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ORIGINAL ARTICLE

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 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.

Keywords Androgenesis · Crested wheatgrass · Doubled haploidy · Hybrid bromegrass · Meadow bromegrass · Microspore culture

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 (*Lolium perenne* L.) 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 perennial forage breeding

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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 (*Bromus riparius x B. inermis*), meadow bromegrass (*Bromus riparius* Rehm.), and crested wheatgrass (*Agropyron cristatum* (L.) Gaertn.), (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, hybrid bromegrass, and meadow bromegrass.

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 min, then rinsed four times with 400 ml of cold sterile water. The microspores from the spikelets/florets were isolated with FHG-2 medium [FHG (Hunter 1988) medium without micronutrients, organics and hormones (Wang et al. 2019)] in a blender cup. The filtrate was centrifuged at 1000 rpm in a refrigerated centrifuge (4 °C) for 5 min. 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 (Zheng et al. 2001) + GLC [glutathione (Asif et al. 2013a), Larcoll (Letarte et al. 2006), cefotaxime (Asif et al. 2013b)] with 0.2 mg/l 2,4-Dichlorophenyoxyacetic acid (2,4-D), 0.2 mg/l kinetin, and 1.0 mg/l phenylacetic acid (PAA) (Wang et al. 2019) was added to achieve a density of 1×10^5 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 for co-culture as per the wheat microspore culture protocol. 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 medium (Gamborg et al. 1968) with the addition of 1.0 mg/l kinetin and 1.0 mg/l IAA. The plates were incubated at 22 °C, 16 h photoperiod, and 100 µmol m^{-2} s^{-1} 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 (Sysmex Partec). Prior to transferring the plants to soil, a section of leaf tissue (approximately 0.5 cm^2) was sampled from the plantlets. This material was chopped with a razor blade and stained using Sysmex CyStain UV Precise P and filtered (30 µm filter) into a tube. The filtrates were analyzed using the Cell Counter Analyzer. Plantlets were hardened off in an environmentally controlled chamber then transferred to the greenhouse. In the greenhouse, the light period was 18 h,

supplemented with high pressure sodium halogen lamps to a total of 490–550 µmol m^{-2} s^{-1} light intensity. 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 cultivars used included Kirk (crested wheatgrass), Fleet (meadow bromegrass), and AC Success (hybrid bromegrass). The spring wheat protocol has a 21 day 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 2: Twelve plants from each of the three species (crested wheatgrass c.v. Kirk, meadow bromegrass c.v. Fleet, hybrid bromegrass c.v. AAC Torque) 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 3: 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 4: Following the protocol described above, 9% sucrose was substituted for 9% maltose in the NPB99 + GLC medium. Experiments were conducted with crested wheatgrass c.v Newkirk and hybrid bromegrass c.v. AAC Torque.
Experiment 5: Evaluation of five concentrations (7, 9, 13, 15, 17%) of maltose as the carbohydrate source in the NPB99 + GLC medium. Crested wheatgrass c.v. Kirk and meadow bromegrass c.v. Fleet were evaluated.
Experiment 6: 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 (0.2 mg/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). The cultivars used were Kirk (crested wheatgrass), Fleet (meadow bromegrass), and AAC Torque (hybrid bromegrass).
Experiment 7: 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. The same cultivars that were utilized in Experiment 6 were evaluated in this experiment (i.e., Kirk, Fleet, AAC Torque).

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 (ANOVA) were performed on experimental means using completely randomized designs and differences between means was determined by Duncan's Multiple Range Test. Statistical analyses were done using the XLSTAT statistical platform for Microsoft Excel.

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 (21 day cold pretreatment) and winter wheat (28 day cold pretreatment) microspore culture protocols (Wang et al. 2019) on the three forage grasses, crested wheatgrass, meadow bromegrass, and hybrid bromegrass. Crested wheatgrass is a wild relative of wheat and comparative genomic analysis between the *A. cristatum* transcriptome and other grass species show that there is a closer relationship between crested wheatgrass and wheat than with crested wheatgrass and barley (Zhang et al. 2015). The bromegrass species belong to the tribe Bromeae, a different tribe from wheat and barley (Triticeae) and therefore a greater taxonomic distance from the cultivated crop species. For crested wheatgrass c.v. Kirk, microspore-derived structures were observed using both the spring and winter wheat protocols, with the majority of the structures from the spring wheat (21 day cold pretreatment) 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

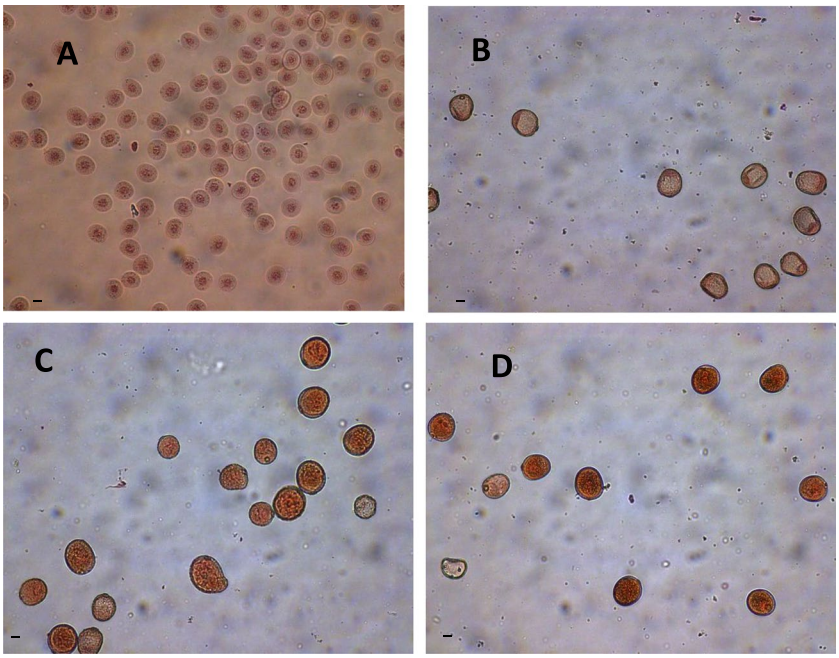

Fig. 1 Developmental stage of microspores in hybrid bromegrass c.v. AAC Torque on Day 0 (day of microspore extraction after 21 days of cold treatment of the spikes) using acetocarmine staining. **A**: early uninucleate, **B**: mid uninucleate, **C**: late uninucleate, **D**: very late uninucleate and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

spring (21 day cold pretreatment) or winter wheat (28 day cold pretreatment) 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 (21 day cold pretreatment) protocol but the quality of the structures was abnormal (i.e., friable callus). From these initial studies, 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

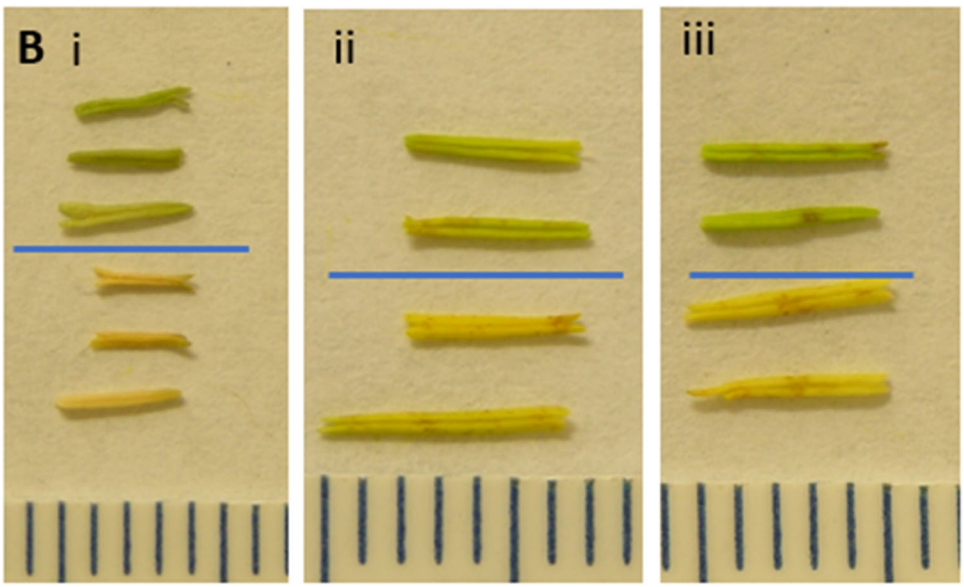

Fig. 2 Anthers from i: crested wheatgrass c.v. Kirk, ii: hybrid bromegrass c.v. AAC Torque, iii: meadow bromegrass c.v. Fleet. Those anthers above the blue line represents anther colour desired when cutting plant material, and those anthers below the blue line represents the desired anther colour at the end of the pretreatment stage

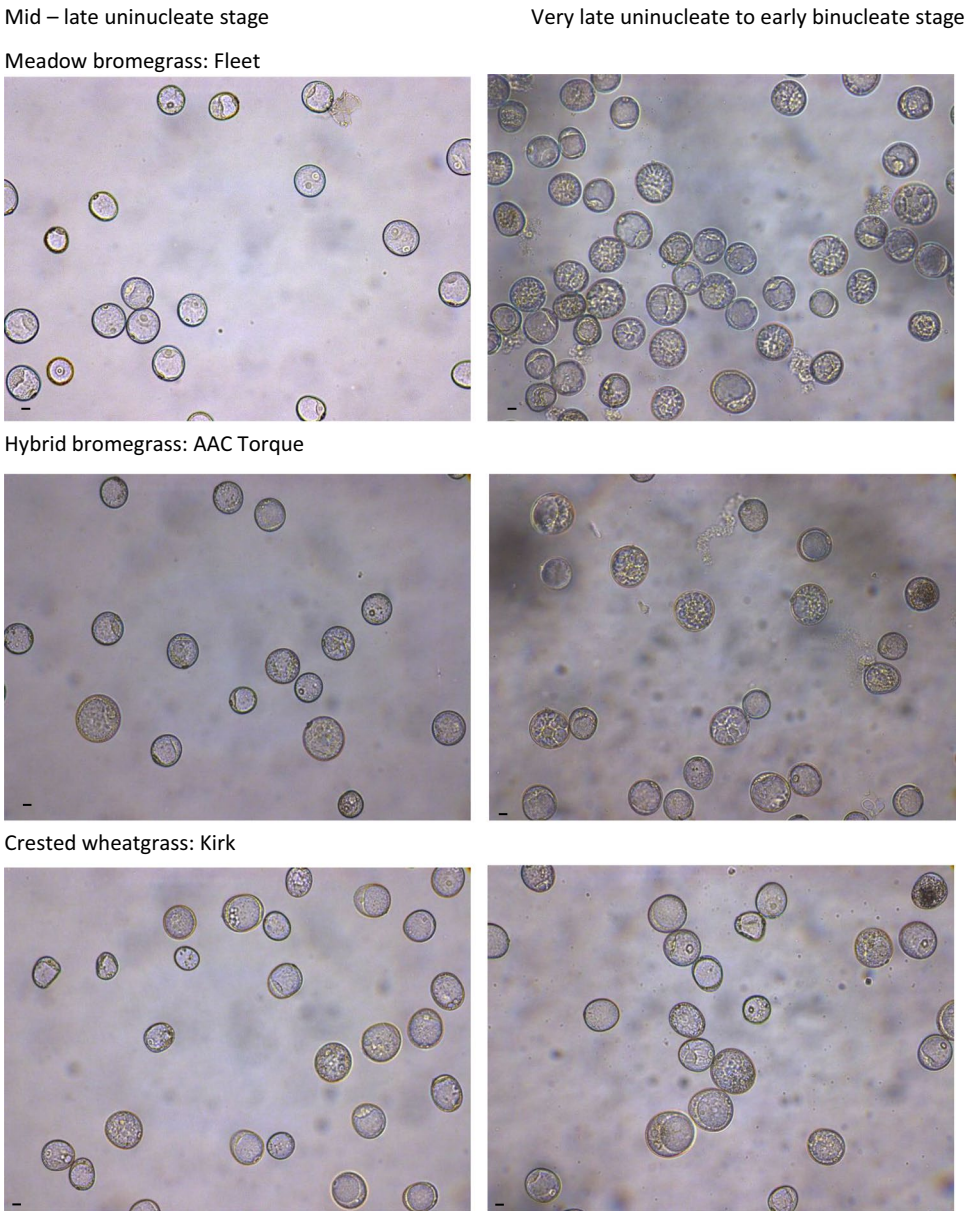

Fig. 3 Induction of forage grass microspores at the mid-late uninucleate stage and the very late uninucleate to early binucleate stage on Day 0 (day of microspore extraction after 21 days of cold treatment of the spikes)

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

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 Kirk	35	91
Hybrid bromegrass AAC Torque	15	44
Meadow bromegrass Fleet	1	29

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

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. 1A, 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. 1D). 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 utilizing the late uninucleate to early binucleate stage of microspore development. 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.

Table 3 Evaluation of five maltose concentrations on microspore embryogenesis of crested wheatgrass c.v. Kirk and meadow bromegrass c.v. Fleet

	Crested wheatgrass Kirk	Meadow bromegrass Fleet
% 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

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 (Newkirk) and hybrid bromegrass (AAC Torque) 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 (Andersen 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).

Table 4 Evaluation of phenylacetic acid (PAA) and 2,4-Dichlorophenyoxyacetic acid (2,4-D) on microspore embryogenesis of three forage grasses (crested wheatgrass c.v. Kirk, hybrid bromegrass c.v. AAC Torque, and meadow bromegrass c.v. Fleet)

PAA (mg/l)	2,4-D (mg/l)	Crested wheatgrass Kirk	Hybrid bromegrass AAC Torque	Meadow bromegrass Fleet
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

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 (Fleet), 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 (Kirk), 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 (Andersen 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).

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)

Species and cultivar	Number of green plants regenerated	Number of albino plants regenerated	Total number of plants regenerated	Percent green plants
Crested wheatgrass				
Newkirk	567	2713	3280	17.3
Kirk	131	252	383	34.2
AC Goliath	6	308	314	1.9
Meadow bromegrass				
Fleet	156	1036	1192	13.1
Hybrid bromegrass				
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 and cultivar	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					
Newkirk	197	197	61 (31%)	68 (35%)	68 (35%)
Kirk	141	98	17 (17%)	27 (28%)	54 (55%)
Hybrid bromegrass					
AAC Torque	65	65	1 (2%)	60 (92%)	4 (6%)
Meadow bromegrass					
Fleet	80	26	19 (73%)	7 (27%)	0 (0%)

*Doubled haploid– similar to the donor plant; haploid – having half the chromosome number of the donor plant; other—mixoploid

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, 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 (Kirk) and hybrid bromegrass (AAC Torque) but not meadow bromegrass (Fleet). 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),

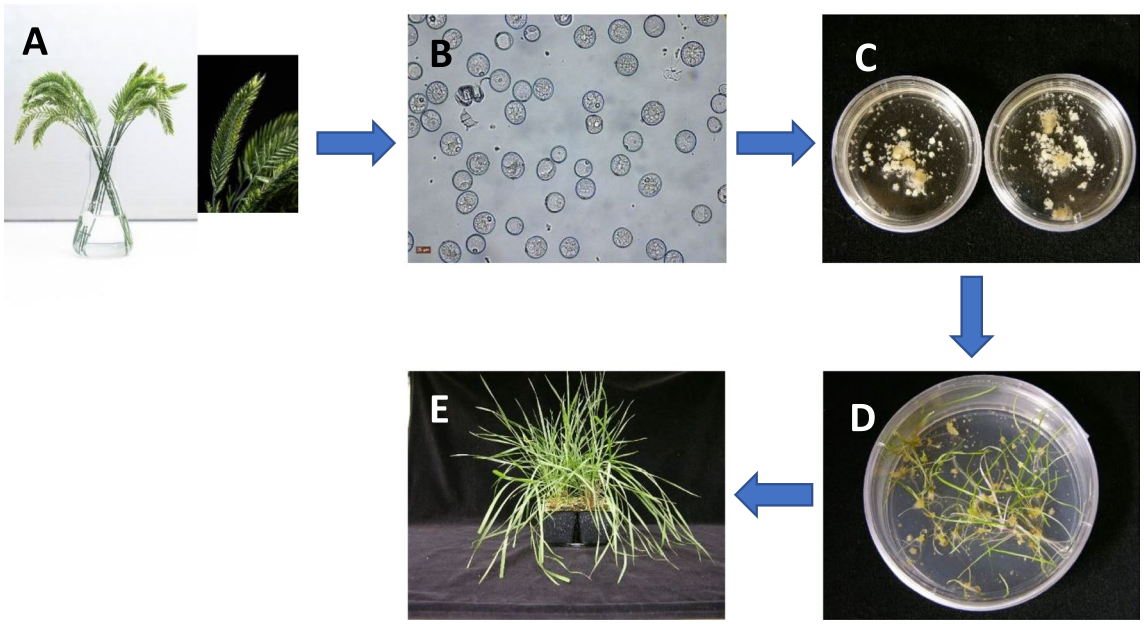

Fig. 4 The doubled haploidy process for crested wheatgrass: **A**. Spikes from donor plants, B. induced microspores, **C**. microspore-derived embryos, **D**. plantlet regeneration, **E**. Doubled haploid plants

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 medium (Gamborg et al. 1968) with the addition of 1.0 mg/l kinetin and 1.0 mg/l IAA]. 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, 483 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 c.v.Fleet was high (73%), whereas it was low (2%) for hybrid bromegrass c.v. AAC Torque (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). The parental cultivars had a range of ploidy levels, Newkirk and Kirk are tetraploid, Fleet is a decaploid and AAC Torque is an octoploid – decaploid.

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 (Table 6, Fig. 4). 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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Data availability Data will be made available on reasonable request.

Declarations

Competing interests AF is associate editor for the journal and, as such, has not been involved in any stage of the evaluation of the submission. This status had no bearing on the editorial consideration of the manuscript.

Conflict of interest The authors declare no conflict of interest.

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