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Isolated microspore culture of wheat (*Triticum aestivum* L.) with Hungarian cultivars

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ABSTRACT The most important steps of microspore-plant system were checked in isolated microspore culture of CY-45 spring genotype. A lot of microspore derived embryos were produced via embryogenesis and green and albino plantlets were regenerated from these structures. This is the first publication which reported about the androgenesis of numerous Hungarian agronomically important varieties in isolated microspore culture. Nine Hungarian varieties (GK Mini Manó, GK Garaboly, GK Hargita, GK Csongrád, GK Délibáb, GK Élet, GK Kata, GK Bán, Mv Palotás) were tested in isolated wheat microspore culture. Every genotype was responsive, embryos and embryo-like-structures were developed in cultures. Plantlets – albino and green – were regenerated in case of every Hungarian cultivar. Green plantlets were produced from six genotypes.

KEY WORDS

cultivars
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Haploid plant production in anther culture of hexaploid ($2n=6x=42$) common wheat (*Triticum aestivum* L.) have been developed as a routine method in the last 25-30 years. *In vitro* wheat haploid induction in anther culture was published by Ouyang et al. (1973) in China more than 30 years ago. From the induced haploids to produce induced doubled haploids (DH) is a relatively easy and effective method using colchicine treatment (Jensen 1974). The genetically homozygous doubled haploids (DHs) in crop breeding would enhance its improvement by accelerating breeding programmes, improving selection efficiency. It means the research of haploid and doubled haploid production is in the highlight of breeders. In Europe, the DH lines of variety improvement at the end of the breeding process are very important tools in protection of intellectual property as a plant variety patent after DUS tests.

Nowadays, there are two plant biotechnology method supported ways to produce large number of haploids and DH plants: (i) anther culture and (ii) haploid production via wide (maize) cross (Szakács et al. 2002; Inagaki 2003). Today there is a new cell culture based approach under development including new advantages (direct study of embryogenesis from the first cell division, selection on cell level etc.). While the previously mentioned two methods are today routinely used in doubled haploid production systems, the isolated microspore culture technology basing upon direct embryogenesis of isolated microspores wheat is described as more recalcitrant.

Laboratories are working with problem of microspore culture (Mejza et al. 1993; Tuveesson and Öhlund 1993; Zheng et al. 2001; Zheng et al. 2002), such as genotype dependency and albinism in regeneration of microspore derived pro-embryos of different cereal species (wheat, triticale, rice). But otherwise dicots as rapeseed (*Brassica napus* L.) and monocots as barley (*Hordeum vulgare* L.) can produce a lot of embryos in isolated microspore culture and thousands of green plants from regenerated embryos of microspore culture induced embryoids (Custers 2003).

Wheat is one of the most important small grain cereal of the world (Swaminatan 2001). This fact motivates biotechnologists to search new approaches - like haploid technology - in improvement methods. In literature, the first data on the isolated microspore culture-derived wheat plantlet can be found from Datta and Wenzel (1987). However, their regenerated plantlets were not real isolated microspore culture-derived ones, because they used float anther culture for induction of microspore embryogenesis. That phenomenon was an embryogenesis of shaded micropores in float anther culture. First reports of isolated microspore-derived wheat green plants were published by two different laboratories (Mejza et al. 1993; Tuveesson and Öhlund 1993) almost at the same time. Different methods – treatment with inducer chemicals, ovary-conditioned medium – were developed to improve the efficiency of embryo production of isolated microspores (Zheng et al. 2001; Zheng et al. 2002). Importance of ovary co-culture in successful microspore culture of wheat was first published by Mejza et al. 1993. They found a posi-

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tive effect of ovary co-cultivation on embryogenesis in float anther culture of wheat. A significant positive effect of ovary co-culture was found by our laboratory, too (Puolimatka et al. 1996; Indrianto et al. 2001; Lantos et al. 2005).

In this paper we summarise the most important result of our laboratory in improving wheat microspore culture. The response of different wheat genotypes in isolated microspore have been in highlights.

Materials and Methods

Plant material

In our experiment, one spring wheat genotype (CY-45) and nine winter wheat genotypes were used to test androgenesis of Hungarian cultivars. Winter genotypes (GK Mini Mamó, GK Garaboly, GK Hargita, GK Csongrád, GK Délibáb, GK Élet, GK Kata, GK Bán, Mv Palotás) were grown in the nursery of Cereal Research Non-profit Company, Szeged, Hungary while spring type in greenhouse. The donor shoots were collected when the anthers of the middle-part of the spikes contained the microspores from mid- to late-uni-nucleate. All leaves were cut except the flag leaf. The tillers were put into Erlenmeyer flasks which contained fresh tap water and were covered by a PVC bag to assure high humidity. Donor tillers were cold pre-treated for about two weeks at 3-4°C. After cold treatment, the donor spikes were surface-sterilised for 20 minutes in 2% sodium hypochlorite solution using 2-3 drops 'Tween 20' solution. The spikes were rinsed three times with sterile water after sterilization.

Isolation of microspores from pre-treated anthers

Hundred and fifty anthers were isolated into 55 mm diameter plastic Petri dishes contained 5 ml 0.3 M mannitol solution and 200 mg l⁻¹ antibiotic (cefotaxim SIGMA-ALDRICH C 7912, CAS No. 64485-93-4). The isolated anthers were cultured for three days at 32°C in the dark. Microspores were synchronized by osmotic pre-treatment and starvation.

After osmotic pre-treatment, wheat anthers consisted of a lot of microspores in late uni- and early bi-nucleate developmental stages. The anthers were homogenized by a glass rod upon a nylon filter (200 µm pore size) and the suspension was filtered again through 80 µm nylon sieve. The tissue and microspore suspension was centrifuged at 80 g for 5 min and resuspended in 2-3 ml 0.3 M mannitol solution. The viable microspores were collected by mannitol/maltose gradient centrifugation using 0.3 M mannitol and 21% maltose (SIGMA-ALDRICH M 5885 CAS No. 6363-53-7) solutions (Fig. 1a). The separated and cleaned microspores were washed in 0.3 M mannitol solution and centrifuged again at 60 g for 5 min. The collected microspores were re-suspended in culture medium. Viable microspores were counted using a Burkert chamber. The density of isolated microspores was diluted to

approximately 30-35 000 microspores ml⁻¹.

Culture of isolated microspores

Microspores were cultured into 35 diameter Corning plastic Petri dishes containing 1.5 ml culture medium. On the first three days, microspores were cultured at 32°C in the dark thermostat and given a hot stress treatment.

After heat shock, the cultures were kept in the darkness at 28°C, developing microspores and microspore-derived colonies were observed by CK-2 Olympus inverted microscope.

Wheat microspores of all genotype were cultured in modified W₁₄ basic media (Jia et al. 1994) contained 1000mg/l Glutamin, 0.5 mg/l 2.4-D and 0.5 mg/l Kinetin- with ovary co-culture. Ten ovaries were put into every Petri dish.

Regeneration of green plants and their transfer to the greenhouse

Microspore culture derived structures were plated on the regeneration medium. Wheat pro-embryos were put on Gelrite (2.8 g l⁻¹) solidified 190-2Cu regeneration medium which contained 0.5 mg l⁻¹ CuSO₄·5H₂O (Zhuang and Xu 1983; Pauk et al. 1991; Purnhauser and Gyulai 1993).

Green plantlets were transferred into glass tubes which contained the regeneration medium. Rooted plantlets were transferred to the greenhouse and wrapped with PVC bag during period of acclimatization.

Statistical analysis

Experiments were carried out in three replications at least and the data were analysed using appropriate programmes from the Minitab Release 14 statistical package (Minitab Inc.). In the table, the different alphabets after the dates mean significant difference at 95% probability.

Results

Isolated wheat microspore culture

The most critical steps of isolated wheat microspore culture were checked by cultivation of CY-45 genotype. The ideal microspore development stage is the first important step of androgenesis induction in microspore culture. The collected donor tillers consisted of microspores with mid-uni-nucleate stage in middle part of spikes.

After cold pre-treatment, the microspores of anthers were synchronised in 0.3 M mannitol solution for 3 days at 32°C. Osmotic pre-treatment and starvation increased the number of viable isolated microspores. These stress pre-treatments changed the gametophytic pathway to sporophytic. After treatments, the microspores were in late uni- and early-bi-nucleate microspore stage (Fig. 1b).

The first cell divisions were obtained at the third and fourth days of culture in the isolated microspore culture. The sister cells inside the microspore wall could be observed

under an inverted microscope at the end of the first week of culture (Fig. 1c). Some structures died when their cells got away from microspore wall (Fig. 1d) but a lot of multi-cellular structures overgrew the microspore wall on the second week of culture (Fig. 1e). Most of them grew very intensively. The haploid microspore-plant system is based on the using of ovary co-cultivation which protected the developing structures against the death (Fig. 1f). After one month, microspore-derived embryos and embryo-like structures were developed in liquid medium (Fig. 1g).

The induced pro-embryos were plated on the solidified regeneration medium and green, albino plantlets were regenerated on the first or second week of regeneration (Fig. 1h). The green plantlets were well rooted and shoot in individual glass tubes (Fig. 1i). The plantlets were transplanted into greenhouse and grown to maturity (Fig. 1j). On the harvested plants, three different kind of spikes were found: fertile, partial fertile and sterile (Fig. 1k).

Isolated wheat microspore culture with Hungarian cultivars

The efficiency of this culture system was studied by nine Hungarian cultivars (GK Mini Manó, GK Garaboly, GK Hargita, GK Csongrád, GK Délibáb, GK Élet, GK Kata, GK Bán, Mv Palotás) and CY-45 spring wheat.

In case of every genotype, androgenesis was induced in isolated microspore culture with ovaries. The microspores developed very intensively and different numbers of embryoids were counted in the cultures of cultivars. Best results were achieved by CY-45, GK Élet, GK Csongrád and GK Mini Manó genotypes but the other varieties also produced numerous embryoids (Table 1).

The developed embryoids were plated on the regeneration medium and the regeneration rate of cultivars was checked. Plantlets were regenerated from every genotype. The most plantlets were produced by CY-45 and GK Élet genotypes. Green plantlets were regenerated from seven genotypes while

three genotypes produced only albino plantlets (Table 1). Green plant production was higher in case of CY-45 and GK Délibáb than at other varieties.

Discussion

Androgenesis of wheat in isolated microspore culture

Characteristic steps of microspore culture were checked by using CY-45 genotype. Our results are in harmony with the data of previous publications in wheat (Mejza et al. 1993; Indrianto et al. 2001). Osmotic pre-treatment of donor anthers and ideal microspore stage (late uni-nucleate and early bi-nucleate) are very important to successful culture (Reynolds 1984). Ovary co-culture was necessary to production of a lot of embryoids (Mejza et al. 1993; Zheng et al. 2002) because ovaries extracted some chemical substances which protected the developing structures (Letarte et al. 2006).

In our experiments the gametofitic pathway of wheat microspores was changed to sporophitic and developed via direct embryogenesis (Zheng et al. 2003). This morphogenesis pathway in wheat was observed by Indrianto et al. (2001) too. Large numbers of pro-embryos appeared in cultures after one month but green plant regeneration rate was to low. After the increase of green plant production, this technique will open up new approach in genetic transformation of haploid cells and tissues (Folling and Olsen 2001).

Microspore culture of Hungarian cultivars

Androgenesis and in vitro embryogenesis was successfully induced in isolated microspore culture of all varieties. Numerous embryos were developed in cultures and green plants were regenerated from seven genotypes. On the one hand, significant differences were found among genotypes in embryo production and plant regeneration. On the other hand, similar results were achieved by ten genotypes. Microspore culture was usable method in case of wide range of our va-

Table 1. Androgenesis of Hungarian varieties in isolated microspore culture. Different letters show the significant differences ($P < 0.05$).

Genotype	Number of ELS/ Petri Dish	Number of plantlets/ Petri Dish	Albino plantlets/ Petri Dish	Green plantlets/ Petri Dish
CY-45	413.5 a	117 a	99.25 a	17.75 a
GK Mini Manó	176.75 c	46.75 b	46.5 b	0.25 c
GK Garaboly	100.25 d	20.25 c	15.75 c	4.5 c
GK Hargita	88.75 d	21.25 c	20.75 c	0.75 c
GK Csongrád	224 bc	27 bc	24.25 bc	2.75 c
GK Délibáb	98.75 d	30.25 bc	18.25 c	12 bc
GK Élet	289.25 b	117.75 a	117 a	0.75 c
GK Kata	65 d	3 c	3 c	0 c
GK Bán	31.75 d	8 c	8 c	0 c
Mv Palotás	72.75 d	4.5 c	4.5 c	0 c
SzD _{5%} =	69.98	23.1	23.1	5.15

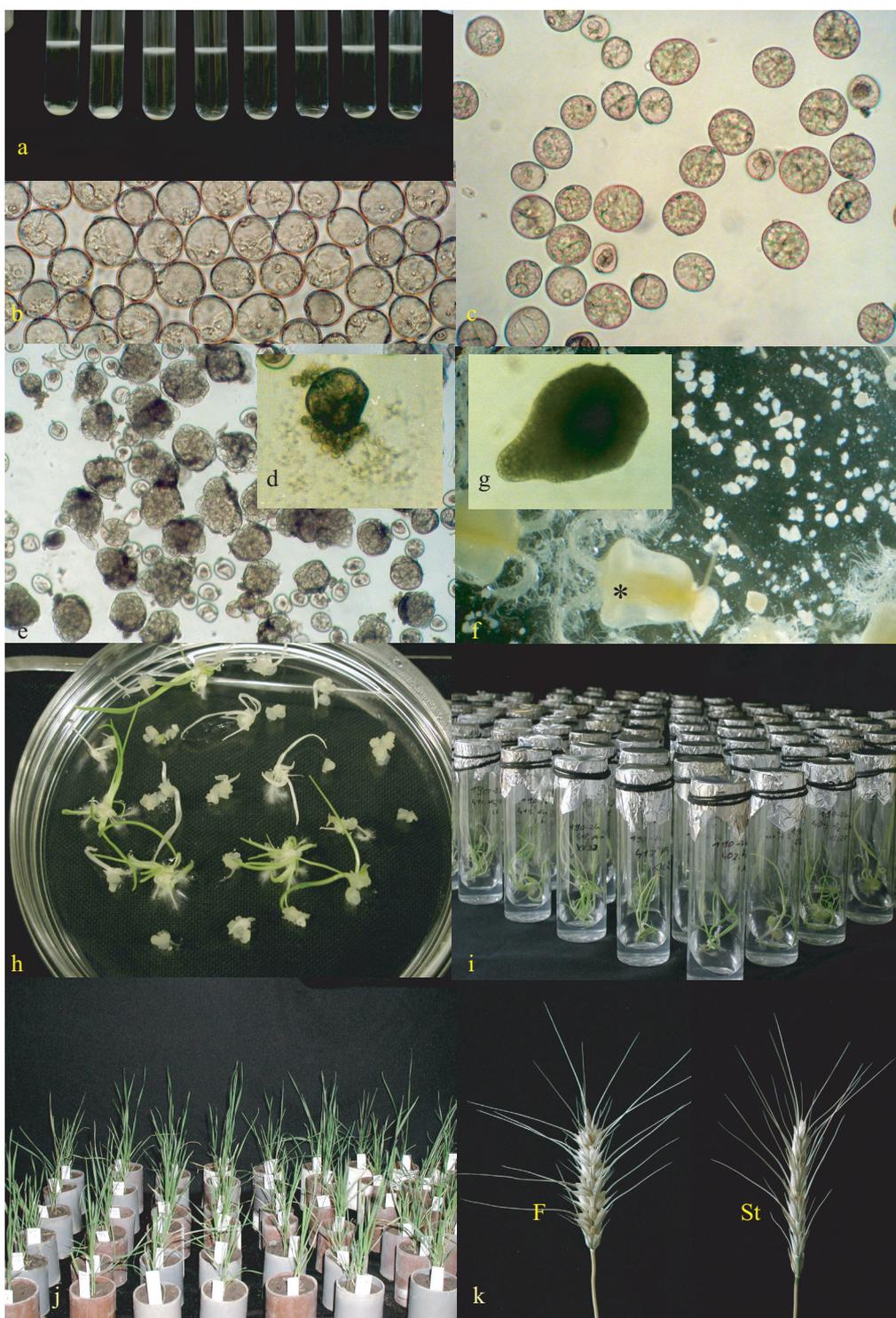


Figure 1. Characteristic steps of microspore-plant system of wheat microspore culture: a, The collected viable microspores in white bands between two solutions after gradient centrifugation (0.3 M mannitol, 21% maltose), b, the ideal (late uni- and early binucleate) stage of wheat microspores after isolation, c, Microspores with sistercells at 7th day of microspore culture. d, 10 day-old multi-cellular structures broke out the wall of microspore. e, 14 day-old multi-cellular structures grew intensively, f, 21 day-old pro-embryoids in microspore culture with wheat ovary (*) co-culture, g, microspore-derived wheat embryo. h, Green and albino plantlets were regenerated on regeneration medium. i Microspore culture-derived green plantlets for individual culturing in glass tubes, j, green plantlets were acclimatized in greenhouse. k, Fertile (F) and sterile (St) wheat spikes were obtained on transplanted microspore-derived plants in greenhouse.

rieties. The green plant regeneration rate has to increase for plant breeding. After optimization of culture conditions, this method will be beneficial technique in our wheat breeding programme like anther culture (Szakács et al. 2002).

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