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Microspore culture for production of doubled haploid plants of Nebraskan winter wheat cultivars.

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Introduction. Microspore culture is a cell (haploid) culture-based approach for producing completely homozygous doubled haploid (DH) plants from immature pollen grains in a single generation. In plant breeding, the single-seed descent (SSD) method is often used to hasten the development of homozygous breeding lines; however, six generations of self-pollination are required to reach 98% homozygosity. Microspore culture is more efficient than the SSD method, results in the recovery of genotypes with 100% homozygosity in a single generation, and can be performed at any stage of the breeding process. Because winter wheat requires 6 to 8 weeks of vernalization to induce flowering in every generation of advancement, microspore culture may prove useful for improving the efficiency of winter-wheat breeding programs, because only one vernalization cycle is required to obtain completely homozygous lines. Therefore, DH technology enables shortening the time required for developing cultivars when applied in traditional plant breeding (Forster and Thomas 2005).

Two basic methods of androgenesis for the production of DH plants are (i) anther and (ii) isolated microspore (immature pollen) cultures. Microspore culture is defined as isolating the microspores from the anther prior to culture, whereas anther culture involves culturing the whole anther (Ferrie and Caswell 2011). The advantage of isolated microspore culture is that microspores can be isolated in greater amounts, providing large number of potentially embryogenic single haploid cells, which can undergo androgenesis, thereby producing thousands of genetically different homozygous plants in one season from a single hybrid plant. Microspore culture consists of three major steps (i) pretreatment (process of sporophytic development from immature microspores), (ii) induction (developing embryoids from embryogenic microspores), and (iii) regeneration (regeneration of microspore-derived embryos).

Our long-term objective is to develop a high-throughput procedure for production of DH plants from major winter wheat cultivars of Nebraska to complement the current breeding program in Nebraska. We report here preliminary results of androgenic response of three Nebraskan winter wheat cultivars to two different pretreatment methods.

Materials and methods. ***Plant materials and growing conditions.*** The three Nebraskan winter wheat cultivars used in the study were Anton, Antelope, and Camelot. Plants were grown in pots in a controlled green house regulated with a photoperiod of 16–17 h light/7–8 h dark. Temperature was set at 22°C day and 15°C night. Humidity was not maintained. Plants were watered on alternate days and fertilized once a week.

Microspore culture. The whole procedure of microspore culture was according to Kasha et al. (2003) except that the pretreatment method was modified. Anthers from four sterilized spikes (half emerged when most of the microspores were at the late uninucleate stage) were removed and put in '60 x 15 mm' sterile petri dishes containing 4 mL of solution B. The petri dishes were kept at 25°C for 4–5 days (no cold treatment). For a cold treatment, the plates were incubated for additional five days at 4°C. The cell density was counted with a haemocytometer and adjusted to a range of 2–4 x 10^5 cells/mL. The suspended microspores were cultured in 35-mm (2.0 mL) or 60-mm (4.0 mL) petri dishes depending on the volume of microspores obtained. Ten to twelve ovaries were put into each petri dish. The petri dishes were incubated in dark at 28°C for 10 days and then transferred to a shaker in the dark at 28°C. The microspore-derived, multicellular structures were observed after 7–10 days. After 21 days, 1–2 mm size embryos were transferred to 90-mm petri dishes containing a modified, semisolid, MMS5 media fortified with ascorbic acid (Santra et al. 2012). The petri dishes were kept at 25°C in the dark for 4 days and then transferred to a light cabinet at 25°C. After 1–2 weeks, plantlets with well-developed roots and shoots were transferred to magenta boxes containing a modified MS media without hormones, which were kept at 22°C in light cabinets for 2–3 weeks.

Results and discussion. The correct microspore development stage is the most important step of androgenic response in isolated microspore culture (Ferrie and Caswell 2011). The right stage of the spike (Fig. 1a, p. 243) was determined based on mid- to late uninucleate stage of the microspores (Fig. 1b, p. 243) in anthers from the middle part of the spike.

The morphological marker for the embryogenic microspores is appearance of star-like structures (Fig. 2a; Shariatpanahi et al. 2006). After putting such star-like microspores in induction media, three things were observed among the isolated microspores (i) more than ~90% of the cells shrunk in the medium, (ii) ~4–5% of the cells did not progress but remained static, and (iii) ~1–2% of the cells enlarged and developed into multicellular structures, which subsequently advanced to embryo-like structures (ELS) (Fig. 2b) and pro-embryoids. The induced pro-embryoids eventually developed into 1–2 mm sized embryos (Fig. 2c), which were transferred to 90 mm petri dishes (Fig. 2d). The regenerated green plantlets (Fig. 2e) from the transferred embryos were transferred to magenta boxes (Fig. 2f).

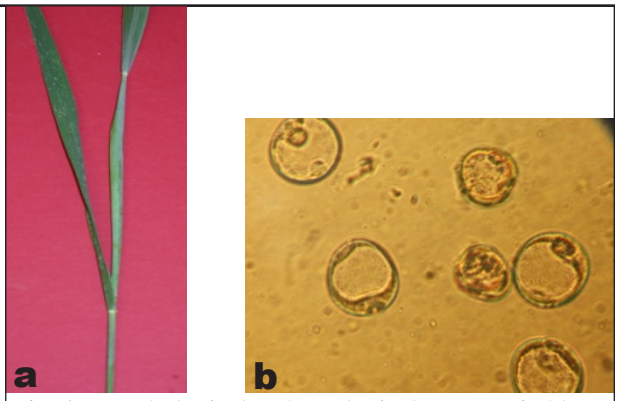

Fig. 1. Morphological and cytological stages suitable for isolated microspore culture in winter wheat. (a) Ideal morphological stage of spike, which carries late, uninucleate microspores in the middle portion; (b) microspores at late, uninucleate stage as seen by the vacuole being formed.

The androgenic response towards two different pretreatment methods of the three winter wheat cultivars is summarized in Table 1. Compared to no cold pretreatment, a cold pretreatment increased the number of embryogenic microspores in Anton by two fold, but no such differences were observed between the two pretreatments in Camelot and Antelope. *In vitro* development of microspores into multicellular and embryo-like structures was quicker in Camelot than in Anton and Antelope. Green plants were regenerated in all three cultivars following both cold and noncold pretreatments (Table 1). In Antelope, the number of green plantlets was higher with a cold (8) than with a noncold (4) pretreatment; because the number was much less, a further experiment is in progress.

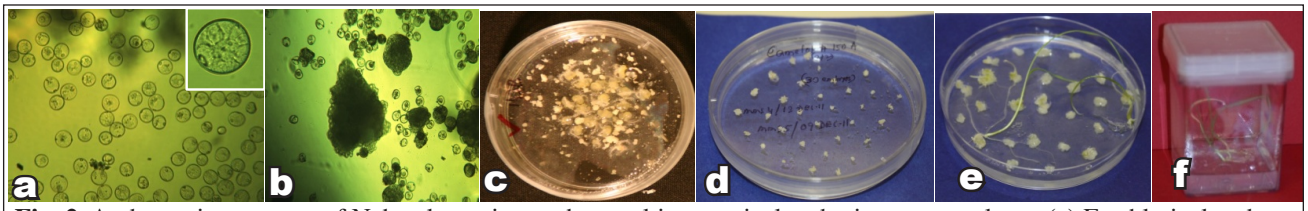

Fig. 2. Androgenic response of Nebraskan winter wheat cultivars to isolated microspore culture. (a) Freshly isolated embryogenic microspores (star-like structure; inset is an enlarged view of a star-like cell; (b) embryo-like structures after two weeks in induction medium; (c) embryoids formed after 20–21 days in induction medium; (d) 1–2-mm embryos transferred to MMS5, semisolid media; (e) regeneration of plantlets in petri dishes, and (f) transfer of green plantlets into magenta boxes.

Table 1. Androgenic response of three Nebraskan winter wheat cultivars to isolated microspore culture.

Cultivar	Treatment	Total number of microspores cultured (x 10^5)	Number of multicellular + embryo-like structures	Number of transferred embryos	Number of regenerated plantlets	Number of regenerated green plantlets
Anton	No cold	5.74	3,776	7	1	1
	Cold	10.8	736	7	1	1
Antelope	No cold	10.0	576	17	5	4
	Cold	8.84	960	37	13	8
Camelot	No cold	11.0	544	57	7	1
	Cold	6.48	640	59	1	1

Because isolated microspore culture depends on the genotype and a number of other factors (Ferrie and Caswell 2011), we studied the androgenic potential of the cultivars to produce DH plants. The higher number of embryogenic microspores woth a cold pretreatment in Anton was not regenerated into a proportional number of green plants. Repli-

cated experiments are under progress to comprehensively compare the two pretreatments in the three cultivars. We hope to establish DH production methods on these and other major Nebraskan winter wheat cultivars so that this method will be a beneficial tool in our wheat breeding efforts.

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2011 Wheat Production in the Commonwealth of Virginia.

Growing conditions. Following an extremely dry summer and corresponding low yields in most of the Commonwealth in 2010, small grain growers experienced a generally drier and warm early start to planting (Fig. 1 and Fig. 2, p. 245). Many farmers were able to get an early start on wheat planting, since the harvest season for corn and soybeans was abbreviated greatly. By 20 September, about 9% of the wheat crop was seeded, compared to the average of 4%. By 20 October, most areas had received enough rainfall so that 65% of the state was rated adequate for topsoil moisture. The trend toward early seeding and early emergence continued with 46% of intended acreage reported as already planted, and 18% of acres emerged compared with the 5-year average of 8% by this date. The end of the first week of November showed continued cool and relatively wet weather throughout much of the state. Still growers managed to have 77% of acres planted. Conditions for early season growth

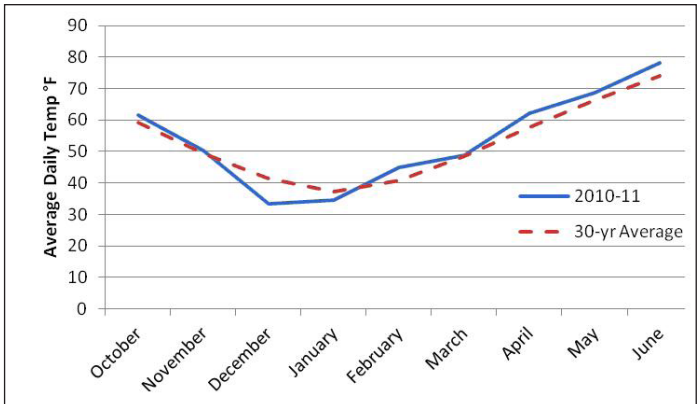

Fig. 1. 2010–11 and 30-yr mean monthly average temperatures in the Commonwealth of Virginia.