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Study of Some Factors Influencing the Production of Durum Wheat Haploids by Cross Wheat x Maize

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Abstract:** Crosses were carried out in Tunisia between seven genotypes of durum wheat used as female parent and a local population of maize as a male parent. Spikes were treated 24 h after pollination with different hormonal solutions containing 2,4-Dichlorophenoxyacetic acid (2,4-D), gibberellin (GA_3) or silver nitrate ($AgNO_3$). The production of haploid embryos was made using embryo rescue technique. Spikes were castrated manually or not castrated and then pollinated early before anthesis. Fruit setting, embryos formation, and regeneration of haploid plants were studied. The parental varieties 'Mahmoudi' and 'Khiar' gave the highest fruit setting rates 54.3 and 53.1%, respectively. The embryo rates were variable for parents and hybrids. The line 'LSA2' gave the highest regeneration rate (90%). The post-pollination treatment using 10 mg/l 2,4-D was the most favorable for fruit setting, embryos formation and haploid plant regeneration. The post-pollination treatment by spraying spikes by the hormonal solution was more favorable for fruit setting and embryos formation than soaking them in this solution.
***Keywords:** Triticum durum; Zea mays; embryo formation; haploid; pollination; regeneration.*

1. Introduction

Durum wheat (*Triticum durum* Desf. 2n = 4x = 28 = AABB) is decidedly an important cereal for human consumption. Traditional methods of improving this self-pollinating species involve the introduction of plant material, hybridization, selection of desired plants, and evaluation. This expensive process can take up to 12 years to reach a desirable level of homozygosity (Campbell *et al*., 2000). The production of doubled haploids saves a lot of time by reducing the time to obtain homozygosity from 3 to 4 years compared to conventional improvement methods (Jauhar *et al*., 2009; DePauw *et al*., 2011).

The method of production of doubled haploids is currently used as a routine technique to generate an improved homozygous material and new varieties of species grown in several countries of the world. Several hundred varieties of cereals produced by androgenesis or gynogenesis have been registered during the last years (Cupta, 1999; Singh *et al*., 2016).

Durum wheat haploids can be obtained by several techniques including the cross system wheat x maize, anther culture and isolated microspore culture. Durum wheat is a recalcitrant species to anther and microspore cultures (Jauhar, 2003; Cistué *et al*., 2009; Bokore *et al*., 2016; Lazaridou *et al*., 2016). In addition, *in vitro* androgenesis is confronted to the problem of albinism and genotypic dependence (Labbani *et al*., 2006). Some genotypes do not respond to the *in vitro* androgenesis and are considered recalcitrant. Even for genotypes having a good response, most of the regenerated plants are albina and have no interest. The cross system wheat x maize is considered to be a preferred method for producing durum wheat haploids because of a low albinism frequency and a better genotypic response compared to *in vitro* androgenesis (Knox *et al*., 2000; Jauhar, 2009).

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The objective of this research was to study the effects of post-pollination treatments, the method of application of these treatments and the castration of spikes on the production of durum wheat haploids by cross wheat x maize.

2. Materials and Methods

2.1. Plant material

Durum wheat: The plant material used in this study consists of 3 varieties: 'Karim', 'Khiar', 'Mahmoudi', from the INRAT (National Institute of Agronomic Research of Tunis – Tunisia) durum wheat breeding program, a line 'LSA2' provided by « The State Plant Breeding Institute Hohenheim, Germany » as well as the hybrids 'Khiar x Mahmoudi', 'Karim x LSA2' and 'Khiar x LSA2'. Sowing was carried out at the INRAT field in Tunis on November 3, 2016. Fertilization consisted of 67 units of P_2O_5 in the form of triple superphosphate before sowing and 80 units of nitrogen in the form of ammonium nitrate in two equal amounts, one at the emergence and the other at the tillering. Weed control was done chemically using Granstar at the seedling stage.

Maize: A local population of maize harvested in Béja region (northwest of Tunisia) has been used as a male parent. Maize was sown under plastic house in 20 cm peat-filled pots. Sowing was realized weekly from December, 2016 to January, 2017 to coincide wheat heading with maize flowering.

2.2. Castration and pollination

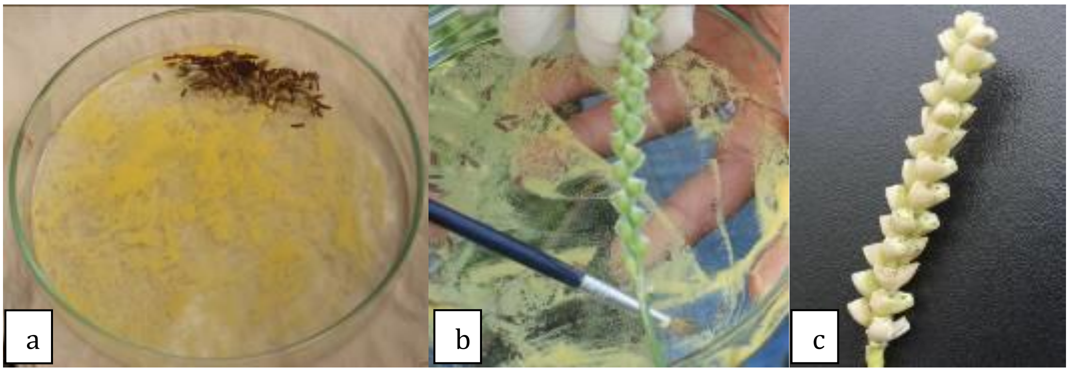
The manual castration of durum wheat spikes was just made at their exit from the sheath. The upper third of spikelets were cut with fine forceps and anthers still green were extracted. 24 hours after castration, receptive stigmas were pollinated with freshly harvested maize pollen. Pollination was done between 9 and 10 am.

2.3. Post-pollination treatment

Spikes were treated 24 h after pollination with different hormonal solutions containing 1% of Tween 20.

T1= 10 mg/l 2,4-D
T2= 10 mg/l 2,4-D + 75 mg/l GA_3
T3= 10 mg/l 2,4-D + 120 mg/l $AgNO_3$
T4= 10 mg/l 2,4-D + 180 mg/l $AgNO_3$
Treatments were done by spraying or soaking spikes in the hormonal solution.

2.4. Embryo Rescue



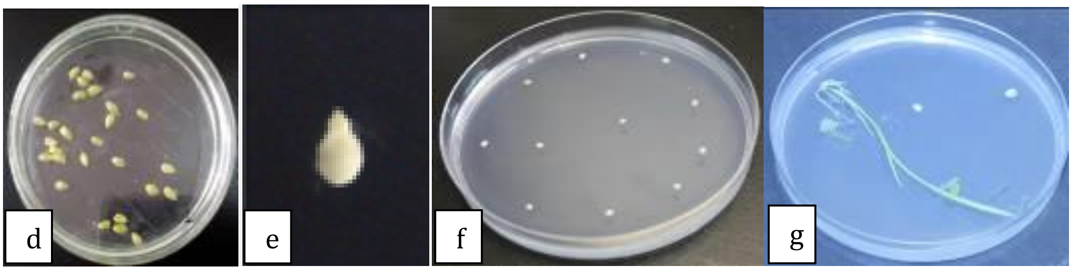


Figure 1. Production haploid plants of durum wheat through wheat x maize crosses. a) collecting maize pollen, b) pollination of a durum wheat spike, c) spike obtained from wheat x maize crosses 15 days after pollination, d) caryopses formed 15 days after pollination, e) haploid embryo of durum wheat, f) haploid embryos cultured on B5 medium, g) haploid green seedling.

Spikes containing caryopses were harvested 15 to 16 days after pollination (Figure 1c). Caryopses were sterilized with 2% sodium hypochlorite solution for 15 minutes, then rinsed 3 times with sterile distilled water under a laminar flow hood.
Haploid embryos were mined under aseptic conditions and cultured in 100 mm x 15 mm Petri dishes containing the B5 culture medium (Gamborg *et al.*, 1968) supplemented with 30 g/l sucrose and 8 mg/l agar (Figure 1f) .
Embryos were placed in dark for 4 to 6 weeks. As soon as coleoptiles and primary roots appeared, the embryos were transferred to light in an incubator set at 25°C for 4 to 6 weeks with a photoperiod of 16 h until they developed into green seedlings (Figure 1g).

3. Results

3.1. Genotype effect on crosses durum wheat x maize

Average rates of fruit set, of formed embryos and of regenerated plants of parental varieties and F1 hybrids are presented in Table 1. The parental varieties 'Mahmoudi' and 'Khiar' gave the highest setting rates, while the line 'LSA2' gave the lowest rate. The hybrid 'Khiar x Mahmoudi' had a fruit set rate lower than those of parents whereas for the hybrid 'Karim x LSA2', this parameter was higher than the parent's ones. The hybrid 'Khiar x LSA2' had an intermediate fruit set rate between the two parent's ones (42%).
The average embryo rate was variable for parents and hybrids. The parental varieties 'Mahmoudi' and 'LSA2' gave an embryo rate of 90% followed by the varieties 'Khiar' and 'Karim' (63.3 and 25.5%, respectively). With the exception of the hybrid 'Khiar x LSA2', the embryo rates of hybrids were intermediate between the parent's ones.
The regeneration rate was also variable. The line 'LSA2' gave the highest regeneration rate (90%) followed by the varieties 'Karim' (34.1%) and 'Khiar' (31.0%). The hybrid 'Khiar x Mahmoudi' transgressed his both parents and gave a regeneration rate of 53.1%.

Table 1. Rates of fruit set, of embryos and of haploid plants obtained by cross wheat x maize for 4 varieties of durum wheat and their hybrids F1.

Genotype	Fruit set rate	Embryo rate	Regeneration rate
Khiar	53.1 a	63.3 b	31.0 d
Mahmoudi	54.3 a	90.0 a	45.0 bc
Karim	32.0 bc	25.5 c	34.1 d

LSA2	15.9 d	90.0 a	90.0 a
Khiar x Mahmoudi	28.4 cd	66.1 b	53.1 b
Karim x LSA2	36.7 bc	34.1 c	33.4 d
Khiar x LSA2	42.0 ab	22.0 c	40.0 cd

Averages followed by the same letter are not significantly different.

3.2. Effect of post-pollination treatment on crosses durum wheat x maize

The 4 post-pollination treatments had significant effects on rates of fruit set, haploid embryos and regenerated plants. The treatment T1 (10 mg/l 2,4-D) was the most favorable for fruit setting, embryos formation and haploid plant regeneration (Table 2). Treatments T2 (10 mg/l 2,4-D + 75 mg/l GA_3) and T3 (10 mg/l 2,4-D + 120 mg/l $AgNO_3$) were less favorable than the treatment T1 and gave rates of embryos and of plant regeneration not significantly different. The treatment T4 (10 mg/l 2,4-D + 180 mg/l $AgNO_3$) was the least favorable for fruit set, embryos formation and plant regeneration.

Table 2. Rates of fruit set, of embryos and of haploid plants obtained with different post-pollination treatments.

Treatment	Number of pollinated flowers	Fruit set rate	Embryo rate	Regeneration rate
T1	918	21.4 a	32.9 a	28.5 a
T2	930	16.1 b	20.1 b	1.9 b
T3	906	8.8 c	16.6 b	1.8 b
T4	932	6.3 c	3.9 c	0.1 c

Averages followed by the same letter are not significantly different.

3.3. Effect of the pretreatment method

Results of the application of the post-pollination treatment by spraying or soaking spikes in the hormonal solution are presented in Table 3. The application of the post pollination treatment by spraying spikes was more favorable for fruit setting and embryos formation than soaking them in the hormonal solution. However, soaking spikes allowed a better conversion of embryos into haploid plants than spraying them.

Table 3. Rates of fruit set, of embryos and of haploid plants obtained by spraying or soaking spikes in the hormonal solution.

Post-pollination treatment	Fruit set rate	Embryo rate	Regeneration rate
Spraying	35.4 a	64.6 a	58.0 a
Soaking	16.1 b	57.3 a	79.2 b

Averages followed by the same letter are not significantly different.

3.4. Effect of spike castration

Rates of fruit set, of embryos formation and of regeneration of haploid plants with and without manual castration of spikes are presented in table 4. Fruit setting and embryo formation were better for spikes castrated manually than for those not castrated and pollinated early before anthesis. However, the rate of regeneration was better for spikes not castrated.

Table 4. Rates of fruit set, of embryos and of haploid plants obtained with and without manual castration of spikes.

Method of spike castration	Fruit set rate	Embryo rate	Regeneration rate
Manual castration	78.3 a	93.9 a	21.8 a
No castration	55.0 b	75.4 b	63.8 b

Averages followed by the same letter are not significantly different.

4. Discussion

The production of durum wheat haploids was addressed through the study of various factors such as the need for the manual castration of spikes, the post-pollination treatments and the methods of application of hormonal treatments.

4.1. Effect of durum wheat genotype on the Production and germination of haploid embryos

The obtained embryo rates were relatively high. The varieties 'Mahmoudi' and 'LSA2' gave particularly a germination rate of 90%. Differences in the production of haploid embryos were observed in the parent varieties of durum wheat and their F1 hybrids. This confirms previous studies that showed differences in the number of embryos among the genotypes of durum wheat (Garcia-LIamas *et al.*, 2004; Mujeeb-Kazi *et al.*, 2006). However, other studies have noted the lack of a genotypic effect on the number of haploid embryos in hexaploid wheat (Campbell *et al.*, 2000; Matzk and Mahn, 1994; Zhang *et al.*, 1996).

Significant differences in embryo germination and plant regeneration were also observed among parental varieties and their F1 hybrids. The variety 'LSA2' has especially given a high rate of 90% for these parameters. The other genotypes gave relatively low embryo rates. The low conversion rate of embryos from crosses wheat x maize in plants has been reported in previous studies (Jauhar, 2009; Campbell *et al.*, 2000).

4.2. Effect of growth substances on embryo formation and germination

The absence of nourishing endosperm in pseudo caryopsis resulting from crosses durum wheat x maize threat the formation and viability of haploid embryos. Treatments with growth substances applied after the pollination permitted to prevent the abortion of embryos that could continue to grow to 1 to 1.5 mm, an *in vitro* rescue stage on the medium B5. The treatment with 2,4-D at 10 mg/l was very favorable for the formation of embryos and their evolution into haploid plants.

This result confirms the beneficial effect of the auxin 2,4-D on the stimulation of the development and size increasing of embryos resulting from crosses wheat x maize that has been revealed by previous studies (Zhang *et al.*, 1996; Al Muslem *et al.*, 1998; Garcia LIamas *et al.*, 2004). The combination of 2,4-D with silver nitrate $AgNO_3$ decreased embryo formation and plant regeneration compared to 2,4-D alone. This result confirms other studies (Laurie *et al.*, 1991; Inagaki *et al.*, 1995; Escarnot *et al.*, 2014) that revealed that 2,4-D alone gave better results than 2 ,4-D used in combination with other growth substances. However, other studies showed a beneficial effect of a combined treatment with 2,4-D and silver nitrate on the formation and size of embryos (Al Muslem *et al.*, 1998, Jauhar, 2003).

4.3. Effect of the method of application of post-pollination treatments

In this study, fruit set was better when the pollinated spikes were sprayed with growth substances. The embryo formation was not modified by the application method of growth substances. However, the conversion of embryos into haploid plants was higher when spikes were soaked in the hormonal solution. Spikes' soaking has been used successfully in previous studies (Ltifi *et al.*, 2016). Several studies showed that spraying of growth substances is beneficial for the formation and germination of embryos resulting from crosses wheat x maize (Al Muslem *et al.*, 1998; Chlyah *et al.*,1999; Daniel *et al.*, 2005). Campbell *et al.* (2000) compared the spraying and injection of growth substances on two wheat varieties 'Rata' and 'Belfield'. They showed that, for the variety 'Belfield', spraying gave a better production of haploid embryos than injection. However, for the variety 'Rata', the numbers of embryos obtained by injection and by spraying were not significantly different.

4.4. Effect of spike castration

In this study, rates of fruit setting and of embryos obtained with manual castration of spikes were higher than those obtained without castration by pollinating wheat spikes whereas anthers were still green and their pollen grains were not mature. These results confirm those obtained by Hussain *et al.* (2012) who showed that the expensive operation of castration can be substituted by an early pollination or the soaking of spikes in hot water at 43°C for 4 minutes.

Regeneration of embryos from non-castrated spikes and early pollinated was better than that of embryos from spikes castrated manually. Embryos formed in non-castrated spikes would be better developed and of better quality than those castrated just before anthesis.

Conclusions

The cross between durum wheat and maize was largely influenced by the genotype of durum wheat. Thus fruit setting, embryos formation, and regeneration of haploid plants were the highest for a variety of durum wheat than for the hybrids F1. Post-pollination treatments as using auxin (2,4-D) on spikes and their manual castration have beneficial effects mainly on fruit set rate and embryo formation.

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