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Efficiency of three haplomethods in durum wheat (*Triticum turgidum* subsp. *durum* Desf.): isolated microspore culture, gynogenesis and wheat × maize crosses

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Abstract: This study presents the first report comparing the efficiency of microspore culture, gynogenesis and durum wheat × maize crosses for haploid plant production from three durum wheat genotypes (Razzek, Karim and Jneh Khotifa). The results showed that the best induction, calli or embryos formation and plant regeneration rates for the three genotypes were obtained with gynogenesis (47.2, 7.6, 0.8%), followed by interspecific crosses (33.1, 1.7, 0.4%) and isolated microspore culture (8.2, 0.05, 0.01%). Interestingly, all plants regenerated by gynogenesis and durum wheat × maize crosses were green whereas all plants obtained by isolated microspore culture were albino. In the haploid production system, all steps of the process are important for the three methods. The critical steps that have greatly reduced the number of regenerated haploid plants were induction, embryogenesis and regeneration for microspore culture, forming and regeneration of calli or embryo and haploid regeneration for interspecific crosses and gynogenesis. Genotypes with good capacity of induction have not necessarily a good capacity of haploid plantlets regeneration and *vice-versa*. However, calli or embryos formation seems to be an indicator of the haploid production. Overall, Razzek showed a good ability to produce haploids using the three methods. Each haplomethod showed a specific advantage. Although gynogenesis is the less used method for durum wheat, it has proved to be a successful approach for green haploid plant production.

Keywords: androgenesis; haploidization; interspecific crosses; unpollinated ovary culture; wheat

The production of doubled haploid plants (DH) is a proven method for obtaining complete homozygous lines in a one-step process, as opposed to conventional line production, which requires several cycles of self-pollination. This method how to make breeding faster has a several advantages and is a useful tool in genetic research, including plant transformation, gene mapping and mutation studies (Lu *et al.*

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2016). The successful use of the DH in most crops to develop commercial cultivars relies highly on an efficient protocol for inducing haploids. Haploids are plants with male or female gametophytic chromosome number that can be doubled (Niu *et al*. 2014).

In gynogenesis, haploid plant development is induced by unpollinated ovary culture. Gynogenesis has been shown to be a successful method for producing haploid plants of many species, such as onion (Campion & Alloni 1990) and sugar beet (Gürel *et al.* 2000). Unpollinated ovary culture is practised more rarely in wheat breeding programs (Mdarhri-Alaoui *et al*. 1998; Sibi *et al*. 2001).

Androgenesis (anther or microspore culture) based on the culture of male gametophyte, is the most used method for several cereal species, particularly barley (*Hordeum vulgare* L.) and bread wheat (*Triticum aestivum* L.) (Makowska & Oleszczuk 2014; Castillo *et al*. 2015; Sriskandarajah *et al.* 2015; Makowska *et al.* 2017). The efficiency of androgenesis has reached an adequate level and has led to the creation of commercial bread wheat varieties such as Florin (Picard *et al*. 1994). In durum wheat (*Triticum turgidum* subsp. *durum* Desf.), however, this method is less used due to the low regeneration rate and high frequency of albino plants (Cistué *et al*. 2009).

Another alternative to obtain haploid plants is "wide crossing" followed by embryo rescue. This involves pollination with a male partner genetically distant or a pollinator of the same genus having desired traits. Interspecific or intergeneric crosses has emerged as an efficient method for producing haploid wheat plants (Garcia-Liamas *et al*. 2004; Ushiyama *et al*. 2007; Ayed *et al.* 2011b). Several crosses, wheat × *Hordeum bulbosum* (Barclay 1975), wheat × maize (Laurie & Bennett 1988), wheat × sorghum (Inagaki & Mujeeb-Kazi 1995) and wheat × *Imperata cylindrica* (Chaudhary *et al*. 2015) were shown as valid hybridization systems. In recent years, wheat × maize crosses has become a main approach for haploid production in durum wheat (Niu *et al*. 2014). The success of this method, however, is still limited since a great number of zygotes abort during the early development stages (Laurie & Bennett 1988). Efforts to optimize various protocol factors, including pre-treatment, medium and growth regulators have been made to improve the three methods for durum wheat haploid plants regeneration (Sibi *et al.* 2001; Cistué *et al.* 2006, 2009; Slama-Ayed & Slim-Amara 2007; Ayed *et al.* 2011a, b), but there have been no reports of comparative studies of the three haplomethods.

In this study, our objective was to compare isolated microspore culture, gynogenesis and interspecific wheat × maize crosses in order to determine the most efficient method for producing durum wheat green haploid plants.

MATERIAL AND METHODS

Donor plants and growth conditions

The most cultivated genotypes of durum wheat (*Triticum turgidum* subsp. *durum* Desf.) in Tunisia, Karim and Razzek, and one landrace, Jneh Khotifa, were used as donor plants for comparing the three haplomethods. The maize (*Zea mays* L.) genotype, Pioneer 37Y15 was chosen for the interspecific crosses.

For microspore culture, the plants were cultivated in pots and grown in a controlled glasshouse at 22/15°C day/night temperature, 70% relative humidity, 16 h light/8 h dark photoperiod and 1150 µmol/m^2/s light intensity. For gynogenesis and interspecific crosses, the seeds were sown in the first week of November in an unconditioned glasshouse (15–20°C, 12–14 h day length) at the National Agronomic Institute of Tunisia and collected in March. Fresh tillers contained microspores at the binucleate stage were selected for gynogenesis and maize crosses. However, the late uninucleate stage was the microspore developmental stage appropriate for the isolated microspore culture. Microspores were examined microscopically by aceto-carmine staining.

Haploid plant production

Isolated microspore culture. As a pre-treatment, fresh tillers containing microspores were maintained at 4°C for 5 weeks (Slama-Ayed *et al.* 2010). Tillers of each genotype were surface-sterilized in 12% sodium hypochlorite for 10 min and washed three times with sterilized water. Florets from the central part of the spike were isolated. Three-microspore isolation per genotype were performed as described by De Buyser *et al.* (2002). The extracted microspores were cultivated in liquid CHB3 medium, as described by Chu *et al.* (1990), modified by adding 90 g/l of maltose. The average number of microspores per isolation was ~103 000 for all combined genotypes. The microspores were adjusted by CHB3 medium at a density of 50 000 microspores/ml. One ml of aliquots

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was cultured in 35 × 15 mm Petri dish. Immature ovaries were added to the culture at a density of four per milliliter, before incubation. The Petri dishes were sealed and incubated in the dark at 27°C. The embryos obtained (1–2 mm diameter) were aseptically transferred to a solid MS medium (Murashige & Skoog 1962) without growth regulators at a density of 30 embryos per Petri dish (100 mm diameter). The dishes were incubated in a growth chamber at 25°C with a 16 h light/8 h dark photoperiod and a light intensity of 80–100 µmol/m^2/s. About 2 weeks after embryo transfer, the number of regenerated plantlets (green and albino) was counted.

Gynogenesis. For unpollinated ovary culture, the collected tillers of each genotype were pre-treated with ordinary water at 4°C for 14 days in the dark (Slama-Ayed & Slim-Amara 2007). Spikes were sterilized with sodium hypochlorite (12%) for 10 min and washed three times with sterilized water. The ovaries (1–1.5 mm diameter) were carefully extracted from the central part of the spike, and 20 of them were then placed in 5.5 cm diameter Petri dishes containing the induction medium proposed by Sibi *et al.* (2001). The cultures were confined and kept in the incubator under dark conditions (Mdarhri-Alaoui *et al.* 1998; Sibi & Fakiri 1994) at 27°C for 5–6 weeks. The regenerable calli obtained were transferred to a differentiation medium (Sibi *et al.* 2001) for 6 weeks at 25°C, 16 h light/8 h dark photoperiod and 80–100 µmol/m^2/s light intensity. Thereafter, calli with emerging shoots were placed on a development medium (DevM) (Sibi *et al.* 2001) and maintained under the same conditions of regeneration. After plantlet regeneration, the cultures were transferred into beakers containing 125 ml of development medium and grown into plantlets.

Interspecific crosses (durum wheat × maize). Crosses were carried out between a maize genotype (Pioneer 37Y15) as the male parent and three-durum wheat genotypes (cv. Razzek, Karim and Jneh Khotifa) used separately as the female parent. Before anthesis, the wheat spikelets were emasculated. After two or three days, spikelets were pollinated with maize pollen. The stems of the pollinated spikes were cut in the middle of the third internode and covered with a paper bag. The detached tillers were then cultured in a solution of 40 g/l sucrose, 8 ml/l sulphurous acid, 100 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 75 mg/l $AgNO_3$ (Ayed *et al.* 2011b). After 18 days of pollination, embryos that grew over two thirds of the glume length were isolated from caryopses and sterilized with sodium hypochlorite (12%) for 10 min and washed 3 times with sterilized water. Thereafter, the embryos were placed in Petri dishes (100 mm diameter) with 5 ml of B5 medium (Gamborg & Eveleigh 1968). The embryos were maintained in a growth chamber at 25°C in the dark until germination. Haploid wheat seedlings at the three-leaf stage (1–2 cm) were transferred into beakers in the same medium at 25°C, 16 h light/8 h dark photoperiod and 80–100 µmol/m^2/s light intensity.

Chromosome counting

For the three haplomethods, the ploidy level of all regenerated plantlets was checked 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).

Data collection

The percentages of induction, regenerable calli or embryos and regenerated plants per 100 florets were

Table 1. Analysed parameters for microspore culture, gynogenesis and interspecific crosses

Microspore culture	Gynogenesis	Interspecific crosses
% of induction = (No. of induced microspores/No. of microspores in 100 florets) × 100	% of induction = (No. of induced ovaries/100 florets) × 100	% of induction = (No. of developed ovaries/100 florets pollinated) × 100
% of embryos produced = (No. of embryos/No. of microspores in 100 florets) × 100	% of calli produced = (No. of calli/100 florets) × 100	% of embryos produced = (No. of embryos formed/100 florets pollinated) × 100
% of haploid plantlets produced = (No. of haploid plantlets/No. of microspores in 100 florets) × 100	% of haploid plantlets produced = (No. of haploid plantlets/100 florets) × 100	% of haploid plantlets produced = (No. of haploid plantlets/100 florets pollinated) × 100.

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Table 2. Analysis of variance of the different parameters according to the haplomethods, durum wheat genotypes and their interaction

Factors	df	% of induction		% of calli or embryos		% of regenerated plants	
		F	*P*	*F*	*P*	*F*	*P*
Haplomethods (H)	2	25.08	< 0.001	9.49	0.002	29.23	0.000
Genotypes (G)	2	0.18	0.833	3.06	0.072	0.22	0.806
H × G	4	1.44	0.261	2.99	0.047	1.92	0.151

recorded for microspore culture, gynogenesis and interspecific crosses (Table 1). For each haplomethod, three replicates per experiment were used for each genotype. All the experimental data were subjected to analysis of variance (ANOVA) using SPSS statistical software (Ver. 16.0, 2007). In figure, graph bar represents mean ± standard error (SE). The statistical significance was tested using Tukey test at a 5% level of probability. A linear regression analysis ($y = mx + c$) was also performed between the percentage of regenerated plants and the percentage of induction and calli or embryos in order to establish their mutual relationship.

RESULTS AND DISCUSSION

Three haplomethods (isolated microspore culture, gynogenesis and interspecific crosses) were compared for their efficiency in regenerating haploid plants. The ANOVA revealed a highly significant ($P < 0.01$) differential response for all tested parameters among the three haplomethods (H) (Table 2). No significant genotypic variation (G) was obtained for the three parameters. However, a significant interaction (H × G) was observed for calli or embryos formation.

The induction was manifested by the swelling of microspores and ovaries in microspore culture and gynogenesis, respectively, and the growth of the developed seeds in. interspecific crosses. This study showed that the best rate of induction for the three genotypes was obtained with gynogenesis (47.2%), followed by interspecific crosses (33.1%) and isolated microspore culture (8.2%) (Figure 1a, Table S1 in Electronic Supplementary Material (ESM)). Several authors have reported good induction rates in bread and durum wheat for the unpollinated ovary

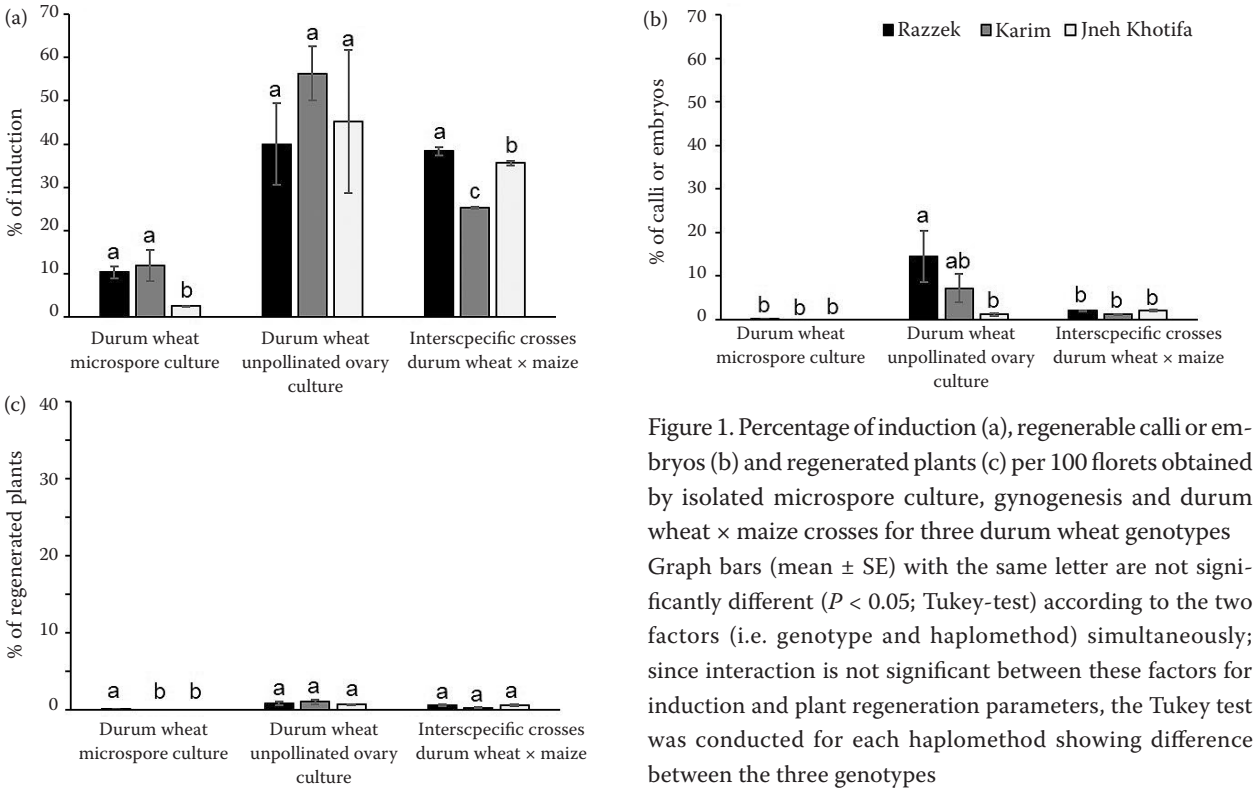

Figure 1. Percentage of induction (a), regenerable calli or embryos (b) and regenerated plants (c) per 100 florets obtained by isolated microspore culture, gynogenesis and durum wheat × maize crosses for three durum wheat genotypes
Graph bars (mean ± SE) with the same letter are not significantly different ($P < 0.05$; Tukey-test) according to the two factors (i.e. genotype and haplomethod) simultaneously; since interaction is not significant between these factors for induction and plant regeneration parameters, the Tukey test was conducted for each haplomethod showing difference between the three genotypes

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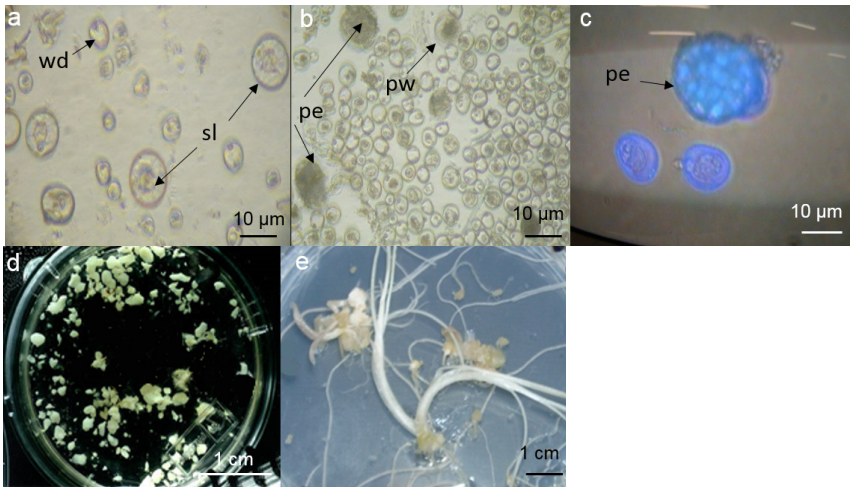

Figure 2. Isolated microspore culture process: induction at 7 days of culture: embryogenic microspore with star-like (sl), microspore without development (wd) (a), pro-embryos derived from microspores at 10 days: pro-embryos beginning to break the exine (pe), pro-embryos trapped in the pollen wall (pw) (b), blue fluorescence of pro-embryo (stained with DAPI) emerging from the exine (pe) (c), mature embryos obtained after 28 days of culture (d), regenerated albino plantlets (e)

culture and interspecific crosses (Inagaki *et al.* 1997; Mdarhri-Alaoui *et al.* 1998; Cherkaoui *et al.* 2000; Sibi *et al.* 2001; Ushiyama *et al.* 2007). The induction stage showed a marked effect on the isolated microspore culture response. The low rate of induction might be explained by the decrease in the viability of microspores during culture. It is worth noting that a small fraction of the microspores swells, others remain in the initial stage, while a big fraction degenerates. Only enlarged microspores with star-like (i.e. induced microspores; Figure 2a) appeared after 7 days of culture can be embryogenic and evolve into pro-embryos. As described by Bal *et al.* (2012), the star-like microspores contained several compartments surrounding a circle in the center (Figure 2a). Compared with the other methods, the microspores culture seems to be more influenced by the growing conditions and the physiological stage of mother plants (Cistué *et al.* 2009; Rituraj *et al.* 2013), genotypes (Cistué *et al.* 2006), stress treatment (Jacquard 2007; Soriano *et al.* 2008), composition of induction medium (Cistué *et al.* 2009), and the inclusion of co-culture ovaries (Castillo *et al.* 2015) that might explain the low rate of induction. There were no significant differences in induction rates between the three genotypes for the three methods (Table 2). The three genotypes have practically the similar rate of induction for each method (Figure 1a).

After induction, microspore culture and interspecific crosses generated embryos directly, while calli were developed from unpollinated ovaries. For gynogenesis, the rate was 7.6% for all genotypes, followed by 1.7% for interspecific crosses and 0.05% for isolated microspore culture (Figure 1b, Table S1 in ESM). Embryogenesis seems to be one of the critical steps of haploid production for the three methods. After 10 days of culture, most of the pro-embryos obtained from induced microspores remain trapped in the pollen wall and were unable to break the exine (Figure 2b, c), which could explain the low rate of embryos formation. The other pro-embryos, however, emerge from the exine wall to transform into mature embryos (Figure 2d). In interspecific crosses, ovule fertilization is followed by paternal chromosome elimination in hybrid embryos. The endosperms are absent or poorly developed, and embryo rescue and further *in vitro* embryo culture are needed (Figure 4a, b). Most of the embryos abort during the initial stage of development. This lack of zygote

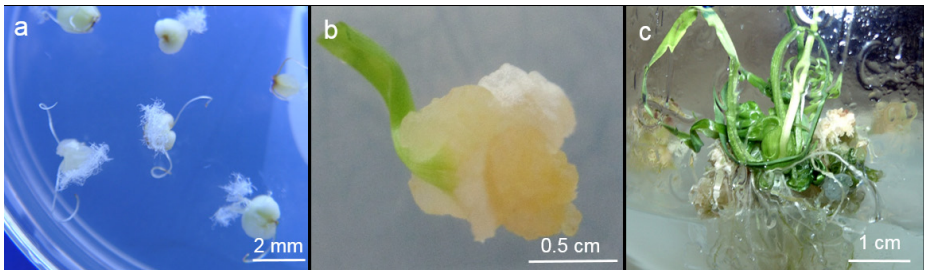

Figure 3. Gynogenesis process: unpollinated ovaries induced after 1 week of culture (a), callus with green shoot after 10 weeks of culture (b), regenerated green plantlets (c)

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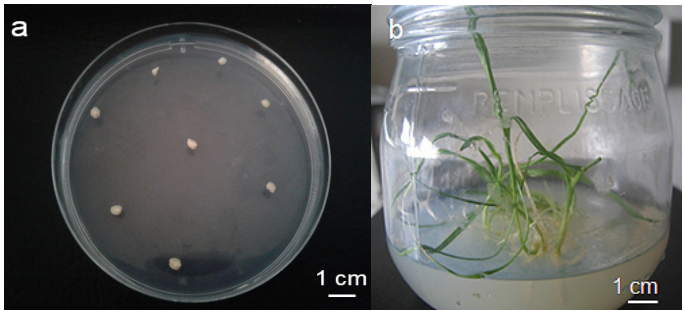

Figure 4. Interspecific crosses process: embryo rescue in solid medium culture (a), regenerated green plantlets (b)

viability affects the conversion of embryos into plantlets. In addition, the ability to form haploid embryos might depend on the application of silver nitrate and hormone treatment, alone or in combination (Ayed *et al.* 2011a; Chaudhary *et al.* 2015).

Based on the morphological appearance, gynogenesis method produce two types of calli structures: embryogenic and non-ambryonic calli. The embryogenic calli were friable and have generated roots and green shoots (Figure 3b). Two types of non-embryogenic calli were obtained: the first type was compact and did not develop roots and green shoots, and eventually became necrotic (Figure 5a). However, the second type did not produce shoots, but only long and enrolled roots without embryogenesis (Figure 5b).

A significant interaction genotype x haploid methods was obtained for calli or embryo formation (Table 2). For example, the rates of calli or embryo formation for Razzek were 14.5%, 2.0% and 0.13% in response to gynogenesis, interspecific crosses and microspore culture, respectively (Figure 1b). However, Karim and Jneh Khotifa were recalcitrant genotypes (0%) to microspore culture method.

For the three genotypes, a higher production of haploid plants was obtained using unpollinated ovary culture (0.8%) and the interspecific crosses (0.4%) compared to isolated microspore culture (0.01%) (Figure 1c, Table S1 in ESM). No spontaneous chromosome doubling was observed for green and albino haploid plantlets in any of the three haplomethod (Figure 6). The regeneration of durum wheat plants seems to be another key step for the three methods. Our results showed that Razzek haploids regenerated by microspore culture were albino, while Karim and Jneh Khotifa did not response at all to this method (Table 3). In fact, durum wheat is a recalcitrant species to androgenesis, with a low regeneration rate and a high frequency of albino plants (Cistué *et al.* 2009). For gynogenesis and durum wheat × maize method, all the regenerated plants were green for the three genotypes (Table 3; Figure 3c, 4b). This work showed that genotypes with good capacity of induction have not necessarily a good capacity of haploid regeneration for the three methods and *vice-versa* (Table 4). For example, Karim presented, despite its high rate of induction *via* gynogenesis, a haploid regeneration capacity similar to that of Razzek and Jneh Khotifa. However, calli or embryos formation seems to be an indicator of the haploid production. Interestingly, Razzek showed a good ability to produce haploid plants using the three haplomethods.

To our knowledge, this is the first study to compare isolated microspore culture, gynogenesis and inter-

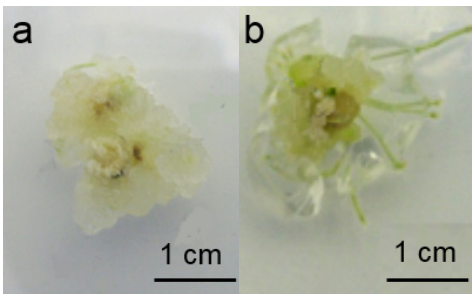

Figure 5. Non-embryogenic callus from ovary culture features after 11 weeks: compact (a), with roots without embryogenesis (b)

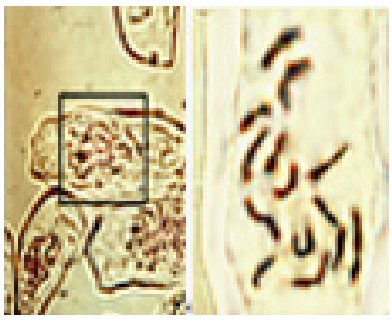

Figure 6. Check of ploidy level of haploid durum wheat plants

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Table 3. Percentage of green and albino durum wheat plants obtained by isolated microspore culture, gynogenesis and durum wheat × maize crosses

Haplomethod	Genotype	Green plants	Albino plants
Microspore culture	Razzek	0	100
	Karim	0	0
	Jneh Khotifa	0	0
Gynogenesis	Razzek	100	0
	Karim	100	0
	Jneh Khotifa	100	0
Interspecific crosses	Razzek	100	0
	Karim	100	0
	Jneh Khotifa	100	0

specific crosses. Overall, our results showed differences between the haplomethods and each of them had a specific advantage. Isolated microspore culture remains interesting despite the low rate of regenerated plantlets, the albinism problem, the complexity of microspore isolation and the dependency on pretreatment and environmental factors. It enables the development of microspores to be monitored from the early stages. In addition, the formation of embryos deriving from microspore culture means that the regeneration of somatic tissues is avoided. The process of this method also takes less time (about six weeks from *in vitro* culture to haploid plant regeneration than maize pollination (about eight weeks) or gynogenesis (about 16 weeks). Microspore culture seems, however, to be very influenced by many factors (Cistué *et al.* 2006) as previously reported, makes it challenging to compare the results from different laboratories (Castillo *et al*. 2000). Further work is needed to (*i*) develop reproducible protocol of microspore culture and (*ii*) improve durum wheat green plants regeneration from a wide range of genotypes including low responding ones.

The method of interspecific durum wheat × maize crosses proved to be efficient to produce green haploid plants. The efficiency of haploid production in wheat × maize crossing system than microspore culture is due mainly to different factors including the absence of albinism, the high embryo formation and plant regeneration rates. For these reasons, currently, the interspecific crossing with *Z. mays* is the more frequently used method to produce durum wheat haploid plants (Jauhar 2003).

Although gynogenesis is the less used haplomethod, it has proved to be a successful method for regenerating haploid plants in durum wheat. The haploid regeneration rates by this method were slightly higher compared to those obtained by interspecific crosses. Unpollinated ovary culture has the advantage that all regenerated plants are green. The albinism problem has not arisen in a wide range of genotypes tested (Mdarhri-Alaoui *et al.* 1998; Sibi *et al.* 2001; Slama-Ayed & Slim-Amara 2007; Slama-Ayed *et al.* 2010). Microspore culture should theoretically have more potential in view of the large number of microspores per florets versus one egg cell per ovary for gynogenesis (Mishra & Goswami 2014). However, the low regeneration rate and the albinism problem hinder the potential of this technique and allows gynogenesis to be more interesting in terms of the number of gametophytic cells cultivated and the number of plant regenerated. Furthermore, the formation of calli using gynogenesis offers the possibility to obtain gametoclonal variation under selection pressure, which could be exploited in durum wheat breeding programs (Huang 1996; Germana 2011). In addition to the interspecific crosses, the performance of these haplomethods reside in the production of green plants for durum wheat compared to androgenesis, which produce chlorophyll-deficient plants (albino). Both nuclear and chloroplast genomes encode proteins for chloroplast development and function responsible to obtain green plants. Despite the fact that the majority of chloroplast proteins are encoded by the nuclear genome, the deletion in the chloroplast genome is also a reason for the expression of albino phenotype (Hofinger *et al.* 2000). In this context, our results

Table 4. Correlation coefficient (R^2) between the percentage of durum wheat haploid plants and the percentage of induction and calli or embryos

Haplomethod	% of induction – % of regenerated plants	% of calli or embryos – % of regenerated plants
Microspore culture	0.05^{ns}	0.98^{***}
Gynogenesis	0.08^{ns}	0.46^{*}
Interspecific crosses	0.37^{ns}	0.53^{*}

$^{ns}P > 0.05$; $^{*}P < 0.05$; $^{***}P < 0.001$

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suggest that the ability of maternal haploids methods (i.e. interspecific crosses and gynogenesis) to avoid albinism might be controlled by chloroplast genes transferred during the maternal heredity.

Overall, the present investigation showed that gynogenesis and interspecific crosses were efficient to produce green haploid lines and showed less genotype-specificity. These haplomethods make it possible to avoid the problem of albinism and the very marked recalcitrance in Tunisian durum wheat. For its simplicity and efficacy, unpollinated ovaries is a method highly recommended to produce durum wheat haploid plants. However, this method needed improvements to increase the quantity of haploid regenerants. The haploid and DH production offers significant potential in wheat breeding programs and requires an efficient and a reproducible plant regeneration system, which can overcome several problems such as albinism, low frequencies of calli or embryos induction and regenerated plants. Nevertheless, the progress made regarding haploid research encourages its future applications in plant breeding.

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