Improvements in the production of doubled haploids in durum wheat (*Triticum turgidum* L.) through isolated microspore culture

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Abbreviations:

NAA: naphthalene acetic acid

BAP: 6-benzylaminopurine

PEG: polyethylene glycol

ELS: embryo like structure

Abstract

The objective of this study was to produce durum wheat doubled haploid plants through the induction of microspore embryogenesis. The microspore culture technique was improved to maximize production of green plants per spike using three commercial cultivars. Studies on factors such as induction media composition, induction media support and the stage and growth of donor plants were carried out in order to develop an efficient protocol to regenerate green and fertile doubled haploid plants. Microspores were plated on a C₁₇ induction culture medium with ovary co-culture and a supplement of glutathione plus glutamine; 300 g/l Ficoll Type-400 was incorporated to the induction medium support. Donor plants were fertilized with a combination of macro and microelements. With the cultivars 'Ciccio' and 'Claudio' an average of 36.5 and 148.5 fertile plants were produced, respectively, from 1,000 anthers inoculated. This technique was then used to produce fertile doubled haploid plants of potential agronomic interest from a collection of 10 F₁ crosses involving cultivars of high breeding value. From these crosses 849 green plants were obtained and seed was harvested from 702 plants indicating that 83% of green plants were fertile and therefore were spontaneously doubled haploids. No aneuploid plant was obtained. The 702 plants yielded enough seeds to be field tested. One of the doubled haploid lines obtained by microspore embryogenesis, named 'Lanuza', has been sent to the Spanish Plant Variety Office for Registration by the Batlle Seed Company. This protocol can be used instead of the labor-intensive inter-generic crossing with maize as an economically feasible method to obtain doubled haploids for most crosses involving the durum wheat cultivars grown in Spain.

Introduction

The production of doubled haploid (DH) plants has become a key tool in advanced plant breeding (Devaux and Pickering 2005). Plant breeders are increasingly using this system in their mainstream pure-line programs to reduce the number of years needed from crosses to commercial variety registration (for an extensive review of the techniques most commonly used in a large number of major crops see the landmark publication of Kasha and Maluszynski (2003).

In barley (*Hordeum vulgare* L.) and in hexaploid bread wheat (*Triticum aestivum* L.) efficiencies have increased to a level adequate for the application of these techniques in commercial breeding programs. However, important difficulties still challenge commercial DH production in durum wheat (*Triticum turgidum* ssp. *Durum* (Desf.), Husn). In this species green plants have been regenerated at a low frequency because most of the genotypes did not respond at all to anther culture, or because most plantlets regenerated were albino (Hadwidger and Heberle-Bors 1986; Foroughi-Wehr and Zeller 1990; Cattaneo et al. 1991; Orlov et al. 1993; Otani and Shimada 1994; Ghaemi et al. 1995; J'Aiti et al. 1999, 2000; Silva-Romano and Canhoto 1999; Dogramaci-Altuntepe et al. 2001; Jauhar 2003b). Furthermore, significant genotype by stress pre-treatments (Mentenwab and Sarrafi 1997), genotype by medium composition (Ghaemi et al. 1994) and genotype by cytoplasm interactions (Ghaemi et al. 1993) have been described. Additionally, when green plants were obtained (Dogramaci-Altuntepe et al. 2001) high numbers of aneuploids were regenerated from the androgenic calli. Cistué et al. (2006) developed a technique which produced green plants from a range of different genotypes and 68% were doubled haploid plants. Other works recently have been published although low success was reported (Labbani et al. 2005; Bakos et al. 2007).

Therefore, two important problems have to be addressed in the durum wheat microspore culture technique before it can be widely applied in commercial breeding programs. The first is the interaction between genotype and microspore culture technique; the second is the high proportion of albino plantlets regenerated. For these reasons, the inter-generic crossing with *Zea mays* L. has been the only method used until now to produce haploid plants (Jauhar 2003a).

Microspore culture of durum wheat was practically ineffective until ovaries were added to the induction medium. Co-culture with ovaries has been tested and found to be a useful method to increase green plant production in hexaploid wheat microspore culture (Puolimatka et al. 1996; Hu and Kasha 1997; Zheng et al. 2002). It has also been used for recalcitrant genotypes in barley (Devaux and Li 2001) and shown to be beneficial in durum wheat (J'Aiti et al. 1999; Cistué et al. 2006).

Brassica napus microspore embryogenesis is being used as a model for studying the molecular mechanisms controlling embryo initiation and early development. Large amounts of embryos can be regenerated from individual single-cell microspores without going through an intermediate callus phase (Supena et al. 2008). For this reason the results obtained with *Brassica*, either morphological or metabolic, can be used for other embryonic studies. Glutathione is a substance added to the induction medium for *Brassica* microspore culture (Coventry et al. 1988; Fletcher et al. 1998). Glutathione has been shown to play an important role during embryo development in both plant and animal systems. Inclusions of reduced glutathione (GSH) in the maturation medium increased the conversion frequency of white spruce somatic embryos without the need of partial drying treatment (Stasolla et al. 2004). However the issue seems to be more complex. Belmonte et al. (2006) showed that the alterations of the endogenous glutathione redox status [defined as the ratio of the concentration of the reduced form (GSH) to the concentration of the oxidized plus reduced forms (GSSG + GSH)] delineate specific stages of embryo development both in vivo and in vitro. The initial phases of embryonic growth generally occurs in a reduce environment, which seems to promote cell division and proliferation. During the second half of embryogenesis, the redox status of the glutathione pool decreases. This is important for a correct embryos development.

The importance of an adequate proportion of glutamine in the induction medium for the regeneration of green plants was clearly shown by Olsen (1987) in the case of barley anther culture. In this species, the initial divisions of microspores, further proliferation, embryo formation and plant recovery could be manipulated independently by altering the nitrogen composition of culture media (Mordhorst and Lörz 1993; Mordhorst et al. 1997).

Anthers can be cultured either in solid or in liquid medium. Both methods have their advantages. Solid medium is less expensive in terms of labor. Plates do not have to be

in a shaker. Liquid seems to be better in the sense that the growing microspore divisions have easier access to nutrients. However in liquid medium it is necessary to avoid submersing the microspores. Kao (1981) used Ficoll Type-400 200 g/l in barley anther culture medium in order to keep microspores from submersing and to avoid an anaerobic medium. Until now, for the production of DH from durum wheat, microspore culture seems to be more productive than anther culture. When microspore culture is used, liquid medium is necessary. When the microspores are floating in a medium with high viscosity, as produced by increasing the concentration of Ficoll Type-400 to 300 g/l, the embryos grow well formed, with scutellum, coleoptyle and coleorhyza (Cistué et al. 2004).

The importance of the physiological stage of the donor plants has been broadly discussed. For cereals some authors believe that field-collected spikes are preferable to spikes grown under artificial conditions. Dahleen (1999) studied the effect of donor plant environment on regeneration from barley embryo-derived callus. Green plant regeneration was higher and less variable from growth chamber-grown donor plants than from plants growing in a greenhouse. Sufficient regeneration from greenhouse-grown donor plants could be reached when natural light levels were high and temperatures were moderate. Jacquard et al. (2006) demonstrated that the number of microspore-derived plants was significantly higher when the anthers were collected from January to July rather than from August to December. Therefore the photoperiod and the light intensity has also influence in the final result. They also found that the physiology of the donor spike is crucial for the success of anther culture in barley. Additionally, the position of the spike on the plant can affect the quality of regenerated plantlets in terms of albinism. However growing conditions can be better controlled in growth chambers so that the number of treatments needed to avoid diseases is lower; and furthermore, different temperatures and controlled fertilizers can be used for the donor plants (Cistué and Kasha 2005).

Górecka et al. (2005) showed that each donor plant of carrot can respond in a different way to anther culture. Other authors had similar results for broccoli (Arnison et al. 1990). These results showed that even the position in the growth chamber is important and each plant can give a different response to anther culture.

The aim of this study was to improve the induction medium developed by Cistué et al. (2006) and to optimize the growth conditions of the donor plants. Two new substances were added to the induction medium. The importance of the support used for the growing of the microspores was studied. One type of fertilizer was selected for growing donor plants. Subsequently, the results obtained were applied to a number of genotypes of agronomic value.

Material and Methods

Seeds of donor plants were sown in a paper-pot with an equal mixture of peat, vermiculite and sand. Plants were vernalized for one month in a growth chamber at 4 °C, with 8/16 h light/dark photoperiod. Following vernalization, plants were transplanted to pots (15 cm diameter) with the same soil mixture and kept in a growth chamber for one month at 12 °C with a 12 h photoperiod. After the first month, the temperature was increased to 20°C and the photoperiod lengthened to 16 h light. Furthermore, all the donor plants used for anther inoculation were grown with similar amounts or quality of light, humidity and fertilization. Relative humidity was kept between 70 to 80%. An N: P: K (15:15:15) fertilizer was mixed into the soil before transplanting.

Spikes were collected when most of the microspores were at the mid to late-uninucleate stage. Collected spikes were removed from their sheaths in a flow bench and sprayed with 70% ethanol. The spike from the primary tiller for each plant was discarded. Three anthers from the flowers from each side of the central part of each spike were plated on a pre-treatment medium containing 0.7 M mannitol (Cistué et al. 1994) and 30 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ solidified with 8 g/L agarose. The number of spikes and anthers are specified for each experiment. Plates were kept for 5-6 days at 24 °C in the dark. Microspore isolation was performed according to the durum wheat protocol described by Cistué et al. (2006).

The number of spikes used for each microspore isolation was not the same. This was due to the fact that spikes that did not properly react during pre-treatment were rejected. The plates, from spikes that after 5-6 days of mannitol pre-treatment did not have the anthers plenty of water and releasing microspores to the medium, were discarded. Selection of spikes at the right stage is more critical for durum than for any other barley or wheat species. Contamination from dead microspores into the culture has already been reported in rapeseed by Kott et al. (1988). This problem is more serious in durum wheat because the stage of the microspores within anthers seems not to be very much synchronized.

Experiment 1

Viable microspores were inoculated on 1.5 ml of C₁₇ medium (Wang and Chen 1986) in 3 cm plates with 200 g/L of Ficoll Type-400. The plates also contained 10 ovaries from another spike, at a more advanced stage of development, but from the same genotype. The control medium was compared with a medium supplemented with 90 mg/L glutathione and 500 mg/L of glutamine. These amounts were based on data from preliminary experiments (data not shown). This medium was named C₁₇ modified medium (C₁₇m).

Three microspore isolations were carried out from durum wheat cultivars ‘Ciccio’ and ‘Claudio’. Viable microspores were separated by centrifugation with 20% maltose (Cistué et al. 1995). Microspores from each isolation were divided equally between two plates: A. with C₁₇ normal medium. B. with C₁₇ modified medium

Experiment 2

Most laboratories use liquid or solid media. Cistué et al (2004) demonstrated that, for barley anther culture, the higher the concentration of Ficoll Type-400 in the medium, the higher the number of well formed embryos obtained. Therefore we decided to assess Ficoll Type-400. The medium C₁₇ with 200 g/L of Ficoll-400 was used as a control and compared with medium C₁₇m with 300 g/L of Ficoll Type-400. In this experiment we used the cultivars ‘Arcolino’ and ‘Claudio’, because ‘Ciccio’ gave a high proportion of albino plants. Microspores were divided evenly between the two treatments. Three microspore isolations were carried out from each cultivar.

Experiment 3

The microspore isolation technique, either with barley or hexaploid wheat, needs growing healthy donor plants to obtain good results. This is even more critical for durum wheat which often has plants with few tillers, not very strong spikes, and many cultivars are very susceptible to different diseases. To avoid the problems mentioned, a

batch of donor plants was fertilized three times per week with 0.5 g (per 15 cm pot), dissolved in 200 ml distillate water, of the medium shown in Table 1. At the same time, and in the same growth chamber, another batch of plants was irrigated with 200 ml of water three times a week.

A similar number of spikes and anthers, but from the two different batches, were inoculated in plates of 3 cm with 1.5 ml of C₁₇m with 300 g/L of Ficoll Type-400. For this experiment we used again the cultivars ‘Ciccio’ and ‘Claudio’. Three microspore isolations were carried out.

Experiment 4

In order to test the improvements in the technique, the new protocol incorporating C₁₇m, 300 g/l Ficoll type-400 and the intensive fertilizer regime was applied over ten F₁ crosses provided by the Spanish Companies Seeds: Batlle S.A. (Lleida) and Agromonegros S.A (Leciñena). These Companies regularly intercross in their breeding programs the cultivars most widely used by farmers the previous years. Therefore none of the crosses was a priori selected for their response to microspore culture.

In this experiment green plants were transferred to J25-8 regeneration medium (Jensen 1983). After 15 days plants were moved to Magenta boxes with J25-8 medium supplemented with 1 mg/L of NAA to promote root growth. After one month of vernalization at 4° C, plants were potted in the greenhouse until seed set. After heading it was easy to separate the doubled haploids from the haploids because the latter were sterile, with many spikes that were shorter than the spikes from the DH plants. None of the plants obtained had strange morphology aspect or strange growing pattern. Therefore the not fertile plants were considered haploids. Flow cytometry was used by Cistué et al. (2006) and the results in terms of DH/haploids obtained with flow cytometry were in complete agreement with those obtained from morphology and seed set.

The durum wheat DH produced by microspore culture were studied in a double line of 30+30 seeds during 2006. The DH lines selected were assayed in Bell lloc (Lleida) and Leciñena (Zaragoza) in trial fields with 4 replicates during the 2007-08 season.

For all experiments the following characters were analyzed: number of embryo like structures (ELS) transferred to regeneration medium; number of albino plants regenerated and the number of green plants regenerated. The number of anthers extracted from each spike was around 50-60. The number of spikes used for microspore isolation was around 15 to 20. All statistical analyses were carried out using SAS/STAT standard procedures (SAS Inst. 2000).

Results

The results of the three experiments are summarized in Figure 1.

Experiment 1: Induction media (Fig. 1A)

For both genotypes, the number of divisions and therefore the number of ELS transferred to regeneration medium significantly increased in the modified medium supplemented with glutathione and glutamine. As expected ‘Claudio’ gave better result than ‘Ciccio’ a more recalcitrant genotype. The ELS obtained from the microspores developed in the modified medium resulted in a higher number of albino and green plants. The increase in the number of green plants was statistically significant. No significant *p* values were detected for any of the three variables recorded, for the interaction genotype by treatment. Although the modified medium produced a higher number of green plants, further improvements were needed.

Experiment 2: Support media (Fig. 1B)

In this experiment, the C_{17m} medium assessed in the previous experiment was used in all treatment combinations. The plants used for this experiment were in a better physiological condition than those used for experiment 1. The number of ELS and green plants increased significantly in both genotypes. In this case the result for fertile plants obtained from the cultivar ‘Arcolino’ increased from 0,8 to 6,0 and in the cultivar ‘Claudio’ the increase was from 25,8 to 55 fertile plants for 1,000 anthers inoculated. The number of albino plantlets was low and not affected by the Ficoll concentration.

Experiment 3: Fertilization of the donor plants (Fig. 1C)

The physiological status of the donor plants is critical in microspore embryogenesis. Under intensive and controlled fertilization, plants developed more, stronger and larger spikes. Only the healthiest spikes were selected for microspore selection, and the rest were discarded. This experiment was carried out using the selected media combination from Experiments 1 and 2. A significant increase in the number of green plants

regenerated was detected in the spikes coming from the batches of plants with the highest fertilization. The number of albino plantlets was significantly different between genotypes, but it was not significant between treatments. As in the previous two experiments, no significant genotype by treatment interaction was detected for any of the three variables recorded. With the genotype 'Claudio', we were able to obtain 1 green plant per every 6 or 7 anthers that is approximately one DH per each set of three flowers. The results for the more recalcitrant cultivar 'Ciccio', an average of 37 fertile plants for 1,000 anthers, were acceptable given the large proportion of albino plants regenerated for this genotype.

Experiment 4: Microspore culture of commercial durum wheat F_1 crosses (Table 2).

Commercial production of fertile DH durum wheat genotypes was possible for ten crosses provided by two Spanish breeding companies. As expected large differences were detected between cross. As mentioned before, only healthy spikes were used for anther extraction. Therefore the number of microspore isolations and the number of anthers varied between crosses. The number of ELS transferred to regeneration medium varied from 164 with the cross 'Carioca' x 'CB' to 1,088 for 'CAV' x 'Ciccio'. The proportion of green/albino plants produced between crosses was also very different. From the 13/109 (10.7%) produced with crossing 4, to 209/69 (75.2 %) produced with crossing 6 (Table 2). When 2,898 anthers were used for three microspore isolations from the cross 'CAV' x 'Carioca', 239 fertile DH genotypes were produced. An average of one fertile plant was obtained for every four flowers put into culture. These numbers are similar, or even better, than the production of DH using the highly labor-demanding technique of the inter-generic crossing with maize. When the numbers were expressed per thousand inoculated anthers, the average of the ten crosses was 31 fertile DH lines, ranging from 2 to 90. Poor results were strongly associated with low numbers of microspore divisions, or with the production of albino plants.

The different steps of the technique are shown in figure 2. Figure 2a shows microspores after 10 days on induction medium. The cytoplasm has followed its normal evolution, the vacuole has disappeared and the microspore is ready to begin division. Figure 2b illustrates a pro-embryo after 20 days in culture which means that the first cell divisions started within first week after microspore isolation. Figure 2c shows a microspore

culture on a 3 cm diameter plate. Ten ovaries from the same cultivar are included for co-culture. After 30 days of culture it is possible to find well developed embryos (Figure 2d), with very well formed scutellum, coleoptyle and coleorizha. The embryo on the left has two coleoptiles, which it is not strange when BAP is used as cytokinin. Figure 2e shows a well formed embryo of durum wheat with bilateral symmetry, which means that will be spontaneous doubling. These kinds of embryos are able to regenerate shoot and roots in 4-5 days on J25-8 medium (Figure 2f). As not all the embryos were well formed, we used the embryo-like-structure terminology. The well formed embryos such as the one shown in figure 2e always develop into a plant with shoot and roots.

Discussion

Somatic and microspore embryogenesis has been used during the last 30 years to produce synthetic seeds and doubled haploids respectively. There has been a great increase of research aimed at developing and optimizing protocols to obtain well developed embryos and, as a consequence, good regeneration rate of plants. In microspore embryogenesis it is very important to produce well developed and fast growing embryos which regenerate vigorous plants. Normally the production of well developed embryos increases the proportion of green/albino plants (Cistué et al. 1995). Weak and not fully developed embryos are not transplanted to regeneration medium.

An important problem for durum wheat is the rate of embryos regeneration. Nowadays, a lot of basic research on microspore embryogenesis is being done with *Brassica napus* L. (Yeung 2002; Belmonte et al. 2006; Supena et al. 2008) but also with *Arabidopsis-Brassica* (Boutilier et al. 2002) with *Picea glauca* (Stasolla and Yeung 2003) and with soft wheat and barley (Hosp et al. 2007). They are studying the metabolic and morphological reasons for the development of well formed embryos. Recently, there have been several studies on the effect and importance of reduced glutathione on morphology and gene expression of white spruce (*Picea glauca*) somatic embryos and its relationship with the abscisic acid (Stasolla et al. 2004). The positive effect we observed in the present study, when glutathione was added to the medium, could be related as in *Brassica* in a first moment in higher number of microspores dividing and later on in the embryo formation and a perfect and rapid conversion with shoot and roots. However, more studies about the proportion and the stage of the glutathione should be accomplished.

An issue with barley, soft wheat, and durum wheat in particular, in terms of microspore embryogenesis, is the large proportion of albino plants. Olsen (1987) demonstrated that in barley glutamine can play a key role to increase the proportion of green plants. We have demonstrated that the C₁₇ medium is a good medium for microspore culture of durum wheat (Cistué et al. 2006). In this study, supplementing the C₁₇ medium with glutamine significantly increased the number of green plants. Mordhost et al. (1993, 1997) discussed the importance of the correct proportion of the ammonium in the

medium. Our results from the present experiments seem to agree with these previous studies.

Kao (1981) used Ficoll-400 in the barley anther culture medium in order to keep microspores from submersion and to avoid the anaerobic medium. When the microspores are floating in a medium with high viscosity as produced by increasing the concentration of Ficoll to 300 g/l, the embryos grow well formed, with scutellum, coleoptyle and coleorhyza. However, most laboratories avoid Ficoll-400 because of its expense and instead use liquid medium, liquid medium with the microspores over a raft, agarose or gelrite (Patel et al. 2004). The results in our experiment with durum wheat microspores, and previous results with barley anther culture (Cistué et al. 2004) showed that the embryos develop much better in a medium with high osmotic pressure or high viscosity, which was translated into an increased number of dividing microspores. Immonen and Robinson (2000) observed similar results increasing the number of green plants regenerated with triticale.

The effect of osmotic potential on anther culture in spring soft wheat was already studied by Kang et al. (2003). Altering the medium osmotic potential by changing the carbohydrate source and concentration or by adding a non-metabolized osmoticum appeared to have the greatest potential for improving anther-derived green plant production. A similar effect is achieved with the use of polyethylene glycol (PEG) in rapeseed. Ilic-Grubor et al. (1998 a, b) cultivated *Brassica napus* with only a very limited amount of sucrose but with high concentrations of mannitol or PEG 4,000. While microspores cultured on high mannitol yielded no embryos and no embryogenic cell divisions were observed, microspores on high PEG concentration developed into embryos within 2 weeks. Somatic embryos of white spruce were also cultured in the presence of PEG, a non-plasmolyzing agent which increases embryo quality and number (Belmonte et al. 2005). They concluded that applications of non-plasmolyzing agents in the culture medium of spruce somatic embryos result in seed-like fluctuations of the ascorbate-glutathione metabolism, which may have positive effects on embryo yield.

Reviews of microspore doubled haploid techniques for different species (Maluszinsky et al. 2003), emphasize that healthy plants are one of the most important prerequisites

for success. Besides, there are some studies that show that, for example, the response of anther culture to culture temperature varies with growth conditions of anther-donor plants of hexaploid wheat (Ouyang et al. 1987) and that the environment of the donor plant particularly changes the rate of callus regeneration in barley (Dahleen 1999). It was also known that seasonal effects had influence over the number of haploid embryos produced through wheat x maize crosses, but Campbell et al. (1998) demonstrated that the temperature and light intensity at which pollinations were made and subsequent fertilization and embryo development occurred significantly influenced the frequency of haploid embryo production.

However the amount of fertilizer must be carefully controlled. An excess of nitrogen could be a problem; fertilizer may already have been optimized and the added nitrogen fertilizer may have become toxic in the high-N treatment (Knox et al. 2000). The fertilizer and the amount that we have used in this study seem to be adequate for durum wheat. We have had two positive results. First, the number of spikes obtained for F1 seed has been doubled, which is important provided that 50% of the spikes are discarded before inoculating the anthers or because the anthers do not react to the pre-treatment. Secondly, as shown with the cultivar 'Claudio', we obtained 148.5 fertile DH from 1,000 anthers inoculated. This is three times greater than with the usual fertilization regime used for donor plants.

Currently, the standard technique to produce haploids of durum wheat is inter-generic crossing with maize (O'Donoghue and Bennett 1994 a, b). In addition to the demanding labor requirements, this system has the additional problem that only haploid plants are recovered. Therefore it is necessary to use colchicine to restore chromosome number and fertility (Thiebaut et al. 1979; Jensen 1983). The use of colchicine is a very labor demanding step. Furthermore, normally not all the spikes are effectively doubled. This means that fewer seeds are harvested from the artificially doubled haploid plants. Although the technique has been significantly improved, the success rate is still low with one doubled haploid per emasculated spike as an average result (Knox et al. 2000), although it can reach three haploids per spike under high humidity (Ballesteros et al. 2003).

According to the results obtained in this work, the rate of spontaneous duplication increased was greater than that obtained by Cistué et al (2006). Kasha et al. (2001) demonstrated that nuclear fusion leads to chromosome doubling during mannitol pre-treatment of barley (*Hordeum vulgare* L.) microspores. With the present protocol the number of divisions increased and microspores were in a better physiological stage. Some of them developed a symmetric first mitotic division. After that, complete embryos with bilateral symmetry (doubled haploids) developed and from these we regenerated plants with shoot and roots. The direct production of doubled haploid plants is a clear advantage over the inter-generic crossing with maize.

The possibility of using microspore culture and male gametes instead of female gametes as in the maize system can also have advantages for breeding purposes. When the meiotic recombination frequencies between wheat doubled haploid populations obtained through maize pollination (MP♀) and anther culture (AC♂) were compared, some differences could be found in the recombination frequencies (Guzy-Wróbelska et al. 2007). The linkage groups in both DH populations showed identical orders of markers, except for one group mapping to chromosome 6B. The MP (♀) and AC (♂) linkage maps differed significantly in the recombination frequencies for corresponding intervals. In total, the AC linkage map (495.5 cM) was 40.5% longer than the MP map (352.8 cM), indicating a significantly higher meiotic recombination rate in pollen mother cells. Besides many applications in gamete and embryo biology, microspores also have applications in mutation induction, in vitro selection and genetic transformation (Forster et al. 2007). The large number of green doubled haploids produced in this study could be utilized in other areas of research.

The co-culture with ovaries is effective. However it is a labor demanding step. A recent report of progress in microspore culture to improve the number of DH obtained involves the addition of arabinogalactan instead of ovaries (Letarte et al. 2006). Approximately 25% of the anthers are discarded after the mannitol period because they do not react to the pre-treatment. Soriano et al. (2008) demonstrated that the addition of n-butanol for a short period during the mannitol pre-treatment increased the number of responding anthers and microspore divisions.

Growing of healthy donor plants, selection of spikes and anthers, co-culture with ovaries, and reduction of rate of albinism are some of the aspects that should be improved. However, the protocol developed in this study can be used to produce large number of doubled haploids from commercial durum wheat genotypes.

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Table 1. Fertilizer liquid composition. Every 15 cm pot was fertilized three times a week with 200 ml of distillate water supplemented with 0.5 g of the following stock.

Macronutrients	w/w	Micronutrients	w/w
Nitric Nitrogen	6%	Boron	0.02%
Ammonium Nitrogen	6%	Copper	0.05%
Nitrogen from Urea	8%	Zinc	0.05%
Phosphoric anhydride	20%	Iron	0.05%
Potassium oxide	20%	Manganese	0.05%
		Molybdenum	0.0025%

Figure 1. ELS (open bar), albino (gray bar), fertile plants (black bar) per 1000 anthers in microspore culture in three different experiments involving three genotypes (Arcolino, Ciccio and Claudio) and two levels of alternative factors: A: C17 vs. modified C17 media (C17m); B: 200 vs. 300 g/l Ficoll-400; C: Normal vs. special fertilizer. The numbers indicate green plants produced per 1000 anthers in the three experiments.

	Experiment 1			Experiment 2			Experiment 3		
<i>p</i> - value ²	ELS	Albino Plants	Green Plants	ELS	Albino Plants	Green Plants	ELS	Albino Plants	Green Plants
Genotype	0.022	0.344	<.001	<.001	0.01	<.001	0.329	<.001	<.001
Treatment	0.037	0.111	0.027	0.016	0.253	0.010	0.110	0.439	<.001
Genotype * Treatment	0.120	0.356	0.584	0.576	0.875	0.304	0.315	0.333	0.383

¹ Number of anthers extracted from one spike, the spikes and anthers per treatment ranged from 50 to 60, from 10 to 28 and from 756 to 1526, respectively.

- ² p -values of the analyses of variance for log-transformed data corresponding to the previous experiments are listed.

Table 2. Microspore culture of 10 different durum wheat F₁ among cultivars of applied breeding interest.

Cross	F ₁	Spikes/Micros pore isolation	Anthers	ELS	Albino plants	Green plants	Fertile plants	<i>Fertile plants/ 1000 anther</i>
1	AGR6 x AGR8 ¹	37/2	1980	252	101	139	111	56
2	Avispa x CB	29/2	1929	185	44	53	47	24
3	CA x CB	29/1	1392	191	26	36	33	24
4	CA x CIT	35/2	1608	294	109	13	10	6
5	Carioca x CB	40/2	1881	164	24	30	23	12
6	CAV x Carioca	42/3	2898	423	69	209	169	58
7	CAV x Ciccio	50/3	2658	1,088	330	280	239	90
8	CB x CIT	45/2	2442	284	86	27	20	8
9	Ciccio x CB	33/2	3007	211	84	7	6	2
10	CIT x Avispa	54/3	3085	479	115	55	44	14
Total		394/22	22880	3979	988	849	702	<i>Mean 31</i>

¹ This cross was carried out under a contract with Agromonegros SA

Figure 2. Production of *Triticum durum* doubled haploids by microspore culture. Division and germination.

- a. Microspores after 10 days in induction medium.
- b. Pro-embryo after 20 days in culture.
- c. Divisions in a microspore culture with ovary co-culture in modified medium.
- d. Two well formed embryos developed in modified induction medium.
- e. Well developed *Triticum durum* androgenic embryo.
- f. Green plantlet with three roots after 5 days on J25-8 regeneration medium.