperatures often occur during the germination of maize under field conditions, leading to protracted germination and patchy emergence, while pests and pathogenic fungi also find it easier to attack the germinating seeds and young seedlings. The germination percentages recorded for the tested genotypes at control and cold temperatures were similar to those reported in the literature (Marton and Kőszegi, 1997). It was found that lines with high resistance factors also had high chilling tolerance indexes. Nevertheless a few genotypes with relatively high resistance factors in other tests had very low values for the chilling tolerance index, while others, whose oxidative stress tolerance was not outstanding, had greater chilling tolerance than the non-selected DH lines. Similar results were obtained

for chilling tolerance in the seedling stage (Ambrus et al., 2008; Darkó et al., 2011), but as these data were only recorded for the A18 hybrid, the results were not included in the thesis. In addition, the research team has detected the development of cross-tolerance in the case of other stress factors (drought, pathogenic infection) after enhancing the tolerance of oxidative stress through the *in vitro* selection of microspores, which could thus be an advantage in the case of other abiotic stress factors. This could be a useful agronomic trait for avoiding damage caused by cold weather at sowing and emergence or by the early occurrence of drought. This, however, will need to be confirmed in several years of field testing.

The results of all the experiments indicate that the *in vitro* selection of microspores can be successfully performed using compounds inducing the formation of reactive oxygen species, and that fertile DH plants resistant to oxidative stress can be regenerated. The technique elaborated works not only on model genotypes, but also for the *in vitro* selection of genotypes suitable for breeding purposes. It can thus be applied to develop valuable breeding material possessing good adaptability.

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6. PUBLICATION ACTIVITIES

6.1. Publications related to the thesis topic:

Papers in scientific journals:

Ambrus, H., Darkó, É., Szabó, L., Bakos, F., Király, Z., Barnabás, B. (2006): *In vitro* selection in maize anther culture with oxidative-stress stimulators. ***Protoplasma,*** 228: 87-94. (**IF:1,333**)

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6.2. Publications not directly related to the thesis topic

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