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RESEARCH ARTICLE

Use of nurse endosperm for the culture of haploid embryos produced by durum wheat x maize crosses

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ABSTRACT

The use of doubled-haploids in plant breeding programs enables accelerating the release of new varieties adapted to climate change. The durum wheat x maize crosses technique is a method of choice for producing durum wheat haploid plants. The haploid embryos produced by this method develop without albumen and their survival is ensured by post-pollination hormonal treatments. In this study, nine post-pollination treatments with 2,4-Dichlorophenoxyacetic acid (2,4-D), Picloram and Dicamba at the concentrations of 10, 50 and 100 mg.L^{-1} were applied to 7 durum wheat genotypes. The effects of genotype and post-pollination treatment on durum wheat haploid embryos produced by durum wheat x maize crosses and the use of the endosperm nursing technique for haploid plantlets regeneration were investigated. The haploid induction parameters varied with the durum wheat genotypes as well as the post-pollination treatments. The phenomenon of polyembryony resulting from durum wheat x maize crosses is reported for the first time in this article. The durum wheat genotypes showed different abilities to produce monoembryo and polyembryos. The post-pollination treatments with 2,4-D (10 mg.L^{-1}) and Picloram (10 and 100 mg.L^{-1}) gave a higher embryo formation frequency than the treatments with Dicamba. The embryo conversion to plantlet was greatly improved, especially in recalcitrant genotypes using the durum wheat endosperm as supplemental nourishment in combination with the Gamborg B5 regeneration medium.

Keywords: Durum wheat; Haploid embryo; Maize; Nurse Endosperm Technique; Polyembryony

INTRODUCTION

In cereal crops, the release of new varieties by conventional methods remains a relatively slow process since breeding in a self-pollinated species is time-consuming, as it is based on several cycles of inbreeding and selection for obtaining an acceptable level of homozygosity. The doubled haploid technology is used to produce in a short time fully homozygous lines from segregating material (Tuvesson et al., 2000; Yan et al., 2017; Devaux, 2021). The use of doubled-haploid technology may increase the efficiency of breeding programs (Schaffer et al., 1979; Knox et al., 2000; Jauhar et al., 2009). Doubled haploids are also used in molecular mapping, genetic studies, and mutation-selection (Falak et al., 1999; Cornish et al., 2001; Jauhar et al., 2009).

Durum doubled-haploids can be produced either by anther/microspore culture or by wheat x maize crosses. In *in vitro* androgenesis, doubled haploid production for breeding purposes is limited by albinism (Almouslem et al., 1998; Holme et al., 1999; Cistué et al., 2009) and genotype dependency (Tuvesson et al., 2000; Lazaridou et al., 2016; Bokore et al., 2017). Although durum wheat is a recalcitrant species for anther and microspore culture, some research had made it possible to produce durum wheat haploids, using durum wheat x maize crosses (Chlyah et al., 1999; Bouatrous et al., 2010; Slama-Ayed et al., 2010). The durum wheat x maize system is preferred because of a low frequency of albinism and less genotype dependency than *in vitro* androgenesis (Kisana et al., 1993; Chlyah et al., 1999; Sadasivaiah et al., 1999; Campbell et al., 2000; Knox et al., 2000; Ushiyama et al., 2007; Jauhar et al., 2009).

To produce haploids through the wheat x maize system, three types of phytohormones were usually used after pollination of wheat spikes with maize pollen. These hormones are 2,4-Dichlorophenoxy acetic acid (2,4-D),

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Picloram, and Dicamba. 2,4-D has been frequently applied as post pollination treatment to obtain haploid plants in cereals (Kisana et al., 1993; Campbell et al., 2000; Jauhar et al., 2009). Wedzony et al. (1998) reported that the hormones Picloram and Dicamba stimulate the growth and development of embryos. More than one embryo in a single caryopsis is found in the course of haploid production by wheat x maize crosses. This phenomenon is called polyembryony and there is a lack of knowledge about it. Besides, there are no reports on polyembryony in durum wheat x maize crosses. The polyembryony added extra advantage to obtain an increased number of haploid plants. Hexaploid wheat cultivars show different abilities to produce twin embryos produced by wheat x maize crosses (Linacero et al., 1996 and Khan et al., 2004).

However, the ability of durum wheat genotypes to produce haploid polyembryos is poorly understood. In addition, the regeneration of embryos from durum wheat x maize crosses is challenging because durum wheat is a recalcitrant species for *in vitro* culture. Germination of these embryos in artificial culture media is low, and therefore their conversion to plantlets is not satisfactory (Almouslem et al., 1998; Jauhar, 2003; Devaux, 2021). The embryo germination can be enhanced by using durum wheat and maize genotypes that are efficient in haploid embryo production and choosing appropriate culture media.

Therefore, the objective of this study is to examine the effect of durum wheat genotypes and post-pollination treatments on polyembryony in durum wheat x maize crosses and to improve the recovery of green haploid plantlets from recalcitrant genotypes.

MATERIALS AND METHODS

Plant material

Durum wheat

Seven genotypes of durum wheat (Triticum durum, 2n= 4x= 28): 'Mahmoudi', 'Agili', 'Salim', 'INRAT 100', 'LSA1', 'Nasr' and 'Razzek' were used for the study. Sowing was carried out in the field of the National Institute of Agronomic Research of Tunisia. The fertilization of the plants consisted of 6.7 g m^{-2} of phosphorus in the form of triple superphosphate before sowing and 8 g m^{-2} of nitrogen in the form of ammonium nitrate: 4 g m^{-2} after the emergence stage and 4 g m^{-2} at the tillering stage. Weed control was chemically carried out with Granstar (15 g ha^{-1}) at the seedling stage.

Maize

A local population of maize harvested in the region of Beja (northern Tunisia) was used as the male parent. Maize was sown weekly in 20 cm peat-filled pots to match the emergence of durum wheat ears with maize flowering.

Emasculation and pollination

Manual emasculation of the ears was done during the appropriate stage (when they have fully emerged from the flag leaf), before anthesis, when the anthers are green. The upper part of the spikelet was carefully cut off. Anthers were then gently removed with fine tweezers. The emasculated spikes were covered with glassine bags to prevent unwanted pollination. With a brush, pollen was carefully applied to the stigmas of the emasculated florets. Within two days after emasculation, the receptive stigmas were pollinated with freshly collected maize pollen. Pollination was performed in the morning from 9 to 10 am. The pollinated ears were then covered with glassine bags.

Post-pollination treatments

Pollinated ears were treated twenty-four hours after pollination with 120 mg l^{-1} $AgNO_3$ combined with the following hormonal treatments:

T1= 10 mg l^{-1} 2,4-D; T2= 50 mg l^{-1} 2,4-D; T3= 100 mg l^{-1} 2,4-D; T4= 10 mg l^{-1} Picloram, T5= 50 mg l^{-1} Picloram; T6= 100 mg l^{-1} Picloram; T7= 10 mg l^{-1} Dicamba; T8= 50 mg l^{-1} Dicamba; T9= 100 mg l^{-1} Dicamba.

Treatments were conducted by dipping the spikes in the hormonal solution.

Embryo rescue

The spikes that contained well-developed caryopses were harvested 14 to 16 days after pollination and refrigerated at 4°C for 24 hours. Cold treatments improve the success of embryo rescue by breaking any dormancy. Caryopsis containing haploid embryos were sterilized with a 2% sodium hypochlorite solution for 15 minutes, and then rinsed three times with sterile distilled water under a laminar flow hood. Under aseptic conditions, embryos were dissected from the caryopses and cultured on hormone-free Gamborg B5 medium (Gamborg et al., 1986) supplemented with 30 g l^{-1} of sucrose and 8 g l^{-1} of agarose in Petri dishes (100 mm x 15 mm). Haploid embryos were also cultured using a nurse endosperm of durum wheat. A milky endosperm from a normal durum wheat caryopsis was used as supplemental nourishment in combination with a Gamborg B5 medium. A 14-16 day old caryopsis containing a milky endosperm was sterilized as described above, cleared of its embryo, and placed on the surface of the Gamborg B5 medium. Then, a haploid embryo was placed in the endosperm. The cultured embryos were incubated for 4-6 weeks in the dark at 25°C. As soon as coleoptiles and primary roots appeared, the embryos were transferred to an illuminated incubator

maintained at 25 °C, with a 16 h light regime, until they develop into green plantlets.

Measured parameters

The data on caryopsis formation frequency (number of caryopses obtained/100 durum wheat florets), embryo formation frequency (number of embryos/100 caryopses), monoembryo formation frequency (number of monoembryos/100 caryopses), polyembryos formation frequency (number of polyembryos/100 caryopses), haploid regeneration frequency (number of green haploid plantlets/100 cultured embryos).

Statistical analysis

The data were analyzed by two-way analysis of variance (ANOVA) to evaluate the genotype and post-pollination effects, and means were compared by Fisher's least significant difference (LSD). Statistical analysis was performed using the MSTAT-C software (MSTAT-C 1990).

RESULTS

The pollination of durum wheat genotypes with the maize pollen and subsequent post-pollination treatments have allowed the formation of caryopses containing haploid embryos (Fig. 1d). A phenomenon of polyembryony was observed during the dissection of the embryos from caryopses. Indeed, one, two, and even three twin embryos were observed in one caryopsis (Fig. 2).

The statistical analysis revealed that the effects of the genotype and the post pollination treatments were significant for mono and polyembryos formation at $P < 0.001$ (Table 1).

Genotype effect on haploid embryo formation

The frequency of embryo formation of 9 durum wheat genotypes pollinated with maize is presented in Table 2. The frequency of monoembryo ranged from 3.3 to 35.5/100 caryopses depending on the genotype, while the overall average was 11.4 monoembryo/100 caryopses. The values of polyembryos formation frequency ranged from 3.1 polyembryos/100 caryopses in 'Jawda' genotype to 33.6 haploid polyembryos/100 caryopses in 'Maali' genotype with an average of 7.9 polyembryos/100 caryopses. The genotypes 'Nasr', 'Khiar', and 'Razzak' did not produce polyembryos. The overall average frequency of monoembryo was much higher than the overall frequency of polyembryos (Table 2).

Effect of post-pollination treatments on mono and polyembryony

The nine post-pollination treatments had a significant effect on haploid embryo formation (Table 3). The monoembryo formation frequency ranged from 6.3 to 28.0 monoembryo/100 caryopses. The Polyembryos formation frequency values ranged from 1.3 to 21 haploid polyembryos/100 caryopses. The hormone treatments T1 (10 mg l^{-1} 2,4-D), T4 (10 mg l^{-1} Picloram), and T6 (100 mg l^{-1} Picloram) were the most favorable for monoembryo and polyembryos formation. The treatment with Dicamba (100 mg l^{-1}) showed the lowest production of polyembryos.

Table 1: Statistical analysis of the caryopsis formation and the mono and polyembryos formation for 9 durum wheat genotypes and 9 post-pollination treatments by the tow-way ANOVA

Source of variance	df	Mean squares	
		Monoembryo formation frequency	Polyembryos formation frequency
Replication	2	3.16	7.56
Genotype	8	983.35	489.29***
Treatment	8	644.90	698.84***
Genotype x Treatment	64	112.31	127.53
Error	242	83.55	73.87

*** The values significantly differ at P<0.001

Fig 1. Caryopses formation in wheat x maize crosses, a) mature maize tassel, b) fresh collect maize pollen, c) pollination of emasculated durum wheat spike, d) seed setting (arrows).

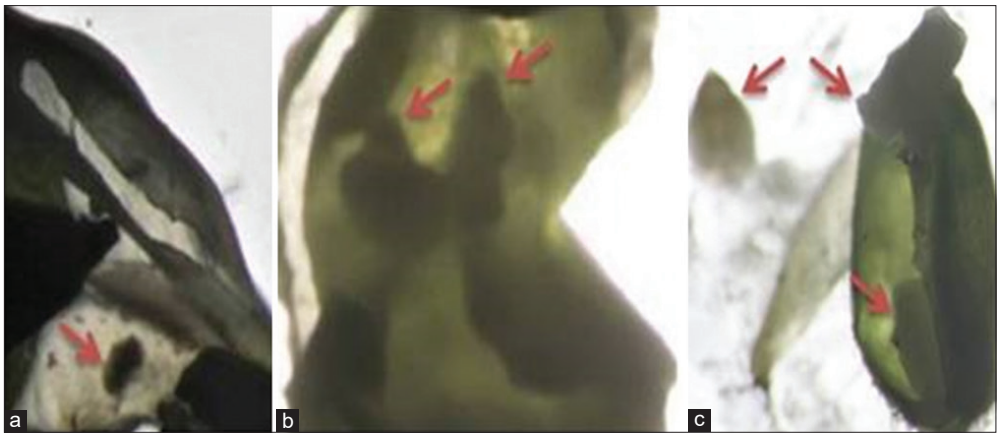

Fig 2. Polyembryony in durum wheat x maize crosses. Caryopses with 1,2, and 3 embryos (arrows).

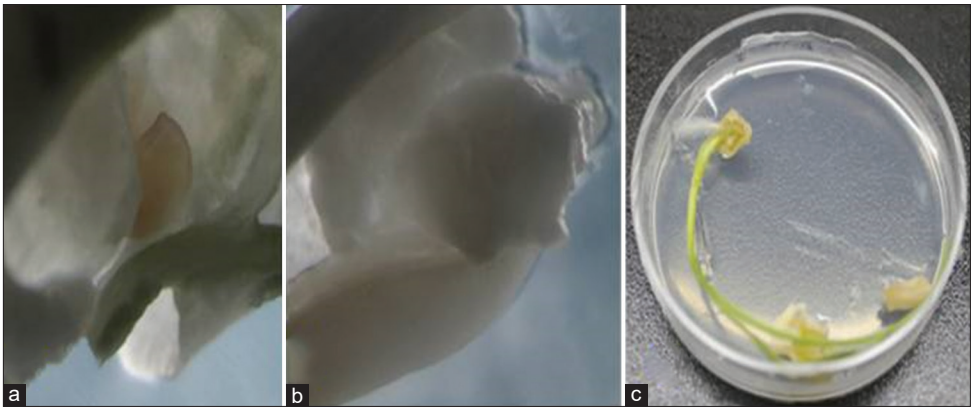

Fig 3. Nurse endosperm technique. a) Haploid embryo inserted into durum wheat endospermplaced on the surface of Gamborg B5 medium; b) Embryo elongation after 5-7 days of culture; c) Plant developing from cultured embryo after 3 weeks of culture.

Table 2: Genotype effect on mono and polyembryos formation through durum wheat x maize crosses

Genotype	Total number of caryopsis	Total number of embryos	Monoembryo formation frequency	Monoembryo over total embryos	Polyembyos formation frequency	Polyembryos over total embryos
Karim	567	103	14.8^{b}	81.6	3.3^{c}	18.4
Maali	107	74	35.5^{a}	51.3	33.6^{a}	48.6
Jawda	385	37	10.5^{b}	89.2	3.1^{c}	10.8
INRAT100	134	46	11.7^{b}	34.8	22.4^{b}	65.2
Salim	746	58	6.1^{c}	79.3	4.6^{c}	20.7
LSA4	136	81	11.0^{b}	71.4	4.4^{c}	28.6
Nasr	555	28	5.0^{c}	100.0	0^{d}	0
Khiar	299	11	4.6^{c}	100.0	0^{d}	0
Razzak	302	7	3.3^{c}	100.0	0^{d}	0
Overall mean			11.4	78.6	7.9	21.4

Values having same letter(s) are statistically identical

Endosperm nursing technique for haploid embryo rescue

The small haploid embryos were difficult to grow on the Gamborg B5 regeneration medium. The nurse endosperm was used as additional nourishment to enhance the conversion of embryos to haploid plantlets (Fig. 3).

The statistical analysis revealed that the effect of the genotype was highly significant for the plantlet's regeneration on Gamborg B5 regeneration medium and Gamborg B5 regeneration medium + nurse endosperm of durum wheat at $P<0.001$ (Table 4). Regenerated plantlets frequency values on the Gamborg B5 regeneration medium + nurse endosperm ranged from 12% for the genotype 'LSA4' to 40% for the genotype 'Khiar' with an average of 25.1% (Table 3). Embryos of recalcitrant genotypes 'Nasr', 'Salim', and 'LSA4' failed to germinate on the Gamborg B5 regeneration medium (Table 5). In these recalcitrant cases, the embryos were successfully rescued using nurse endosperm. The regeneration frequencies of

Table 3: Post pollination treatment effect on polyembryos formation through durum wheat x maize hybridization

Post-pollination Treatment	Total number of embryos	Monoembryo formation frequency	Polyembryos formation frequency
T1	48	16.5[b]	14.1[c]
T2	54	4.1[d]	2.4[f]
T3	17	8.6[c]	2.3[f]
T4	41	28[a]	15.5[b]
T5	64	4.5[d]	3.4[e]
T6	110	25.4[a]	21[a]
T7	43	4.5[d]	8.3[d]
T8	46	4.6[d]	3.1[f]
T9	22	6.3[c]	1.3[g]

Values having same letter(s) are statistically identical

Table 4: Statistical analysis of the plantlets regeneration frequency for 7 durum wheat genotypes by the one-way ANOVA

Source of variance	df	Mean squares	
		Plantlets regeneration on Gamborg B5 medium	Plantlets regeneration on Gamborg B5 medium + nurse endosperm
Replication	4	22.27	16.23
Genotype	6	218.97 ***	468.51 ***
Error	24	68.44	41.80

*** The values significantly differ at P<0.001

Table 5: Plantlets regeneration on Gamborg B5 medium alone and Gamborg B5 medium supplemented with nurse endosperm of durum wheat

Genotype	Plantlets regeneration frequency	
	Gamborg B5 medium	Gamborg B5 medium + nurse endosperm
Maali	21.6[a]	38.0[a]
Khiar	20.10[a]	40.0[a]
Razzak	7.0[b]	15.0[c]
Nasr	0.0[c]	27.0[b]
Salim	0.0[c]	14.0[c]
LSA1	0.0[c]	12.0[c]
INRAT 100	8.2[b]	29.5[b]
Overall mean	8.1	25.1

Values having same letter(s) are statistically identical

the recalcitrant genotypes 'Nasr', 'Salim', and 'LSA4' were 27, 14, and 12%, respectively. The regeneration frequencies obtained on Gamborg B5 medium supplemented with nurse endosperm were significantly higher than those obtained on Gamborg B5 medium alone.

DISCUSSION

The production of haploid durum wheat plants by durum x maize crosses depends significantly on the formation of embryos that can develop into haploid plants. In this study, embryo formation was influenced by the durum wheat genotype and post-pollination treatments. Embryo formation rates were relatively low (0 to 12.9%) compared to much higher rates obtained in bread wheat, a hexaploid species (Bains et al., 1995; Verma, 1999; Devaux, 2021). Nevertheless, the rates of haploid embryos obtained in this study were close to the rates typically obtained by crossing tetraploid durum wheat with maize (Almouslem et al., 1998; Cherkaoui et al., 2000) and a wild perennial grass *Imperata cylindrica* (Chaudhary et al., 2000; Mahato et al., 2015).

Differences in haploid embryo formation were observed in durum wheat genotypes. This confirms previous studies that have shown differences in the number of haploid embryos between durum wheat genotypes (Martins-Lopes et al., 2001; García-Llamas et al., 2004; Niroula and Thapa, 2009). During the process of excision of the haploid embryos, a polyembryos formation was observed. Only 3 genotypes ('Agili', 'INRAT 100', and 'Salim') of the 7 tested produced polyembryos. Therefore, the phenomenon of polyembryony resulting from wheat x maize crosses appears to be genotype dependent. The polyembryony has significant potential that could be used to improve the production of durum wheat haploid plants using durum wheat x maize crosses. As far as we know, the phenomenon of polyembryony resulting from durum wheat x maize hybridization has not been reported before. In bread wheat, the formation of polyembryos resulting from wheat x maize crosses is well documented. Linacero et al. (1996) reported that the genotype 'Thatcher' produced 20% polyembryos when crossed with maize, but the genotype 'Chris' produced only 7% and 'Dollar' produced no polyembryos.

In the absence of nurse endosperm, haploid embryos from durum wheat x maize crosses cannot survive without growth hormone treatment. Post-pollination treatments are the most significant factor influencing embryo formation and growth (Cherkaoui et al., 2000; Jauhar, 2003). In our study, the treatment with 10 mg l^{-1} of 2,4-D was highly favorable for embryo formation. This result confirms the beneficial effect of 2,4-D auxin on stimulation of the development and the increase in embryo size in wheat x maize crosses, reported in previous studies (Zhang et al., 1996; García-Llamas et al., 2004; Niroula et al., 2007). The treatment with 100 mg l^{-1} of Picloram was very favorable for embryo formation and this is in agreement with the results of He et al. (2010). Dicamba auxin has also been used for haploid production in triticale (Wedzony et al., 1998). Treatments with 2,4-D, Dicamba, and Picloram, used in this study, would cause the formation of polyembryos. Interspecific pollination with maize pollen could also induce the formation of haploid twin embryos. However, it is not possible to test either of these two hypotheses because pollination with maize pollen was necessary to obtain haploid embryos of durum wheat and post-

pollination treatments were mandatory for embryo growth. As a result, experiments using only one of these factors are not feasible.

Embryo germination on the Gamborg B5 regeneration medium and conversion of embryos to haploid plantlets were low although genotypic variability was observed. Small embryos (less than 1 mm) could not germinate on the Gamborg B5 medium because their nutritional requirements were not met. In addition, embryos from durum wheat x maize crosses are difficult to germinate due to the recalcitrance of durum wheat to *in vitro* culture (Savaskan et al., 1997; Jauhar, 2003; Chaudhary et al., 2015; Ltifi et al., 2018). To overcome this difficulty, we used the nurse endosperm to stimulate embryo growth.

In the present research, we showed that the technique of culturing embryos in durum wheat endosperm has greatly improved the germination of haploid embryos in the experienced genotypes. The addition of durum wheat endosperm to the Gamborg B5 regeneration medium was instrumental in the conversion of embryos to plantlets, especially in the recalcitrant genotypes 'Salim', 'LSA2', and 'Maali'. The embryo culture using nurse endosperm was a key factor in circumventing recalcitrance in durum wheat. The endosperm provided natural nutrition conditions for the embryos and consequently reduced the chances of their abortion. Indeed, the embryo fed on the endosperm and possibly also on the Gamborg B5 medium on which the endosperm is placed. Thus, the chances of germination and growth of the embryo were increased. In other species, the nurse endosperm technique has been successfully used for embryo rescue in rye (Aydin et al., 2016; Zimny and Michalski, 2019), Hordeum x Triticum hybrids (Kruse, 1974), and forage legumes (Williams, 1980; Williams and Williams, 1983).

CONCLUSION

This research has highlighted the importance of durum wheat genotype and post-pollination treatments in the formation of haploid embryos via durum wheat x maize hybridization. This is the first study, as far as we know, reporting the phenomenon of polyembryony resulting from durum wheat x maize crosses. Haploid embryos that failed to germinate on the Gamborg B5 medium were successfully rescued using the nurse endosperm technique.

Authors's contributions

Anissa Sahli: Carried out the experiment, analyzed the data and wrote the first draft. Sonia Mansouri: performed the statistical analysis, wrote and edited the paper with input from all authors. Faouzi Haouala: performed the statistical analysis, wrote and edited the paper with input from all authors. Ali Ltifi supervised, reviewed and edited the manuscript.

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