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Production of Haploid Wheat via Maize Pollination

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Abstract

One of the ways of haploid production is a cross between wheat and maize. Recently success has been achieved in this method, leading to the production of zygotes; these zygotes generally undergo elimination of maize chromosomes and thus lead to production of wheat haploid plant. The purpose of this study was to develop an efficient method of haploid production via maize pollination in short duration bread and durum wheat cultivars grown in Iran and India. Pollination of agronomically superior varieties of six hexaploid wheat (2n=2x=42): Golestan, Mahdavi MACS-2496, HD-2189, NIAW-34, NI-5439 and four tetraploid wheat (2n=2x=28): MACS-9, MACS-2846, MACS-1967, Raj-1555 with pollen from maize cultivar resulted in haploid embryos by post pollination treatment with four concentration of dichlorophenoxyacetic acid (2,4-D) and silver nitrate ($AgNO_3$). Treatment with 3 mgL^{-1} 2,4-D plus (120-180 mgL^{-1} $AgNO_3$) gave highest frequency of haploid embryos and haploid plants. In this study haploid embryos and haploid plants were produced from all of the ten genotypes, but frequency of haploid embryos (7.45%) and haploid plants (1.06%) in hexaploid wheat was more than haploid embryos (6.16%) and haploid plants (0.83%) in tetraploid wheat. We produced a total of 368 mature haploid plants.

Keywords: Wheat; Maize; Haploid production; Embryo culture

Introduction

The production of haploid plants and complete homozygosity double haploids in crop species helps to accelerate breeding programs, improves selection efficiency and facilitates genetic analysis [6-8,24].

Haploid production from wide crosses between wheat and maize is potentially of great value to wheat breeding programs, because it may reduce the time required to achieve homozygosity in breeding lines. Wide crosses followed by elimination of the maize genome have been an alternate method for inducing haploid zygote embryos and subsequent plants [4,13,14,16]. Zygotes contain one complete haploid chromosome set from each parent, confirming the hybrid origin of the embryos, but maize chromosomes

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have poorly defined centromers and appear to have little affinity for spindle microtubules in zygotes and young embryos. As a result, they are lost during the first few cell division cycles to produce embryos whose cells contain a haploid complement of wheat chromosomes. Treatment of embryo *in vivo* with other growth regulators, such as kinetin, gibberellic acid and silver nitrate, could possibly also enhance embryo survival and development [1]. Pollination with maize is effective in producing haploids in bread wheat [10,12]. This method has also helped to produce haploids of durum wheat, but in low frequency [1,2,20,21,23]. In many studies of pollination, certain genotypes of maize have been used as interspecific pollinator [25,27].

The purpose of this study was to develop an efficient method of haploid production via maize pollination in short duration bread and durum wheat cultivars.

Materials and Methods

Plant Material and Pollination Methods

Six genotypes of hexaploid wheat (*Triticum aestivum* L., 2n = 6x = 42) MACS-2496, NI-5439, NIAW-34, HD-2189, Mahdavi, and Golestan, and four genotypes of tetraploid wheat (*Triticum turgidum* L., 2n =4x =28) Cultivars, including MACS-9, MACS-1967, MACS-2846, and Raj-1555 were used as female parents in crosses with maize (*Zea mays* L., 2n = 2x = 20) including Golden green as male parent. The seeds were planted weekly in pots and fielded under field condition (with temperature range from 7°C to 32°C and 11 h / 13 h light / dark).

Wheat Plants grown for 3 years were used for further analyses. Spikes were emasculated by cutting the top portion of the first two florets (being careful not to cut any of the anthers) and all but the primary and secondary florets were removed. Anthers were then removed with forceps and the spikes covered with glassine bags. One to three days after emasculation, feathery, receptive stigmas were pollinated with fresh maize pollen. Pollination was generally done in the morning and each emasculated spike was pollinated at least twice on successive days.

Post Pollination Treatment and Embryo Culture

Five *in vivo* treatments were started 24 h after pollination and were given once daily for up to 16 days. In these treatments the concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) were equal, but silver nitrate was different. The treatments applied were as follow:

T^0 = control spikes were sprayed with distilled water (dH_2O)

T^1- 3 mgL^{-1} 2,4-D + 60mgL^{-1} $AgNO_3$

T^2- 3 mgL^{-1} 2,4-D +120mgL^{-1} $AgNO_3$

T^3- 3 mgL^{-1} 2,4-D + 180mgL^{-1} $AgNO_3$

T^4- 3 mgL^{-1} 2,4-D + 240mgL^{-1} $AgNO_3$

Spikes treatments were given by spraying the treatment solution and inserting it between the glumes by micropipette. A distilled water (dH_2O) control was used for all of the spikes. Spikes were harvested 16 to 18 days after pollination and chilled for 24 to 48 h at 4°C. The caryopses containing embryos were surface sterilized for 1 min in 70% (vol/vol) ethanol and 20 minutes in 0.1% (w/v) $HgCl_2$ and rinsed in several changes of sterile distilled water. Embryos measuring 1.5-2 mm were dissected aseptically and cultured with scutellum oriented away to hormone free MS medium [17] supplemented with 30 gL^{-1} sucrose and 8 gL^{-1} purified agar in sterile test tubes and Petri dishes for 4 to 6 weeks at 25 ± 2°C, and continue darkness.

Embryos were transferred to fresh media every 3 to 4 weeks. When coleoptiles and small primary roots were evident, the embryos were transferred to a lighted incubator for 2 to 4 weeks at 25 ± 2°C, 16 h / 8 h (light/dark) until they developed into healthy, green plantlets. The green plantlets were transferred to sterile peat moss in small pots. They were kept in a lighted growth room at 21 ± 2°C in a 16 h photoperiod (1500 lux) and daily irrigated with Hoagland solution for hardening roots and shoots for 4 to 6 weeks. The hardening plantlets (20-25 cm high) were transferred to soil in pots and grown to maturity in the field (Fig. 1).

Statistical Analysis

In this study the complete randomized block design was performed and data on percentages of embryos and haploid plants were transformed by Arc sin function to achieve a normal distribution. Data were analyzed by analysis of variance (ANOVA) and the least significant difference method (LSD).

Pollination with *Zea mays* and post pollination treatments with 2,4-D + $AgNO_3$ proved effective in producing haploid embryos and haploid plants. From the 37711 florets of both *aestivum* and *durum* wheat cultivars which were processed during this experiment we produced total of 2626 (6.96%) haploid embryos and 368 (0.98%) mature green haploid plants (Table 1).

Table 1 shows from 23564 florets of hexaploid genotypes 1755 (7.45%) haploid embryos and 250 (1.06%) haploid mature green plants were produced, and from 14147 florets in tetraploid genotypes 871 (6.16%) haploid embryos and 118 (0.83%) haploid

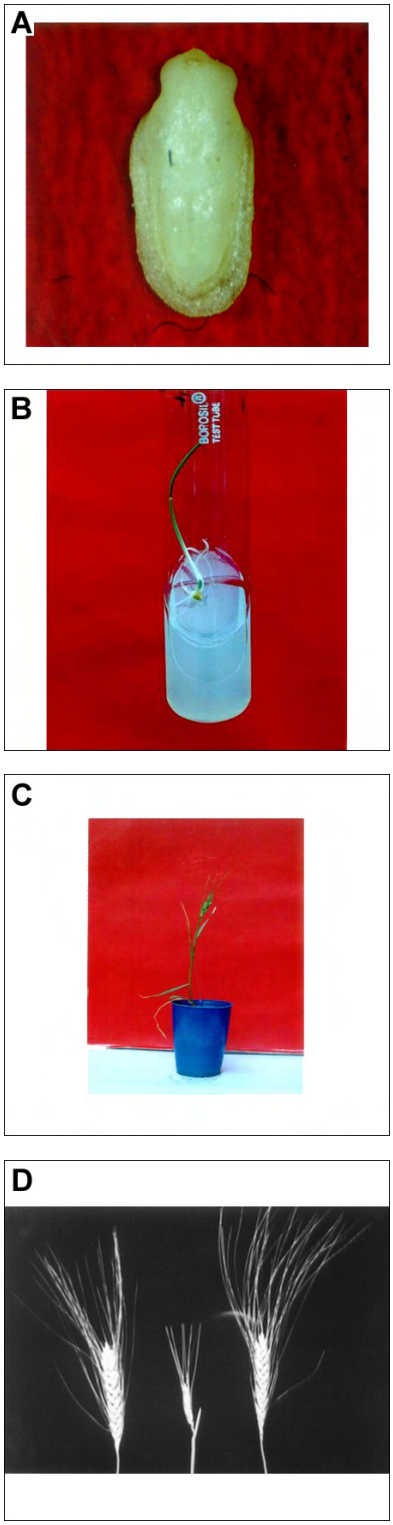

Figure 1. Stages of wheat haploid plant production. A) An embryo sprouting on hormone free MS medium in the dark; B) A young plantlet with several primary roots; C) A haploid plant in small pot with peat-moss; D) Spike of normal and haploid plants.

mature green plants were produce. Lack of endosperm in the caryopses harvested served as the initial criterion for identifying haploid embryos. When the plantlets transferred in the peat moss and then transferred in the soil gave haploid mature green plants (Fig. 1). After 16 days post pollination treatments were effective promoting the yield of haploid embryos (Table 1). In both, hexapod and tetraploid genotypes, the control (T^0) did not show either caryopses swelling or embryo formation. Treatment with 3 mgL^{-1} 2,4-D + 60 mgL^{-1} $AgNO_3$ (T^1) gave haploid embryos, ranging from 1.08% to 10.91% and 1.36% to 6.55% and haploid green plants, ranging from 0.26 to 1.33% and 0 to 1.19% in hexaploid and tetraploid genotypes, respectively. Most of these embryos were small and did not give haploid plants. Treatment with 120 mgL^{-1} $AgNO_3$ (T^2) resulted the good yield of embryos, ranging from 1.48% to 14.65% and 2.04% to 15.69% and consequently of haploid mature plants, ranging from 0.19% to 2.27% and 0.18% to 2.43% in both genotypes of hexaploid and tetraploid. (T^3), treatment with 180 mgL^{-1} $AgNO_3$ gave the highest yield of haploid embryos, ranging from 2.92% to 18.67% and 2.63% to 13.54% and haploid plants, ranging from 0.42% to 2.49% and 0.30% to 1.86% in both genotypes. Treatment with 240 mgL^{-1} $AgNO_3$ (T^4) resulted in caryopses swelling and haploid embryos, ranging from 0.89% to 1.87% and 0.45% to 1.59% in hexaploid and tetraploid genotypes, respectively, but frequency of haploid embryos was very low and did not give mature haploid plants. On the other hand T^2 and T^3 gave haploid plants more than others.

ANOVA analysis showed that the effects of treatments and wheat genotypes both were significantly different (Tables 2 and 4). Using LSD method we conclude that the treatments of 3 mgL^{-1} 2,4-D + 120 mgL^{-1} $AgNO_3$ and 3 mgL^{-1} 2,4-D + 180 mgL^{-1} $AgNO_3$ were not significantly different, but they were significantly different from other treatments. Similarly genotypes MACS-2496 and Golestan did not demonstrate a significant difference, although they were significantly different from the rest of the hexaploid genotypes. Raj-1555 as a tetraploid genotypes was significantly different from the rest of tetraploid genotypes (Tables 3 and 5).

Discussion

Haploids in wheat could be obtained by several techniques including anther culture, microspore culture, ovary culture, chromosome elimination via crosses between wheat and *Hordeum balbosum* or wheat and maize. In the present study hexaploid and tetraploid wheat genotypes were used for haploid production via

Table 1. Number and frequency of embryos and haploid plants recovered after pollination of aestivum and durum genotypes with maize and treatment of florets with 2,4-D and $AgNO_3$

Treatment	***T. aestivum* genotypes**						***T. durum* genotypes**			
	MACS-2496	HD-2189	NIAW-34	NI-5439	Mahdavi	Golestan	MACS-9	MACS-1967	MACS-2846	RAJ-1555
T^0: Control										
No. of florets	150	144	126	142	156	168	120	126	172	126
No. of embryos	0	0	0	0	0	0	0	0	0	0
No. of plants	0	0	0	0	0	0	0	0	0	0
T^1: 3 mgL^{-1} 2,4-D + 60 mgL^{-1} $AgNO_3$										
No. of florets	825	720	651	814	780	896	620	588	748	672
No. of embryos	90	25	7	64	36	78	18	8	16	44
%	(10.91)	(3.47)	(1.08)	(7.86)	(4.62)	(8.71)	(2.90)	(1.36)	(2.14)	(6.55)
No. of plants	11	4	3	9	2	6	3	0	2	8
%	(1.33)	(0.56)	(0.46)	(1.11)	(0.26)	(0.67)	(0.48)	0	(0.27)	(1.19)
T^2: 3 mgL^{-1} 2,4-D + 120 mgL^{-1} $AgNO_3$										
No. of florets	1300	1080	1050	1100	1222	1372	1000	1092	1166	1071
No. of embryos	136	16	59	129	114	201	58	21	118	168
%	(10.46)	(1.48)	(5.62)	(11.73)	(9.33)	(14.65)	(5.80)	(2.04)	(10.12)	(15.69)
No. of plants	27	2	8	25	15	28	9	2	16	26
%	(2.08)	(0.19)	(0.76)	(2.27)	(1.23)	(2.04)	(0.90)	(0.18)	(1.37	(2.43)
T^3: 3 mgL^{-1} 2,4-D + 180 mgL^{-1} $AgNO_3$										
No. of florets	1200	960	1113	1188	1092	1484	1120	987	1078	1197
No. of embryos	176	28	55	109	90	277	80	26	146	138
%	(14.67)	(2.92)	(4.94)	(9.18)	(8.24)	(18.67)	(7.14)	(2.63)	(13.54)	(11.53)
No. of plants	29	4	6	20	14	37	10	3	20	19
%	(2.42)	(0.42)	(0.54)	(1.68)	(1.28)	(2.49)	(0.89)	(0.30)	(1.86)	(1.59)
T^4: 3 mgL^{-1} 2,4-D + 240 mgL^{-1} $AgNO_3$										
No. of florets	750	672	735	748	832	980	720	672	660	756
No. of embryos	12	6	7	14	10	16	7	3	8	12
%	(1.60)	(0.89)	(0.95)	(1.87)	(1.20)	(1.63)	(0.97)	(0.45)	(1.21)	(1.59)
No. of plants	0	0	0	0	0	0	0	0	0	0
%	0	0	0	0	0	0	0	0	0	0
Total No. of florets	4075	3432	3549	3850	3926	4732	3460	3339	3652	3696
Total No. of embryos	414	75	128	316	250	572	163	58	288	362
%	(10.16)	(2.19)	(3.61)	(8.21)	(6.70)	(12.09)	(4.71)	(1.74)	(7.89)	(9.79)
Total No. of plants	67	10	17	54	31	71	22	5	38	53
%	(1.67)	(0.29)	(0.48)	(1.40)	(0.79)	(1.50)	(0.64)	(0.15)	(1.04)	(1.43)

Total No. of florets in hexaploid genotypes : 23564
Total No. of florets in tetraploid genotypes : 14147
Total No. and frequency of embryos in hexaploid genotypes : 1755 (7.45%)
Total No. and frequency of embryos in tetraploid genotypes : 871 (6.16%)
Total No. and frequency of haploid plants in hexaploid genotypes : 250 (1.06%)
Total No. and frequency of haploid plants in tetraploid genotypes : 118 (0.83%)

Table 2. ANOVA on treatments and *T. aestivum and T. durum* haploid embryos

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.080(a)	12	.007	8.925	.000
Intercept	.147	1	.147	198.207	.000
Treatments	.042	3	.014	19.032	.000
Genotypes	.037	9	.004	5.555	.000
Error	.020	27	.001		
Total	.247	40			
Corrected Total	.100	39			

(a) R Squared = .799 (Adjusted R Squared = .709)

Table 3. Mean separation test results based on LSD method on treatments for the Arc sin of percentages of embryos

(I) treatments	(J) treatments	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.0375(*)	.01220	.005	-.0625	-.0124
	3.00	-.0441(*)	.01220	.001	-.0691	-.0190
	4.00	.0373(*)	.01220	.005	.0123	.0623
2.00	1.00	.0375(*)	.01220	.005	.0124	.0625
	3.00	-.0066	.01220	.593	-.0316	.0184
	4.00	.0748(*)	.01220	.000	.0497	.0998
3.00	1.00	.0441(*)	.01220	.001	.0190	.0691
	2.00	.0066	.01220	.593	-.0184	.0316
	4.00	.0814(*)	.01220	.000	.0563	.1064
4.00	1.00	-.0373(*)	.01220	.005	-.0623	-.0123
	2.00	-.0748(*)	.01220	.000	-.0998	-.0497
	3.00	-.0814(*)	.01220	.000	-.1064	-.0563

(*) The mean difference is significant at the .05 level.

pollination with maize and post pollination treatments with 3 mgL^{-1} 2,4-D and four concentration of the ethylene inhibitor $AgNO_3$. The speed of development of the activated cells obtained by maize pollination is much slower than the development of zygotes [28]. Relatively slow rate of development and low survival rate of the rescued embryos suggest that they have special and possibly unknown requirement in their developmental stages. Similar to other studies [3,9] almost all of the isolated zygotes that started to complete the normal embryogenic pathway in vivo were able to grow into plants. Embryo formation and haploid plant production were affected by 2,4-D concentration [29]. Silver ions applied as $AgNO_3$ and other silver salt solution inhibit the action of ethylene exogenously applied to whole plants and plant parts [5]. In this study haploid embryos and haploid plants were produced from all of the ten genotypes of hexaploid and tetraploid wheat, but frequency of haploid embryos (7.45%) and haploid plants (1.06%) in hexaploid wheat X maize crosses was more than haploid embryos (6.16%) and haploid plants (0.83%) in tetraploid wheat X maize crosses (Table 1). These results are consistent with the report of other studies [11,19,25]. Pervious attempts to cross tetraploid wheat and maize resulted in fertilized embryo formation but not in haploid plant production [18,21].

Table 2 shows that many of tetraploid wheat cultivars have fertilized embryo formation and produced haploid plants. Raj-1555 and MACS-2846 cultivars have provided highest relative embryos (15.69% and 10.12%, respectively), but frequency of haploid plants was very low (2.43% and 1.37%, respectively). Kisana *et al.* (1993) have reported a ratio of 4.7% haploid plants [8]. The reason why some of the cultured embryos failed to develop into plantlets is still unknown; however their

Table 4. ANOVA on treatments and *T. aestivum* and *T. durum* haploid plants

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.001(a)	11	.000	8.498	.000
Intercept	.004	1	.004	239.436	.000
Treatments	.000	2	.000	11.011	.001
Genotypes	.001	9	.000	7.940	.000
Error	.000	18	1.539E-05		
Total	.005	30			
Corrected Total	.002	29			

(a) R Squared = .839 (Adjusted R Squared = .740)

Table 5. Mean separation test results based on LSD method on treatments for the Arc sin of percentages of haploid plants

(I) treatments	(J) treatments	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1.00	2.00	-.0071(*)	.00175	.001	-.0108	-.0034
	3.00	-.0071(*)	.00175	.001	-.0108	-.0035
2.00	1.00	.0071(*)	.00175	.001	.0034	.0108
	3.00	.0000	.00175	.991	-.0037	.0037
3.00	1.00	.0071(*)	.00175	.001	.0035	.0108
	2.00	.0000	.00175	.991	-.0037	.0037

(*) The mean difference is significant at the .05 level.

frequency may be influenced by in vitro culture parameters [15]. Embryo culture is probably adaptable to haploid plant. This method could be helpful in tetraploid and hexaploid wheat double haploid production [4]. These results indicates that the frequency of embryo which plantlets form depends on the wheat genotype. Genotypic differences for wheat haploid production efficiency with the maize hybridization method have been reported earlier [1,2,23,26]. Attempts at crossing durum wheat with maize and post pollination treatments with 3 mgL^{-1} 2,4-D resulted in sufficient caryopsis swelling [1,29].

In our study treatments with 3 mgL^{-1} 2,4-D plus (120-180 mgL^{-1} $AgNO_3$) gave highest frequency of haploid embryos and haploid plants (Table 1). These results are consistent with the report of Almouslem *et al.* (1998). The phenomenon seen in untreated ovaries of *Triticum* species pollinated with maize seems to involve abscission of the ovary from the mother plant and senescence. This might be due to ethylene production in the mother plant and the applied $AgNO_3$ might counteract this effect. Auxins can promote ethylene production in plants and the internal level of auxin may regulate the rate of ethylene production. Perhaps $AgNO_3$ inhabits the effect of ethylene produced in response to the exogenously applied 2,4-D [22].

In summary, the results of this study indicate that successful fertilization of hexaploid and tetraploid wheat genotypes with maize can be achieved at relatively high frequencies and that these crosses are characterised by the rapid elimination of the maize chromosomes and consequent formation of haploid embryos.

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