Doubled haploidy methodology for three forage grasses [crested wheatgrass (Agropyron cristatum (L.) Gaertn.), hybrid bromegrass (Bromus riparius x B. inermis), and meadow bromegrass (Bromus riparius Rehm.)]

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Abstract

Doubled haploidy (DH) methodology is used in many plant species to accelerate crop improvement and cultivar development; however not all species are amenable to the tissue culture technique. Experiments were undertaken to develop DH protocols for three perennial grasses [crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), hybrid bromegrass (*Bromus riparius x B. inermis*), and meadow bromegrass (*Bromus riparius* Rehm.)]. The initial experiment screened these forage grass species to established wheat (*Triticum aestivum* L.) microspore culture protocols. Following the initial screen, several factors influencing microspore embryogenesis were evaluated. These included genotype, donor plant conditions, developmental stage of the microspore, pretreatments, media composition, and culture conditions. For regeneration of the embryos to plants, media composition and culture conditions were assessed. Microspore-derived embryos/calli as well as green haploid/doubled haploid plants were regenerated from all three forage grasses. Differences were observed between species and genotypes within species in terms of embryogenic response. Modifications to the initial wheat DH protocol included the donor plant conditions, developmental stage of the microspore to late uninucleate to early binucleate and media composition. Regenerated plants were grown in the greenhouse.

Key words: androgenesis, crested wheatgrass, doubled haploidy, hybrid bromegrass, meadow bromegrass, microspore culture

Key message: Doubled haploidy methodology was developed for the perennial forage grasses, crested wheatgrass, meadow bromegrass, and hybrid bromegrass. Microspore-derived embryos and green doubled haploid plants were produced.

Introduction

Doubled haploidy (DH) techniques have proven to be useful breeding tools in the improvement of crops. The main advantage of using DH plant production technology in a breeding program is to shorten the breeding cycle by three to four years (Ulrich et al. 1984). Haploids and doubled haploids can also be used in mutagenesis, transformation, biochemical, physiological, and genomic studies. Microspore and anther culture are techniques that have been used to generate DH plants in many species and are, therefore, being used to develop cultivars and advanced breeding lines.

Haploid/DH perennial ryegrass plants have been regenerated via anther culture (Olesen et al. 1988; Bante et al. 1990) or microspore culture (Andersen et al. 1997) although the frequency of albinism of the regenerated plants is high. There is also a lack of information on the performance of the resulting DH plants and the potential use of DH techniques in a forage breeding program to develop vigorous hybrid lines. Advances in DH technology in the last decade have resulted in microspore culture response of recalcitrant species including oat (*Avena sativa* L.) and wheat (*Triticum aestivum* L.) (Ferrie et al. 2014; Wang et al. 2019). It has been observed recently that some of the forage grasses (i.e., perennial ryegrass) are responsive to wheat or barley (*Hordeum vulgare* L.) DH protocols (Begheyn et al. 2016). In perennial ryegrass, even though there was an obvious sign of inbreeding depression, researchers have found vigorous, homozygous, and fertile plants (Andersen et al. 1997). Hybrid breeding has significantly accelerated yield gains of many important crops but this system has not been explored in outcrossing perennial grasses.

The most important forage grass species grown in western Canada are hybrid bromegrass, meadow bromegrass, and crested wheatgrass (Baral et al. 2018; Biswas et al. 2020). New varieties that are adapted to changing environmental conditions, with improved yield, quality, re-growth, winter hardiness, and enhanced disease resistance will be beneficial to the beef and forage industries. There are currently no DH protocols available for these grasses, however, this method would be of value to the development of new breeding materials with unique genetic make-up and exploitation of potential heterosis of hybrid breeding populations. In addition, DH plants are useful for genomic studies to understand complex genomics of grasses.

The objective of this project was to develop a reliable, efficient microspore culture protocol for generating DH plants for forage grass species [crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), hybrid bromegrass (*Bromus riparius x B. inermis*), and meadow bromegrass (*Bromus riparius* Rehm.)].

Materials and Methods

Donor plant material

Germplasm of three forage grasses (crested wheatgrass, meadow bromegrass, and hybrid bromegrass) was provided by the Crop Development Center of the University of Saskatchewan. The cultivars used were: crested wheatgrass c.v. 'Kirk', 'NewKirk', and 'AC Goliath', meadow bromegrass c.v. 'Fleet', and hybrid bromegrass c.v. 'AAC Torque' and 'AC Knowles'. Seeds were planted into root trainers using Sunshine Mix #4 (Sun Gro Horticulture, Bellevue, WA, USA) with ~4.5 g of slow-release fertilizer Nutricote 14-13-13 (Plant Products). Growing conditions were set at 20/18°C (day/night temperature), 18 h photoperiod, and 350-420 µmol m^{-2} s^{-1} light intensity. After 2 weeks, the plants were transferred to 5°C, 12 h photoperiod, and 300 µmol m^{-2} s^{-1} light intensity for a 7 - 10 week vernalization period. After vernalization, the plants were transferred to 25.4 cm pots and grown at 24/18°C, 16 h photoperiod and 350-420 µmol m^{-2} s^{-1} light intensity.

General procedure for microspore isolation and culture

Microspore culture experiments were conducted using the wheat microspore culture protocol (Wang et al. 2019) previously developed in the NRC laboratory. The viability and the developmental stage of the pollen grain were determined using Fluorescein diacetate (FDA) and acetocarmine stain, respectively. When the majority of the microspores were at the mid–late uninucleate stage, the spikes were cut and placed in water at 4°C in the dark. After 21 days, the spikelets/florets were placed in an autopsy basket and sterilized in 400 ml of 50% bleach with Tween 80 and shaken on a rotary shaker for 10 minutes, then rinsed four times with 400 ml of cold sterile water. The microspores from the spikelets/florets were isolated with FHG-2 medium (Wang et al. 2019) in a blender cup. The filtrate was centrifuged at 1000 rpm in a refrigerated centrifuge (4°C) for 5 minutes. The media was removed and fresh FHG-2 media was added. This process was repeated to ensure a clean sample of microspores. The microspores were counted using a hemacytometer and cold NPB99 + GLC (glutathione, Larcoll, cefotaxime) (Wang et al. 2019) was added to achieve a density of 1 x 10^5 microspores/ ml. The microspore suspension (1.5 ml) was dispensed into a Petri plate and

to each Petri plate, three immature wheat (c.v. 'Nanda') ovaries were added. The microspores were incubated in the dark at 28°C for 28 – 35 days, at which time, the microspore-derived structures were counted and plated onto solid B5-5 medium (Gamborg et al. 1968, modified by Wang et al. 2019). The plates were incubated at 22°C, 16 h photoperiod, and 100 µE light intensity. After 30 days, the number of green plantlets and number of albino plantlets were counted. The ploidy level of resulting green plantlets was determined by flow cytometry analyses using the Partec Cell Counter Analyzer. Plantlets were hardened off in an environmentally controlled chamber then transferred to the greenhouse. In the greenhouse, the light period was 18h, supplemented with high pressure sodium halogen lamps to a total of 490–550 µM s^{-1} m^{-2} PAR. The temperature was 22 °C and 18°C, for day/night, respectively. Relative humidity was maintained at 48.0 %.

A number of factors influencing microspore embryogenesis were evaluated during the project including: genotype, donor plant conditions, developmental stage of the microspore, pretreatments, media composition, and culture conditions. The focus of this manuscript will be on developmental stage of the microspore and media composition.

Initial evaluation of species utilizing spring wheat and winter wheat protocol.

Experiment 1: Plants were grown as described above. The spring wheat protocol has a 21 days pretreatment at 4°C which was compared to the winter wheat protocol, which has a 28 day pretreatment at 4°C. Microspores were cultured in NPB99 + GLC medium as described above.

Evaluation of the developmental stage of the microspore

Experiment 1: Twelve plants from each of the three species were grown in the growth cabinet at 24/18°C (day/night temperature) as stated above and the developmental stage of the immature pollen grains was determined using anthers from the florets of the middle spikelets and acetocarmine staining.

Experiment 2: Three crested wheatgrass c.v. AC Goliath, Kirk, and NewKirk, and two hybrid bromegrass c.v. AAC Torque and AC Knowles were evaluated for microspore culture response using the later developmental stage (late uninucleate – early binucleate).

Evaluation of media composition

Experiment 1: Following the protocol described above, 9% sucrose was substituted for 9% maltose in the NPB99+GLC medium.

Experiment 2: Evaluation of five concentrations (7, 9, 13, 15, 17%) of maltose as the carbohydrate source in the NPB99+GLC medium.

Experiment 3: Evaluation of the plant growth regulators (PGRs) phenylacetic acid (PAA), 2,4-Dichlorophenyoxyacetic acid (2,4-D) and kinetin in the NPB99+GLC medium added at culture initiation and kept continuously in the medium. Five treatments and the control were compared: zero PGRs, ½ control-strength PGRs (0.1 mg/I Kin, 0.5 mg/l PAA, 0.1 mg/l 2,4-D), kinetin only (0.2mg/l), PAA only (1.0 mg/l), or 2,4-D only (0.2 mg/l), and control (0.2 mg/l Kin, 1.0 mg/l PAA, 0.2 mg/l 2,4-D).

Experiment 4: Evaluation of auxin effects with four concentrations of 2,4-D (0, 0.1, 0.2, 0.4 mg/l) and PAA (0, 0.5, 1.0, 2.0 mg/l) tested in the culture medium NPB99+GLC. Kinetin was excluded from the medium.

With all factors evaluated, three replicate experiments, using 3-5 spikes/tillers each, with three to five plates per treatment for each experiment, were conducted. Analyses of variance were performed on experimental means using completely randomized designs and differences between means was determined by Duncan's Multiple Range Test.

Results and discussion

Initial screening using wheat microspore culture protocol

Except for a responsive DH methodology for perennial ryegrass, as described by Begheyn et al. (2016), this study was one of the first experiments to evaluate the effectiveness of the spring and winter wheat microspore culture protocols (Wang et al. 2019) on the three forage grasses, crested wheatgrass, meadow bromegrass, and hybrid bromegrass. For crested wheatgrass cultivar Kirk, microspore-derived structures were observed using both the spring and winter wheat protocols, with the majority of the structures from the spring wheat protocol; however, most of these structures consisted of friable calli and were not embryos. For meadow bromegrass c.v. Fleet, there were no significant

differences in embryogenic response when the spring or winter wheat protocols were used with low success of microspore-derived structures. For hybrid bromegrass c.v. AC Success, there was no viable pollen from the plants after a 28-day cold pretreatment (winter wheat protocol). Embryos/calli of hybrid bromegrass were observed from the spring wheat protocol but the quality of the structures was abnormal (i.e., friable callus). For all three forage species, green plant regeneration was poor or non-existent. Using the wheat microspore culture protocol as a base, experiments were undertaken to enhance the production of microspore-derived embryos of the forage grasses by evaluating the factors influencing embryogenesis. The focus of the manuscript is on developmental stage and media components.

Evaluation of developmental stage of the microspore:

The most responsive microspore developmental stage for induction of embryogenesis varies with species but generally ranges from the early uninucleate to early binucleate stage. In some species, the presence of binucleate microspores can result in unfavourable conditions for microspore development and the consequent reduction in frequency of embryogenesis and the production of abnormal and stunted embryos (Kott et al. 1988). The spring wheat or winter wheat protocols utilized mid to late uninucleate microspores (Wang et al. 2019). The mid to late uninucleate microspores have also been used in anther culture of x Festuloium (*Festuca pratensis* x *Lolium multiflorum)* (Leśniewska et al. 2001), perennial ryegrass (Olesen et al. 1988), and orchardgrass (*Dactylis glomerata* L.) (Christensen et al. 1997). In barley, it was observed for some genotypes that using an earlier developmental stage of pollen grain resulted in fewer albino plants and more regenerated green plants (Gajecka et al. 2020).

Initially, we evaluated two microspore developmental stages; early to mid-uninucleate and mid-late uninucleate (control) (Fig. 1 A, B, C). The results indicated that the early to mid-uninucleate stage of microspore development was not beneficial for microspore culture in the three perennial grasses and the mid - late uninucleate stage was more responsive (data not shown). Further studies evaluated the response of very late uninucleate to early binucleate stage (Fig. 1 D). Correlation of the microspore developmental stage with anther colour eased the selection of microspores with the desired developmental stage (Fig. 2). The anthers that were light yellow to yellow in colour had a higher frequency of late to early binucleate cells. There was more fractionation of the vacuoles in the microspores at time of culture when the late uninucleate to early binucleate stage of microspore was used compared to the mid-late uninucleate stage for all three grasses (Fig. 3). Microspore fractionation is associated with embryogenicity (Indrianto

et al. 2001), resulting in a higher number of microspore-derived structures as observed from the cultures (Table 1). The late uninucleate to early binucleate microspore stage was also used in both anther culture (Guo et al. 1999) and microspore culture of timothy (*Phleum pratense* L.) (Guo and Pulli 2000). For timothy, embryo production was observed at the early uninucleate stage to the middle binucleate stage but the highest frequency of embryo development was at the late uninucleate to early binucleate stage.

Microspore culture response varies among genotypes within a species as is commonly found with many tissue culture methodologies. In some cases, there are differences in embryogenic response between the plants within the genotype (Phippen and Ockendon 1990; Hiramatsu et al. 1995). There have been many studies which have screened genotypes for embryogenic response; however this has not been done in the perennial grasses. It is desirable to have a protocol that is genotype independent.

An evaluation of crested wheatgrass cultivars (Kirk, AC Goliath, and NewKirk) and two cultivars of hybrid bromegrass (AAC Torque and AC Knowles) was conducted utilizing the late uninucleate to early binucleate stage of pollen development. Embryogenic response as well as green plant production was observed for all genotypes at this stage. There was no significant differences observed among the genotypes (Table 2). This indicates that the microspore culture protocol is amenable to several perennial grass species and cultivars.

Evaluation of media composition

Media composition is a major factor influencing the androgenic response of crops. The basal medium, carbohydrate source and concentration, macro- and micro-nutrients, growth regulators, and other additives can contribute to the embryogenic response. There are countless papers evaluating media modifications to enhance androgenic response as well as subsequent embryo development and plantlet regeneration. In this study, maltose is more effective than sucrose as the carbohydrate source for crested wheatgrass and hybrid bromegrass as there was no response to sucrose (data not presented). For perennial ryegrass, anther culture studies have shown that maltose was more beneficial than sucrose, but there was still response from the cultures containing sucrose (Anderson et al. 1997). A similar response was observed in microspore culture of timothy as a comparison between sucrose, maltose, and glucose indicated that

maltose was the best carbohydrate source, but embryos were still produced when sucrose or glucose was used as the carbohydrate source (Guo and Pulli 2000).

In addition to the type of carbohydrate, the concentration of carbohydrate is very important. In our study, comparisons were made between the control (9%) and 7, 13, 15, or 17% maltose. For meadow bromegrass, results showed that 7% maltose was not advantageous in terms of number of embryos produced (Table 3). There was an increase in embryogenic response as the maltose concentration increased; although not statistically different from the control (9%), the most embryogenic cultures were in medium containing 13% maltose,. For crested wheatgrass, 9% maltose was significantly better than the other maltose concentrations evaluated (Table 3). A maltose concentration of 9% is used in wheat microspore culture or anther culture (Wang et al. 2019; Castillo et al. 2021) as well as triticale microspore culture (Maheshwari and Laurie 2021), oat anther culture (Warchol et al. 2021) and was the most responsive concentration in perennial ryegrass (Anderson et al. 1997). Lower maltose concentrations have been beneficial in some species with 6.2% maltose being the best for barley microspore cultures (Cistué and Echávarri, 2021) and 8% maltose for spelt wheat (Lantos and Pauk, 2021).

Growth regulators such as auxins (e.g., 2,4-D, NAA [α-naphthaleneacetic acid]) and cytokinins (e.g., BA [benzyl adenine], kinetin) are used extensively to promote callus or embryo development from anthers or microspores. The growth regulators, kinetin, PAA (phenylacetic acid), and 2,4-D have shown to be beneficial in wheat (Zheng et al. 2001; Wang et al. 2019) and triticale (Maheshwari and Laurie, 2021) microspore culture. PAA (4.0 mg/l) alone had a positive effect on wheat microspore culture (Hu et al. 1995) and kinetin and 2,4-D have been used in anther culture of spelt wheat (Lantos and Pauk, 2021) and oat (Warchol et al. 2021). In our study, experiments compared the control (0.2 mg/l Kinetin, 1.0 mg/l PAA, 0.2 mg/l 2,4-D), zero growth regulators, ½ strength growth regulators (0.1 mg/I Kinetin, 0.5 mg/l PAA, 0.1 mg/l 2,4-D), kinetin only (0.2 mg/l), PAA only (1.0 mg/l), or 2,4-D only (0.2 mg/l). There were no significant differences between the treatments in terms of the number of structures and green plant regeneration was very poor overall (data not shown).

Observations of the quality of embryos/calli indicated that kinetin was not beneficial, therefore further studies evaluated four concentrations of 2,4-D (0, 0.1, 0.2, 0.4 mg/l) and PAA (0, 0.5, 1.0, 2.0 mg/l) (Table 4). There was

significant difference among the growth regulator treatments for both crested wheatgrass and hybrid bromegrass but not meadow bromegrass. The most responsive treatment for crested wheatgrass was the media formulation with no growth regulators (61.1 embryos/calli per Petri plate), although this treatment was not significantly different from the control (46.8 embryos/calli per Petri plate). For hybrid bromegrass, the best response was from the treatment with 0.4 mg/l 2,4-D only (100.6 embryos/calli per Petri plate), although there was no significant difference between this treatment and the control (63.9 embryos/calli per Petri plate). For meadow bromegrass, the control and 1 mg/l PAA and 0.4 mg/l 2,4-D were the best growth regulator formulations (61.8 and 61.2 embryos/calli per Petri plate, respectively) but there was no significant difference between any of the treatments. Andersen et al. (1997) evaluated both 2,4-D and PAA and their effect on microspore culture of *L. perenne* and observed that 0.375 mg/l 2,4-D and no PAA was the best treatment. They concluded that 2,4-D had a stimulating effect on the microspores but PAA had a negative effect as embryogenesis decreased as PAA concentrations increased.

Conversion of microspore-derived embryos to plants

As embryos or calli developed from the microspores, these were plated on solid media (B5-5 medium [Gamborg et al. 1968, modified by Wang et al. 2019]). Embryo culture experiments evaluated media composition and culture conditions. Although plants were regenerated, it was difficult to determine the best conditions due to the variation in response.

Albinism, a common problem with the regeneration of plantlets in cereals and grasses (Żur et al. 2021), was also observed in the forage grasses. From the conducted experiments, green plant production ranged from 1.9% for crested wheatgrass AC Goliath to 34.2% in crested wheatgrass Kirk (Table 5). Despite poor regeneration and albinism, 473 plants were regenerated from the microspore-derived embryos/calli of the three forage species and transferred to soil (Table 6). Prior to planting in soil, plantlets (386) were tested for ploidy. Spontaneous doubling rates can vary between species and treatments. From the experiments conducted, the spontaneous doubling rates in meadow bromegrass was high (73%), whereas it was low (2%) for hybrid bromegrass (Table 6). Variation in the ploidy levels of resulting anther culture and microspore culture derived plants have been observed in the literature from haploid, diploid, and higher ploidy levels (Andersen et al. 1997; Christensen et al. 1997; Guo and Pulli 2000).

Conclusions:

Microspore-derived embryos/calli as well as green haploid/doubled haploid plants from all three forage grasses (crested wheatgrass, hybrid bromegrass, meadow bromegrass) were produced (Fig. 6). Differences in embryogenic response were observed between species and cultivars. Modifications to the initial wheat DH protocol include the developmental stage of the microspore to late uninucleate to early binucleate as well as changes in growth regulator concentrations. Albinism is a problem with many of the cereals and this was also observed with the forage grasses. To date, the production of high-yielding hybrid varieties from DH system are unavailable in perennial grass breeding. Single cross hybrid, double cross hybrid or population based hybridization have been proposed in polyploid grasses (Riddle and Birchler 2008; Wilkins 1991). This study generated many DH plants of the three common grasses which will be a valuable germplasm source for further studies including single cross or polycross population development to create new hybrid populations and exploit the phenomenon of heterosis.

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References

Andersen SB, Madsen S, Roulund N, Halberg N, Olesen A (1997) Haploidy in ryegrass. In: Jain SM, Sopory SK, Veilleux RE (eds.), In vitro haploid production in higher plants, Springer Netherlands, pp 133–147

Bante I, Sonke T, Tandler RF, van den Bruel AMR, Meyer EM (1990) Anther culture of *Lolium perenne* and *Lolium multiflorum*. In: Sangwan R, Sangwan-Norreel BS (eds) The Impact of Biotechnology in Agriculture; Springer: Dordrecht, The Netherlands, pp 105–127

Baral K, Coulman B, Biligetu B, Fu Y-B (2018) Genotyping-by-sequencing enhances genetic diversity analysis of crested wheatgrass [*Agropyron cristatum* (L.) Gaertn.]. International Journal of Molecular Science, 19, 2587; doi:10.3390/ijms19092587

Begheyn RF, Lübberstedt T, Studer B (2016) Haploid and doubled haploid techniques in perennial ryegrass (*Lolium perenne* L.) to advance research and breeding. Agronomy DOI:10.3390/agronomy6040060

Biswas D, Coulman B, Biligetu B, Fu Y-B (2020) Advancing bromegrass breeding through imaging phenotyping and genomic selection: a review. Frontiers in Plant Science 10:1673. doi: 10.3389/fpls.2019.01673

Castillo AM, Valero-Rubira I, Allué S, Costar MA, Vallés MP (2021) Bread wheat doubled haploid production by anther culture. In: Seguí-Simarro JM (ed) Doubled Haploid Technology: Volume 1: General Topics, Alliaceae, Cereals. Methods in Molecular Biology, Springer, vol 2287. pp 227-244

Cistué L, Echávarri B (2021) Barley isolated microspore culture. In: Seguí-Simarro JM (ed). Doubled Haploid Technology: Volume 1: General Topics, Alliaceae, Cereals. Methods in Molecular Biology, Springer vol 2287. pp 187-198

Christensen JR, Borrino E, Olesen A, Andersen SB (1997) Diploid, tetraploid, and octoploid plants from anther culture of tetraploid orchard grass, *Dactylis glomerata* L. Plant Breeding 116:267-270

Ferrie AMR, Irmen KI, Beattie AD, Rossnagel BG (2014) Isolated microspore culture of oat (*Avena sativa* L.) for the production of doubled haploids: effect of pre-culture and post-culture conditions. Plant Cell Tiss Org Cult 116:89-96

Gajecka M, Marzec M, Chmielewska B, Jelonek J, Zbieszczyk J, Szarejko I (2020) Plastid differentiation during microgametogenesis determines green plant regeneration in barley microspore culture. Plant Science. 291. 110321. 10.1016/j.plantsci.2019.110321

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Guo Y-D, Pulli S (2000) An efficient androgenic embryogenesis and plant regeneration method through isolated microspore culture in timothy (*Phleum pratense* L.). Plant Cell Reports 19:761-767

Guo YD, Sewón P, Pulli S (1999) Improved embryogenesis from anther culture and plant regeneration in timothy. Plant Cell Tissue Organ Cult 46:219-225

Hiramatsu M, Odahara K, Matsue Y (1995) A survey of microspore embryogenesis in leaf mustard (*Brassica juncea*). Acta Hortic. 392:139–145

Hu TC, Ziauddin A, Simion E et al. (1995) Isolated microspore culture of wheat (*Triticum aestivum* L.) in a defined media I. Effects of pretreatment, isolation methods, and hormones. In Vitro Cell Dev Biol - Plant 31: 79–83 https://doi.org/10.1007/BF02632241

Indrianto A, Barinova I, Touraev A, Heberle-Bors E (2001) Tracking individual wheat microspores in vitro: identification of embryogenic microspores and body axis formation in the embryo. Planta 212:163–174

Kott LS, Polsoni L, Ellis B, Beversdorf WD (1988) Autotoxicity in isolated microspore cultures of *Brassica napus.* Can. J. Bot. 66: 1665- 1670

Lantos C, Pauk J (2021) In vitro anther culture for doubled haploid plant production in spelt wheat. Methods Mol Biol. 2287:257-266. doi: 10.1007/978-1-0716-1315-3_13. PMID: 34270035

Leśniewska A, Ponitka A, Ślusarkiewicz-Jarzina A, Zwierzykowska E, Zwierzykowski Z, James AR, Thomas H, Humphreys MW (2001) Androgenesis from *Festuca pratensis* x *Lolium multiflorum* amphidiploid cultivars in order to select and stabilize rare gene combinations for grass breeding. Heredity 86:167-176

338 Maheshwari P, Laurie JD (2021) Triticale isolated microspore culture for doubled haploid production. In: Seguí-
339 Simarro JM (ed). Doubled Haploid Technology: Volume 1: General Topics, Alliaceae, Cereals. Methods in Molecular
340 Biology, Springer, vol 2287. pp 295-312
341
342 Olesen A, Andersen SB, Due IK (1988) Anther culture response in perennial ryegrass (*Lolium perenne* L.). Plant
343 Breed. 101: 60–65
344
345 Phippen C, Ockendon DJ (1990) Genotype, plant, bud size and media factors affecting anther culture of cauliflowers
346 (*Brassica oleracea* var. *botrytis*). Theor. Appl. Genet. 79:33–38
347
348 Riddle NC, Birchler JA (2008) Comparative analysis of inbred and hybrid maize at the diploid and tetraploid levels.
349 Theor. Appl. Genet. 116: 563–576
350
351 Ulrich A, Furton WH, Downey RK (1984) Biotechnology and rapeseed breeding: Some economic considerations. Sci.
352 Counc. Can. Rep., Ottawa, 67pp
353
354 Wang HM, Enns JL, Nelson KL, Brost JM, Orr TD, Ferrie AMR (2019) Improving the efficiency of wheat microspore
355 culture methodology – evaluation of pretreatments, gradients, and epigenetic chemicals. Plant Cell Tissue and Organ
356 Culture 139:589-599
357
358 Warchol M, Juzoń K, Dziurka K, Czyczylo-Mysza I, Kaploniak K, Marchińska I, Skrzypek E (2021) The effect of
359 zinc, copper, and silver ions on oat (*Avena sativa* L.) androgenesis. Plants 10:248
360
361 Wilkins PW (1991) Breeding perennial ryegrass for agriculture. Euphytica 52: 201–214
362
363 Zheng MY, Liu W, Weng Y, Polle E, Konzak CF (2001) Culture of freshly isolated wheat (*Triticum aestivum* L.)
364 microspores treated with inducer chemicals. Plant Cell Rep 20:685–690
365

366 Żur I, Gajecka M, Dubas E, Krzewska M, Szarejko I (2021) Albino Plant Formation in Androgenic Cultures: An Old
367 Problem and New Facts. In: Segui-Simarro JM (ed) Doubled Haploid Technology. Methods in Molecular Biology,
368 vol 2288. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-1335-1_1
369
370

Statements and Declarations

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Competing interests

AF is associate editor for the journal. The other authors (KN, BB) have no relevant financial or non-financial interests to disclose.

Author Contributions

AF conceptualized the study and both AF and BB applied for funding. KN provided technical support and conducted all experimental work. AF and KN interpreted results and all authors assisted in writing and critical review of the manuscript and approved of the submitted version.

Conflict of interest

The authors declare no conflict of interest.

Data availability

Data will be made available on reasonable request.

Table 1: Effect of microspore developmental stage on embryo production per plate in three perennial grass species.

Species	Mid-late uninucleate stage	Very late uninucleate to early binucleate
	Embryos per Petri plate	
Crested wheatgrass	1	29
Hybrid bromegrass	15	44
Meadow bromegrass	35	91

Table 2: Genotype evaluation of three crested wheatgrass genotypes (AC Goliath, Kirk, NewKirk) and two hybrid bromegrass genotypes (AAC Torque, AC Knowles) utilizing late - early binucleate microspores in microspore culture

Species and cultivar	
	Average number of embryos/calli per plate
Crested wheatgrass	
AC Goliath	25.8 a
Kirk	29.0 a
NewKirk	29.0 a
Hybrid bromegrass	
AAC Torque	13.8 a
AC Knowles	11.5 a

Means within a column within a species, followed by different letters are significantly different at the P = 0.05 level as determined by Duncan's Multiple Range Test.

Table 3: Evaluation of five maltose concentrations on microspore embryogenesis of crested wheatgrass and meadow bromegrass.

	Crested wheatgrass	Meadow bromegrass
% maltose in the culture media	Average number of embryos/calli per plate	
7	11.7 b	7.2 a
9 (Control)	30.8 a	13.1 a
13	12.8 b	14.9 a
15	11.7 b	13.1 a
17	9.0 b	13.8 a

Means within a column within a species, followed by different letters are significantly different at the P = 0.05 level as determined by Duncan's Multiple Range Test.

Table 4: Evaluation of phenylacetic acid (PAA) and 2,4-Dichlorophenyoxyacetic acid (2,4-D) on microspore embryogenesis of three forage grasses (crested wheatgrass, hybrid bromegrass, and meadow bromegrass).

PAA (mg/l)	2,4-D (mg/l)	Crested wheatgrass	Hybrid bromegrass	Meadow bromegrass
		Average number of embryos/calli per plate		
	Control	46.8 ab	63.9 abc	61.8 a
0	0	61.1 a	54.4 abcd	41.2 a
0	0.1	58.0 a	81.3 ab	36.7 a
0	0.2	40.7 abc	83.3 ab	45.8 a
0	0.4	39.8 abc	100.6 a	50.9 a
0.5	0	40.0 abc	40.2 bcd	42.8 a
0.5	0.1	24.1 bcd	48.7 bcd	29.1 a
0.5	0.2	26.2 bcd	88.9 ab	55.0 a
0.5	0.4	24.6 bcd	66.0 abc	40.5 a
1.0	0	22.7 bcd	52.4 abcd	29.7 a
1.0	0.1	28.3 bcd	45.8 bcd	46.0 a
1.0	0.2	21.7 bcd	22.8 d	41.4 a
1.0	0.4	18.9 bcd	65.2 abc	61.2 a
2.0	0	15.1 d	30.1 cd	41.0 a
2.0	0.1	34.1 abcd	18.4 d	35.2 a
2.0	0.2	35.4 abcd	61.5 abc	39.0 a
2.0	0.4	39.6 abcd	28.6 cd	20.0 a

Control: 0.2 mg/l Kin, 1.0 mg/l PAA, 0.2 mg/l 2,4-D
Means within a column within a species, followed by different letters are significantly different at the P = 0.05 level as determined by Duncan's Multiple Range Test.

Table 5: Number of green plants and albino plants derived from doubled haploidy experiments of three forage grass species (crested wheatgrass, hybrid bromegrass, and meadow bromegrass).

Cultivar	Number of green plants regenerated	Number of albino plants regenerated	Total number of plants regenerated	Percent green plants
Newkirk	567	2713	3280	17.3
Kirk	131	252	383	34.2
AC Goliath	6	308	314	1.9
Fleet	156	1036	1192	13.1
AAC Torque	78	187	265	29.4
AC Knowles	0	1	1	0

Table 6: Total number of forage grass (crested wheatgrass, hybrid bromegrass, and meadow bromegrass) microspore-derived plantlets transferred to soil and ploidy level of those plants.

Species	Total number of green plants transferred to soil	Total plants ploidy tested	Doubled haploid *	Haploid	Other
			Total number of plants (% of total analyzed)		
Crested wheatgrass	328	295	78 (26%)	95 (32%)	122 (41%)
Hybrid bromegrass	65	65	1 (2%)	60 (92%)	4 (6%)
Meadow bromegrass	80	26	19 (73%)	7 (27%)	0 (0%)

*Doubled haploid or diploid plants – similar to the donor plant; haploid – having half the chromosome number; other - mixoploid

445 Figure legends

446

447 Figure 1: Developmental stage of microspores in hybrid bromegrass c.v. AAC Torque on Day 0 (day of microspore
448 extraction after 21 days of cold treatment of the spikes) using acetocarmine staining. A: early uninucleate, B: mid
449 uninucleate, C: late uninucleate, D: very late uninucleate and early binucleate.

450

451 Figure 2: Anthers from i: crested wheatgrass, ii: hybrid bromegrass, iii: meadow bromegrass. Those anthers above
452 the blue line represents anther colour desired when cutting plant material, and those anthers below the blue line
453 represents the desired anther colour at the end of the pretreatment stage.

454

455 Figure 3: Induction of forage grass microspores at the mid-late uninucleate stage and the very late uninucleate to
456 early binucleate stage on Day 0 (day of microspore extraction after 21 days of cold treatment of the spikes).

457

458 Figure 4: The doubled haploidy process for the crested wheatgrass: A. Spikes from donor plants, B. induced
459 microspores, C. microspore-derived embryos, D. plantlet regeneration, E. Doubled haploid plants

460

Figure 1: Developmental stage of microspores in hybrid bromegrass c.v. AAC Torque on Day 0 (day of microspore extraction after 21 days of cold treatment of the spikes) using acetocarmine staining. A: early uninucleate, B: mid uninucleate, C: late uninucleate, D: very late uninucleate and early binucleate.

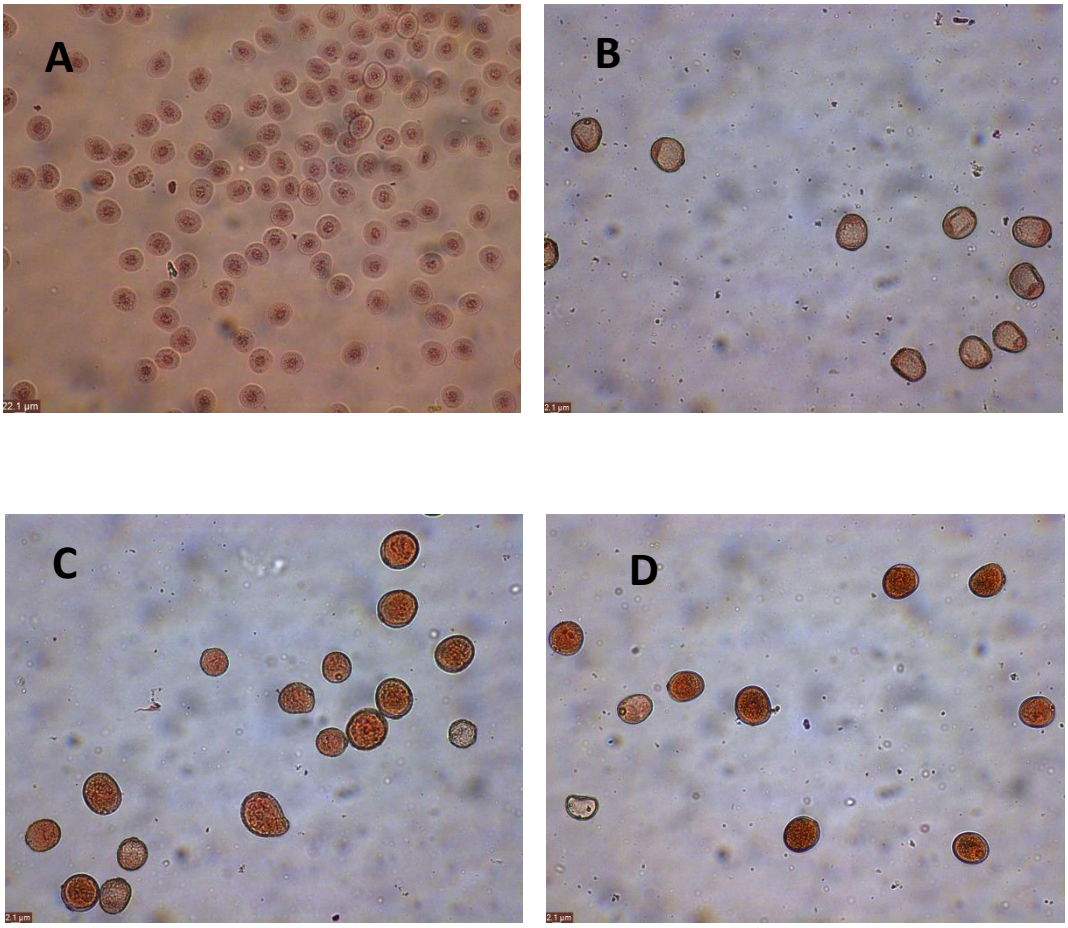


Figure 2: Anthers from i: crested wheatgrass, ii: hybrid bromegrass, iii: meadow bromegrass. Those anthers above the blue line represents anther colour desired when cutting plant material, and those anthers below the blue line represents the desired anther colour at the end of the pretreatment stage. The ruler indicates 1 mm increments.

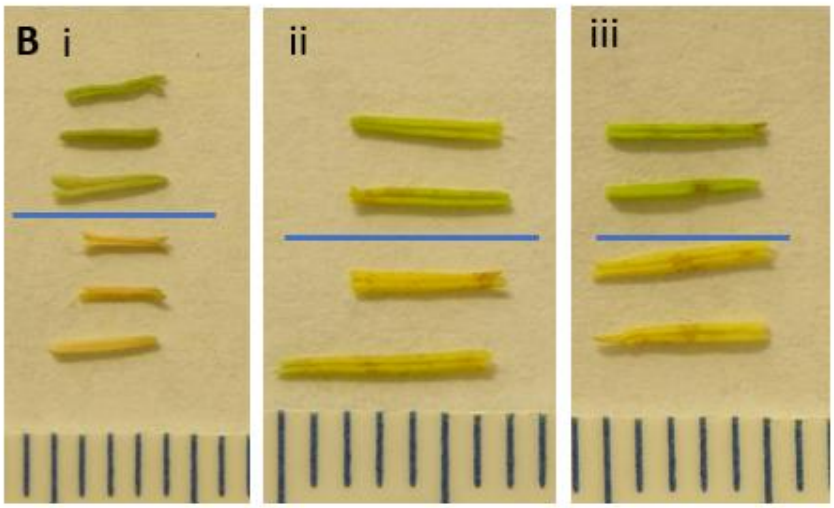
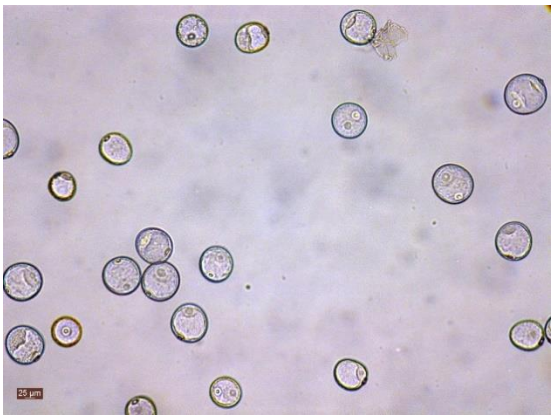


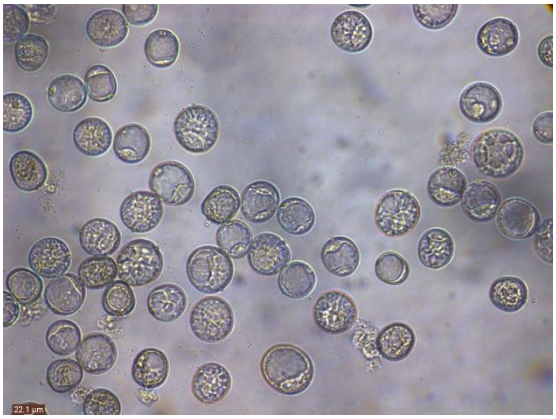
Figure 3: Induction of forage grass microspores at the mid-late uninucleate stage and the very late uninucleate to early binucleate stage on Day 0 (day of microspore extraction after 21 days of cold treatment of the spikes).

Mid – late uninucleate stage

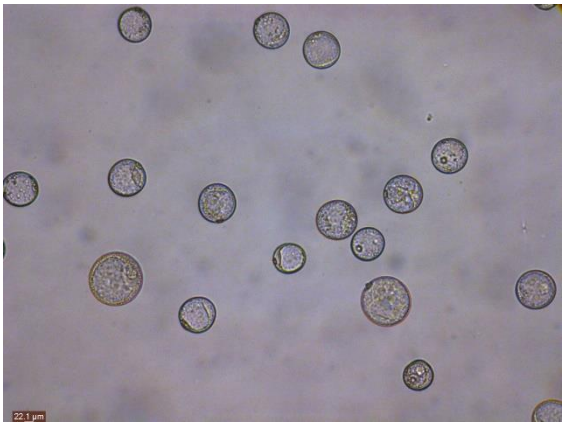
very late uninucleate to early binucleate stage

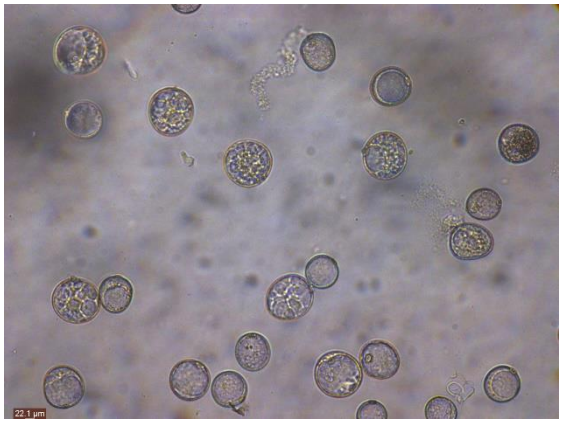
Meadow bromegrass:



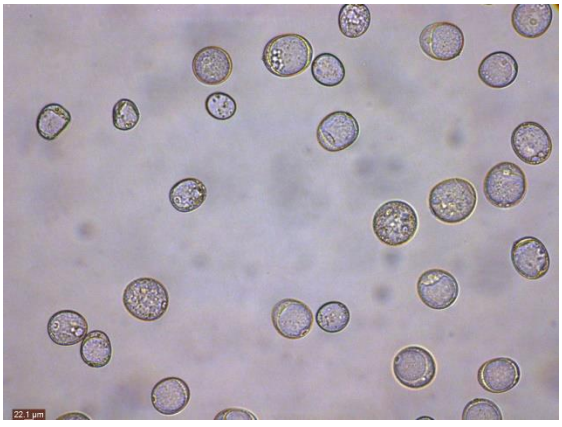


Hybrid bromegrass:





Crested wheatgrass



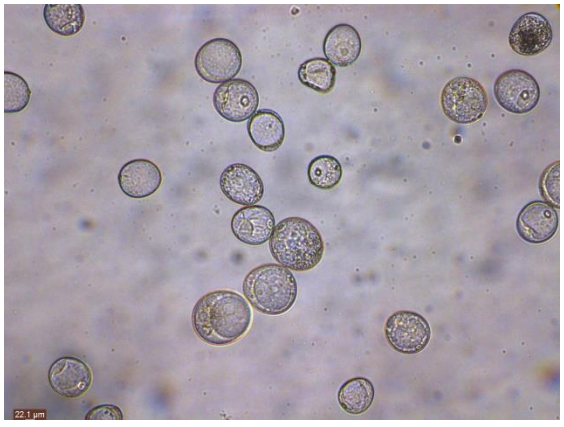


Figure 4: The doubled haploidy process for the crested wheatgrass: A. Spikes from donor plants, B. induced microspores, C. microspore-derived embryos, D. plantlet regeneration, E. Doubled haploid plants