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ORIGINAL PAPER

Production of doubled haploids in durum wheat (*Triticum durum* Desf.) through culture of unpollinated ovaries

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Abstract The objective of this work was to produce doubled haploid plants from durum wheat through gynogenesis using unpollinated ovary culture of three local Tunisian genotypes (Jenah Khotifa, Hmira, Azizi) and three improved cultivars (Karim, Khiar, Razzek). A total of 12,000 unpollinated ovaries were cultured in this study. Spikes were either pretreated at 4°C for 14 days or at 4°C in a mannitol solution (0.3 M) for 7 days. Induction was performed using two media. We showed that ovary development, callus and plantlet regeneration was influenced significantly by genotype and growth conditions. The highest regeneration frequency was obtained when the microspore population was in the late mononucleate to binucleate stage. Our results suggested that the cold pretreatment for 14 days was more efficient than the cold treatment in a mannitol solution. Furthermore, the addition of 2,4-D, vitamins and glutamine, and the use of maltose as sugar source in media improved the ovary culture. When the unpollinated ovaries were cultured under the conditions found to be optimal in the present study, a total of 84 plants were produced, all green and haploid. The best levels for regenerated plants were obtained with the cultivars Khiar (3.5%), Hmira (3.1%) and Karim (1.5%). Fertile doubled haploid plants were obtained by colchicine treatment. This result represents a modern tool for breeders to produce durum wheat homozygous lines in a few months.

Keywords In vitro gynogenesis · Doubled haploid · Durum wheat · Pretreatment

Abbreviations

DH	Doubled haploid plant
2,4-D	2,4 Dichlorophenoxyacetic acid
2ip	6-(γ, γ-Dimethylamino)purine
NAA	Naphtaleneacetic acid

Introduction

One goal of recent biotechnology research is to obtain doubled haploid plants which offer complete homozygosity and phenotypic uniformity in one generation (Zheng et al. 2001). Another advantage is a substantial reduction in the cost and the time required to produce breeding lines by conventional system (Liu et al. 2002). Haploid plants can be obtained from male or female gametic cells. In bread wheat (*Triticum aestivum* L.), androgenesis including anther and isolated microspore culture is one of the most efficient methods for doubled haploid

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production. A great improvement on the isolated microspore culture system has been achieved during the last years (Hu and Kasha 1999; Zheng et al. 2001, 2002, 2003; De Buyser et al. 2002; Liu et al. 2002; Kasha et al. 2003; Cistué et al. 2005). However, in durum wheat (*Triticum durum* Desf.), low regeneration rate and very high frequency of albino plants hinder the application of this technique. Several authors have reported its recalcitrance to anther and isolated microspore culture due to the high level of albino plants regenerated (Slim Amara 2000; Dogramaci-Altunetpe et al. 2001; Cistué et al. 2005; Labbani et al. 2005) reaching in most cases 100% (Labbani et al. 2005).

Gynogenesis has been showed to be an efficient way to ovoid this problem plants and was used to produce green double haploid plants in barley and wheat (San Noeum 1976; Mdarhri-Alaoui et al. 1998; Sibi and Fakiri 1994; Sibi et al. 2001). In sugar beet, it has been the most successful method for the production of doubled haploid plants (Gürel et al. 2000).

In order to use the doubled haploid production system into a routine technique, many problems must be solved and then optimized (pretreatment, induction medium, genotype dependence, growth conditions). Pretreatment has an important effect on the production of haploid plants. Indeed, in androgenesis, cold or mannitol pretreatment can influence the embryogenic development of the male gametophyte of wheat and induce the sporophytic development of embryos and callus (Hu and Kasha 1999). In gynogenesis, only cold treatment was used on durum wheat (Mdarhri-Alaoui et al. 1998; Sibi et al. 2001). In our present work, a 14-day cold pretreatment at 4°C or a 7-day cold mannitol pretreatment was applied to the spikes.

Induction medium is another important factor for the development of embryoïds and plantlets (Slim Amara 2000; Pauk et al. 2003). In fact, the successful use of unpollinated ovaries for such application requires optimized induction procedures to achieve a high regeneration frequency and refinements for each species. In durum wheat, two studies have been directed towards the cultivation of unpollinated ovaries: Mdarhri-Alaoui et al. (1998) and Sibi et al. (2001). The induction media component of these two studies are considerably different in the concentration of macro component, 2,4-D, vitamins, amino acids and carbohydrate source. In our study, we have tested these two media to compare their efficiency. Finally, genotypic differences in gynogenesis do exist; some genotypes naturally react better than others in response to unpollinated ovary culture (Mdarhri-Alaoui et al. 1998; Sibi et al. 2001). About six important agronomical Tunisian genotypes will be tested for their gynogenetic ability.

The overall aim of this work was to define an optimized protocol to produce doubled haploid durum wheat plants by gynogenesis. This protocol would be efficient on a wide range of genotypes.

Materials and methods

Donor plants

A total of Six cultivars of Tunisian durum wheat were used: Jenah Khotifa, Hmira, Azizi, Karim, Khiar and Razzek. The first three cultivars were local (hardy) and drought-resistant (Ben Salem et al. 1995); the others were improved cultivars chosen for their high productivity (Maamouri et al. 1988).

Plants were grown in the experimental fields during the normal season at the National Agronomic Institute of Tunisia; seeds were sown on the first week of November. Spikes were collected in March when microspores are at the late uninucleate or binucleate stage. Subsequently, additional tillers were collected when they reached similar morphological development stage.

Pretreatments

Two different pretreatments were used: cold and cold mannitol treatment. For the first pretreatment, collected tillers were placed in ordinary water and maintained at 4°C for 14 days in the dark; for the second, fresh tillers were placed in a 0.3 M mannitol solution and maintained at 4°C for 7 days in the dark.

Culture media

The culture media are presented in Table 1. They are based on Sibi et al. (2001) for M1 media (Induction medium: Ind M1; differentiation medium: Diff M1)

and on modified Mdarhri-Alaoui et al. (1998) for M2 media (Ind M2 based on (MS/2) Murashige and Skoog (1962); and Diff M2 based on MS without growth regulators). The development medium (Dev M) has the same composition as Diff M1, but lacks growth regulators. All components of the media were adjusted to pH 5.8, and solidified with 0.7% purified agar (Sigma A 7992), sterilized by autoclaving at 120°C for 20 min except that vitamins, amino-acids and growth regulators were filter-sterilized using 45 μm pore size filter and added to the autoclaved media.

Ovary culture and plant regeneration

The tillers used for ovary culture were pretreated as described above. After pretreatment, the spikes were sterilized with 12% bleach for 10 min and washed 3 times with sterilized water. The ovaries of 1–1.5 mm length were carefully extracted, and 20 ovaries were placed in 5.5 cm diameter Petri dishes of induction medium (Ind M1 or Ind M2) (Table 1). A total of 12,000 unpollinated ovaries were used for this study. Cultures were sealed and kept in incubator under the dark condition (Sibi and Fakiri 1994; Mdarhri-Alaoui et al. 1998) at 27°C for 5–6 weeks. Calli obtained were transferred to a differentiation medium (Diff M1 or Diff M2) (Table 1) for 6 weeks at 25°C with a 16 h photoperiod at light intensity of 80–100 μE m^{-2} s^{-1}. The calli with emerging shoots were placed on development medium (Dev M) and kept in the same conditions for regeneration. After plantlet regeneration, the cultures were transferred into jars containing 125 ml of development medium and grown to plants.

Chromosome studies

In our work, we determined the ploidy level using chromosome counts protocol from mitotic cells of root tips grown on development medium. Root pretreatment, fixation, hydrolysis and chromosome staining for counting were performed following the protocol described by Jahier et al. (1992). Ploidy level was determined before and after colchicine treatment for all regenerated plantlets.

Chromosome doubling

Colchicine treatment of plantlets at the three-leaf stage was achieved after washing the roots and carefully wiping with filter paper. The roots were placed in 0.1% colchicine solution (1 g of colchicine in 1,000 ml of distilled water) in the greenhouse under a lamp for 4 h. Then they were carefully rinsed with running water for 0.5–1 h. After chromosome doubling was checked, these plants were transferred to soil.

Transfer of plants to soil

Doubled haploid plantlets were potted in small pots containing a mixture of sand and peat (2:1) and transferred into a growth room at 25°C day/night temperature, a 16-h photoperiod, at light intensity of 350–450 μE m^{-2} s^{-1}. To prevent plants from water stress they were covered with glass caps for about 1 week. When plants established a vigorous growth and roots overgrow the soil, they were transferred into bigger pots with the mixture of sand and peat (1:2).

Gynogenetic parameters used

The following percentage values were obtained:

- Percentage of responding ovaries: Number of responding ovaries (swelling ovaries)/Number of cultured ovaries × 100
- Percentage of calli: Number of calli/Number of responding ovaries × 100
- Percentage of green shoots: Number of green shoots/Number of responding ovaries × 100
- Percentage of haploid plantlet development: Number of haploid plantlets/Number of responding ovaries × 100
- Percentage of doubled haploid plants: Number of doubled haploid plants obtained after colchicine treatment/Number of responding ovaries × 100

Data analyses

Analysis of variance (ANOVA) was conducted using SAS computer software (1988). The data were analyzed as two factors for media/pretreatment and as one factor for genotype.

Table 1 Composition of media used for durum wheat unpollinated ovary culture

Components	Ind M1	Ind M2	Diff M1	Diff M2	Dev M
Macroelements g/l					
NH_4NO_3	0.160	0.825	0.160	1.650	0.160
$CaCl_2{\cdot}4H_2O$	0.440	0.220	0.440	0.440	0.440
$MgSO_4{\cdot}7H_2O$	0.370	0.185	0.370	0.370	0.370
KH_2PO_4	0.170	0.085	0.170	0.170	0.170
KNO_3	1.900	0.950	1.900	1.900	1.900
FeEDTA	0.040	0.060	0.040	0.060	0.040
Microelements mg/l					
KI	0.83	0.83	0.83	0.83	0.83
H_3BO_3	6.20	6.2	6.2	6.2	6.20
$MnSO_4{\cdot}2H_2O$	22.30	16.88	22.30	16.88	22.30
$ZnSO_4{\cdot}2H_2O$	8.60	8.6	8.60	8.6	8.60
$Na_2MO_4{\cdot}4H_2O$	0.250	0.25	0.250	0.25	0.250
$CuSO_4{\cdot}5H_2O$	0.025	0.025	0.025	0.025	0.025
$CoCl_2{\cdot}6H_2O$	0.025	0.025	0.025	0.025	0.025
Vitamins mg/l					
Myo inositol	100	100	100	100	100
Nicotinic acid	1	0.5	0.5	0.5	0.5
Pyridoxine HCL	1	0.5	0.5	0.5	0.5
Thiamine HCL	1	0.1	0.1	0.1	0.1
Pyruvate Na	–	–	–	–	5.0
Aminoacids mg/l					
Glutamine	750	–	146	–	146
Glycine	–	2	2.25	2	2.25
L-asparagine	–	150	–	150	–
Growth regulators mg/l					
2,4-D	2	1	1	–	
NAA	–	–	1	–	
Kinétine	0.5	1		–	
2iPA	–	–	0.1	–	
Maltose g/l	60	–	–	–	–
Sucrose g/l	-	30	30	30	30
Purified agar (A 7992)	7	7	7	7	7
pH	5.8	5.8	5.8	5.8	5.8

Ind M1 and Ind M2, induction media; Diff M1 and Diff M2, Differentiation media; Dev M, development medium

Results and discussion

The maturity of ovaries was determined by the development stage of the microspore. The staining of microspores from the oldest anthers on a single spike in aceto-carmine has been used to determine the stage of the microspore based on the location of nucleus relative to the microspore pore (Kasha et al. 2001). Spikes were collected when microspores were at the late uninucleate (Fig. 1a) or binucleate stage (Fig 1b). Subsequently, tillers containing spikes at the desired development stage could be pre-selected on the basis of their morphology (Fig. 1c)

Effect of media-pretreatment

Ovaries of each of the three cultivars Hmira, Azizi and Khiar were used to test the two pretreatments procedures and the two sets of induction and

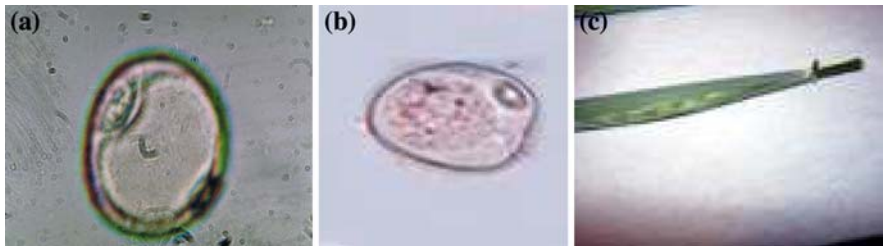

Fig. 1 (**a**) Microspore at the late uninucleate stage; (**b**) Microspore at the binucleate stage; (**c**) Spike at the corresponding morphological stage for maturity of the ovaries of durum wheat

differentiation media (Fig. 2). The highest percentage of responding ovaries was obtained with Ind M1 (Sibi et al. 2001) associated with cold pretreatment for 14 days (Fig. 2a). Ind M1 showed increase in responding ovaries, which seems to be explained by lower macro nutriments, higher organic nitrogen (glutamine), vitamins, 2,4-D and the use of maltose rather than sucrose. The callus percentage was significantly superior on the Diff M1 than on the Diff M2 medium for both pretreatments (Fig. 2b). Considering the percentage of responding ovaries, the combination of cold pretreatment and media M1 was more appropriate for induction stage of gynogenesis.

Unpollinated ovary response and haploid plant production

About 15 days after culture in induction medium on the dark, a fraction of ovaries responded to culture with increased volume (Fig. 3a). Many of these produced calli (Fig. 3b). After their transfer to differentiation medium and exposure to light these calli showed an abundance of shoots (Fig. 3c). The developed haploid plantlets (Fig. 3d) were counted. Doubled haploid plants (Fig. 3h) were obtained after treatment with colchicine 0.1% (Fig. 3f). Ploidy level was tested for haploid plants (Fig. 3e) and for colchicine treated plants (Fig. 3g).

It was shown that pretreatment interacted strongly with induction medium; the successive developmental phases must be fulfilled to initiate shoots able to grow into plants. When this process is stopped, either the pretreatment did not prepare the material to give shoots and plants, or the conditions and sequence of successive media were non optimal (Sibi et al. 2001). We noted that selected induction medium M1 and cold pre-treatment enabled a large number of ovaries to develop into good quality calli that were competent for regeneration into haploid plants.

Effect of genotype

Evaluation of gynogenetic ability and regenerability was based on percentage of responding ovaries, calli, plantlets and regeneration of haploid and doubled haploid plants.

Table 2 shows significant genotypic differences on unpollinated ovary culture for each gynogenetic

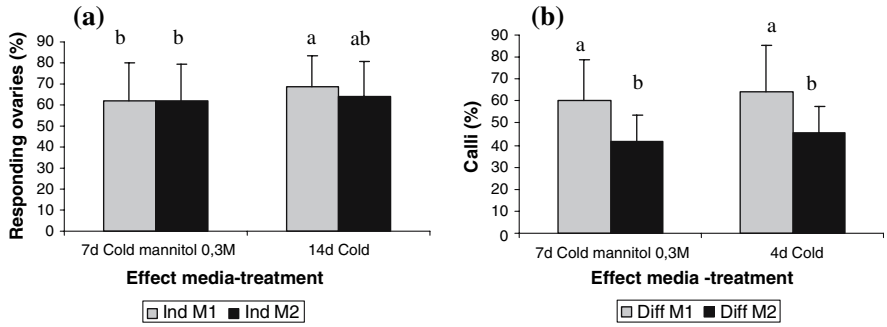

Fig. 2 The relationship between pretreatment and media based on responding ovaries frequency (**a**) and calli frequency (**b**) d: number of days at 4°C Media were based on Sibi et al. (2001) for M1(Ind M1 and Diff M1) and modified Mdarhri-Alaoui et al. (1998) for M2 (Ind M2 and Diff M2). Bars represent the standard deviation of the mean data; data with different letters are significantly different at $P \leq 0.05$

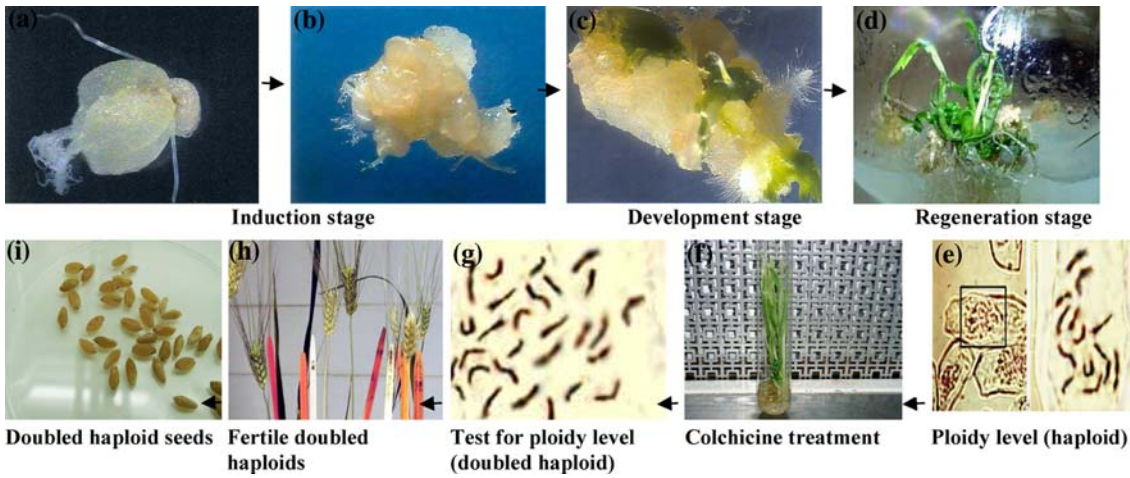

Fig. 3 Different stages in the development of the unpollinated ovaries culture of durum wheat and production of fertile doubled haploids plant (**a**) Ovary after 2 weeks in induction medium in the dark at 27°C (**b**) Callus obtained after 6 weeks of in vitro induction culture before transferring to differentiation medium (**c**) Callus with green shoots obtained after 3 weeks in differentiation medium with a 16-h photoperiod at 25°C (**d**) Green plantlets regenerated after 4 weeks in development medium with a 16-h photoperiod at 25°C (**e**) Test for ploidy level of haploid plants (**f**) Roots soaked for 4 h in 0.1% colchicine solution (**g**) Test for ploidy level of doubled haploid plants (**h**) Fertile doubled haploid plants (**i**) Seeds obtained by fertile doubled haploids

Table 2 Response to ovary culture of six Tunisian durum wheat genotypes

Genotypes	Responding ovaries	% Responding ovaries[1]	% Calli[2]	% Green shoots[3]	% Haploid Plantlets[4]	% Doubled haploid plants[5]	% Doubled haploid total /haploid plants regenerated[6]
Hmira	1,221	68.4 a	19.4 b	8.5 a	3.1 a	0.67 a	15.38
Azizi	1,256	60.7 ba	6.9 b	3.4 dc	0.0 b	0.00 b	0
Razzek	1,120	49.1 bc	21.9 a	4.8 bdc	1.2 ba	0.49 ba	50
Jenah Khotifa	1,221	49.0 bc	3.4 b	1.6 d	0.7 b	0.55 ba	60
Karim	796	48.1 bc	12.6 ba	7.1 ba	1.5 ba	0.32 b	36.36
Khiar	788	41.7 c	11.7 ba	6.1 bac	3.5 a	0.84 a	23.81

Percentage values are the means of six replicates

Means in the same column followed by different letters are significantly different at 0.05 level

[1] % of responding ovaries: number of responding (sweeling) ovaries/ number of cultured ovaries ×100

[2] % of calli: number of calli/number of responding ovaries ×100

[3] % of green shoots: number of green shoots/number of responding ovaries ×100

[4] % of haploid plantlets: number of regenerated plantlets/number of responding ovaries ×100

[5] % of doubled haploid plants: number of doubled haploid plants/number of responding ovaries ×100

[6] % of doubled haploid total/haploid plants regenerated: number total of doubled haploid plants transferred to soil/number total of haploid plants ×100

parameter. The best percentage of responding ovaries was obtained from Hmira with 68.4%. Razzek yielded the highest percentage of calli (21.9%) and was significantly better than all other genotypes. The highest percentage of green shoots was obtained for Hmira with 8.5 %. The best values for plant haploid were obtained for khiar, Hmira and Karim with the respective values of 3.5%, 3.1% and 1.5%. The yield of doubled haploid plants in all genotypes is lower than those of haploids. The percentages of doubled

haploid obtained for Khiar, Hmira and Karim were respectively 0.84%, 0.67% and 0.32%. For Jenah Khotifa the percentage was very low for the two parameters with respectively 0.7% and 0.55%. Only Azizi was recalcitrant for gynogenesis (no plant was produced), however, this genotype tested by anther culture showed very good androgenetic ability (Slim Amara 2000). For the genotype Hmira, ovaries responded relatively regularly with good values for all gynogenetic parameters but it presented a high mortality after colchicine treatment and transfer to soil. For all the genotypes, 84 haploid plants were obtained. It is important to note that, in accordance with Mdarhri-Alaoui et al. (1998) and Sibi et al. (2001) findings, all regenerated plantlets were green.

Haploid plant regeneration rates obtained in this study were comparatively good according to previous studies with durum wheat. Sibi et al. (2001) using the same conditions, obtained for five durum wheat cultivars (Cham1, Cocorit, Isly, Jori and Sarif) a regeneration rates of 0%, 18.1%, 0%, 4% and 0%, respectively. Cocorit, which gave the highest value, did not react at all in other conditions and showed problems in surviving through transfers. In Mdarhri-Alaoui et al. (1998) study, working with eight genotypes and using 15 days of cold pretreatment, the lowest rate obtained was 1.08%. According to Mdarhri-Alaoui et al. (1998) and Sibi et al. (2001), genotype plays an important role in durum wheat gynogenetic response and haploid production.

In our study, among the six genotypes tested five were responsive and produced calli, regenerated plantlets, haploid and doubled haploid plants.

Ploidy levels and fertility of regenerants

Regenerated plantlets were evaluated for ploidy via chromosome counts. A total of 84 plantlets were obtained and their chromosome number determined. We have showed that all were haploid and thus were the result of regeneration of haploid unpollinated ovaries and not of diploid ovary tissues. All genotypes included, the treatment with colchicine produced 22 of doubled haploid plants (Table 3). This procedure is one of the most critical steps in the doubled haploid breeding process. Moreover, colchicine treatment has disadvantages: it may cause a reduction in plant fertility up to substantial plant mortality (Barnabàs and Szakàcs 2000). It is possible that mortality caused by colchicine treatment can be influenced by the genotype.

Following transfer to soil, eight plantlets survived, were all fertile (Fig. 3h). None showed any type of abnormality in the chromosome number or in the morphology of the plant or of the spike. These plantlets gave a total of 15 fertile spiklet tillers and set seeds (Fig. 3i).

Unpollinated ovary culture appears to be an effective method for green double haploid production in durum wheat. In our system, we have showed that tillers should be collected when microspores were at the late uninucleate or binucleate stage. Then unpollinated ovaries were subjected to a cold pre-treatment (14 days at 4°C) and cultured in induction medium of Sibi et al. (2001) (M1) in the dark at 27°C. Green shoots were transferred to development medium for plant regeneration with a 16-h photoperiod, and room temperature of 25°C.

Table 3 Number of spikes and seeds obtained by survived doubled haploid plants after colchicine treatment and soil transfer

Genotypes	Number total of haploid plants	Number total of doubled haploid plants transferred to soil	Number of survived plants after transfer to soil	Number of tillers spiklet		Number of seeds
				Fertiles	Steriles	
Hmira	39	6	0	0	0	0
Azizi	0	0	0	0	0	0
Razzek	8	4	3	5	0	86
Jenah Khotifa	5	3	2	3	0	44
Karim	11	4	0	0	0	0
Khiar	21	5	3	7	1	124

The number of haploid plants was based on the number of regenerated plantlets

The number of doubled haploid plants was based on the number of transferred plantlets in soil after colchicine treatment

This system offers great potential and has the advantage that all the haploid plants obtained are green. Generally, the system used to obtain durum wheat haploid plants has been anther culture (Slim Amara 2000; Dogramaci-Altunetpe et al. 2001) and microspore culture (Picard et al. 1998; Jaiti et al. 2000; Labbani et al. 2005; Cistué et al. 2005) but albinism is a major problem of androgenesis. Gynogenesis is the method of choice to avoid the albino problem and produce green haploid lines in durum wheat. We will continue improving the rate of doubled haploid green plants obtained by amending the culture conditions and chromosome doubling. Nevertheless, the current technology itself may be widely applicable to durum wheat breeding.

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