PRODUCTION OF MICROSPORE DERIVED PLANTS AND SOMATIC HYBRIDS FROM MAIZE GENOTYPES

PhD theses

Béla Szarka

Gödöllő, 2002

PHD SCHOOL

Title:	PhD School of Plant Science
Science:	Agriculture Science
Coordinator of PhD school:	Dr. Ferenc Virányi, university and MTA professor, SZIE, Agriculture and Environmental Science, Crop Production Institute
Supervisor:	Dr. Sándor Mórocz, PhD, Cereal Research Institute, Szeged Maize Breeding Dpt.

…….. ……..

PhD school leader's confirmation Supervisor's confirmation

1. THE ANTECEDENTS AND GOALS OF THE WORK

The development of the *in vitro* tissue and cell culture methods widened the range of tools, which can support the plant breeding. The somatic hybridization and the haploid plant production techniques became useful breeding tools and gained important practical role. The protoplast fusion technically broke the limitations of sexual incompatibility. Production of haploid plants incorporated in several practical breeding projects increasing the breeding efficiency. However both techniques are still challenged by genotype limitations in case of corn.

In the present thesis's maize genotypes, which were used for microspore culture and the somatic hybridization showed unique tissue culture traits (Mórocz et al.1990). The genotypes were maintainable on hormone free N6M media producing calli and regenerating fertile plants on long run. We supposed these phenomena gave advantage during both the microspore culture and the somatic hybridization.

The main goals of the presented experiments were:

1. Production of fertile DH (doubled haploid) maize plants, using simplified method lacking preliminary anther isolation, nurse culture and colchicin treatment.

1.1. Increase the post-isolation viability of the microspores using culture media, which proved to be suitable for protoplast culture of the tested androgenic maize genotype.

1.2. Examine the effect of pH, osmolality, and 2,4-D applied in the culture media on the microspore viability.

1.3. Compare the androgenic response of the PH TC and microspore derived MH SC plants.

2. PEG mediated fusion of the albino maize protoplasts with wheat mesophyl protoplasts.

2.1. Selection of green hybrid colonies and plant regeneration.

2.2. Morphological and molecular characterization of the regenerates.

2. MATERIALS AND METHODS

2.1. Maize microspore isolation and culture

2.1.1. Donor plant growth

The donor plant material for the first experiment was the three-way cross maize hybrid 3AL/95 produced from parent lines obtained via anther culture by one of the authors (S. Mórocz) in earlier experiments. Donor plants were grown under field and greenhouse conditions. In greenhouse the plants were grown in 2 l pots filled with 1:1:1 mixture of turf, sand and Floraska B (commercially available soil for ornamental plants). The temperature regime was 25/18 ^{o}C (day /night). In addition to the natural daylight, plants were exposed to nighttime illumination (from 8 pm to 7 am) with Na and HGLR OSRAM Mercurium vapor lamps (400 W), thus plants were under nearly continuous light. Plants were watered daily and supplied with nutrients (Volldünger) weekly. Tassels, containing the responsive late uninucleate – early binucleate microspores, were harvested when they were tangible through the covering 3-4 upper leaves. This developmental stage was reached 60-78 days after sowing for greenhouse grown plants (in winter) and 68-75 days in the field (in summer). The collected tassels were wrapped in plastic and paper bags and treated at 7^{o}C in the dark for 14-28 days.

2.1.2. Microspore isolation and culture conditions

Prior to isolation, florets were removed from the tassel branches by forceps and were sterilized with 50 % dilution (v/v) of commercial sodium hypochlorite solution for 10 min, followed by three rinses in sterile deionized water. Microspores were isolated mechanically with a mixer mounted on a commercial coffee mill. Spikelets (300-400 pcs /sample) were blended in 100 ml ISO (Gaillard et al. 1991) for 8 seconds (12000 rpm). To clean the microspores from the debris, a 210 μm (Tetex) sieve was used. The appropriate microspore fraction was collected with a 60 sieve. This fraction of microspores was suspended in 2 ml of ISO and over layered on a two-phase gradient, containing 20 % and 30 % Percoll (v/v) diluted in ISO, and centrifuged at 1000 rpm for 3 min. Only the microspore fraction floating at the top of 20 % Percoll gradient was used for further steps consisting of suspending in the culture medium of Galliard et al (1991) with 0.44 M sucrose and centrifuged at 1000 rpm for 3 min. This step was repeated with the culture medium of Galliard et al. (1991) containing 0.09 M sucrose. The microspores were cultured at 6x10^{5}/ml density in separately sealed 35 x 10 mm Greiner Petri dishes containing 2 ml YPM-G without co-culture with ovaries or intact anthers. For media tests 24-well Greiner macro plates were used. Microspore cultures were kept in the dark at 28^{o}C for 14 days, then at 20 to 24^{o}C under varying illumination until colony formation. All the microspore derived colonies (MDCs) reaching 1mm in diameter were transferred individually onto an N6M^{-} (Mórocz et al. 1990) hormone-free medium with 5g/l Gelrite as gelling agent, and were subculture every 21 days to establish SMC as well as to regenerate plantlets. The SMC cultures grew at 20^{o}C in continuous light. The differentiated well-rooted plants with 6-10 cm shoot were removed from the Petri dish, cleaned from the rest of medium, washed with tap water, and transferred to soil. The young plants were covered with plastic

bags in order to keep high humidity during the first 10 days of adaptation to greenhouse conditions. Microspore-derived plants were grown as donor plants. Mature plants were either self-pollinated or cross-pollinated with plants from other microspore-derived clones.

2.1.3 Plant regeneration

The microspore calli, which grew up to the size of one mm in the liquid culture medium, were transferred onto 50 ml solid N6M hormone free medium. In order to maintain the intensive growth I transferred the calli to fresh culture media every 10-14 days. Reduction of the passage cycle to 3-4 weeks initiated the plant regeneration. In the first experiment, microspore culture response of tassels representing different F_1 families from PH-TC was investigated, and well-growing, microspore derived regenerates (RSMCs) were established. Effort was made to regenerate plants and to obtain seeds from all the redeemable SMCs under our greenhouse conditions.

2.1.4. Effect of medium modification on microspore viability

The main goal of this media experiment was to increase microspore viability. The experiment involved maize protoplast culture medium ppN6M/89 (Mórocz et al. 1990) (0.600 Osmol/kg, pH 3,0, 0.2 mg/l 2,4-D), its mN6M modification and YPM-G (Galliard et al. 1991) (0.475 Osmol/kg, pH 5.8, 0 mg/l 2,4-D). The mN6M was prepared like the ppN6M/89 except of pH value, osmolality and 2,4-D content that referred to YPM-G. The osmolality of mN6M was adjusted to 0.475 Osmol/kg with 0.1 g/l sucrose and adding water.

The N6 type microspore culture media and the vitamins of the YPM-G were filter sterilized (through 0,2 μm pore size membrane), while the remaining components of the YPM-G were autoclaved. All the solutions were stored and used at 4 ^{o}C. The donor plants were grown in similar condition as described earlier for 3AL/95. Tassels were used from both the original 3AL/95 and the ML SC hybrid plants that were produced by intermating two microspore-culture derived lines from the first experiments.

Viability of microspores was estimated by counting minimum 1.500 microspores per experiment. Post isolation viability of microspores was identified by fluorescein dictate (FDA) staining in the preliminary experiments (data not shown). To simplify the evaluation of culture treatments at day 1 only enlarged microspores with dense cytoplasm were regarded as viable ones. At 7-day viability recordings dividing microspores and the visually assessed viable cells were summed, since they could be easily differentiated from the dead, shriveled microspores.

2.1.5. Statistical analysis

The culture media experiments were designed in randomized complete blocks. Individual tassels were used as repetitions. The Microsoft Excel software was used for the analysis of variance.

2.1.6. Culture media

Table 1.: Culture media applied for microspore culture

	ppN6M/89	**mN6M**	**mYP**
	mg/l	**mg/l**	**mg/l**
KNO_3	2830	2830	2500
$(NH4)_2SO_4$	463	463	0
KH_2PO_4	400	400	510
$MgSO_4x7H_20$	370	370	3700
$CaCl_2 x2H_2O$	300	300	176
NH_4NO_3	0	0	165
	ml	**ml**	**ml**
N6 vitamins+ glicin (Chu et al 1975)	1	1	0
Strauss vitamins –L asparagin (Green 1975)	0	0	1
MS microelements (Murahige and Skoog 1962)	1	1	1
$FeNa_2EDTA$	5	5	2,5
	mg/l	**mg/l**	**mg/l**
Fructose	30000	30000	0
Glucose	50000	50000	0
Maltose	2500	2500	0
Galactose	2500	2500	0
Galacturonic acid	500	500	0
Glucoronic acid	500	500	0
L-asparagin	500	500	0
L-glutamin	100	100	0
L-serin	100	100	99
Inositol	0	0	100
Szacharose	10000	100	29950
2,4 D	0,4	0	0
Naftil acetic acid	0,7	0	0
Zeatin (mixed isomers)	0,7	0,7	0
PH	2,9-3,0 (without adjustment)	5,8 (KOH)	5,8 (KOH)
	mOsmol/kg	**mOsmol/kg**	**mOsmol/kg**
Osmolality	700	475	475

2.2 Maize and wheat somatic hybridization

2.2.1. Plant materials

Maize protoplasts were isolated from H 1160 albino maize cell line, which derived from anther culture of H229 x C2-A-18 maize hybrid (Mórocz 1991). Wheat plants were grown in sterile conditions from seeds of GK Öthalom variety (Cereal Research Inst. Szeged, Hungary).

2.2.2. Suspension culture

The suspension culture was maintained and cultured weekly for protoplast isolation in 2 g/50 ml N6M medium (Mórocz et al. 1990).

The osmolarity, pH and cell mass growth curves were set up in order to control the cell culture repeatability and try to find a specific optimum stage for fusion. As a preliminary experiment forty-two 2 g/50 ml cultures were initiated and every day three samples were measured for osmolarity, pH and cell mass growth. After recording the data calli were mixed from the three dishes for protoplast isolation and fusion.

Plant cells were sedimented and the remaining liquid media was maximally removed by electric pipette, then the packed cell weight was recorded. In the waste culture medium pH was measured and the osmotic value with cryoscopy osmometer (Osmomat 030-D) respectively.

2.2.3. Donor wheat plant production

Prior to germinating wheat seeds underwent surface sterilization by treating with absolute ethanol (1 min), 0.1 % Mercuric chloride (3 min), 50 % sodium hypo chlorite (15 min) then rinsed 3 times with sterile deionized water. Wheat plants were grown 8 days in 18 cm Schott tubes under continuous light and 22 ^{o}C.

2.2.4. Protoplast isolation

Isolation of maize protoplasts was done according to Mórocz et al. (1990), except that we used split incubation: 14 hrs (overnight) at +4 ^{o}C without shaking, followed by a two hours treatment at room temperature with gentle (15 rpm) shaking.

Prior to wheat protoplast isolation, leaves of 8 day old plants were cut off sterile and immersed into 10 ml solution A (Sarhan and Cesar 1988). Each leaf were than laid into 2-3 drops of the same solution in the middle of a Petri dish, and the epidermis was removed from the reverse side of leaves by fine forceps. Mesophyl protoplasts were released by floating 2 g of leaf tissue for 4 hours in 10 ml digestion solution, which was prepared according to Sarhan and Cesar (1988) without heat treatment.

2.2.5. Protoplast fusion and culture

Isolated maize and wheat protoplasts were mixed at 2:1 ratio and suspended in 10 ml UM solution (Uchimaya and Murashige 1974), than centrifuged in swing bucket rotor (3 min, 1000

rpm). Dense suspension of (1.5 x 10^6 /400 µl UM) protoplast was incubated for 20 minutes in a single droplet of 10 mm diameter on a vibration free place. One ml of PEG solution was added in a very slow continuous flow to the protoplasts (40 w/v% of 3500 MW Sigma PEG, dissolved in solution D according to Kao and Michayluk 1974). Careful pipetting was important to avoid flotation of the protoplasts, which adhered to the bottom of a 35 mm Greiner Petri dish. Elution of the PEG solution started after first fused cells had appeared (5-10 minutes) that was controlled under microscope. Fusion mixture was eluted drop wise with 10 ml of Kao C solution (Kao and Michayluk 1974). Finally the washing solution was replaced by 1 ml ppN6M/89 culture medium (Mórocz et al. 1990). Effectiveness of the elution was controlled at each step by taking 50 µl samples and measured by osmometer.

Depending on the amount of the isolated protoplasts after each experiment one to six PEG treated and three non-treated control (maize, wheat and mixed protoplasts) samples were made.

Protoplasts were cultured in liquid ppN6M/89 medium at room temperature in dark. The positions of visible hybrid cells were recorded by embedding cultures in agarose after first divisions had appeared (fresh 1:1 rate mixture of double concentrated culture medium and 2.4% low gelling temperature agarose, both containing normal concentration sugar composition and 2,4-D of the ppN6M /89 medium).

Developing small calli of 0.5-1 mm size (two month after fusion) were transferred onto hormone free N6M regeneration medium. Amount of dedifferentiated and embryogen calli was regulated by the subculture frequency using the same medium.

The differentiated well-rooted plants with 6-10 cm shoot were removed from the Petri dish, cleaned from the rest of medium, washed with tap water, and transferred into soil. The young plants were covered with plastic bags in order to keep high humidity during the first 10 days of adaptation to greenhouse conditions. Plants developing ears or tassels were self and also cross-pollinated with other maize varieties.

2.2.6 Identification of the hybrids

The number of hybrid cells was counted under light microscope immediately after fusion. Cultures were embedded with low gelling temperature agarose and the positions of the visible hybrid cells were marked on the bottom of the dish.

The chromosome number of the putative hybrid calli and plants were revealed by Feulgen staining method.

The total DNA for RAPD analysis was extracted with CTAB procedure (Bousquet et al. 1990). In the PCR reaction 10 ng DNA was used in 20 µl reaction mix containing 5 pmol primer (Operon Alameda), 2 µl from 2 mM dNTP solution (Boehringer Mannheim), and 1unit Taq polimerase (Boehringer Mannheim). DNA was amplified in Hybaid Omnigene thermocycler at 94 ^{o}C for 1 min followed by 35 cycles each with 35 ^{o}C for 30 sec, 72 ^{o}C for 1 min, 94 ^{o}C 5 sec with a final cycle of 35 ^{o}C for 30 sec and 10 min 72 ^{o}C. The reaction products were separated on 2.0 % agarose gel (Boehringer Mannheim) that was stained for 30 min in 0.5 % ethidium bromid bath.

2.2.7. *In situ* hybridization

The albino maize chromosome plates were prepared for in situ hybridization according to Kao (1982), with minor modifications. The protoplast isolation was carried out according to Mórocz et al. (1990), and the cells were stained with acetocarmine. In the case of wheat and the hibrid plants we used squash preparatum.

The FISH analysis was performed according to Reader et al. (1994). Total genomic DNA was isolated from wheat tissue (GK Öthalom) and broken with sonication to 1000-1500 bp. Labeling procedure was the following: 5 µl Nick translation puffer (0.5 M Tris HCl, pH: 7.8; 0.05 M $MgCl_2$; 0.5 mg/ml Bovine Serum Albumin), 5 µl unlabelled nucleotide mixture (0.5 mM solution of each dCTP, dGTP, dATP in 100 mM Tris HCl, pH: 7.5), 3.5 µl fluorochrome-labelled nucleotide mix (1 µl 0.05 mM dTTP; 2.5 µl Fluorogreen [Amersham]); 1 µl 100 mM dithiotreitol; 200 ng/µl sonicated wheat DNA were mixed, and the volume was made up to 45 µl with sterile water. 5 µl DNA polymerase/DNase I (Gibco) was added, and incubated for 3.5 hours at 15 ºC. At the end of the incubation, the enzime activity was arrested with 5 µl 0.3 M EDTA (pH: 8.0). Unlabelled maize DNA was shared by autoclaving for 20 minutes and used as a competitor in 30 times the quantity of the probe amount in order to block common sequences in the hybridization step. 1/10 volume of 3 M sodium acetate, and 3 volumes of ice-cold ethanol were added. Mixing of the contents followed by precipitation at –80 ºC for 1 hour. The supernatant was removed after spinning the tubes, and pellet was incubated for 30 min at 4 ºC with 500 µl 70% ethanol. After centrifuge and discarding supernatant, the pellet was dried overnight. The pellet was dissolved in 20 µl TE buffer.

Fifty µl of hybridization solution containing 20 µl 25 % dextran sulphate, 5 µl 20x SSC, 1,25 µl 10 % sodium dodecyl sulphate and 50 ng labelled probe together with the competitor DNA, were loaded per slide and incubated for 2 h at 65 ºC. Following appropriate washes the slides were counterstained with DAPI (4',6-diamino-2-phenylindole, 1 µg/ml).

The chromosomes were examined with a Zeiss Axioskop 20 epifluorescence microscope equipped with Filter 10 for FITC and triple band filter (25) set for DAPI. The images were captured with a SPOT CCD camera using the appropriate SPOT software (Diagnostic Instruments, Inc.) and processed with the Image Pro Plus software.

3. RESULTS

3.1. Production of microspore derived maize plants

3.1.2. Production of sustainable regenerable cultures from PH TC microspores

Forty microspore colonies (25 %) PH TC grew up to callus size (>1 mm), and 24 (15%) proved to be develop into of sustainable regenerable cultures on hormone free N6M media (Table 2.) (Picture 2.A.).

Table 2: Microspore reaction of PH TC maize genotype evaluated on 160 tassels

Developmental stage	Sum of microspore response /160 F_1 tassel pcs (%)	Responsible F_1 tassel pcs	Microspore response pcs /F_1 tassel
Microspore derived callus	40 (25)	non applicable	non applicable
Sustainable culture	24 (15)	6	2...12
Regenerable culture	6 (3.8)	4	1...3
Fertile plant production	4 (2.5)	4	1-1
Self fertile DH lines	2	2	1-1

Six microspore derived culture resulted in green plants. Four plants were fertile. Albino plants did not develop.

3.1.3. Fertile microspore derived plants and their progeny

Twenty, twenty- five days culture maintenance cycles of initiated 1- 1.5 g calli, resulted in 10-12 plants, which were suitable for transferring into soil (Picture 2.B.). Among the fertile DH lines two (ML-8, ML-15) set seeds only in field condition. The “greenhouse effect” reduced the seed production of the two other lines (ML-1, ML-2) as well. In contrary their hybrids were fertile and viable both in greenhouse and field conditions (Picture 2.C,D,E,F). The ML-1 and ML-2 plants produced selfed seeds.

3.1.4. Effect of media modifications on microspore viability

The application of the mN6M media increased the microspore viability in the early stage, as compared to mYP and ppN6M/89 media (Table 3).

Table 3.: Effect of culture media on microspore viability (% ±SE), microspore colonies (MSPK, ±SE), and regenerable calli (RK, ±SE).

Media	Viability %		Microspore colonies (pcs)	Regenerable calli (pcs)
	Day 1	Day 7	Day 30	Month 6
ppN6M/89	6.1±1.5	1.0±0.3	2.5±0.9	0.0
mM6M	25.3±6.2	5.8±0.7	246.0±28.2	5.3±1.8
mYP	13.2±2.9	4.1±0.8	165.3±15.9	1.5±0.5

The media experiment did not yield fertile plants.

The media comparison revealed the significant (P=1%) advantage of pH 5.6 as compared to the pH 3.0. The effects of tested two osmolality and 2,4- D doses did not affect the microspore viability significantly (Table 4.).

Table 4: effects of pH, 2,4-D and osmolality (Osmol/kg) on the microspore viability after the first day of culture in mN6M (%, ±SE) (n=12). Data marked with star (*) indicate significant differences.

pH	Viability after day1 day %±SE	2,4-D mg/l	Viability after day 1 day %±SE	Osmolality Osmol/kg	Viability after day 1 day %±SE
3.0	9.77±1.87*	0.2	13.98±1.71	0.475	13.94±2.28
5.8	16.98±1.72*	0	13.47±2.39	0.600	12.76±1.90

The MH SC microspores showed higher viability (29,7%) than the PH TC (18,64%) in the early phase of development. The androgenic reaction of F_1 donor plants grown in identical conditions showed high variation (Table 5.).

Table 5.: Variability of the androgenic reaction of PH TC F_1 tassels grown in identical conditions (78 day old donor plant, 18 day cold treatment at +7°C).

Sample	Cover Leaves (pcs)	Quantity of Separated microspores (mm)			Microspore reaction				
					Division	Microspore colonies	Sustainable callus	Plant regeneration	Fertile plant
		20%	20/30 %	30%					
/29	4	2	5	8	-	-	-	-	-
/30	5	8	0.5	0	-	-	-	-	-
/31	4	0.5	3	3	+	+	-	-	-
/32	5	2	5	5	+	+	-	-	-
/33	5	6	1	0.5	+	+	+	+	+
/34	5	2	3	3	+	+	+	+	+
/35	3	1.5	4	6	+	+	+	+	-

3.2. Maize x wheat somatic hybridization

3.2.1. Protoplast isolation, fusion, culture

The survival hybrid cells usually started to divide on the 10 day, 5-7 days after intact maize cells. The wheat chloroplasts remained visible in the maize cytoplasm until 3-4 weeks post fusion (Picture 3.D.). The 10-15 cells colonies were not differentiable from the albino maize colonies.

3.2.2. Regeneration of green plants

Seven green callus line were selected which derived from 3 independent experiment. One clone resulted green plants, which shoved stepwise improving morphological appearance between the months 6-12 of the regeneration phase. The plants were sterile despite of the continuous attempt to pollinate them with either self or foreign pollen. Green calli appeared only from hybridized cultures.

3.2.3. Analysis of the putative hybrid plants

The green calli developed to normal maize plants (Picture 3.F.). The cytological study revealed variable number (47-56) of chromosomes in different samples. Prior to plant regeneration there were pro-metha phase chromosomes in the callus samples instead of more reliably countable metaphase stage. Later the root tip samples confirmed the number of 56, without intact or fragmented wheat chromosome.

Appearance of the green chloroplasts and the significant plant size increase (8-10 times plant height) indicated first the hybrid nature of the regenerates.

The RAPD test revealed hybrid type bands with the Operon 07, 09, 10 primers (Picture 4.A,B,C.). Two experiments resulted in similar fragments using the whole DNA extract for template from the green plants.

The *in situ* DNA hybridization revealed wheat DNA in the maize chromosomes (Picture 3.G,H,I). The hybrid plants did not show morphological intermediary traits.

3.3. New scientific results

I consider as new scientific results:

1, Production of first Hungarian maize lines from isolated microspore via simplified method as compared to preliminary results (without application of anther isolation, -preculture, nurse cultures and colchicin).

2, Presentation of the second generation of microspore plants (PH SC) and their advantages over the initial material (PH TC).

3, Presentation of the role of pH in the culture media experiments.

4, completion of maize and wheat fusion protocol.

5, Production of viable maize x wheat hybrid cell line and plant regeneration.

6, Characterization of putative hybrid plants with molecular tools.

4. CONCLUSIONS AND SUGGESTIONS

4.1. Culture of maize microspores and haploid plant production

The viability of PH TC microspores was similar to that of published by Gaillard et al. (1991) while Coumans et al. reported lower (50%) viability (1989). Same amount of microspores stayed alive in mN6M media on the first day of culture as Coumans et al. (1989) and Pescitelli et al. (1990a) reported. The two other media were less suitable for culture. In case of mYP this can be explained with the possible adaptation of the genotypes to N6 type media (Mórocz et al. 1990), and with the very low pH of the ppN6M/89.

The responsive microspores developed into sustainable callus cultures rather than embryos (Pescitelli et al. 1989, Gaillard et al. 1991). This increased the chance of seed setting, because in case of embryo producing genotypes, each embryo develops one plant, thus in case of fertilization failure the whole new DH genotype can be lost. The production of fertile progeny is a guarantee for the complete microspore- plant system. Part of the produced seeds was occasionally unable to germinate, or produced only rudimentary sterile plants. Early results of Miao et al. (1978) supported this observation, because out of 65 selfed DH seed they obtained, only 8 contained embryo, and only one germinated. Such level of microspore-derived plants does not make progress over a model system, either for breeding or transformation purposes. Jardinaud et al. (1995) successfully transformed the highly effective microspore – plant system developed by Gaillard et al. (1991) but could not regenerate fertile transgenic plants.

The viability of the isolated microspores decreased rapidly in the first few days of culture. Three media were tested in order to improve the culture efficiency. The ppN6M/89 media was richer than the mYP, with low pH (3,0) (Table 1.), and the protoplasts of HE/89 genotype (Mórocz et al. 1990) grew very well in it. The PH TC contained HE/89 in its pedigree; I assumed that microspores would react positively for the ppN6M/89 media. In reality I obtained opposite results (Table 2.). This observation was the basis for composition of mN6M (Table 2.) With this modification made the mN6M provided better survival for the microspores than the mYP, although the advantage reduced during the culture. The pH had the highest impact on the microspore viability, and the positive effect of pH 5.8 got confirmed.

I observed high variation of microspore induction rate among the tassels grown in standard conditions (Table 5.). A seasonal variation was also present among the tassels of PH TC and MH SC. I concluded that beside the genetic background the actual physiological state is also critical in the androgenic response.

The anther derived PH TC and microspore derived MH SC genotypes presented here increased the limited number of genotypes (Brettel et al. 1981, Petolino et al. 1988, Barloy et al. 1989, Genovesi and Yingling 1990, 1994) suitable for direct microspore culture. These genotypes, which are result of agronomical and androgenic selection, may be useful for further studies on genetic background or linkages of microspore induction. The breeding application requires further studies whether the responsible genes for microspore induction ability are linked with positive or negative breeding traits.

4.2. Maize somatic hybridization

The H1160 morphogenic albino maize cell suspension was good object for fusion experiments. No green revertant was observed either in maintained albino culture or the PEG treated albino protoplast control. However we do not study the genetic background of the albino genotype, thus the option of spontaneous reversion cannot be excluded.

Expectation of a collaborating maize and wheat genome would be unrealistic because of the somatic incompatibility (Dudits 1982), the phylogenetic distance and different physiological systems of the two donor species. However there was a chance for transferring chromosome fragments or gene sequences.

The microscopic observation after the PEG mediated fusion showed the division of the fused cells. The mixture of wheat chloroplasts and maize cytoplasm was clearly visible. Out of few hundred viable fusions 7 green callus clones were selected and one regenerated green plants. These data showed that the albino defect could be recovered in very rear cases.

The availability of a single hybrid makes very difficult to interpret the molecular and cytological processes that can results in the formation of nuclear or cytoplasm hybrids. The present observation that a prolonged *in vitro* culture period improved the viability and regeneration potential is in a good agreement with the early studies on carrot hybrids (Dudits et al. 1977, Dudits et al. 1980). This might be related to chromosomal loss or rearrangements. The extended influence of incompatible responses was evidently shown by the lack of normal function of sexual organs. Up to now we were able to propagate this genotype only *in vitro* cultures. Therefore we might expect a continuous alteration in genomic constitution of these plant materials. In addition to the phenotypic

characterization showing maize traits, the chromosome studies were expected to provide insight to the genetic nature of the selected green tissue and plant material. Since the wheat and maize chromosomes are significantly different in size and morphology we can safely state that the selected tissues carry only maize chromosomes. The actual chromosome number was found to be 56 in the green regenerant. Fusion of more than two protoplasts was frequently observed in the mixture of different cells during PEG treatment. This observation can explain the high chromosome number in the analyzed cells. The failure of recognition of intact wheat chromosomes emphasized the need for the additional molecular tools to uncover the origin of selected genotype.

The RAPD analysis indicated the presence of wheat specific sequence elements. The size of amplified PCR fragments suggested the combination of the two parental DNA representing either nuclear and/or organelle markers.

The existence of wheat DNA in the genome of these plants was visibly shown by in situ studies. Since total wheat DNA was labeled we can suggest that repetitive sequence elements were integrated into the maize genome.

Considering the high number of signals distributed to several chromosomes we can predict an extensive rearrangement between the parental genomic DNAs. In interpretation of the origin of genomic constitution we can rely on the results of early studies on fusion between mitotic and interphase plant protoplasts (Szabados and Dudits 1980.) The premature chromosome condensation (PCC) can cause complete fragmentation of interphase nucleus. The cytological pictures showed formation chromatin droplets that can be incorporated into the nuclear DNA of the hybrid cells during the subsequent division cycles. However the S-phase related PCC is expected to occur only in very rare cases the above cytological events might provide a hypothetical explanation for the formation of DNA islands from wheat.

Despite of the fact that unique, unknown molecular and cellular events produced the described new genotype with maize and wheat DNA, the regenerated plants exhibit several potentials for applications in functional genomic and stress research. Further studies are in progress to search for expression of wheat specific genes or characters.

The genotype does not carry direct breeding value. A successful fusion procedure was presented for mixing of the maize and wheat genomes. Since the green regenerates selection efficiency was extremely low, application of mesophyl protoplasts with selectable marker (phosfinotrycin acetyl transferase) would be desirable in case of further fusion experiments (Pauk et al. 1998).

The selection pressure may activate wheat DNA more efficiently, than in the present system where recovery of the green chloroplast was the only marker. This would serve with direct proofs for the presence of wheat derived DNA in the putative hybrids as well.

5. PUBLICATIONS

Mórocz S., Omirulleh S., Donn G., Szarka B., Ladányi M., Albrecht L., Dudits D. (1999): Field experiments with transgenic maize in Hungary in 1993-1994. In: Use of Agriculturally Important Genes in Biotechnology, NATO science Series A: Life Sciences- Vol 319.

Pauk J., Szarka B., (1991): Protoplast isolation and culture investigation in common wheat (Triticum aestivum L.) Cereal Research Institute, Szeged, POB. 391, H-6701 Szeged, Hungary In: Abstracts 8th International Protoplast Symposium, Uppsala, Sweden, 16-20 June, 1991, *Physiologia plantarum*, 82(1), A1-A42

Pauk J., Szarka B., Kertész Z. (1991): In vitro methods in wheat breeding. Conference on Plant Biotechnology, organized by CEC and Hungarian Technical and Developmental Organization, Gödöllõ, Report EUR 13415 EN: 92-93.

Pauk J., Szarka B., Simon I.K. (1991): Rapid suspension establishment and efficient protoplast-plant system of agronomically important rice varieties In: Abstracts 8th International Protoplast Symposium, Uppsala, Sweden, 16-20 June, 1991, *Physiologia plantarum*, 82, (1), A1-A42.

Pauk J., Szarka B., Simon I.K. (1994): Rapid and efficient suspension development system using agronomically important rice genotypes. FAO MedNet Rice Breeding and Biotechnology Groups, Proceedings of the Workshops, Volume 8., Szarvas 7-10/ II. /1994, Hungary

Szarka B. (1997): Intergeneric somatic hybridization between maize and wheat in book of abstracts, 8th European Congress on Biotechnology Budapest 17-21 August 1997.

Szarka B. (1997): Maize microspore culture in book of abstracts, 8th European Congress on Biotechnology Budapest 17-21 August 1997.

Szarka B., Dévényi M., Mórocz S. (1999): Termékeny kukorica növények előállítása izolált mikrospórákból. *Növénytermelés*, 48/6: 521-582.

Szarka B., Dévényi M., Mórocz S. (2001): Fertile maize lines obtained from isolated microspores. *Euphytica*, 122, 53-60.

Szarka B., Mórocz S., Fehér A., Dudits D. (1995): Kukorica és búza protoplasztok szomatikus hibridjei. Növénynemesítési Tudományos Napok `94 , 89. 1995. január 16-17.

Szarka B., Mórocz S., Omirulleh S., Ábrahám M., Ladányi M., Dudits D., (1994): Szabadföldi kísérletek idegen gént hordozó genotípusokkal. Növénynemesítési Tudományos Napok `93, 1994. jan 11-12.

Szarka B., Göntér I., Molnár L. M., Mórocz S., Dudits D. (2002): Mixing of maize and wheat genomic DNA by somatic hybridization in regenerated sterile maize plants. *Theor. Appl. Genet.*, közlésre elfogadva (TAGB970).