Highly Efficient Doubled-Haploid Production in Wheat (*Triticum aestivum* L.) via Induced Microspore Embryogenesis

Weiguo Liu,* Ming Y. Zheng, Enrique A. Polle, and Calvin F. Konzak

ABSTRACT

An efficient doubled-haploid production technology that induces homozygosity can greatly reduce the time and cost of cultivar development. Low efficiency of doubled-haploid production previously has limited exploitation of this method for crop improvement. This study aimed to develop a more efficient and effective isolated microspore culture system for generating doubled-haploid wheat (*Triticum aestivum* L.) plants. We report here the development and testing of a new chemical formulation for its efficiency to induce microspore embryogenesis, and the development of a system for double haploid production, in which the induction of embryogenesis in microspores was followed by isolating embryogenic microspores, and culturing them under optimized growth conditions to produce high embryoid yields. Up to 50% of the total treated microspores in the whole spike were converted from their preprogrammed gametophytic to a sporophytic pathway by a chemical inducer formulation consisting of 0.1 g L^{-1} of 2-hydroxynicotinic acid, 10^{-6} mol L^{-1} 2,4-dichlorophenoxyacetic acid, and 10^{-6} mol L^{-1} 6-benzylaminopurine. The isolated embryogenic microspores were cocultivated with live wheat ovaries in a liquid NPB 99 media with an osmolality of about 300 mOsmol kg^{-1} H_2O, resulting in the regeneration of 50 to 5500 green plants per single spike of eight wheat genotypes. The high efficiency and simplicity make the system practical for biological research and for accelerating cultivar development in wheat breeding programs.

Androgenesis, the process by which pseudoembryos (embryoids) able to germinate into plants are produced from microspores (pollen embryogenesis), is of significant interest for developmental and genetic research as well as for plant breeding and biotechnology, since the process is a means for producing genetically true-breeding, doubled-haploid (DH) plants. By producing DH progeny, the number of possible gene combinations for inherited traits is more manageable (Konzak et al., 1987). An efficient DH technology can greatly reduce the time and the cost of cultivar development (Hu and Yang, 1986; Hu, 1997).

Low efficiency in DH production previously has limited exploitation of this potentially powerful method for crop improvement. Several methods of haploid production have been investigated and reported in the literature, including microspore and/or anther culture (androgenesis), ovule culture (gynogenesis), *Hordeum bulbosum* L. or maize (*Zea mays* L.) pollination methods (alien species chromosome elimination), and an alien cytoplasm system (Dunwell, 1985; Kasha, 1989). Microspore and anther culture methods have the potential to produce more than a thousand haploid plants per cultured anther; all other methods are limited to one haploid plant per floret (Devaux, 1988). Androgenesis induction in microspores may be affected by various factors which cause low induction efficiency and by genotype dependence (Dunwell, 1985). Most advances toward improving anther–microspore culture methods have been focused primarily on the concept of using "stress" treatments to induce androgenesis from the preprogrammed gametophytic to the sporophytic pathway (Touraev et al., 1996, 1997; Hu and Kasha, 1999; Zhou and Konzak, 1997; Zheng and Konzak, 1999; Simonson et al., 1997; Reynolds, 1997). Those culture systems have been effective only for a narrow range of responsive genotypes, and other genotypes remain recalcitrant. Thus, more effective methods are needed for inducing androgenesis in large populations of microspores for a wide range of genotypes.

A relationship between microspore embryogenesis and chemical treatment was observed in our experiments and by others (Konzak et al., 2000; Bennett and Hughes, 1972; Rowell and Miller, 1971; Picard et al., 1987). Although Picard et al. (1987) described improvements in androgenesis with wheat (*T. aestivum*) anther cultures, their treatments were less effective than those in use for anther culture at that time (Zhou and Konzak, 1989). We envisioned that according to the signal system concept of Ryan and Balls (1962) and Constabel et al. (1995) some chemical formulations could effectively induce a large proportion of microspores to become embryogenic, if the correct formulations were developed. We recognized, however, that after embryogenesis was induced, the induced microspores require an optimal physiological environment to develop further into embryoids able to germinate and develop into green plants.

The objectives of this work were to develop a method for efficiently initiating microspore embryogenesis by a chemical inducer formulation, and for producing large quantities of microspore-derived green plants from a wide spectrum of genotypes under optimal culture conditions.

MATERIALS AND METHODS

Growing Wheat Plants

The spring wheat genotypes 'Chris', 'Pavon 76', WED 202-16-2,'Yecora Rojo', 'Calorwa', 'Waldron', 'Wawawai', and winter wheats 'Capo' and 'Svilena', were used. These genotypes differed in responsiveness to embryogenesis and green plant regeneration on the basis of previous anther culture experiments. One to three plants per pot (20 by 25 cm in diameter) were grown in a greenhouse controlled at 27 ± 2°C, at a light regime of 17 h light and 7 h dark. Winter wheat

Northwest Plant Breeding Company, 2001 Country Club Road, Pullman, WA 99163. Received 10 April 2001.*Corresponding author (weiguo_liu@yahoo.com).

Published in Crop Sci. 42:686–692 (2002).

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2-HNA, 2-hydroxynicotinic acid; BAP, 6-benzylaminopurine; DH, doubled haploid; NPB, Northwest Plant Breeding Company.

seedlings were artificially vernalized at 6°C for 2 to 2.5 mo before growing in the greenhouse. Fertilizers (N, P, K) were premixed with soil at the time of sowing, and additional fertilizer was applied by daily watering, which contained liquid forms of nitrogen (N), phosphorus (P) and potassium (K).

Collecting Tillers

Fresh tillers containing microspores at an appropriate developmental stage were cut from below two nodes, counting from the top of the tiller, and immediately placed in a clean container with distilled water. All leaves were removed by cutting at their bases. The time between the collection of tillers and their treatment was minimized to reduce the possibility of contamination by microorganisms. Microspores enclosed within the anthers in the middle section of a spike preferably were collected at the mid to late-uninucleate stage of development. Morphological features of tillers containing microspores at these stages were established for each cultivar via microscopic examination of microspores in an anther sample with acetocarmine stain.

Pretreatment of Spikes

After removing the lower nodal section, the collected tillers were placed in an autoclaved sterile flask, containing 50 mL of sterile (autoclaved) distilled water (the control treatment) or 50 mL of the desired amount of sterile (autoclaved) inducer formulations [0–1 g L^{-1} 2-HNA (Sigma-Aldrich Co., St. Louis, MO), with or without 10^{-6} mol L^{-1} 2,4-D and 10^{-6} mol L^{-1} BAP]. The open end of a plastic bag (thin-walled, grocery store bag) was placed over the spikes, wrapped around the neck of the flask, and sealed around the flask with masking tape to limit microbial contamination and excessive loss of water. The flask was placed in an incubator at 33°C for a desired period of time, ranging between about 48 h and about 72 h with different genotypes, until microspores enclosed within the anthers from the center section of a spike showed a characteristic embryogenic structure, i.e., the fibrillar-appearing cytoplasm of induced microspores (Fig. 1a).

Microspore Isolation

After the tillers were pretreated, they were removed from the treatment flask in a laminar flow hood. All foliage beneath the first tiller node was removed, keeping only the boot containing the spike. Isolated boots were then placed on a paper towel and sprayed with 750 g L^{-1} ethanol to saturation. The boots were wrapped in the towel and placed in the hood for approximately 45 min, or until the ethanol fully evaporated. The spikes were aseptically removed from each disinfected boot and placed on top of a 125-mL kitchen blender cup. Awns (if present), and the upper spikelets were removed with sterile forceps and scissors. Florets were cut from their bases and allowed to drop into the open blender cup. Florets obtained from one to three spikes were used for each run of the blending process. Forty milliliters of a 0.3 mol L^{-1} mannitol solution (autoclaved) was added to the blender cup, and a sterilized cap was placed on the blender cup, which was assembled on the blender. The florets were blended for 20 s at 2200 rpm to release most microspores. The blended slurry was poured from the blender cup into a sterile filter (a container with 100-μm stainless steel mesh at the bottom), and the blender top was rinsed twice with 5 mL of a 0.3 mol L^{-1} mannitol solution per rinse, and the mannitol solution was poured into the filter. Residue trapped on top of the filter was discarded, and the filtrate was pipetted into 15-mL sterile centrifuge tubes and centrifuged at 100 × *g* for 3 min. The supernatant was discarded from the tubes, and the pellets were combined and resuspended in 2 mL of 0.3 mol L^{-1} mannitol solution. The resuspended pellets were layered over 5 mL of a 0.58 mol L^{-1} maltose solution (sterile) and centrifuged at 100 × *g* for 3 min. Three milliliters of the upper band (containing microspores) was collected and resuspended in 10 mL of a 0.3 mol L^{-1} mannitol solution in a 15-mL centrifuge tube. The lower band (pellet) was resuspended (for counting purposes) in 12 mL water in a separate 15-mL centrifuge tube. Both centrifuge tubes were centrifuged at 100 × *g* for 3 min. The supernatant was discarded and the pellet resuspended in 3-mL culture medium for upper band microspores, or 3 mL water for lower band microspores. The number of microspores

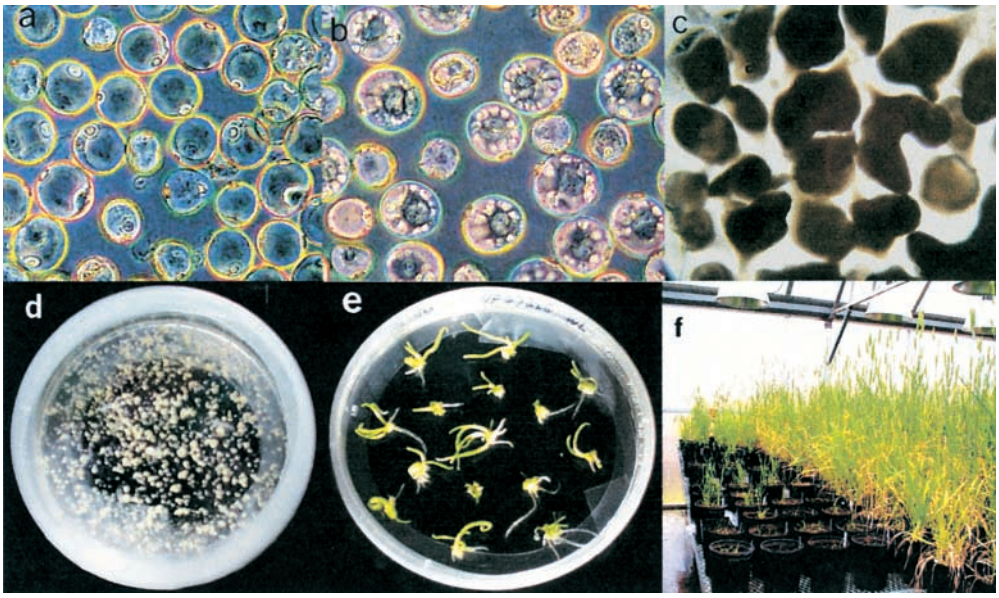

Fig. 1. Process of isolated microspore liquid culture for doubled-haploid production, genotype Chris. (a) Mid- to late-uninucleate microspores from the freshly harvested spikes. (b) Embryogenic microspores with fibrillar cytoplasm induced by 2-HNA treatment at high temperature for 65 h. (c) Microspore derived developing embryoids cultured in liquid media for 18 d. (d). Continuous production of mature embryoids upon liquid culture from 21 d on. (e) Germination of embryoids on solid media in Petri dish at 10 d. (f) Thousands of doubled-haploid plants derived from microspores of a single spike.

in each band was counted with a hemocytometer, and after counting the lower band microspores were discarded. The total of microspores isolated was the sum of the microspores from both the upper band and the lower band. Only the microspores from the upper band were used for culture. The lower band microspores appeared to be those that were too young, or too old and containing starch; thus, they had not developed sufficiently or had developed beyond the stage of development useful for DH production. The upper band microspores were resuspended in 10 mL of culture medium in a 15-mL centrifuge tube and centrifuged at 100 × *g* for 3 min. The supernatant was discarded and the pellet resuspended in culture medium at a concentration of approximately 1×10^4 microspores mL^{-1}.

Culture of Isolated Microspores

Isolated microspores were cultured as a suspension in liquid NPB 99 medium, which contains 232 mg L^{-1} $(NH_4)2SO_4$, 1415 mg L^{-1} KNO_3, 83 mg L^{-1} $CaCl_2$-$2H_2O$, 200 mg L^{-1} KH_2PO_4, 93 mg L^{-1} $MgSO_4$-$7H_2O$, 5 mg L^{-1} H_3BO_3, 0.0125 mg L^{-1} CoCl-$6H_2O$, 0.0125 mg L^{-1} $CuSO_4$-$5H_2O$, 0.4 mg L^{-1} KI, 5 mg L^{-1} $MnSO_4$-$4H_2O$, 0.0125 mg L^{-1} Na_2MoO_4-$2H_2O$, 5 mg L^{-1} $ZnSO_4$-$7H_2O$, 37.3 mg L^{-1} Na_2EDTA, 27.8 mg L^{-1} $FeSO_4$-$7H_2O$, 50 mg L^{-1} myo-inositol, 0.5 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} pyridoxine-HCl, 5 mg L^{-1} thiamine-HCl, 500 mg L^{-1} glutamine, 0.2 mg L^{-1} 2,4-D, 0.2 mg L^{-1} kinetin, 1 mg L^{-1} phenylacetic acid (PAA), and 90 g L^{-1} maltose, adjusted to pH7 and filter sterilized. An aliquot of 2 mL media per 35 by 10 mm Petri dish, or 5 mL media per 60 by 15 mm Petri dish, at a density of approximately 1×10^4 microspores mL^{-1} was used. Immature ovaries were added to the culture at a density of one per milliliter, immediately preceding the incubation. Ovaries were aseptically dissected from fresh and disinfected spikes. The ovaries from the cultivar Chris were used as convenient sources for supporting embryogenesis of the nine wheat lines tested. The Petri dish was sealed with Parafilm (American Can Co., Greenwich, CT) and incubated in the dark at 27°C.

After embryoids had grown to 1 to 2 mm in diameter, they were transferred aseptically to solid 190-2 medium (Zhuang and Xu, 1983) at a density of 25 to 30 embryoids in each 100- by 15-mm Petri dish for germinating into plants. The embryoids were incubated under continuous fluorescent light at room temperature (22°C). In approximately 2 wk, green plants developed and subsequently transferred to soil and grown to maturity in the greenhouse. To avoid bias, the first available 200 embryoids from each Petri dish were transferred for evaluating plant germination rate and DH percentage. Green and albino plants with well-developed roots and shoots were counted at 14 d after embryoids were transferred to germination culture media. Plant fertility was evaluated on the basis of seed set. More than 20 plants per replication were evaluated for seed fertility.

Data Analysis

All experiments were analyzed as completely randomized designs. There were two to six replications for each treatment. For the 2-HNA dose experiment, similar Chris tillers were assigned to each flask, and each treatment was randomly applied twice to the flasks. Microspores from each of the two flasks with the same treatment were separately isolated and cultured in the same Petri dish (replication), and each Petri dish was separately evaluated. For the experiments on osmolality or ovary source, microspores from six Pavon 76 or six Chris spikes were first isolated, and equally distributed to each Petri dish, and each treatment was randomly applied twice to the Petri dishes. Each of the two Petri dishes with the same treatment was considered as a replication and was evaluated separately. NPB99 media with different osmotic pressures were made by adjusting concentrations of maltose and mannitol. For the genotypic response experiment, the same pretreatment regime with 50 mL of the inducer formulation (0.1 g L^{-1} 2-HNA, 10^{-6} mol L^{-1} 2,4-D and 10^{-6} mol L^{-1} BAP) was applied to eight genotypes, and data were pooled means of two to six replications per genotype. General linear model (Lentner and Bishop, 1993) was used to analyze the data. Analysis of variance was conducted, followed by a 5% least square difference analysis for the three properties, i.e., number of embryoids, green plant percentage, and DH percentage.

RESULTS AND DISCUSSION

Embryogenesis Triggered by a 2-Hydroxynicotinic Acid Formulation

Over 50% of the total microspores in a spike routinely can be induced to become embryogenic by treatment with a formulation, including the chemical 2-HNA at 33°C, leading to the development of thousands of green plants originating from the microspores of a single wheat spike (Table 1; Fig. 1). After the treatment, the embryogenic microspores typically have eight or more small vacuoles immediately enclosed by the cell wall (Fig. 1a and b). These vacuoles surround the condensed cytoplasm in the center, forming a fibrillar structure. The embryogenic microspores are usually, but not always, of a larger size (about 50 μm) than the average nontreated or noninduced microspores (25–45 μm).

The optimal concentration of 2-HNA in the formula-

Table 1. Genotypic response to NPB isolated microspore culture system: the number of green plants obtained from microspores of a single spike of one recalcitrant and seven medium to highly responsive genotypes.

Name	Type†	No. of embryoid‡	Regeneration	Green plant	DH
			%		
Chris	HRS	6294	90	99	50
Pavon76	HWS	4965	50	60	65
WED202-16-2	HWS	4305	61	70	80
Svilena	SWW	2809	90	90	30
Wawawai	SWS	1020	50	48	73
Capo	HRW	2056	50	75	30
Calorwa	SWS, Club	2210	48	8	20
Waldron§	HRS	68	80	99	55

† HRS = Hard Red Spring, HWS = Hard White Spring, SWW = Soft Red Winter, SWS = Soft White Spring, HRW = Hard Red Winter, SWS = Soft White Spring.
‡ Data were based on 200 most advanced mature embryoids and estimation of developing embryoids.
§ Recalcitrant genotype.

tion for treating microspores to induce embryogenesis and form mature embryoids was determined to be approximately 100 mg L^{-1} (Table 2). The number of induced embryoids increased with increasing concentrations of 2-HNA up to a threshold of 100 mg L^{-1}, while the percentage of germinated green plants (expressed as a percentage of the number of embryoids transferred to germination medium) did not significantly differ between different concentrations of 2-HNA. Spontaneous chromosome doubling percentage reached 65% with 2-HNA treatment at 100 mg L^{-1}. A toxic level of 2-HNA in the pretreatment formulation was observed at the dose 1 g L^{-1}, when the tiller stem tissues deteriorated and microspores died. Microspores, when isolated from tillers pretreated without chemical inducer formulation (in distilled H_2O), appeared to develop toward pollen maturation, and died when cultured in induction medium.

The chemical, 2-HNA, was previously identified to increase the efficiency of androgenesis in anther cultures when applied to wheat spikes at a critical developmental stage (Konzak et al., 2000). It can be effectively and conveniently delivered to act on microspores by the described method. The chemical inducer formulation is absorbed by the vascular system of the stem and transported to the anthers and into the microspores, and the 33°C temperature speeds up the efficiency of chemical delivery to microspores. Lower temperatures may be employed, but adjustments must be made for the slower rates of chemical uptake and tiller growth. The optimal period of pretreatment appears to vary somewhat with the genotype and the treatment temperature, ranging between about 48 h and about 72 h at 33°C (Konzak et al., 2000). Tillers can be stored for convenience in a refrigerator at 4°C for up to 1 mo before subjecting the microspores to pretreatment with the chemical inducer formulation and temperature, and with nutrient stress. Because the microspore viability falls sharply, tillers should not be stored in a refrigerator at 4°C after the temperature-nutrient stress treatment. The influence of microspore developmental stages on androgenesis was very strong. The mid to late uninucleate microspores were the most responsive to chemical induction of androgenesis. Since microspores in wheat spikes are not synchronized in their developmental stage, one can only expect a portion of the microspores from a given spike to be inducible to embryogenesis. The task is to synchronize the maximum number of microspores in a spike at the appropriate developmental stage. Success can be determined by staining microspores of an anther in the middle section of the spike.

Table 2. Optimization of 2-HNA concentrations for inducing androgenesis. Means of embryoid yields and percentages of green plants and spontaneous doubled-haploids from microspores treated with various concentrations of 2-HNA.

2-HNA (mg L^{-1})	0‡	1	10	100	1000
Number of embryoids†	2a	118b	322b	1 238c	0a
Green plant, %	0a	100b	97b	100b	0a
Doubled-haploid, %	0a	0a	64b	65b	0a

† No. of embryoids with 0.2 to 2 mm diameter was counted at Day 40 after incubation and smaller structures (developing embryoids) were ignored. Actual figure would be 5-fold higher if all potential embryoids were cultured to maturity. Means followed by the same letter in the same row are not significantly different on the basis of ANOVA and 5% LSD analysis.

‡ Microspores developed in their gametophytic pathway during pretreatment, and died upon culture in induction medium.

Isolation Methods and Isolated Microspore Purification

Once microspores are embryogenic, it is necessary to separate them from spikes and culture them in liquid nutrient media. Liquid culture of isolated microspores provides many advantages over anther culture. First, the entire process of microspore embryogenesis in the culture plate can be easily monitored under an inverted microscope and the process of embryogenesis followed over time, as desired. Microscopic examination provides an effective way to observe development. Second, all embryoids formed in the culture plate are certain to be microspore derived, and plants regenerated are either haploid or DH, because the only cells placed in the culture plates are microspores (Fig 1 a and b).

The isolation process should minimize damage to microspores. Different microspore isolation methods were tested, including use of a vortexer, stirring bars, glass bar grinding, and blending. While all methods seemed to work (Konzak et al., 2000), only isolation by use of a blender gave us repeatable results, and high yields of viable and responsive microspores, especially when fixed mechanical conditions such as blend speed and time were monitored. Use of a blender is a rapid and efficient means for processing large numbers of samples. Despite these advantages, we have observed that using a blender damages over 50% of the embryogenic microspores, resulting in early abortion of development toward mature embryoids. Future research should aim to improve the isolation methods to reduce damage to the induced embryogenic microspores. Nevertheless, since as many as 50% of the microspores in the spikes can be induced to be embryogenic, the current procedure already can produce large numbers of microspore-derived green plants. In fact, so many embryoids are usually produced that the transfer of the embryoids to germination media can be the factor limiting the number of plants recovered.

Purifying embryogenic microspores is another important step for which results are repeatable. The dead, nonembryogenic microspores or debris may interfere with developing embryoids by releasing phenolics and by changing media composition, such as pH and osmolality. Several purification methods were found to work, but the combination of a simple gradient centrifugation by 0.58 mol L^{-1} maltose and mesh-filter filtration proved to be most effective and efficient.

Effect of Osmolality in Culture Media

With a large population of embryogenic microspores isolated, the task is to provide a favorable environment to enable them to develop into mature embryoids. In the described method, embryogenic microspores began their first cell division after approximately 12 h in cul-

Table 3. Optimization of osmotic pressure in the induction media for androgenesis.

	Media†							
	1	2	3	4	5	6	7	8
Osmolity, mOsmol kg^{-1} H_2O‡	152.5	202.5	252.5	299	347	402.5	449.5	497.5
Maltose, g L^{-1}	40	57	76	90	90	90	90	90
Mannotol, g L^{-1}	0	0	0	0	8	17	24.5	34.5
Size of calli on Day 14, mm	0.05	0.05	0.2	0.2	0.1	0.1	0.1	0.05
Number of embryoids§	5a	22ab	90c	100c	10a	0a	0a	0a

† Osmolality was adjusted by changing concentrations of maltose and mannitol in NPB 99 media.
‡ Osmolality of each medium was measured by Osmette S Model #4002 (Precision Systems, Inc., Natick, MA).
§ Actual figure would be 5-fold higher if all potential embryoids were cultured to maturity. Means followed by the same letter in the same row are not significantly different on the basis of ANOVA and 5% LSD analysis.

ture. Multicellular proembryoids, still enclosed within the microspore wall or exine, were formed in approximately 1 wk. After an additional week, the exine wall ruptured and immature embryoids emerged, which grew into mature embryoids within about 10 to 14 d (Fig. 1, c and d). This process was affected by a number of factors, of which culture media composition and osmolality were critical (Table 3).

Several media including MS and MN 6 (Murashige and Skoog, 1962; Chu and Hill, 1988) seem to work successfully for embryoid development, and the composition of most media contains adequate nutrients to feed developing embryoids. Thus, the physical constraint of osmolality becomes critical in the development of embryoids. As demonstrated in Table 3, the number and size of calli–embryoids were influenced by osmotic pressure in the culture media. The number and size of calli–embryoids increased with increasing osmotic pressure up to 300 mOsmol kg^{-1} H_2O, then decreased with higher osmotic pressure. The results indicated that the optimal osmotic pressure in culture media for embryoid formation is about 300 mOsmol kg^{-1} H_2O.

Effect of Ovary Coculture and Sources of Ovaries

The female part of the wheat reproductive system definitely plays an essential role for the reprogrammed sporophytic development. In our studies, even large populations of embryogenic microspores were obtained and cultured in nutrient media, but the majority of the developing embryoids ceased cell division in the process toward forming mature embryoids in media without the presence of live ovaries. Extracts of ovaries were not active, indicating that female nurse substances, only synthesized by live ovaries, were responsible for nursing the majority of embryogenic microspores to become mature embryoids (Table 4).

Table 4. Effects of ovary source and coculture methods on androgenesis.

Ovary source†	Ovary per plate	Number of embryoids	Green plant percentage
Pavon/fresh live	2	225a‡	100a
Chris/fresh live	2	259a	100a
WED202/fresh live	2	230a	100a
Pavon/extract	2	0b	0b
Chris/extract	2	0b	0b
Yecora Rojo/extract	2	0b	0b
Pavon/extract	10	0b	0b
Chris/extract	10	0b	0b
Yecora Rojo/extract	10	0b	0b

† Ovaries were either freshly isolated right before coculture from freshly harvested spikes, or extracted from fresh ovaries of the three genotypes by grinding them in liquid nitrogen and filter-sterilized with 0.22-μm filter (Minipore, INC).
‡ Means followed by the same letter in the same column are not significant on the basis of ANOVA and 5% LSD procedure. Actual figure would be 5-fold higher if all potential embryoids were cultured to maturity.

The results also showed that there were no significant differences in the nurse function for androgenesis among the live ovaries of the three different wheat genotypes tested. In fact, we found that live ovaries from other genotypes, including those of low or nonresponsive genotypes and even oat and barley ovaries, were found to have similar nurse effects for androgenesis (Konzak et al., 2000). This finding indicates that a universal mechanism, present in ovaries of any given wheat genotype effectively provides nurse factors for androgenesis. Thus, we can take the advantage of the finding such that Chris ovaries, for example, can be used for androgenesis of all genotypes. This is especially valuable in situations where only a limited number of spikes from a target genotype are available for isolated microspore culture, as often may occur in breeding programs.

Genotype Effect on Albinism and Chromosome Doubling

All eight genotypes responded to the described method and formed green plants, and possible genotypic differences in response to 2-HNA and the stress treatments, ability to form embryoids and green plants as well as spontaneous chromosome doubling were observed (Table 1). The overall efficiency in terms of numbers of green plants/single spike ranges from 50 to 5500, indicating that the procedure would be effective for use in breeding and research programs. Winter wheat genotypes responded similarly to spring wheats when they were fully vernalized. Experiments are in progress to overcome the albinism problems occurring with a few genotypes, which otherwise produce high numbers of embryoids. The proportion of spontaneous chromosome doubling among the isolated microspore-derived plants varied for different genotypes, and often was about 50%. Values were recorded as low as 10% or as high as 80% for genotypes such as WED 202-16-2, which is a Pavon 76 derivative of a *T. dicoccoides* (Koern. ex Asch. & Graebner) Aarons. backcross with Pavon 76 (Personal Communication, 1991, Dr. Adriana Grama Dvosea, Volcani Institute, Bet Degan, Israel). If plants appear to be haploid as determined by examining the stomata size and plant morphology, colchicine can be

Table 5. Additional genotypes and F_1 crosses tested with NPB isolated microspore culture system.

Name	Embryoid induction†	Green plants per spike‡	Name	Embryoid induction	Green plants per spike
HWSP 00003§	A	H	SWSW 00001§	B	M
SWS 00003§	A	H	SWSW 00002§	B	M
HWWR 99005§	A	H	Alpowa	B	M
HWWR 99006§	A	H	WPB 926	B	M
HRSR 99003§	A	H	Chinese Spring	B	M
HRSR 99005§	A	H	SWWR 99007§	B	M
SWSC 99004§	A	M	SWWR 99110§	B	M
SWSC 99004-1§	A	M	Bob White	B	M
SWSC 99006§	A	M	BonPain	B	M
Red Bob	B	M	Grandin	B	M
Platte	B	M	Claire	C	L
Chris/WPB 926	B	M	Eltan	C	L
WED 202-16-2/WPB 926	B	M	Madsen	C	L
SWWR 99002§	B	M	Enola	C	L
SWWR 99003§	B	M	HWSW 98025-1§	C	L
GK Delibab	B	M	Rulo	C	L
Rio Blanco	B	M	Sapphire	C	L
SWWR 99008§	B	M	Soisson	C	L

† A = Highly responsive (more than 1000 embryoids per spike); B = Moderately responsive (100–1000 embryoids per spike); C = Less responsive (less than 100 embryoids per spike).
‡ H = More than 500 green plants per spike; M = 50 to 500 green plants per spike; L = 5 to 50 green plants per spike.
§ NPB proprietary F_1 crosses.

applied to induce chromosome doubling. Progeny produced on plants are expected to be homozygous, and so can be used for further evaluation, i.e., seed increase, possible screening in a small plot, then selection and increase, followed by yield trial and multilocational testing in a breeding program.

We applied the described method for DH production to 36 additional genotypes and F_1 crosses at Northwest Plant Breeding Company (NPB). We obtained embryoids and green plants from all 36 genotypes or F_1 crosses (Table 5). Because large quantities of spikes were processed for producing DHs in the actual breeding program at NPB, we did not attempt to repeat all the experiments. Since, genotypic responses may vary when different greenhouse growth and culture conditions are used, the data must be considered preliminary. Most genotypes responded moderately, and produced over 100 embryoids and over 50 green plants per spike. For less responsive genotypes, we observed that the use of ovary-preconditioned culture media were beneficial to promote embryoid production (Konzak et al., 2000). The results demonstrate efficient wheat DH production via induced microspore embryogenesis as described in this paper.

This culture system represents a major breakthrough for DH production in wheat. Two factors are important. First, the procedure provides an effective and efficient means to obtain large populations of embryogenic microspores, as demonstrated for the eight genotypes tested. The chemicals 2-HNA and others (Konzak et al., 2000) can effectively trigger microspore embryogenesis. Second, large populations of isolated embryogenic microspores are cultured at optimal conditions to form embryoids and green plants. The optimal conditions include purification of embryogenic microspores from dead microspores and tissue debris, a liquid culture medium with an osmolality around 300 mOsmol kg^{-1} H_2O, and coculture with ovaries for providing the nurse factors, which the embryogenic microspores apparently cannot efficiently synthesize. When this procedure was applied for production of DHs for breeding materials at NPB, microspores of a single spike from moderately responsive crosses have yielded over 50 DHs. Responsiveness to androgenesis appears to be largely dominant inherited on the basis of results from tested crosses between responsive and low or nonresponsive genotypes (Konzak et al., 2000).

In conclusion, the present system provides methods for generating DH and/or haploid plants from microspores with high efficiency. A very high frequency of green plants can be obtained from microspores of single spikes. Significantly, even more green plants might be produced if the efficiency of the embryogenic microspore isolation method can be further improved to minimize damage to the embryogenic microspores that are isolated. Results indicate that 44 genotypes–crosses tested are all responsive to the culture system, and generate embryoids–green plants. The high efficiency and simplicity of the system described, make DH production practical for biological research and wheat breeding. Although the genetic diversity of the wheats tested in the reported experiments indicates the method to be widely applicable for wheat breeding applications, we recognize that genetic diversity of wheats extends beyond that of the sampling we have tested, thus a wider base of wheat genetic diversity extending beyond that of our recently tested germplasm resources will need to be evaluated. Nevertheless, even if recalcitrant or low responders are discovered in the course of further testing, breeders using the described system can readily incorporate the low, or nonresponding germplasm in breeding populations via the process of germplasm improvement through cross combinations with high responding gene sources, including some of those described herein. The advantages of DH technology for accelerating wheat germplasm improvement and cultivar development programs include reduced costs for cultivar development, via the greater efficiency by which the homozygous DH lines can be evaluated for selected traits, including responses to pathogens, mineral imbal-

ances (as Al or Mn toxicities), herbicides, disease-causing toxins, simulated drought stresses, and other criteria. Our preliminary experiments indicate also the vernalization of winter wheats can be achieved while plantlets are germinating, and possibly earlier, allowing greater efficiency in the use of laboratory space and equipment. A myriad of other trait screening applications also may be feasible and practical, and none need to be limited by the effects of heterozygosity on the genetic stability of the traits for which screening is applied. Moreover, even the time and generation of yield potential evaluations may be shortened considerably, because of the genetically fixed status of the DH lines produced from the F_1 generations of crosses.

REFERENCES

Bennett, M.D., and W.G. Hughes. 1972. Additional mitosis in wheat pollen induced by Ethrel. Nature 240:566.

Chu, C.C., and R.D. Hill. 1988. An improved anther culture method for obtaining higher frequency of pollen embryoids in *Triticum aestivum* L. Plant Sci. 55:175–181.

Constabel, C.P., D.R. Bergey, and C.A. Ryan. 1995. Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. Proc. Natl. Acad. Sci. (USA) 92:407–411.

Devaux, P. 1988. Comparison of anther culture and the *Hordeum bulbosum* method for the production of doubled haploids in winter barley. Plant Breed. 100:181–187.

Dunwell, J.M. 1985. Anther and ovary culture. p. 1–44. *In* S.W.J. Bright and M.G.K. Jones (ed.) Cereal tissue and cell culture. Matinus Nijhoff/Dr. W. Junk Publishers, Dordrecht, the Netherlands.

Hu, H. 1997. In vitro induced haploids in wheat. *In* M.S. Jain et al. (ed.) In vitro haploid production in higher plants. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Hu, T., and K. Kasha. 1999. A cytological study of pretreatments used to improve isolated microspore cultures of wheat (Triticum aestivum L.) cv. Chris. Genome 42:432–441.

Hu, H., and H.Y. Yang. 1986. Haploids of higher plants in vitro. China Academic Publishers, Beijing/Springer-Verlag, Berlin.

Kasha, K.J. 1989. Production of haploids in cereals. p. 71–80. *In* M. Maluszynski (ed.) Current options for cereal improvement. Kluwer Academic Publishers, London.

Konzak, C.F., D.W. Sunderman, E.A. Polle, and W.L. McCuiston. 1987. Spring wheat plant design for conservation tillage crop management systems. p. 247–273. *In* L. Elliott (ed.) STEEP—Conservation Concepts and Accomplishment. Washington State University Publications, Pullman, WA.

Konzak, C.F., E.A. Polle, W. Liu, and Y. Zheng. 2000. Methods for generating doubled-haploid plants. Published international patent application. PCT/US99/19498.

Lentner, M., and T. Bishop. 1993. Experimental design and analysis. Valley Book Company, Blacksburg, VA.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant 15:473–497.

Picard, E., C. Hours, S. Gregoire, T.H. Phan, and J.P. Meunier. 1987. Significant improvement of androgenetic haploid and doubled-haploid induction from wheat plants treated with a chemical hybridization agent. Theor. Appl. Genet. 74:289–297.

Reynolds, T.L. 1997. Pollen embryogenesis. Plant-Mol.-Biol. 33(1): 1–10.

Rowell, P.L., and D.G. Miller. 1971. Induction of male sterility in wheat with 2-chloroethylphosphonic acid (ethrel). Crop Sci. 11: 629–631.

Ryan, C.A., and A.K. Balls. 1962. An inhibitor of chymotrypsin from Solanum Tuberosum and its behavior toward trypsin. Proc. Natl. Acad. Sci. (USA) 48:1839–1844.

Simonson, R.L., P.S. Baenziger, and V.D. Gustafson. 1997. Wheat anther culture as affected by various cultural changes and supplements. J. Appl. Genet. 38:381–392.

Touraev, A., A. Indrianto, I. Wratschko, O. Vicente, and E. Heberle-Bors. 1996. Efficient microspore embryogenesis in wheat (*Triticum aestivum* L.) induced by starvation at high temperature. Sexual Plant Reproduction 9(4):209–215.

Touraev, A., O. Vicente, and E. Herberle-Bors. 1997. Initiation of microspore embryogenesis by stress. Trends Plant Sci. Rev. 2(8): 297–302.

Zheng, M.Y., and C.F. Konzak. 1999. Effect of 2-4-dichlorophenoxyacetic acid on callus induction and plant regeneration in anther culture of wheat (*Triticum aestivum* L.). Plant Cell Rep. 19:69–73.

Zhou, H., and C.F. Konzak. 1989. Improvement of anther culture methods for haploid production in wheat. Crop Sci. 29:817–821.

Zhou, H., and C.F. Konzak. 1997. Influence of genetic and environmental factors on anther culture response of wheat. J. Appl. Genet. 38:393–406.

Zhuang, J.J., and J. Xu. 1983. Increasing differentiation frequencies in wheat pollen callus. p. 431. *In* H. Hu and M.R. Vega (ed.) Cell and tissue culture techniques for cereal crop improvement. Science Press, Beijing, P.R. China.