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Doubled Haploid Production in Tunisian Durum Wheat

Abstract – To ensure food security with climate change, the challenge in durum wheat breeding is a higher yielding in a shorter time. Haplomethods produced perfect homozygous lines in one generation and has several uses in plant breeding, as opposed to conventional line production, which requires several cycles of self-pollination. Androgenesis, gynogenesis or intergeneric hybridization are the main methods to produce doubled haploid lines. For durum wheat, the major problem is the low efficiency of regenerated plants and albinism. In order to determine the more efficient method to regenerate green haploid plants, the efficiency of these technics were compared using cv. Razzek. The rates of induction, callus/embryo development and plant regeneration were investigated. The best induction rate (76.67%) was obtained for maize method. A differential response was also obtained for the percentage of green plantlets regenerated. The highest rate of durum wheat green haploid plantlets was regenerated by gynogenesis and maize method with respectively 26.09% and 20.00%. Results showed that gynogenesis was the most efficient technique to obtain durum wheat green haploid plants. The production of such pure lines offers potential in wheat breeding programs.

Keywords: durum wheat; gynogenesis; haploid plants; *in vitro* culture; maize method; microspore culture.

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Introduction

The production of haploid plants is a proven method for obtaining perfect homozygous lines in a one-step process, as opposed to conventional line production, which requires several cycles of self-pollination. This method of speed breeding have a several advantages and is a useful tool in genetic research [15]. The haploid lines can be obtained using androgenesis, gynogenesis or intergeneric hybridization. Androgenesis (anther or microspore culture) is frequently used for several cereal species, particularly barley (*Hordeum vulgare* L.) and bread wheat (*Triticum aestivum* L.) [4, 16, 27]. In durum wheat (*Triticum turgidum* subsp. *durum* Desf.), however, this technic is less used due to the low regeneration rate and high frequency of albino plants [7]. Gynogenesis has been shown to be a successful method for producing haploid plants of many species, such as onion [3] and sugar beet [11]. Unpollinated ovary culture (gynogenesis) is practised more rarely in wheat breeding programs. Over the past 15 years, interspecific or intergeneric hybridization has emerged as an efficient technique for producing haploid wheat plants [2, 10, 28]. In durum wheat, however, a great number of zygotes abort during the early development stage [14]. 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 [1, 2, 4, 7, 21, 24, 27], but there have been no reports of comparative studies of the three methods.

In this study, our objective was to compare three haploid technologies – isolated microspore culture, gynogenesis and intergeneric hybridization – in order to determine the most efficient method for producing durum wheat green haploid plants.

Material and methods

Donor plants and growth conditions

An improved durum wheat variety «Razzek» was used as the donor plant for comparing the three haploid techniques. One of the most cultivated varieties in Tunisia, it was tested beforehand for its responsiveness to these techniques following the protocols optimized in our laboratory [1, 2, 22]. Donor 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 1 150 μE m^{-2} s^{-1} light intensity. The spikes were collected in March when microspores were at the late uninucleate stage for isolated microspore culture and the binucleate stage for gynogenesis and intergeneric hybridization.

Haploid plant production

Isolated microspore culture. As a pre-treatment, 7 fresh tillers containing microspores were maintained at 4°C for 5 weeks [22]. The tillers were sterilized and

microspores were isolated as described by DE BUYSER *et al.* [8]. The extracted microspores were cultivated in CHB3 medium, as described by CHU *et al.* [6], modified by adding 90 g l^{-1} of maltose [22]. The microspores were adjusted at a density of 50 000 microspores m l^{-1} and were cultivated in 35 x 15 mm Petri dishes. Immature ovaries were added to the culture at a density of four per milliliter, before incubation. The inclusion of ovaries in these culture systems seemed to enhance embryogenesis and/or improve the quality of the embryos [4]. 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 [19] without growth regulators at a density of 30 embryos per Petri dish. 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 $\mu E\ m^{-2}\ s^{-1}$. About 2 weeks after embryo transfer, the number of regenerated plantlets (green and albino) was counted.

Gynogenesis. For unpollinated ovary culture, 7 collected tillers were pre-treated with ordinary water at 4°C for 14 days in the dark [21]. The 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, and 20 of them were then placed in 5.5 cm diameter Petri dishes containing the induction medium proposed by SIBI *et al.* [24]. A total of 920 unpollinated ovaries were used for this study. The cultures were confined and kept in the incubator under dark conditions [17, 23] at 27°C for 5-6 weeks. The regenerable calli obtained were transferred to a differentiation medium [24] for 6 weeks at 25°C, 16 h light/8 h dark photoperiod and 80-100 $\mu E\ m^{-2}\ s^{-1}$ light intensity. Thereafter, calli with emerging shoots were placed on a development medium (DevM) [24] 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.

Intergeneric hybridization (durum wheat x maize). Crosses were carried out between a durum wheat genotype (cv. Razzek) used as the female parent and a maize (*Zea mays* 2n = 2x = 20) genotype (Pioneer 37Y15) as the male parent. The maize plants were grown in pots in an unconditioned glasshouse at temperature slightly warmer than outside. Two or three days before anthesis, wheat 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 7 detached tillers were then cultured in a solution of 40 g l^{-1} sucrose, 8 ml l^{-1} sulphurous acid, 100 mg l^{-1} 2,4-D and 75 mg l^{-1} $AgNO_3$ [2]. After 18 days of pollination, the embryos were placed in Petri dishes with 5 ml of B5 medium [9]. The embryos were maintained in a growth chamber at 25°C in the dark until germination and the regeneration of haploid plantlets.

Data collection

The percentages of induction, embryos/regenerable calli and regenerated plants were recorded for each method. For the isolated microspore culture, three parameters were determined:

– % induction = [number of induced microspores / number of cultured microspores] x 100

– % calli/embryos produced = [number of embryos / number of induced microspores] x 100

– % haploid plantlets produced = [number of haploid plantlets / number of embryos] x 100.

For each stage, three replicates per experiment were used with about 24 spikes per replicate. All the experimental data were subjected to analysis of variance (ANOVA) using SPSS statistical software 16.0 (SPSS for Windows, 2007, Chicago, USA). In figure, each graph bar represents mean ± standard error (SE). The statistical significance was tested using Tukey test at a 5% level of probability.

Results and discussion

Three haplomethods (isolated microspore culture, gynogenesis and intergeneric hybridization) were compared for their efficiency in regenerating Razzek haploid plants. ANOVA revealed a significant ($P < 0.01$) differential response for induction and haploid plantlet regeneration with the three techniques, but there was no significant difference in callus/embryo formation (Table 1).

Factors	df	% of induction	% of calli/embryos	% of regenerated plants
Haplomethods	2	36.01^{**}	1.45^{ns}	11.96^{**}
R^2		0.89	0.10	0.73
$^{ns}P > 0.05$; $^{*}P < 0.05$; $^{**}P < 0.01$				

Table 1. Analysis of variance of the three haplomethods (isolated microspore culture, gynogenesis and durum wheat x maize cross) of durum wheat.

This study showed that the best rate of induction (76.7%) was obtained with intergeneric hybridization, followed by gynogenesis (39.9%) and isolated microspore culture (10.3%) (Fig. 1a). Several authors have reported good induction rates in bread and durum wheat for the maize method and unpollinated ovary culture [5, 12, 17, 24, 28]. The low rate of induction using isolated microspore culture might be explained by the decrease in their viability during culture. Only microspores with fibrillar cytoplasms (Fig. 2a) can be embryogenic and evolve into pre-embryos. Compared with the other techniques, microspore culture seems to be more influenced by mother plants, growth conditions [20] and, in particular, the pre-treatment [13, 25].

There were no significant differences in callus/embryo development among the three methods (Table 1). For isolated microspore culture, the rate was 48.3%, followed by 36.3% for gynogenesis and 24.3% for intergeneric hybridization (Fig. 1b). In the durum wheat, isolated microspore culture generated embryos directly (Fig. 2d). During embryogenesis, some of the pre-embryo remains became trapped in the pollen wall and were unable to break the exine (Fig. 2b, c) and produce a mature embryo (Fig. 2d). In intergeneric hybridization, 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 (Fig. 4a, b). Most of the embryos abort during the initial stage of development. This lack of zygote viability affects the conversion of embryos into plantlets. In addition, the ability to form haploid embryos might depend on the application of osmotic stress and hormone treatment, alone or in combination [1]. The gynogenesis method produced two types of callus structures: regenerable calli (i.e. friable; Fig. 3b) and calli with no embryogenic development. The non-regenerable calli were not very differentiated and were compact, and eventually became necrotic or produced roots without embryogenesis.

The successful production of Razzek haploid plantlets using unpollinated ovary culture (26.1%) and the maize method (20.0%) was compared with isolated microspore culture (11.9%) (Fig. 1c). All plants regenerated by the first two methods were green (Fig. 3c and 4c), whereas those obtained with isolated microspore culture were albino for this genotype (Fig. 2e). With regard to androgenesis, durum wheat is a recalcitrant species, with a low regeneration rate and a high frequency of albino plants [7].

This was the first study to compare isolated microspore culture, gynogenesis and intergeneric hybridization. Overall, our results showed that each technique 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 pre-treatment 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. These structures have been observed in gynogenesis [18]. Microspore culture also takes less time (about 6 weeks) than maize pollination (about 12 weeks) or gynogenesis (about 16 weeks) to produce haploid plants. Further work is needed, however, to improve durum wheat green plants regeneration. Gynogenesis was an efficient method for producing Razzek haploid plants. It has the advantage that all regenerated plants are green. The albinism problem has not arisen in a wide range of genotypes tested [17, 21, 22, 24]. Gynogenesis is also very interesting in terms of the number of gametophytic cells cultivated and the number of plant regenerated. A single ovary per floret was used in gynogenesis, whereas a greater number of haploid microspores per flower was used in isolated microspore culture that showed a lower rate of plant regeneration and albinism

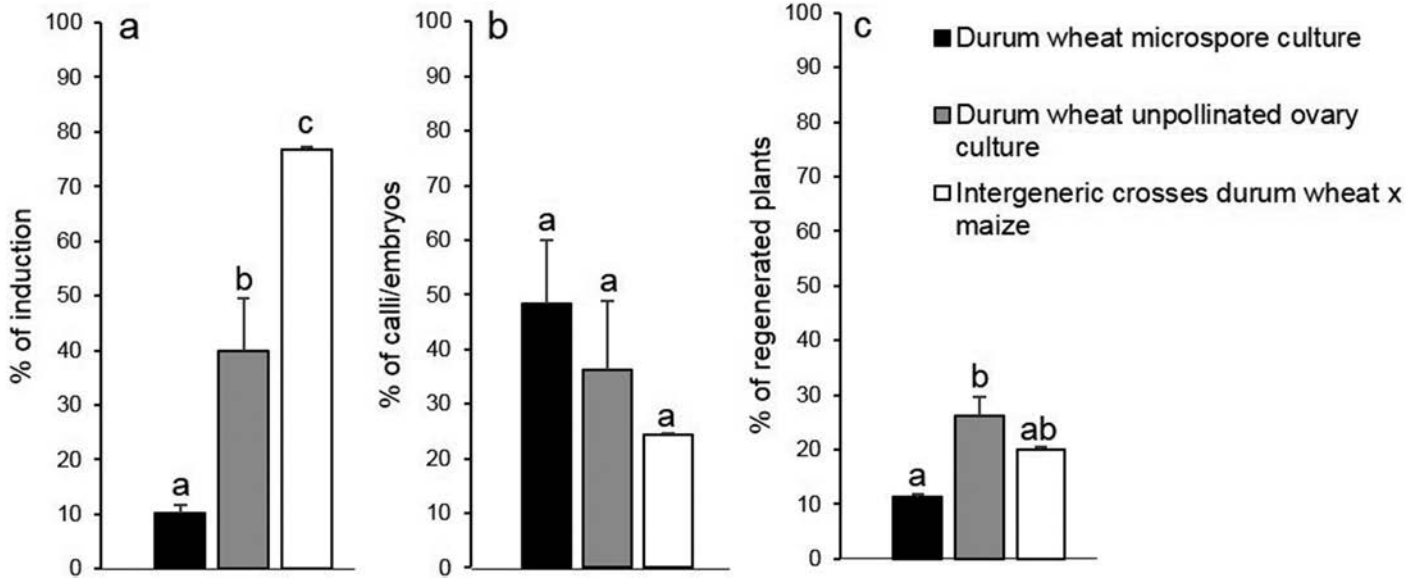

Fig. 1. Percentage of induction (a), regenerable calli/embryos (b) and regenerated plants (c) of Razzek durum wheat obtained by isolated microspore culture, gynogenesis and durum wheat × maize crosses Graph bars (mean ± SE) with the same letter are not significantly different ($P < 0.05$; Tukey-test).

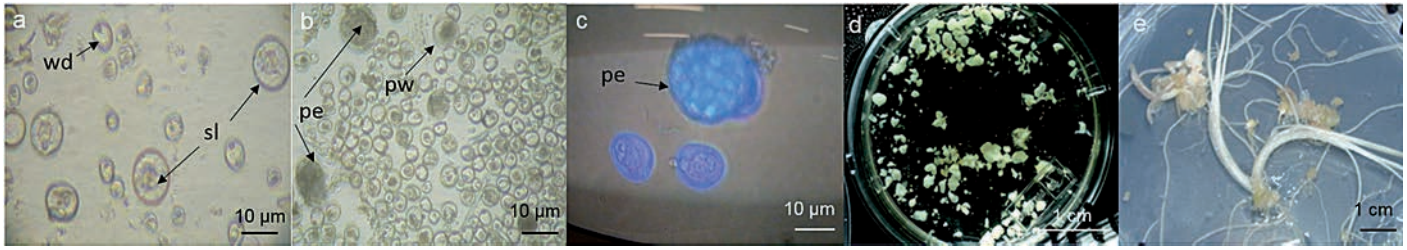

Fig. 2. Isolated microspore culture process: (a) Induction at 7 days of culture: embryogenic microspore with fibrillar structure (fs), microspore without development (wd), (b) Pre-embryos derived from microspores at 10 days: pre-embryos beginning to break the exine (pe), pre-embryos trapped in the pollen wall (pw), (c) Blue fluorescence of pre-embryo (stained with DAPI) emerging from the exine (pe), (d) Mature embryos obtained after 28 days of culture, (e) Regenerated albino plantlets.

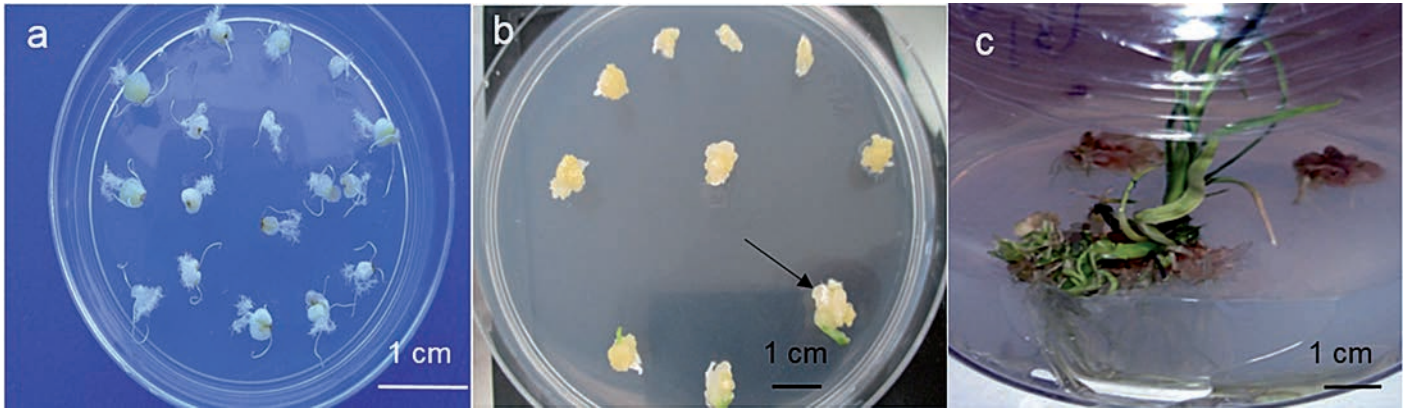

Fig. 3. Gynogenesis process: (a) Unpollinated ovaries induced after 1 week of culture, (b) Callus with green shoot after 10 weeks of culture, (c) Regenerated green plantlets.

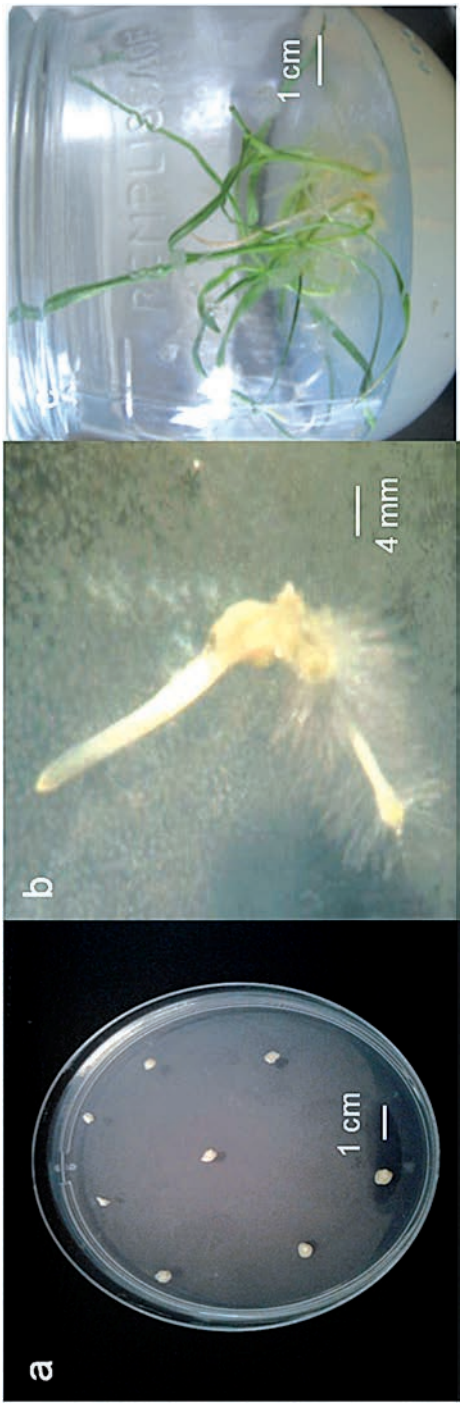

Fig. 4. Intergeneric hybridization process: (a) Embryo rescue in solid medium culture, (b) Embryo after 7 days of culture, (c) Regenerated green plantlets.

problem. In addition, the formation of calli offers the possibility of obtaining gametoclonal variation under selection pressure, which could be exploited in durum wheat breeding programs. Currently, however, gynogenesis is less frequently used to regenerate haploid plants of durum wheat than other methods. The intergeneric maize x durum wheat hybridization method also proved to be efficient in producing Razzek green haploid plants. More haploid plantlets of durum wheat were regenerated using maize method than has been reported in previous studies [5, 29]. Our results suggest that the ability of intergeneric hybridization and gynogenesis methods to avoid albinism might be controlled by cytoplasmic factors.

Overall, the present investigation showed that the three methods were all efficient in regenerating homozygous lines, with gynogenesis and intergeneric hybridization proving to be the most efficient. The production of such pure lines offers significant potential in wheat breeding programs.

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