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Establishing isolated microspore culture to produce doubled haploid plants in Brazilian wheat (*Triticum aestivum* L.)

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Abstract

The objective of this study was to establish an isolated microspore culture (IMC) protocol in wheat (*Triticum aestivum* L.) for use in genetic studies and to evaluate its potential for routine use in the Brazilian Wheat Breeding Program at Embrapa Wheat. Important steps of the method were identified and plant physiology of microspore mother plants and ovary co-culture were considered as key factors for effective establishment. Three Brazilian wheat genotypes were tested (Toropi, BRS 194 and F_1 wheat cross 020037 × 020062), and two other genotypes were used as controls (Bobwhite and Fielder). Spikes containing uninucleated microspores were subjected to cold pretreatment (4°C) for 21 days in the dark. Number of embryos, green and albino plants were recorded for each genotype. The method was successfully established, and several fertile green plants were produced by using tissue culture and responsive controls. However, the results greatly differed among Brazilian wheat genotypes, suggesting a strong genotype-dependent effect. Microspore induction medium alone did not promote embryogenesis; ovary co-culture was a necessary step for embryo development and green plant formation, for all genotypes. The F_1 wheat cross (020037 × 020062) produced a total of 85 green plants (out of 108 spikes), 64% of which were spontaneous diploids. BRS 194 produced many embryos, exhibiting a good androgenic response, but only a few grew into green plants. Toropi behaved as a recalcitrant genotype, and zero plants were produced. To our knowledge, this is the first report on wheat IMC from Brazilian genotypes resulting in androgenic embryogenesis and plant regeneration.

Keywords: androgenesis; doubled haploids; microspore culture; *Triticum aestivum* L.; wheat breeding.
Abbreviations: APR_Adult Plant Resistance; CIMMYT_ International Maize and Wheat Improvement Center; DH_ Doubled Haploids; IMC_ Isolated Microspore Culture.

Introduction

The production of doubled haploid (DH) plants is an important biotechnological tool used in many plant breeding programs, especially for speeding up the release of new major agronomic varieties. DH plants also play an important role in molecular and genetic studies at several research institutions, providing an excellent source of material for genome mapping (Jauhar et al., 2009). Haploid plants can be obtained *in vitro* using female and male gametes, leading to the formation of totally pure plants in a single generation (Kölliker et al., 2010; Murovec and Bohanec, 2012; Tadesse et al., 2012). The use of DH methodology makes it possible to achieve complete homozygosity for all the *loci*, avoiding the numerous cycles of self-pollination necessary for conventional breeding approaches (Dunwell, 2010; Santra et al., 2012). Furthermore, the expression of recessive alleles is not blocked or masked by dominant alleles when using DH technology (Touraev et al., 2001), offering a more precise picture of the diversity present in the gametic cells. Because of its efficiency, the use of DH plants can significantly decrease the time, cost and labor involved in developing new varieties (Barkley and Chumley, 2012). Several methods for producing DHs in cereals have been used in the past, mostly through intergeneric crosses, by using *Hordeum bulbosum* or *Zea mays* as pollen donors. However, employing an haploidization methodology with intergeneric crosses (using female gametes) is not only more labor intensive but also results in very low spontaneous duplication rates, requires several colchicine treatments and results in high mortality rates (Soriano et al., 2007; Broughton, 2008; Castillo et al., 2009). In addition, when compared to androgenesis, maize pollination offers a lower meiotic recombination frequency, a trait that is especially important for genetic studies (Broughton, 2008). Therefore, intergeneric crosses have been slowly replaced by androgenesis using either anther culture in some species, or, more recently, microspore culture (Ferrie and Caswell, 2011). Cereal anther culture (barley and wheat, more in particular) has two major limitations, which are specifically related to the genotype response (strongly genotype dependent) and to the production of albino plants (Forster et al., 2007; Kumari et al., 2009; Torp and Andersen, 2009; Dunwell, 2010). Although the occurrence of albinism is strongly linked to genetic causes, other factors such as temperature, light intensity and media composition (types of sugar and plant growth regulators) are also associated with albinism, hampering the production of DH plants (Kumari et al., 2009). Therefore, albinism could be avoided to some degree by promoting adequate environmental conditions for growing donor plants and by refining the medium and culture

conditions. A great number of studies have been conducted to improve the number of green plants per spike, leading to the so-called “resurgence” of interest in haploids (Forster et al., 2007; Murovec and Bohanec, 2012). As a consequence, better results have been observed as newer and more reliable tissue culture protocols have been developed (Germanà, 2011). Currently, the application of isolated microspore culture (IMC) makes it possible to produce a larger number of fertile plants per spike (Zheng, 2003; Li and Devaux, 2005; Cistué et al., 2009; Ferrie and Caswell, 2011) and is associated with less interference of maternal sporophytic tissues from the anther wall, because these tissues are discarded before culturing isolated cells (Chugh and Eudes, 2008; Murovec and Bohanec, 2012). Culturing purified cells without anther somatic tissues offers the most striking advantage over anther culture, as all of the regenerated plants are derived from a gametic source and not from maternal tissues (Bal et al.,2009). Although cereal microspore culture has been practiced for many years (Gustafson et al., 1995) and is routinely adopted in many countries (Lantos et al., 2006), after several improvements (Hu and Kasha, 1997; Liu et al., 2002a, b; Shariatpanahi et al., 2006; Cistué et al., 2009; Slama Ayed et al., 2010), the establishment of IMC in many laboratories has been very limited mainly because of difficulties associated with the extraction and purification procedures involved and a lack of facilities. Protocols describing IMC methodology may vary among laboratories, but some fundamental and critical phases include the following: growing donor plants, collecting spikes at the right stage, purifying and inducing microspores, regenerating embryos and doubling the chromosomes (Ferrie and Caswell, 2011). However, the growing conditions of donor plants, the medium composition and the genotype response are among the key factors for a successful DH production system of microspore culture. Implementing any type of DH technique for genetic studies, or more importantly, applying this technique to a breeding program, requires an efficient haploid production system. Moved by this motivation, to improve DH production efficiency, the objective of this research was to establish an IMC protocol following a “standard procedure” for microspore purification (Eudes and Amundsen, 2005). Two wheat genotypes responsive to tissue culture and androgenesis (Bobwhite and Fielder, respectively) were used. The same protocol was applied to evaluate the androgenic response of three Brazilian wheat genotypes (Toropi, BRS 194 and one F_1 local cross named 020037 × 020062). To our knowledge, this is the first report on the establishment of wheat IMC and its use for the production of DH plants in Brazil. Possible routine IMC applications to the wheat breeding program were also evaluated.

Results

Donor plants and growth conditions

In our studies, microspore donor plants were kept in a growth cabinet with temperatures ranging from 18 to 14°C (day/night) with a photoperiod of 16 h, resulting in healthy and vigorous plants. No chemicals were applied to the plants after tillering, only during seed treatment (Gaucho - Imidacloprid, Bayer and Baytan - Triazole, Bayer), which was enough to provide a pathogen/insect-free environment until the end of each cycle. Attempts to produce healthy and vigorous plants under semi-controlled conditions (in greenhouses) failed, as insect and disease management was relatively more difficult to achieve.

Spike pretreatment for triggering microspore embryogenesis

In preliminary studies, the use of mannitol combined with cold for a shorter period (4-7 days at 4°C) did not promote embryogenesis.. Therefore, only cold shock was applied to the spikes by incubating wheat tillers for a three-week period at 4°C in the dark, varying whenever possible the duration of spike pretreatment time (starting from 15 up to 29 days). Our results showed that this variation in time did not affect the embryogenesis induction or plant regeneration.

Microspore regeneration on different wheat genotypes

Microspore purified cells were observed under an inverted microscope showing a mixture of cells at different stages of development, but uninucleated microspores were predominant. Typical uninucleated microspores plated in the semi-liquid NPB 99 culture medium exhibited a large central vacuole, with the nucleus located against the cell wall and at the opposite side of the pore. After an average of 6–8 days of incubation in the dark at 27°C, the uninucleated microspores from the initial isolation procedure started to float, converting the large and single vacuole into smaller ones, and enclosing a fibrillar or star-like structure inside them. The first divisions were observed shortly after this stage. After 21 days of incubation, it was possible to observe multicellular structures ready to break out the exine in all tested genotypes. However, significant differences across the genotypes were observed, especially when counting the number of multicellular structures per genotype and the number of days during which they were produced after plating. The great majority of work using wheat IMC describes the isolation procedure steps using microspores at the mid- to late-uninucleate stages (Jähne and Lörz, 1995; Letarte et al., 2006; Shirdelmoghanloo et al., 2009). However, better results were achieved in our studies when using early to mid-uninucleate stages, mostly because a long duration of pretreatment was applied to the spikes (21 days ± 3 days, 4°C). The results revealed a good yield of cells (number of purified cells mL^{-1}) for most genotypes, although some anther tissue debris was observed at some level during the isolation procedures. Damaged cells combined with plant debris were, in most cases, eliminated after centrifugation with maltose solution (20%). Cleaner cell samples were obtained when purification was carried out by using anthers alone instead of spikelets or flowers (Figure 1). The growing condition of the donor plants is a very important feature, as it also impacts the amount and quality of microspore and pollen grains, directly affecting the competence of these cells. High cell yields were difficult to obtain when using donor plants (of all genotypes) kept under uncontrolled conditions (or semi-controlled conditions) using soil with copper and zinc deficiency and aluminum toxicity (early cell purification attempts failed; data not shown). A minimum density of viable cells is necessary to ensure microspore regeneration (Hoekstra et al., 1993; Castillo et al., 2000). Our data were obtained with a cell density varying from 6–9 x 10^4 cells mL^{-1}, but embryos and green plants (Figure 1) were also observed when the cell concentration was as low as 3.8 x 10^4 cells mL^{-1} (for Fielder). Microspore-purified cells from Bobwhite and Fielder produced the largest number of multicellular structures in a shorter period of time, followed by F_1 wheat cross 020037 × 020062. The total number of green plants (haploids and diploids) per genotype followed the same proportion (192 for Bobwhite, 136 for Fielder and 85 for F_1 cross 020037 × 020062). BRS 194 exhibited an intermediate number of embryos and timing but

Table 1. Production of regenerated plantlets (green and albino plants, haploids and diploids) from five different wheat genotypes using microspore culture co-cultivated with four ovaries per Petri dish. Absolute numbers obtained from three different cycles. Mean numbers are in parenthesis. Values followed by the same letter do not differ significantly (in the column) according to the Scott-Knott test at a significance level of $p < 0.05$.

Genotype	Green Plants	Albino Plants	Haploids	Diploids
Bobwhite	192 (21.3) a	462 (6.3) a	42	150
Fielder	136 (15.1) a	309 (5.1) a	117	19
PF 020037/020062	85 (9.4) a	41 (1.9) b	28	57
BRS 194	5 (0.5) b	38 (1.2) b	2	3
Toropi	0 (0) b	7 (0.5) b	0	0

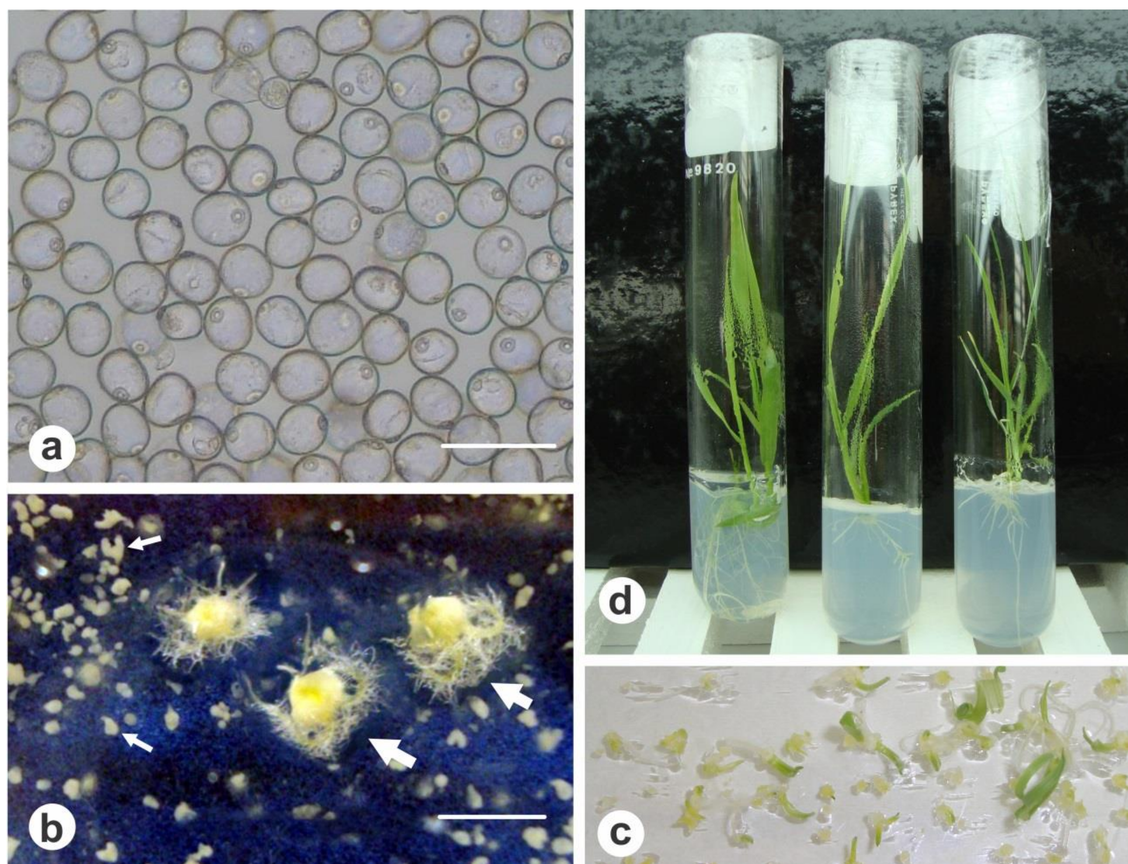

Fig 1. Androgenesis steps in wheat during *in vitro* isolated microspore culture (Fielder): **a)** High concentration of clean and purified cells (uninucleated microspores), scale bar represents 50 µm; **b)** Developing embryoids (smaller arrows) obtained from co-cultivation with ovaries (larger arrows), scale bar represents 5 mm; **c)** Formation of green plantlets in a Petri dish after 8 days on solid GEM culture medium; **d)** Green plants developed and ready for transplantation to vermiculite.

did not produce the same proportion of green plants. Toropi produced the lowest rates in terms of embryo formation, and zero green plants were produced (Table 1). The very few embryos originated from Toropi gave rise only to albino plants. The regeneration percentage obtained from larger embryos (≥ 2.0 mm) was significantly higher than with small (0.5–1.0 mm) embryos. However, the embryo size was not the only factor affecting regeneration. Time combined with size also influenced the embryo capacity. The embryos that first developed to a size of 2.0 mm exhibited the best plant regeneration responses. Embryos that were kept more than 40 days in the semi-liquid culture medium failed to regenerate into green plants when they had reached a size of only 1.0 mm. The regeneration process (embryo development into green plants) was more efficient with Bobwhite and Fielder genotypes, followed by the F_1 cross 020037 × 020062 (number of embryos/green plant). BRS 194 produced an average of 193 embryos for every green plant, exhibiting a limitation in the conversion of embryos into plants.

Morphological differences and frequency of spontaneous diploids

Several factors play important roles during the plant regeneration phase among different tissue culture systems. The composition of induction and regeneration media, embryo types and sizes are some of the criteria used to ensure success during this phase. From our studies, we observed that the great majority of embryos developed directly into plants (as initiated by direct embryogenesis) and did not pass through the callus stage. Large embryos that were transferred to GEM solid medium became green plantlets shortly after plating (3–5 days). Green plantlets were transferred to rooting medium and, after exhibiting a set of 2–3 leaves and sufficient root growth, were individually transplanted to pots with vermiculite and placed in a growth cabinet with the same conditions as the donor plants. Stable and fertile green plants were regenerated from Bobwhite, Fielder and F_1 wheat cross 020037 × 020062.

Table 2. Analysis of variance (ANOVA) obtained from the mean values of total green plants.

ANALYSIS OF VARIANCE					
Source	DF	SS	MS	F	Pr>F
Genotypes	4	117.412563	29.353141	11.892	0.000
Replications	8	25.303246	3.162906	1.281	0.2877
Error	32	78.985898	2.468309		
Total	44	221.701706			
Mean	2.0885843	Number of observations: 45			

Number of Green Plants Transformation: Square root - SQRT (Y)

Table 3. Analysis of variance (ANOVA) obtained from the mean values of total albino plants.

ANALYSIS OF VARIANCE					
Source	DF	SS	MS	F	Pr>F
Genotypes	4	232.976928	58.244232	10.907	0.000
Replications	8	48.256565	6.032071	1.130	0.3707
Error	32	170.881084	5.340034		
Total	44	452.114577			
Mean	2.995756	Number of observations: 45			

Number of Albino Plants Transformation: Square root - SQRT (Y)

Albino plants were obtained from all genotypes, and F_1 wheat cross 020037 × 020062 showed the lowest rates (albino plants/spike). No significant differences in albino plants were observed between Bobwhite and Fielder, totaling 537 and 408 albino plants respectively (tables 1, 2 and 3). The great majority of plants from all genotypes did not present morphological alterations, resulting in genetically stable and normal plants. Some morphological abnormalities were observed in very few plants. Plants from Bobwhite, Fielder and F_1 wheat cross 020037 × 020062 presented the "grass-like" type morphology, exhibiting a longer vegetative stage. Spikes from these plants were, in most cases, sterile. "Grass-like" plants are usually associated with aneuploidy, presenting a mixture of different chromosome numbers in the same root tip cells (Hu and Kasha, 1997; Immonen and Robinson, 2000). Bobwhite plants also presented some spikes with morphological alterations (doubled spikelets), and no seeds were produced from these spikes. Morphological alterations are usually originated from somaclonal variation during the callus phase (Larkin and Scowcroft, 1981; Ahloowalia and Sherington, 1985; Dahleen et al., 2001). In our study, green plants were originated from direct embryogenesis without passing through the callus phase. This finding explains why very few plants presented morphological alterations. No colchicine treatment was applied to the resulting plants; thus, the ploidy level was estimated by checking the number of seeds per plant. The frequency of spontaneous diploids was significantly higher in Bobwhite. Our results showed a varied percentage of spontaneous duplication between Bobwhite and Fielder (78% and 14%, respectively). For wheat F_1 cross 020037 × 020062, 55 out of 85 (64%) plants were spontaneous diploids. The frequency of spontaneous chromosome duplication was shown to be genotype dependent. However, further studies with a wide-ranging collection of genotypes should be conducted to confirm this preliminary study. BRS 194 and Toropi did not produce enough plants for this type of evaluation. The wheat cultivar Toropi is of great pre-breeding importance because of its known disease-resistance traits, but its massive production of albino plants will hamper its use in microspore culture.

Discussion

Although wheat IMC has been previously described, adjustments are always necessary to establish a reliable and efficient protocol, especially when dealing with different genotypes with unknown androgenic responses. As expected, the results of this study showed significant differences among the genotypes regarding embryo formation, green-albino plant frequency and spontaneous duplication. Important factors affecting the establishment of IMC were evaluated. The growing conditions of microspore mother plants are an important factor in the production of DH plants, regardless of the chosen methodology. Thus, the strong effect of donor plants on microspore induction and embryo formation has been reported by several authors (Jähne and Lörz, 1995; Zheng, 2003; Broughton, 2008; Cistué et al., 2009). According to these studies, the androgenic capacity of the microspores can be severely affected by the physiological status of the donor plants, even when using responsive genotypes. Microspore embryogenesis and regeneration can vary within the same genotype simply from changes in the environmental conditions of the mother plants (Jähne and Lörz, 1995; Zheng, 2003). Raising donor plants under controlled environment conditions (growth chambers) would eliminate most types of biotic and abiotic stresses that could negatively affect the plant´s androgenic response (Devaux and Pickering, 2005). Semi-controlled conditions could also be an option; however, photoperiod and light intensity varies greatly by season and latitude. Studies conducted by Jacquard et al. (2006) demonstrated that the number of barley microspore-derived plants was significantly higher when the anthers were collected from January to July than from August to December, varying from 55.3 to 72.8 for the barley cultivar Igri and between 45.7 to 58.6 for the cultivar Cork, confirming the seasonal influence on the response. Moreover, whenever establishing a new protocol, as in the case of the present report, enabling a stress-free environment promotes more reliable results. In addition, negative results obtained from donor plants kept in greenhouses could be erroneously associated with recalcitrant genotypes.

Adjustments will be always necessary when considering genetic differences present in the germplasm and their climatic variations (Broughton, 2008). Spike pretreatments are necessary to switch the genetic program of the microspores from gametophytic to sporophytic developmental pathways, a phenomenon also known as *in vitro* androgenesis (Barnabás et al., 2001). Different pretreatments have been described as triggers to promote androgenesis, such as temperature (high or low), osmotic shock and carbohydrate/nitrogen starvation (Touraev et al., 1997; 2001; Zheng, 2003; Islam and Tuteja, 2012). However, cold shock is by far, the most common method used for many plant species. Mannitol and cold combined with mannitol have also been used as alternative pretreatments and provided better results with regard to embryo formation and green plant production (Hu and Kasha, 1999; Labbani et al., 2007; Shirdelmoghanloo et al., 2009). The type, duration and time of application for these pretreatments may vary with different species or even within the same species but with different genotypes. In this study, cold pretreatment shock was used to induce androgenesis promoting positive results in four out five tested genotypes (Bobwhite, Fielder, BRS 194 and F_1 020037 × 020062). However, searching for more effective pretreatments would promote better regeneration rates, improving the method´s efficiency. To obtain a large number of purified microspores, leading to the formation of embryos and green plants, microspore purification should be carried out in such a way as to avoid contamination with nonviable cells and debris from anther tissues. A suspension of purified microspores should be free of contaminants, as they may inhibit embryogenesis and consequently limit the number of embryos (Zheng, 2003). A very efficient purification procedure should also minimize possible damage to the microspore cells, mainly during blending steps (Liu et al., 2002b). Although no positive relationship was established between the number of purified cells mL^{-1} (cell concentration varied from 3.8 to 9.0 x 10^4 cells mL^{-1}) and embryo formation, the embryogenic response of some genotypes could be improved when using a higher number of purified cells during the initial purification and incubation steps. This improvement could have occurred for BRS 194, as it produced very few plants (green and albino) but an extensive number of embryos. This finding indicates that a high percentage of microspores went through androgenic development, and only a small proportion was regenerated into mature green plants. Similar results were found by Broughton (2008) for the wheat cultivar "Datatine," which produced 35.9 embryos per spike but only three green plants. The results presented here showed that the stress pretreatment was able to reprogram the gametophytic route of the microspores to the sporophytic one, allowing BRS 194 microspores to divide and develop into embryos. However, the regeneration efficiency was extremely low (0.75%). It was previously reported that the efficiency of the androgenesis process relies on the following three independent components: the formation of multicellular structures, regeneration ability and frequency of green plants, all of which are regulated by nuclear inheritance and separated by genetic control, although some portion of the regeneration efficiency is also determined by strong environmental influences (Krzewska et al., 2012). Considering the influence of environmental conditions, improved responses regarding embryo formation and the number of green plants could be achieved with the appropriate stress treatment and culture medium optimization (Liu et al., 2002b). Many papers have described the use of ovary co-cultivation for non-responsible genotypes to improve androgenesis efficiency (Zheng et al., 2002; Zheng, 2003; Letarte et al., 2006). Our results were obtained exclusively in the presence of ovaries, even when using responsive genotypes (Bobwhite and Fielder). Our preliminary attempts to avoid ovary co-cultivation did not promote embryogenesis. Other studies have shown that microspores cultured without ovary co-cultivation did not undergo cell division and did not produce embryos with any genotype combination (Patel et al., 2004). Benefits from ovary co-culture are related to a stimulatory effect on microspore regeneration, leading to the release of beneficial and nursing compounds (Zheng et al., 2002). The positive effects of ovary co-culture were also described as behaving like phytohormones (Hu and Kasha, 1997), promoting a stimulatory result on microspore androgenesis in wheat (Broughton, 2008). The decision to adopt any kind of haploidization method should always be considered in terms of its cost of establishment, efficiency and final purpose. The number of fertile green plants per spike is generally an important and decisive quality for selecting which method will be chosen. A method that offers a higher meiotic recombination frequency can also be used as a decisive feature, and it can be achieved by using anther or microspore culture with male gametes, instead of the maize cross pollination method with female gametes (Guzy-Wróbelska et al., 2007). This increased recombination frequency is especially important for genetic studies and for mapping population. Many studies have shown a reduced genotype-dependent effect on wheat DH plants obtained through maize pollination (Tuvesson et al., 2007; Jauhar et al., 2009). However, the extra work involving emasculation and pollination, the application of 2,4 D (or similar growth hormones), the isolation of embryos and colchicine treatment make this method more expensive in comparison to anther/microspore culture (Lantos et al., 2013). Embrapa Wheat has been using DH technology for breeding wheat and barley cultivars for a long time. However, our effort to establish an efficient protocol for wheat IMC was necessary because previous methods for producing DH plants in our laboratory (cross-pollination with maize pollen and anther culture) were more laborious, time-consuming and less effective. The green plant regeneration rate achieved in this report, using microspore culture, however, must increase before implementing it in the wheat breeding program. A very recent study comparing DH application versus conventional breeding strategies at CIMMYT (International Maize and Wheat Improvement Center) has shown no significant advantages for adopting DH technologies (Li et al., 2013). However, maize cross-pollination was the evaluated DH methodology (less cost-effective), and the study was conducted using two growing seasons per year. As recently reported, a tissue culture protocol applicable to a large spectrum of wheat genotypes remains elusive, mainly because of the influence of genetic causes and their response to tissue culture (Santra et al., 2012). The development of less genotype-dependent protocols remains important. To meet the different needs of a breeding program, improvements in the protocol described here will be necessary, especially considering that parental lines are chosen based on agronomic traits rather than compatibility with a specific DH methodology (Snape et al., 1986; Henry et al., 1994). More research is currently in progress on different aspects of this protocol, such as: modification of pretreatment stages to promote androgenesis, regardless of the genotype, and optimization of microspore culture efficiency by minimizing albinism. The results reported here will be used as groundwork for upcoming studies with IMC and its

application for DH plant production for the wheat breeding program. Further experiments will include a comprehensive study of the genetic basis of the response to microspore culture in a wide collection of Brazilian wheat germplasms.

Material and Methods

Donor plants and growth conditions

Two wheat (*Triticum aestivum* L.) genotypes known to be highly responsive to tissue culture and androgenesis were used as "positive controls," namely, Bobwhite (SH 98 97) and Fielder. Three wheat Brazilian genotypes were evaluated: Toropi, an old Brazilian variety that is widely used for mapping population and genetic studies because of its known source of Adult Plant Resistance (APR) to rusts; BRS 194, a fairly modern cultivar with valuable agronomic traits; and one F_1 wheat cross named 020037 × 020062, which was obtained from parental lines with unknown androgenic response. All seeds were chemically treated with Gaucho at 0.004g L^{-1} (Gaucho FS, Imidacloprid, Bayer Crop Science, D-41538, Dormagen, Germany) and Baytan at 2.5 ml L^{-1} (Baytan SC, Triazole, Bayer Crop Science, D-41538, Dormagen, Germany) and sown at monthly intervals for each experiment. Two treated seeds were sown per pot (two plants per pot; each pot was 20 cm in diameter) containing a mixture of soil:vermiculite:substrate (1:1:1). Plants were grown in a controlled-environment room (Conviron PGW 36) at 18/14°C (day/night) with a 16 h photoperiod and fertilized weekly with Hoagland's nutrient solution. The relative humidity was maintained at 70-80%. Spikes were harvested when microspores reached the mid-uninucleate stage (as observed under a microscope from a median floret using acetocarmine staining). A microscopic determination of the correct stage of uninucleated microspores was applied only for the first two spikes of each genotype. Spikes were further chosen based on the length between the flag leaf and the second leaf and by the tiller thickness (Jähne and Lörz, 1995). All the data were collected from three extraction cycles (each cycle was separated by monthly intervals) and obtained from a group of ten pots (20 plants of each genotype per cycle). Two or three tillers per plant were harvested at a more advanced stage for ovary co-culture, as this method improves plant regeneration efficiency (Liu et al., 2002b; Lantos et al., 2006; Broughton, 2008).

Spike pretreatment for triggering microspore embryogenesis

Only cold treatment was applied to the spikes. Tillers were kept in the refrigerator (4°C) in a large beaker containing deionized water and wrapped in aluminum foil for 21 (±3 days) unless otherwise stated. Ovary donor tillers were kept under the same conditions.

Microspore isolation

Microspores were isolated and cultivated according to the protocol described by Eudes and Amundsen (2005) with some modifications. After pretreatment, the tillers were sprayed with 70% ethanol. After drying, the remaining leaves and awns were aseptically removed and the spikes were placed in an autoclaved beaker and sterilized in a 15% commercial bleach solution (2.5% sodium hypochlorite) for 10 min at constant agitation. The spikes were rinsed four times with autoclaved deionized water and left to dry in a sterile Petri dish inside the laminar flow hood for 5 min. Anthers or spikelets were aseptically collected from a group of 10-12 spikes for each isolation procedure and placed in an autoclaved mini-blender (Waring Micro Blender, Eberbach Corporation, USA) containing approximately 40 mL of cold liquid NPB 99 extraction medium (Liu et al., 2002b). The upper and lower florets from each spike were discarded. Anthers were blended twice for 7 s at low speed, and the suspension was filtered using a cell strainer with 100 µm sterile mesh (BD Falcon 352360, USA). The blender cup containing the debris was rinsed with 40 mL of cold NPB 99 extraction medium and poured through the 100 µm mesh. The filtered solution was placed into two 50 mL tubes, and microspore cells were pelleted by centrifugation (100 x g for 5 min at 4°C). The supernatant was discarded, and each pellet was resuspended in 35 mL of NPB 99 liquid medium. Microspores from both tubes were collected after a new cycle of centrifugation, and the resulting pellet was resuspended in 2.0 mL of NPB 99 liquid medium. Microspore suspension was carefully layered over a sterile maltose solution (20%), and purified cells were obtained after centrifugation. The final pellet was resuspended in 1.0 mL of the same culture medium, and the cell concentration was determined with a hemocytometer. After counting, the microspore cells were diluted in NPB 99 semi-solid medium (NPB 99 + 10% Ficoll from Sigma F-4375, + 10 mg L^{-1} gum arabic from Sigma, G-9752), and the concentration was adjusted to an average of 6–9 x 10^4 cells mL^{-1}. After the cells were distributed into 30 mm Petri dishes, four ovaries were aseptically removed from plants of the same genotype and added to each Petri dish. The small dishes containing the purified microspores were sealed with Parafilm, placed in a larger Petri dish (150 mm) containing sterile distilled water and incubated in the dark at 27°C for 20–40 days. Purified cells were observed using an inverted light microscope. Embryo-like structures were daily evaluated confirming its gametic source (not derived from sporophytic tissues from the anther wall).

Embryo induction and plant regeneration

Once embryos reached ≥ 1.0 mm, they were aseptically transferred to solid GEM medium (Eudes et al., 2003) in 100 mm Petri dishes (20 mL per Petri dish). The dishes were placed in a growing chamber at 20°C with a 16 h light period. Regenerated green plantlets were transferred to rooting medium (Eudes et al., 2003) in test tubes under the same light and temperature conditions. The average detection time for the first green plantlets varied between 10 and 21 days; however, some embryos became green before or after that time. When plantlets had reached a set of two to three leaves and adequate root growth was observed, they were individually transplanted to pots with vermiculite and placed in a growth cabinet with the same conditions as the donor plants (and fertilized weekly).Chromosome counting is a time-consuming activity that involves substantial effort, so ploidy level of the green plants was estimated after harvesting by checking the seed set. The frequency of spontaneous DH plants was recorded, and no colchicine treatment was used.

Statistical analysis

Each extraction consisted of one experimental unit with 12 spikes per extraction and three extractions were carried out per cycle, for three total cycles (three different planting dates, which were separated by monthly intervals), totaling nine replicates. Comparisons were carried out between genotypes. Counting data (number of green and albino plants per genotype) were square root transformed before analysis. Analysis of variance (ANOVA, Tables 2 and 3) and

comparisons among means (Table 1) were carried out by using SISVAR statistical software (Ferreira, 2011) and Scott-Knott test at $p < 0.05$ level of significance.

Conclusions

In the present study, we have established a protocol for producing doubled haploid plants based on Isolated Microspore Culture using Brazilian wheat genotypes and responsive controls. A strong genotype effect was observed. Green and fertile doubled haploid plants were produced from responsive (Fielder and Bobwhite) and from two Brazilian genotypes, out of three tested. Two steps were considered as key factors for an efficient establishment: growing conditions of microspore donor plants and ovary co-culture. In order to apply a DH methodology based on microspore culture in the Wheat Breeding Program, on a routine basis, the protocol used here should be improved, in order to enhance embryogenesis response from recalcitrant genotypes and minimizing albinism.

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