Doubled Haploid Production from Spanish and Central European Spelt by Anther Culture

A. M. Castillo*, S. Anuél, A. Costar, F. Alvaro, and M. P. Valles

ABSTRACT

In recent years, spelt (Triticum aestivum (L.) ssp. Spelta) has become an added-value alternative crop to modern wheat. Spanish spelt constitutes a unique separate gene pool from central European germplasm. The availability of spelt Doubled Haploid (DH) production protocol is a great advantage to speed up breeding programs. This is the first study evaluating the ability of DH plant production, by anther culture, of five Spanish spelt landraces and three F5 lines derived from Spanish spelt x bread wheat crosses. Two central European commercial varieties were also included in the analysis. DH plants were obtained from all material with the exception of one F5 line. The Spanish spelt landraces produced more embryos/100 anthers (73-166.3) than the two European varieties (8.6-22.2). The main bottleneck in the Spanish germplasm was the high number of albino plants regenerated, with percentage of green plants lower than 13% in three of the landraces. Nevertheless, up to 15.6 and 1.8 green plants/100 anthers were obtained from the Spanish and the central European germplasm, respectively. A great variation in the percentage of spontaneous chromosome doubling was obtained, with 4 lines producing around 80% and 2 lines less than 15%. The ovary genotype used for anther co-culture is a critical factor to increase the efficiency of the system. Bread wheat ‘Caramba’ ovaries increased almost 6 times the number of green plants as compared to spelt landrace ‘BG-1987’ ovaries. This study shows that DH plants can be produced efficiently from Spanish spelt to be used in breeding programs.

Keywords: Ancient species, Anther culture, Spanish germplasm, Spontaneous chromosome doubling, Triticum aestivum (L.) ssp. Spelta.

INTRODUCTION

Spelt (Triticum aestivum (L.) Thell. ssp. Spelta) is an ancient hulled hexaploid wheat currently grown in Europe on more than 100,000 ha (Longin and Würschum, 2014). In recent years, spelt has gained renewed interest in developed countries due to current consumer trends towards regional crops that provide nutrients for a healthy diet (Longin and Würschum, 2016). In this sense, spelt has higher protein, lipid, and mineral (Mg, Fe, Cu, Zn and P) content than bread wheat (Triticum aestivum (L) (Ruibal-Mendieta et al., 2005; Escarnot et al., 2012). Furthermore, spelt produces more in less favorable growing conditions, and has lower fertilizer, herbicide and pesticide requirements than bread wheat (Campbell, 1997). Hence, spelt is suitable for stress conditions and organic farming systems (Konvalina et al., 2014). It is, therefore, an added-value alternative crop to modern wheat for farmers, millers, and bakers.

The exploitation of landraces and ancient species is a key factor to drive genetic improvements in plant breeding. Spelt has become a valuable gene resource for modern

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wheat breeding as it hybridizes easily with common wheat. Genetic diversity studies in Spanish spelt landraces and central European and Middle Eastern accessions showed that Spanish spelt landraces constitute a compact and separate gene pool from central European material (Elía et al., 2004). Two new alleles detected in Spanish spelt for the Glu-B1 locus, four alleles for Glu-D1, and one waxy allele have not been described previously (Caballero et al., 2001; Guzmán et al., 2010), and could contribute to distinct bread- and noodle-making quality. This gene pool may also provide new sources of adaptation to environments in southern Europe that are poor in resources. Therefore, the Spanish spelt collection is a valuable and unique genetic resource that can be used in European bread wheat and spelt breeding programs.

The spelt or spelt x bread wheat breeding programs based on Doubled Haploid (DH) plants are of great interest to shorten the time needed to develop new varieties. Intergeneric crosses with maize and anther culture are the two methods with the highest potential for DH production in wheat. The number of bread wheat varieties selected from DH lines obtained by anther or interspecific crosses is increasing every year (Tadesse et al., 2012). In spelt, only one study was reported using intergeneric crosses, obtaining an average of 6.1 haploid plants/100 flowers from 6 breeding lines (Escarnot et al., 2014). Spelt anther culture response has been evaluated in two previous studies using central European germplasm and protocols developed for bread wheat. An average of 21 embryo-like structures and 0.9 green plantlets/100 anthers were produced from ten lines (Schmid, 1990) and, more recently, a high frequency of green plantlets/100 anthers (30.6) was obtained from the Hungarian genotype ‘GK Fehér’ (Lantos et al., 2017). Since Spanish spelt constitutes a separate gene pool from the central European germplasm, evaluation of anther culture response should be assayed before setting up DH breeding programs with these materials.

One of the critical steps to achieve highly efficient wheat DH plant production by anther culture is to use ovary pre-conditioned medium and/or ovary co-culture, as well as the ovary genotype used for the co-culture (Castillo et al., 2015). Spelt contains higher levels of Cu and Zn than bread wheat (Ruibal-Mendieta et al., 2005), and both micronutrients are known to induce anther culture response in barley (Wojnarowiez et al., 2002; Echavarri et al., 2008). Therefore, spelt ovaries could be good candidates for co-culture.

The aim of this study was to evaluate the ability to produce doubled haploids by anther culture from Spanish spelt germplasm, using the standard bread wheat protocol developed in our laboratory. Furthermore, the potential of spelt ovaries to induce anther culture response was assayed.

**MATERIALS AND METHODS**

**Material and Growing Conditions of the Donor Plants**

In this study, we used five Spanish spelt landraces from the CRF-INIA (National Plant Genetic Resources Center): ‘BG-1952’ (L1), ‘BG-1953’ (L2), ‘BG-1954’ (L3), ‘BG-1967’ (L4) and ‘BG-1987’ (L5), meeting the spelt quality characteristics (high protein content and high extensibility), with shorter growth cycles than spelt cultivars from other European countries (Elia, 2007); three F5 lines from spelt x bread wheat crosses: BG-1969 x ‘Ingenio’ (F5-1), BG-1969 x ‘Innov’ (F5-2), BG-13840 x Innov (F5-3); two modern, high-yielding commercial spelt varieties developed by the University of Hohenheim, ‘Zollernspelz’ (L6) and ‘Divimar’ (L7); and the bread wheat commercial variety ‘Caramba’. Donor plants were grown as described by Soriano et al. (2007).
Preparation of Ovary Pre-Conditioned Medium and Ovary Co-Culture (OVPCM)

Ovaries were excised from flowers that contained microspores at a late binucleate stage of development. Ovaries were cultured in 2 mL of MS3M [MS medium modified by Hu and Kasha (1997), containing 62 g L⁻¹ maltose, 1 mg L⁻¹ 2,4-Dichlorophenoxyacetic acid (2,4-D), 1 mg L⁻¹ BenzylAdenine (BA)], supplemented with 200 g L⁻¹ Ficoll Type 400 (Sigma) (MS3MF200) (Soriano et al., 2007). Cultures were kept in the dark at 25ºC for 5 days before anther culture. The ovaries (6 ovaries per 2 ml) were kept in MS-MF200 during anther culture (OVPCM).

Anther Culture

Anthers containing the majority of microspores at the mid- to late-uninucleate stage were pre-treated with 127.5 g L⁻¹ mannitol (Sigma), 5.9 g L⁻¹ CaCl₂ plus FHG macronutrients (Hunter, 1987) solidified with 0.8 g L⁻¹ Sea Plaque Agarose (Lonza) (Soriano et al., 2007). After 5 days, swollen anthers were selected and treated with 0.2% n-butanol for 5 hours in MS3M liquid medium as described by Soriano et al. (2008). After n-butanol treatment, anthers were cultured in 2 mL of OVPCM containing 7 ovaries in 3 cm Ø Petri dishes. After 10 to 12 days, plates were replenished with 2 mL of the MS3MF400 (MS3M supplemented with 400 g L⁻¹ Ficoll Type 400). Cultures were kept in the dark at 25ºC.

Experiments

Experiment 1: DH Production from Spanish and Central European Spelt by Anther Culture

Anthers from five Spanish spelt landraces, three F₅ lines from spelt x bread wheat crosses, and two central European commercial varieties were pre-treated and cultured as described above. Ovaries from bread wheat cultivar Caramba were used for OVPCM.

Experiment 2: Potential of Spelt Ovaries to Induce Spelt Anther Culture Response

Anther culture response from spelt landraces “BG-1953” and “BG-1967” was evaluated in OVPCM medium, using ovaries from the spelt landrace BG-1987 or the bread wheat cultivar Caramba (treatments).

Plant Regeneration, Soil Transfer, Ploidy Analysis and Colchicine Treatment

Embryos developed after 30 days were transferred to 6 cm Ø Petri dishes containing J25-8 medium (Jensen, 1977) for regeneration. Embryos were kept in the dark at 25ºC for 2 days and then transferred to the light. After 20 days, plants were transferred to Magenta boxes containing the same medium plus 2 mg L⁻¹ NAA for root development. Soil transfer and ploidy analysis were performed as described by Soriano et al. (2007). DH plants were transferred to greenhouse for seed production. For colchicine treatment, haploids plants with 2-3 tillers were removed from the soil and roots were washed and cut back to about 2 cm below the crown. Plants were then immersed to a depth of 5 cm in a 0.1% aqueous colchicine (Sigma D-4540) solution containing 2% DMSO for 5 hours at 24ºC (modified from Devaux, 2003). Plants were rinsed in running tap water for 15 minutes, potted in soil and transferred to the greenhouse at 24ºC for seed production.

Statistical Analysis

Twelve to 23 replicates of 35 anthers per genotype were used in each experiment.
Table 1. ANOVA of Experiments 1 and 2 for the variables numbers of embryos (NEmb), green plants (NGP), albino plants (NAP) and percentages of regeneration (Reg) and green plants (GP). The variables NEMB and NAP were transformed with square root (X+0.5) and NGP with log (X+1).

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>Mean squares</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NEmb*</td>
</tr>
<tr>
<td><strong>Exp 1</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>9</td>
<td>159.37***</td>
</tr>
<tr>
<td>Error</td>
<td>121</td>
<td>11.62</td>
</tr>
<tr>
<td><strong>Exp 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anther genotype</td>
<td>1</td>
<td>106.80*</td>
</tr>
<tr>
<td>(AG)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ovary genotype</td>
<td>1</td>
<td>72.64*</td>
</tr>
<tr>
<td>(OG)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AG X AO</td>
<td>1</td>
<td>17.05 ns</td>
</tr>
<tr>
<td>Error</td>
<td>48</td>
<td>15.65</td>
</tr>
</tbody>
</table>

* values based on 100 anthers; ns, *, **, *** Not significant, significant at 0.05, 0.01 and 0.001 levels of probability, respectively
Figure 1. Number of embryos and green plants obtained per 100 anthers from 5 Spanish spelt wheat landraces BG-1952, BG-1953, BG-1954, BG-1967 and BG-1987 (L1-L5, respectively), the central European commercial spelt cultivars Zollernspelz and Divimar (L6 and L7, respectively) and 3 F₅ lines from spelt wheat x bread wheat crosses (BG-1969×Ingenio, BG-1969×Innov, and BG-13840×Innov, F₅-1 to F₅-3, respectively).

A great anther and ovary genotypic effect was shown by ANOVA (Table 1). Anther genotype strongly affected all the variables studied with the exception of number of green plants and percentage of spontaneous doubling (Tables 1 and 3). Statistically significant differences were also observed from BG-1967 and F₅-3 with less than 15%. Haploid plants from BG-1952, BG-1954, BG-1967, and BG-13840 x Innov were treated with colchicine (Table 2). Rates of duplication higher than 75% were obtained in 3 out of the 4 lines and 40% mortality in BG-1967. An average of 55-126 seed/plant was harvested from colchicine treated plants. DH plants from all lines and colchicine-treated haploids plants were transferred to the greenhouse for seed production (Figure 4-a). F₅₋₂ and F₅₋₃ DH plants were given to breeders for agronomical characterization in the field (Figure 4-b).

Experiment 2: Potential of Spelt ovaries to Induce Spelt Anther Culture Response

A great anther and ovary genotypic effect was shown by ANOVA (Table 1). Anther genotype strongly affected all the variables studied with the exception of number of green plants and percentage of spontaneous doubling (Tables 1 and 3). Statistically significant differences were also observed...
between ovary genotypes for all the variables, excluding the number of albino plants. Caramba ovaries produced almost a 6-fold increase in the number of green plants and percentage of green plants, as compared to BG-1987 (Table 3). Interaction of anther x ovary genotype was observed for percentage of green plants (Table 1). A 14- and a 3-fold increase in the value of this variable was obtained in BG-1953 and BG-1967 anther culture, respectively, with Caramba ovaries (data not shown).

DISCUSSION

In this study, the ability of DH plant production from five Spanish spelt landraces and three F₅ lines from Spanish spelt x bread wheat crosses was evaluated for the first time using the protocol developed in our laboratory for bread wheat. The Spanish landraces produced a higher average number of embryos/100 anthers (104) than the two central European commercial varieties included in this study (15). A low number of embryos was also reported with central

Figure 3. Microspore embryogenesis and plant regeneration from BG-1952 (L1) (a, b), BG-1954 (L3) (c, d), the European spelt cultivar Divimar (L7) (e, f), and F₅ line from BG-1969xInnov (F5-2) (g, h). (a, c, e, g= 10X magnification; b, d, f, h= 6 cm Ø Petri dishes).
Figure 4. Spelt doubled haploid plants growing in the greenhouse for seed production (a) and agronomic evaluation of DH plants from BG-1969×Innov (F$_5$-2) and BG-13840×Innov (F$_5$-3) (b) (arrows, dark green plants).

Table 2. Percentages of plant duplication and mortality, and number of seeds after colchicine treatment in different lines of spelt wheat.

<table>
<thead>
<tr>
<th>Line</th>
<th>Colchicine Treatment</th>
<th></th>
<th>N Seeds/Duplicated plant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N Plants</td>
<td>Duplication (%)</td>
<td>Mortality (%)</td>
</tr>
<tr>
<td>BG-1952</td>
<td>3</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>BG-1954</td>
<td>16</td>
<td>75</td>
<td>12</td>
</tr>
<tr>
<td>BG-1967</td>
<td>25</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>BG-13840 x Innov</td>
<td>17</td>
<td>94</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3. Effect of the anther and ovary genotype on spelt wheat anther response.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>NEmb**/ 100 Ant</th>
<th>NGP/ 100 Ant</th>
<th>NAP/ 100 Ant</th>
<th>Reg (%)</th>
<th>GP (%)</th>
<th>Spontaneous DH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anthers</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BG-1953</td>
<td>130.1 b</td>
<td>3.5 a</td>
<td>22.6 b</td>
<td>18.9 b</td>
<td>9.0 a</td>
<td>40.0 a</td>
</tr>
<tr>
<td>BG-1987</td>
<td>203.5 a</td>
<td>2.2 a</td>
<td>48.8 a</td>
<td>27.9 a</td>
<td>3.9 a</td>
<td>25.0 a</td>
</tr>
<tr>
<td>Ovary</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caramba</td>
<td>195.2 a</td>
<td>5.1 a</td>
<td>34.6 a</td>
<td>19.5 b</td>
<td>12.1 a</td>
<td>37.1 a</td>
</tr>
<tr>
<td>BG-1987</td>
<td>140.0 b</td>
<td>0.9 b</td>
<td>36.3 a</td>
<td>26.8 a</td>
<td>1.6 b</td>
<td>25.0 a</td>
</tr>
</tbody>
</table>

** NEmb = number of embryos; NGP = number of green plants; NAP = number of albino plants; Reg % = percentage of plant regeneration; GP % = percentage of green plants. * Values followed by the same letter within anther genotype and ovary genotype are not significantly different (P<0.05)
European material used in previous studies (Schmidt, 1990; Lantos et al., 2017).

A low embryo-plantlet conversion rate has been described to be a major bottleneck in the anther culture of central European spelt (Schmidt, 1990), which is normally associated with small and/or non-differentiated embryos (Kim et al., 2013). Our results with the two European varieties are in accordance with previous studies, producing the lowest percentage of plantlet regeneration (16-20%). However, three Spanish lines showed more than 40% plant regeneration.

The regeneration of chlorophyll-deficient (albino) plants did not seem to be a major problem in previous studies of spelt anther culture, since 80-90% of regenerated plants were green (Schmidt 1990; Lantos et al., 2017). However, albinism seems to be the main bottleneck in Spanish spelt, with 3 of the landraces showing less than 13% of green plantlets. These results could indicate that Spanish spelt germplasm could have alleles that favor the production of albino plants in anther culture. In wheat anther culture, albinism is a major hurdle as compared to intergeneric crosses, where almost no albino plants are produced (Wedzony et al., 2009). Genetic and physiological factors are known to influence the frequency of albino plants (Dwivedi et al., 2015).

In this study, green plantlets were obtained from all the lines, except the F3-1, despite the low percentages of plantlet regeneration and green plants from most of the material. The number of green plantlets/100 anthers obtained from the 5 Spanish landraces (3-16) is quite similar to those described previously with different bread wheat genotypes (0.04-28.7 green plant/100 anthers) by Lantos et al. (2013). In the central European varieties, the small number and low quality of embryos could condition the lowest number of green plants (1.5-1.8). These results are in accordance with those reported in 10 central European spelt lines (Schmidt, 1990).

Our results suggested that, in general, the Spanish spelt germplasm could respond better than the central European germplasm despite its high percentage of albinism. Differences in anther culture response according to the origin of the material (Eastern vs. North Western Europe) have been described previously in bread wheat (Holme et al., 1999). Further studies with a larger number of Spanish landraces would be desirable to confirm these results, since certain genotypes such as GK Fehér produced a higher number of green plants/100 anthers (30.6) than the best Spanish landrace (Lantos et al., 2017).

Identification of materials with a high androgenetic response is important when DH technology is being used in crop improvement. In this sense, landraces BG-1954 and BG-1967, that rendered the highest number of green plants, showed similar values for almost all variables. In addition, a short genetic distance between both landraces was reported in a genetic analysis performed on a collection of 100 Spanish spelt landraces (Elia, 2007). Therefore, it is possible to suggest that these lines had similar alleles for these traits. Also, Innov bread wheat cultivar could have alleles that favor the percentage of green plants since F3-2 (BG-1969 x Innov) and F2-1 (BG1969 x Ingenio) produced the highest and lowest percentage of green plants (62% and 0%, respectively).

The obtaining of a high percentage of spontaneous chromosome doubling in anther or microspore culture is a clear advantage over interspecific crosses or gynogenesis, where 10-15% rates have been reported (Maluszynski et al., 2003; Bohanec, 2009). In bread wheat anther culture, rates of 25-70% have been described (Maluszynski et al., 2003). In this study, a great variation in the percentage of spontaneous doubling (from 6-84%) was obtained depending on the material. The central European varieties showed percentages (around 80%) similar to that reported from GK Fehér (Lantos et al., 2017). However, this percentage was significantly higher than 30% reported in ten
central European spelt lines (Schmid, 1990) and 40% of the five Spanish landraces. Other factors besides the genotype have been reported to influence the percentage of spontaneous doubling such as the stage of microspore development and the type of stress treatment (Castillo et al., 2009).

Spelt lines also showed different responses to colchicine treatment. Plants from 3 out of the 4 lines treated with colchicine showed high rates of survival and seed set (over 75%). These results agreed with those described in bread wheat (Castillo et al., 2009). The genotype, species, and the health and vigor of the plant material has been reported to influence the rates of induced duplication in cereals (for review see Castillo et al., 2009).

In bread wheat anther culture, ovary co-culture has been proved to increase the efficiency of DH production. Furthermore, the ovary genotype used is a critical factor to increase embryogenesis and spontaneous doubling (Castillo et al., 2015). Spelt ovaries were expected to enhance DH production due to their high Zn and Cu content (Ruibal-Mendieta et al., 2005), which are known to increase the efficiency of barley anther culture (Wojnarowicz et al., 2002; Echavarri et al., 2008). However, bread wheat Caramba ovaries produced almost a 6-fold increase in the number of green plants/100 anthers as compared to spelt BG-1987 ovaries. These results agree with those reported by Castillo et al. (2015), showing that Caramba and Tigre ovaries were the most efficient in comparison with other bread wheat cultivars. More spelt genotypes should be screened in order to confirm the low promoting effect of spelt ovaries on anther culture response.

This study demonstrated that DH plants from Spanish and central European spelt germplasm can be efficiently produced with a bread wheat anther culture protocol developed in our laboratory, using bread wheat ovaries for co-culture. Spanish germplasm produced higher numbers of embryos and green plants and higher percentages of plant regeneration than the two central European commercial varieties evaluated. The number of green varieties obtained ensures the viability of Spanish spelt or spelt x bread wheat DH-based breeding programs.

ACKNOWLEDGMENTS

This work was supported by Projects AGL2013-46698-R and AGL2016-77211-R from the National Plan for Agrofood Resources and Technology of Spain (Plan Nacional de Recursos y Tecnologías Agroalimentarias) of Spain and the Regional Government of Aragon (Diputación General de Aragón) (Grupo A06).

REFERENCES


تولید هاپلوئید مضاعف از گندم پوشان (spelt) اسپانیایی و اروپای مکزی با کشت

باک

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چکیده

در سال های اخیر، گندم پوشان (spelt) (Triticum aestivum L.) ssp. Spelta) با نا م علمی برای گندم مدرن گرایه ای با ارزش افزوده شده است. گندم پوشان اسپانیایی دارای خصائص زنی منحصر به فرد و مجزا از زمین پلاسم های اروپای مرکزی است. برای سرعت دادن به برنامه های اصلاح نزار، دردسترس بودن دستورالعمل تولید هاپلوئید مضاعف گندم پوشان (DH) بسیار مفید است. این تحقیق، به تولید گیاه DH با روش کشت گیاه با 5 رقم بومی F5 مشتق از تلاقی گندم پوشان اسپانیایی X گندم نان را ارزیابی می کند. این تحقیق دو رقم تجاری اروپای مرکزی را نیز شامل بود. از همه مواد (گیاهی) مطالعه شده، به استثنای یک بسیار مفید است. این گیاهان DH ها از یک بعد سوم در رقم های بومی گندم پوشان اسپانیایی T. aestivum (L.) ssp. Spelta) در مقایسه با دو رقم اروپای مرکزی ( درصد 166.3-73 درصد) بسیار بود. این گیاهان اصلی در زمین پلاسم اسپانیایی زیاد بودند. درصد گیاهان Zal (البینو) با یک بعد سوم در رقم های بومی کمتر از 13% بود. با این وجود، از زمین پلاسم های اسپانیایی و اروپای مرکزی، به ترتیب درصد 15/8 و 100 سیب به استفاده درصد این بسیار زیاد بود. در روش همراه کشت (co-culture) با سیب، زنگولتی جنین مورد استفاده عمده حاصل از افزایش سامانه جنین. این گیاهان نان 'Caramba' تعداد گیاهان سیب را تا 6 برابر جنین رقم بومی گندم پوشان 'BG-1987' افزایش داد.
این پژوهش نشان می‌دهد که برای استفاده در برنامه‌های اصلاح نزاد، می‌توان گیاهان DH را از گندم بسیار به‌عنوان درمانی با کاراپی بالا تولید کرد.