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Effect of 2,4-Dichlorophenoxyacetic Acid and Nitrate Silver on the Efficiency of Haploid Production in Durum Wheat × Maize Crosses

Ayed Sourour* • **Slama-Ayed Olfa** • **Slim-Amara Hajer**

Genetic and Plant Breeding Laboratory, Department of Agronomy and Biotechnology, National Agronomic Institute of Tunisia, 43, Avenue Charles Nicole, 1082 Tunis, Tunisia

Corresponding author: ***ayedsourour@yahoo.fr**

ABSTRACT

In order to develop an efficient method of haploid production via interspecific cross between durum wheat and maize genotypes, the effect of different concentrations (0, 25, 50, 75, 100, 125, 150 and 175 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4-D) and nitrate silver ($AgNO_3$) in sucrose solution and the combination of these two treatments (100 mg/l of 2,4-D and 75 mg/l of $AgNO_3$) were tested. Four Tunisian durum wheat genotypes (female parent): two local cultivars ('Jenah khotifa' and 'Biskri') and two improved varieties ('Karim' and 'Razzek') were crossed with a maize genotype ('Pioneer 37Y15') (male parent). The best results of embryo formation and haploid plants were obtained when the concentration of 2,4-D was 100 mg/l in combination with 75 mg/l of $AgNO_3$. Embryogenesis and the regeneration rates reached 26.01 and 22.22%, respectively. Significant differences in the frequencies of developed ovaries, embryos and haploid plant production were observed for all durum wheat genotypes used. The local genotypes 'Jenah khotifa' and 'Biskri' showed the highest frequency of developed ovaries, formed embryos and haploid plant. A total of 877 haploid plants were regenerated with the method outlined in the present study.

Keywords: $AgNO_3$, interspecific hybridization, 2,4-D
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; **$AgNO_3$**, nitrate silver; **SD**, standard deviation

INTRODUCTION

The production of haploid/doubled haploid plants can shorten the time required for cultivar development by conventional programs such as pedigree or backcross system. Another advantage is the production of homozygous and pure breeding lines in one generation. Haploid plants express all the genetic characters which facilitate selection of useful traits (Cherkaoui *et al.* 2000).

Among haplo-methods, anther and isolated microspore culture are mainly used for producing wheat doubled haploid plants. However, many authors reported the recalcitrance of durum wheat through androgenesis because they produce a high frequency of albino plants (Cistué *et al.* 2006; Slama Ayed *et al.* 2010). Therefore, intergeneric hybridisation can be an efficient method to avoid the problem of albinism. This system has the advantage that all the haploids obtained are green. The *bulbosum* method is the most commonly used method to produce doubled haploids in intergeneric crosses (Snape *et al.* 1988; Ballesteros *et al.* 2003) although, in some wheat genotypes, the Kr_1 and Kr_2 genes situated on chromosomes 5B and 5A, respectively, were responsible for an incompatibility between these two species reducing the frequency of fertilisation (Inagaki and Snape 1982; Sitch and Snape 1987). The hybridization wheat × maize method was largely unaffected by the presence of dominant alleles at the Kr loci which restrict crossability with *Hordeum bulbosum* (Laurie and Bennett 1987; Kisana *et al.* 1993; O'Donoughue and Bennett 1994). Wide crosses followed by elimination of the maize genome have been an alternate method for inducing haploid zygote embryos and subsequent plants (Laurie and Bennett 1990; Bakos *et al.* 2005).

Zygotes, resulting from this crossing, contain one complete haploid chromosome set from each parent, confirming the hybrid origin of the embryos. The viability of these zygotes is low, and most of them abort during the initial stages of development (Laurie and Bennett 1988).

Despite significant improvements in hexaploid wheat × maize crossing methods (Inagaki and Mujeeb-Kazi 1995; Brazauskas *et al.* 2005; Bidmeshkipour *et al.* 2007), the success in durum wheat remain limited since the viability of zygotes is low and most of them abort during the initial stage of development (Laurie and Bennett 1988; Amrani *et al.* 1993; O'Donoughue and Bennett 1994). Hormonal treatment can induce ovary enlargement and enhance the survival and development of haploid embryos to a stage that enables their culture onto nutrient media (Laurie and Reymondie 1991).

In durum wheat, the application of different types and concentrations of hormones following pollination such as 2,4-dichlorophenoxyacetic acid (2,4-D), gibberellic acid and silver nitrate ($AgNO_3$) notably affects the capacity to produce embryos and haploid plants (Fedak *et al.* 1997; Almouslem *et al.* 1998; Garcia-Llamas *et al.* 2004).

The objective of this study was to test the influence of different hormonal treatments and concentrations on the production of developed ovaries, embryos and haploid plants, via maize × durum wheat crosses. Two treatments (2,4-D and $AgNO_3$) were studied alone or in combination to compare their efficiency in haploid plant development.

MATERIALS AND METHODS

Plant materials

Crosses were carried out between 4 durum wheat (*Triticum durum* 2n = 4x = 28) genotypes: 2 landraces ('Jenah khotifa' and 'Biskri') and 2 improved varieties ('Karim' and 'Razek') used as the female parent. Durum wheat was planted every 2 weeks in the field under

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Table 1 The composition of durum wheat embryo regeneration medium used.

Media components	B5 (mg/l)
Macro salts	
KNO_3 (Potassium nitrate, Sigma)	2500
$CaCl_2 \cdot 2H_2O$ (calcium chloride dihydrate, Fisher Scientific, UK)	150
$MgSO_4 \cdot 7H_2O$ (magnesium sulfate, Chemi-Pharma)	250
$(NH_4)_2SO_4$ (ammonium sulfate, Sigma)	134
$NH_4H_2PO_4$ (ammonium sulfate monobasic, Sigma)	150
Macro salts	
KI (potassium iodide, Sigma)	0.75
H_3BO_3 (boric acid, Fisher Scientific)	3
$MnSO_4 \cdot 4H_2O$ (manganese sulfate, Fluka)	10
$ZnSO_4 \cdot 4H_2O$ (zinc sulfate, Brix Worth-Northants, UK)	2
$Na_2MOO_4 \cdot 5H_2O$ (sodium molibdate, Siegfried Handel)	0.25
$CuSO_4 \cdot 5H_2O$ (copper sulfate (Cryst Pure, Northampton UK)	0.025
$CoCl_2 \cdot 6H_2O$ (cobalt chloride, Fisher Scientific)	0.025
Iron source	
Na_2·EDTA (ethylenediamine-tetraacetic acid, Sigma)	37.3
$FeSO_4 \cdot 7H_2O$ (ferric sulfate, Brix Worth-Northants, UK)	27.8
Vitamins	
Nicotinic acid	1
Thiamine hydrochloride (Sigma)	10
Pyridoxine hydrochloride (Sigma)	1
Myo-inositol (Sigma)	100
Others components	
Sucrose (Applichem, Germany)	20000
Agar (ICN, Biomedicals)	7500
pH	5.5

natural growing conditions. A maize (*Zea mays* 2n = 2x = 20) genotype ('Pioneer 37Y15') was used as the male parent. Maize plants were grown in pots in an unconditioned greenhouse at temperatures slightly warmer than those outside.

Crosses and culture of detached tillers

Two or three days before anthesis, the apical and basal spikelets of wheat, and all florets except for the two outermost florets, and the remaining florets were emasculated and receptive stigmas were pollinated with freshly collected maize pollen. These pollinated spikes with stems were cut in the middle of the third internode and covered with a paper bag to maintain humidity after emasculation.

Treatments application and embryo rescue

Detached tillers were cultured in a solution contained 40 g/l sucrose and 8 ml/l of sulphurous acid (Inagaki *et al*. 1997). Different concentrations of 2,4-D or $AgNO_3$ were added at this solution as follows:

Experiment 1: 2,4-D: 0, 25, 50, 75, 100, 125, 150, 175 mg/l

Experiment 2: $AgNO_3$: 0, 25, 50, 75, 100, 125, 150, 175 mg/l

Experiment 3: 100 mg/l of 2,4-D and 75 mg/l of $AgNO_3$

For each treatment with 2,4–D (Sigma-Adrich) or $AgNO_3$ (Park Scientific Ltd., UK) in each cross, 10 spikes (approximately 200 florets) per replication were pollinated with fresh pollen. Twelve, 14, 16, 18 and 20 days after pollination, the embryos were isolated from caryopses that grew over two thirds of the glume length and were sterilized with 12% bleach for 10 min and washed 3 times with sterilized water. On a sterile working surface, embryos were removed from caryopses with the aid of a X20 stereoscope. Embryos were placed into Petri dishes with 5 ml B5 medium containing 20 g/l sucrose and 7.5 g/l agar (Gamborg *et al*. 1968) (**Table 1**). Embryos were kept in the dark at about 25°C in a growth room until germinated. After further growth, haploid wheat seedlings at the three-leaf stage (1-2 cm) were transferred to a bottle with the same medium at a light intensity of 350–450 µE m^{-2} s^{-1} under a 16-h photoperiod.

Transfer of plants to soil

Plants were transferred to sterile peat moss in small pots. They were kept in a lighted growth room at 25°C in a 16-h photoperiod and irrigated daily with Hoagland solution (FAO 1984) (**Table 2**) for hardening roots and shoots for 4-6 weeks. The number of plants developed was counted one month after embryo rescue. Root tips from each plant were sampled and examined mitotically according to the method of Jahier *et al*. (1992).

Ploidy analyses

Somatic chromosome number was examined in root-tip cells of the seedlings (**Fig. 1**). At least two root tips from each seedling were cut to a length of 1 cm, kept in distilled water at 0°C for 24 h, fixed in 3:1 absolute ethanol/glacial acetic acid, then hydrolysed in 1 HCl for 12 min. Root tips were stained with 1% acetocarmine solution and chromosomes were observed after squashing.

Data analyses

Three traits were measured:

Frequency of developed ovaries = (number of developed ovaries/ total florets pollinated) × 100

Frequency of embryos formed= (number of embryos formed/total florets pollinated) × 100

Frequency of haploid plants = (total of plant regenerated/total floret pollinated) × 100.

In this study a complete randomized block design was used. Data were analysed by analysis of variance (ANOVA) and Fisher's least significant difference (LSD) at 5% after comparison of means using SPSS 10.0 statistical software.

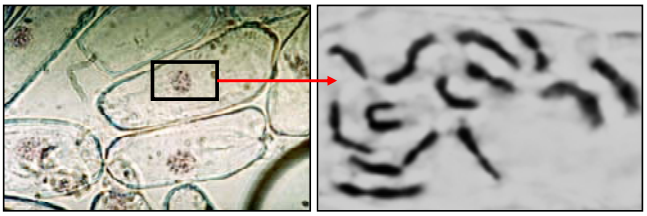

Fig. 1 Somatic chromosomes (2n = 14) of haploid plants obtained from the cross of durum wheat with maize.

Table 2 The composition of Hoagland's solution.

Components	Quantity (mg /l)
Macro salts	
KNO_3 (potassium nitrate, Sigma)	303
$Ca(NO_3)_2 \cdot 4H_2O$ (calcium nitrate, Panreac Barcelona)	902
$MgSO_4 \cdot 7H_2O$ (magnesium sulfate, Chemi-Pharma)	123
$NH_4H_2PO_4$ (ammonium phosphate monobasic, Sigma)	115
Macro salts	
KCl (potassium chloride, Cryst Pure, Northampton, UK)	1.86
H_3BO_3 (boric acid, Fisher Scientific)	7.73
$MnSO_4 \cdot 4H_2O$ (manganese sulfate, Fluka)	0.22
$ZnSO_4 \cdot 7H_2O$ (zinc sulfate, Brix Worth-Northants, UK)	0.29
$CuSO_4H_2O$ (copper sulfate, Cryst Pure)	0.06
$H_2MoO_4H_2O$ (acid molybdate, Sigma)	0.36
Iron source	
Na_2EDTA (ethylenediamine-tetraacetic acid, Sigma)	37.3
$FeSO_4 \cdot 7H_2O$ (ferric sulfate, Brix Worth-Northants, UK)	27.8
pH	5.7

Table 3 Frequency of developed ovaries, embryos and haploid plant production in wheat and maize crosses treated with various concentrations of 2,4-D.

Genotypes	Concentration of 2.4-D (mg/l)	Number of florets pollinated	Frequency of developed ovaries (%)	Frequency of embryos formed (%)	Frequency of haploid plants (%)
Jeneh khotifa	0	101	48.30 d	16.83 c	11.86 d
	25	102	50.23 c	18.90 b	13.26 cd
	50	108	53.83 b	20.13 b	14.33cd
	75	116	54.33 b	19.83 b	16.00 bc
	100	92	71.43 a	27.53 a	22.23 a
	125	103	54.10 b	18.90 b	16.46 bc
	150	95	53.00 b	18.13 bc	14.83cd
	175	83	52.66 b	17.73 c	14.66 bc
Karim	0	107	41.90 d	14.7 c	9.50 d
	25	106	49.66 c	16.26 b	12.33 bc
	50	109	53.00 b	17.43 b	12.87 bc
	75	98	53.30 b	16.83 b	15.27 b
	100	86	64.83 a	21.33 a	19.63 a
	125	116	55.46 b	17.70 b	13.37 bc
	150	114	54.46 b	17.03 b	12.37 bc
	175	94	53.0 b	16.43 b	11.93 c
Razzek	0	122	43.00 d	15.80 c	9.50 c
	25	113	46.97 c	16.00 bc	12.90 b
	50	118	52.67 b	17.00 bc	13.97 b
	75	128	5 3.97 b	17.50 bc	14.50 ab
	100	131	59.60 a	20.33 a	16.67 a
	125	114	53.10 b	17.83 b	14.61 ab
	150	100	52.77 b	16.23 bc	14.57 ab
	175	78	52.77 b	16.66 bc	13.67 b
Biskri	0	124	44.70 d	17.65 c	9.7 d
	25	96	51.96 c	18.23 c	12.5 c
	50	104	54.10 bc	20.10 b	14.93 b
	75	131	54.43 bc	20.17 b	15.63 b
	100	102	67.76 a	25.30 a	20.63 a
	125	112	54.13 bc	18.53 c	16.63 b
	150	94	52.00 c	17.43 c	14.30 b
	175	85	51.91 c	17.40 c	13.07 bc
Mean±SD		Total : 3381	53.39±6.05	18.37±2.66	14.33±2.87

Means in the same column followed by different letters are significantly different at 0.05 level
SD: Standard deviation
2,4-D: 2,4-dichlorophenoxyacetic acid, $AgNO_3$,: nitrate silver

RESULTS AND DISCUSSION

The effect of 2,4-D concentration in durum wheat × maize crosses

Embryo rescue 18 days after pollination showed that 621 (18.37%) and 484 (14.33%) of these florets produced haploid embryos and durum wheat plants, respectively on B5 medium for the four genotypes studied. The application of various concentrations of 2,4-D showed a significant difference ($P < 0.01$) in the frequency of developed ovaries, embryo formation and haploid plant production in 4 different durum wheat genotypes pollinated with maize (**Table 3**). The best yield was obtained with 100 mg/l 2,4-D in developed ovaries (65.90%), embryos (23.62%) and haploid plants (19.79%). However, > 100 mg/l of 2,4-D was considered to be excessive and clearly reduced the percentage of embryos and haploid plants. Kisana *et al.* (1993) noticed that the application of 100 mg/l of 2,4-D to wheat spikes when crossed with maize stimulated the retention and development of embryos. A comparison between the effect of 2,4-D at various concentrations on the percentage of caryopsis development and embryo formation showed that at 10 mg/l 2,4-D, the percentage of caryopsis development was only 11.7% and no embryos were obtained. Caryopsis development was slightly improved at 1000 mg/l (79.1%) compared with 100 mg/l (64.9%) but the efficiency of embryo formation was decreased from 16.1% at 100 mg/l to 4.1% at 1000 mg/l (Suenaga 1994). Garcia-Llamas *et al.* (2004) showed that treatment with 100 mg/l 2,4-D significantly increased the production of embryos (2.6%) and haploid plants (1.3%) per spike in durum wheat × maize crosses. According to Sun *et al.* (1992) and Matzk and Mahn (1994), the optimal concentration of 2,4-D for bread wheat × maize cross was 100 mg/l. Ushiyama *et al.* (2006) suggested that treatment with 2,4-D at 100 mg/l would also be effective for haploid wheat production by *Hordeum bulbosum* method. For the wheat × maize cross, Ushiyama *et al.* (2007) showed that treatment with 50 mg/l 2,4-D increased embryo size but the treatment with > 75 mg/l 2,4-D inhibited embryo development.

The efficiency of our method is dependant on the wheat genotype used (**Table 4**). 'Jenah khotifa' and 'Biskri' showed the highest frequency of embryo formation (19.75, 19.29%) and haploid plant regeneration (15.45, 15.04%). A significant effect of wheat genotype on embryo formation was also demonstrated by Sarrafi *et al*. (1994) on 10 wheat varieties and by Saidi *et al.* (1998) on seven.

The effect of $AgNO_3$ concentration in durum wheat × maize crosses

In this experiment, from 3013 durum wheat florets pollinated with maize pollen and treated with different concentrations of $AgNO_3$ 341 embryos were produced (11.31% of pollinated florets) and 298 haploid plants (9.86% of pollinated florets). **Table 5** indicates a significant difference ($P < 0.05$) among various concentrations of $AgNO_3$ in terms of frequency of developed ovaries, embryo formation and haploid plant regeneration. Treatment with 75 mg/l $AgNO_3$ showed, for all durum wheat cultivars tested, the best frequency of developed ovaries (55.90%), embryos (16.62%) and haploid plants obtained (14.21%). According to Almouslem *et al.* (1998), the application of $AgNO_3$ is likely to delay the abscission process and stimulate embryo development. Beyer (1976) noted that silver ions applied as $AgNO_3$

Table 4 Response to durum wheat x maize cross of four Tunisian durum wheat genotypes treated with various 2,4-D and $AgNO_3$ concentration.

Genotypes	Frequency of developed ovaries (%)		Frequency of embryos formed (%)		Frequency of haploid plants (%)	
Treatment	2,4-D	$AgNO_3$	2,4-D	$AgNO_3$	2,4-D	$AgNO_3$
Jenah khotifa	51.73 a[1)]	44.73 a	19.75 a	12.75 a	15.45 a	10.45 a
Karim	53.22 a	43.22 a	17.21 b	10.21 b	13.38 b	9.05 a
Razzek	51.81 a	41.81 a	17.21 b	10.21 b	13.85 b	9.67 a
Biskri	54.06 a	44.06 a	19.29 a	12.29 a	15.04 a	10.26 a

Percentage values are the means of three replicates
[1)] Means in the same column followed by different letters are significantly different at 0.05 level
2,4-D: 2,4-dichlorophenoxyacetic acid, $AgNO_3$,: nitrate silver

Table 5 Frequency of developed ovaries, embryos and haploid plant obtained by durum wheat x maize cross and treatment of florets with $AgNO_3$.

Genotypes	Concentration of $AgNO_3$ (mg/l)	Number of florets pollinated	Frequency of seed setting (%)	Frequency of embryos formed (%)	Frequency of haploid plants (%)
Jenah khotifa	0	81	38.30 d	9.83 d	7.67 d
	25	94	40.23 c	11.90 bc	8.33 cd
	50	106	43.83 b	13.13 b	10.33 cd
	75	100	61.43 a	20.53 a	15.57 a
	100	138	44.33 b	12.83 b	12.67 b
	125	106	44.10 b	11.90 bc	10.80 bc
	150	88	43.00 b	11.13 bc	9.33 cd
	175	69	42.67 b	10.73cd	8.97 cd
Karim	0	75	32.07 d	8.10 c	6.97 c
	25	94	39.67 c	9.26 bc	7.33 c
	50	106	43.00 b	10.43 b	8.40 bc
	75	100	54.83 a	14.33 a	13.97 a
	100	131	43.30 b	9.83 b	10.30 b
	125	88	45.47 b	10.70 b	9.83 bc
	150	81	44.47 b	10.03 b	8.03 bc
	175	63	43.00 b	9.43 bc	7.63 c
Razzek	0	81	32.67 d	8.80 c	7.33 c
	25	88	36.97 c	9.00 bc	8.63 bc
	50	106	42.67 b	10.00 bc	9.63 b
	75	106	49.60 a	13.33 a	13.00 a
	100	131	43.97 b	10.50 bc	11.63 a
	125	88	43.10 b	10.83 b	9.58 b
	150	88	42.77 b	9.23 bc	8.90 bc
	175	69	42.77 b	9.67 bc	8.67 bc
Biskri	0	75	34.47 d	10.10 c	7.33 d
	25	88	41.97 c	11.23 c	8.83 cd
	50	106	44.10 bc	13.17 b	9.60 bc
	75	100	57.77 a	17.07 a	14.33 a
	100	131	44.43 b	13.17 b	11.67 b
	125	100	44.13 bc	11.53 c	10.97 bc
	150	75	43.67 bc	10.40 c	9.97 bc
	175	63	42.00 c	10.43 c	9.43 bc
Mean±SD		Total : 3013	43.46±6.05	11.31±2.66	9.86±2.66

2,4-D: 2,4-dichlorophenoxyacetic acid, $AgNO_3$: nitrate silver

and other silver salt solutions inhibit the action of ethylene exogenously applied to whole plants and plant parts.

Durum wheat genotypes significantly affect the capacity of production in crosses with maize (**Table 4**). Greatest embryo formation was shown for 'Jenah khotifa' (12.75%) and 'Biskri' (12.29%). Other results such as those of Brazauskas *et al.* (2005) confirm a positive effect on both caryopses and embryo formation in bread wheat × maize crosses.

The effect of the combination of 2,4-D and $AgNO_3$ treatment

With previous results obtained in our study, we noted that in durum wheat the optimal concentrations of 2,4-D and $AgNO_3$ were 100 and 75 mg/l, respectively. **Fig. 2** shows a significant difference between three treatments observed for the four durum wheat lines. The combination of 2,4-D and $AgNO_3$ showed a positive effect on the frequency of seed setting (78%), embryos (26.01%) and haploid plants (22.22%) for all genotypes used. 2,4-D mixed with $AgNO_3$ can successfully increase the production of durum wheat haploid plants by the maize method. These results agree with those reported by Almouslem *et al.* (1998) who found that 120 mg/l $AgNO_3$ and 180 mg/l 2,4-D improved the production of embryos and haploid plant development in durum wheat × maize crosses. Campbell *et al*. (2000) hypothesized that $AgNO_3$ added to 2,4-D solution reduces the emission of ethylene, which consequently promotes increased embryo survival. However, Sharmeen *et al.* (2005) showed an insignificant difference between the application of only 2,4-D and 2,4-D + $AgNO_3$ and suggested that ethylene emission might not be a problem in spring wheat. Bid-

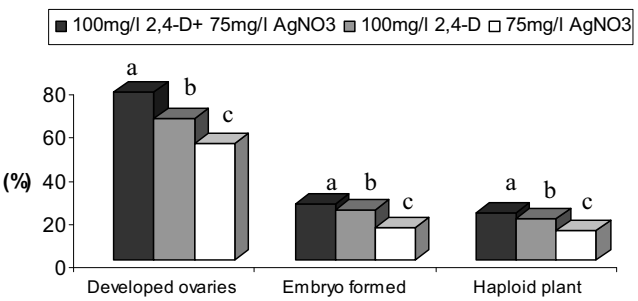

Fig. 2 Effect of 2,4-D (100 mg/l), $AgNO_3$ (75 mg/l) and combination of (100 mg/l 2,4-D + 75 mg/l $AgNO_3$) on frequency of developed ovaries, embryo formation and haploid plant regeneration in durum wheat x maize crosses.

meshkipour *et al.* (2007) showed that treatment with 3 mg/l 2,4-D + 120-180 mg/l $AgNO_3$ gave highest frequency of haploid embryos (7.45 and 6.16%) and haploid plants (1.06 and 0.83%), respectively for hexaploid and tetraploid wheat crossed with maize.

CONCLUSIONS

An efficient plant regeneration system for durum wheat × maize crosses was developed in this study. This system offers great potential with a total of 877 plants regenerated and has the advantage in that all the haploid plants obtained are green. The frequency of haploid plants will be increased by amending the rescue stage, media culture, and pollen source of maize. Nevertheless, the current technology itself may be widely applicable to durum wheat breeding.

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