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FACTORS AFFECTING DOUBLED HAPLOID PLANT PRODUCTION VIA MAIZE TECHNIQUE IN BREAD WHEAT

IOANNIS XYNIAS[1*], ANTONIOS KOUFALIS[2], EVDOKIA GOULI-VAVDINOUDI[2], AND DEMETRIOS ROUPAKIAS[2]

[1]*Department of Agricultural Technology, School of Agricultural Technology and Food Technology and Nutrition, Technological and Educational Institution of Western Macedonia, 53100 Florina, Hellas*
[2]*Laboratory of Genetics and Plant breeding, School of Agriculture, Aristotle University of Thessaloniki, 54124 Thessaloniki, Hellas*

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The effect of two in planta factors (growth conditions, genotype) and two in vitro factors (time of embryo rescue, embryo rescue medium) on doubled haploid (DH) plant production in bread wheat via maize technique was investigated in nine F_1 hybrids produced after crossing four bread wheat cultivars. During the first year one group of F_1 plants was grown in a field and at the proper stage pollinated with maize pollen (sweet corn population). In parallel, a second group of F_1 plants was grown in a growth chamber and pollinated as in the former group. In the second growing season the experiment was repeated but only field-grown plants were used. All the produced haploid embryos were cultured in three different media and the resulting 146 haploid plants were subsequently treated with aqueous solution of colchicine. Finally, 86 doubled haploid plants were obtained. We noted that the growing conditions of the parental plants and the intervening time between day of pollination and day of embryo rescue influenced the percentage of haploid embryo production. Culture medium also influenced haploid and doubled haploid plant production. The two media (MS/2, B5) were found equally effective. Most of the haploid embryos originated from the Penios × Acheloos cross, whereas most of the doubled haploid plants were produced from the KVZ × Penios cross. Doubled haploid plants were produced from all crosses.

Key words: Culture, doubled haploid, embryos, haploid, maize technique, *Triticum aestivum* L.

INTRODUCTION

Doubled haploid (DH) plant production is a very effective method to shorten the time needed to develop a new variety (Henry and de Buyser, 1990; Zhi-Hong 1990; Deyao and Xigan, 1990; Hussain et al., 2012). There are several ways to produce haploid plants: parthenogenesis and apogamy, anther and microspore culture, chromosome elimination techniques, and ovary culture (Kasha, 2005). Of these approaches, anther and microspore culture and chromosome elimination techniques (*bulbosum* technique and maize technique) appear to be the most effective and most popular (Hussain et al., 2012). Despite its effectiveness and convenience, anther culture has a very serious disadvantage: it is strongly genotype-dependant (Almouslem et al., 1998; Niroula and Bimb, 2009). Chromosome elimination techniques, and more precisely maize technique, offer an interesting alternative approach since they are not genotype-dependant (Hussain et al., 2012). Nor is maize technique influenced by the dominant *Kr* wheat crossability genes (Laurie, 1989).

The factors affecting production of doubled haploid plants in wheat via the maize technique can be divided into two classes of factors: in planta (i.e., growth conditions of the wheat and maize plants, mode of growth regulator application, genotype) and in vitro (i.e., time of embryo rescue, embryo rescue medium). The effect of the growth conditions is crucial (Brazauskas and Pasakinskiene, 2001). Matzk and Mann (1994) observed differences in haploid embryo formation resulting from differences in the response of each genotype to different annual climatic conditions. O'Donoughue and Bennett (1994)

*e-mail: xynias@teikal.gr; ioannis_xynias@hotmail.com

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observed differences in the genotype response to maize technique, probably due to differences in the wheat and maize plants' response to the growth conditions. The effect of the growth regulator is also important since it influences the embryo formation percentage, embryo growth rate and final size (Knox et al., 2000). The most common synthetic growth regulator used is 2,4-D (dichlorophenoxy acetic acid). It has been applied in several ways: sprinkling of the spike, as a drop, injection into the upper internode, and spike immersion (Laurie and Bennett, 1988; Cherkaoui et al., 2000). Other successfully applied regulators include dicamba (Savaskan et al., 1997; Campbell et al., 1998), gibberellic acid (Almouslem et al., 1998) and silver nitrate (O'Donoughue and Bennett, 1994; Almouslem et al., 1998).

The most important in vitro factor is the time of embryo rescue. Most papers state that small embryos (≤1.5 mm) must be rejected (Almouslem et al., 1998) and that embryo rescue should be attempted 10–12 days (Inagaki and Tahir, 1990) or 18–21 days (Savaskan, et al., 1997) after pollination of wheat plants with maize pollen (DAP). Cherkaoui et al. (2000) achieved the highest haploid embryo development percentage when embryo rescue was performed at 14 DAP; when embryo rescue was attempted earlier the embryos were smaller and less differentiated, producing fewer green plants.

The embryo rescue medium, which supports the development of the immature embryos to green plants, is the second important in vitro factor. The most common of the various media are MS medium as originally presented (Murashige and Skoog, 1962) or variants of it (MS/2), and B5 medium. Cherkaoui et al. (2000) obtained the highest green plant production from embryos cultured on MS/2 and B5 media. Adding indoleacetic acid and kinetin can increase the rate of green plant production (Zhang et al., 1996; Cherkaoui et al., 2000).

We investigated the effect of certain in planta factors (growth conditions, mode of growth regulator application, genotype) and in vitro factors (time of embryo rescue, and embryo rescue medium) on doubled haploid plant production with maize technique using Hellenic bread wheat germplasm responding well to anther culture (Zamani, 2001; Zamani et al., 2001, 2003; Xynias et al., 2001, 2005).

MATERIALS AND METHODS

PLANT MATERIAL

The study used nine F_1 hybrids produced after intercrossing four bread wheat cultivars (*Triticum aestivum* L. em Thell). Three of the four parental cultivars were developed at the Cereal Institute of Thessaloniki (cvs. Acheloos, Vergina, Penios) (Anonymous, 1991) and the fourth was the Russian cultivar Kavkaz/Cgn kindly provided by the Hellenic Gene Bank. These hybrids have been reported to be effective in producing doubled haploid plants via anther culture (Zamani, 2001; Zamani et al., 2001, 2003; Xynias et al., 2001, 2005). A sweet corn population (origin unknown) kindly provided by the American Farm School of Thessaloniki was used as pollinator.

EXPERIMENTS

Experiment 1 (first year)

The F_1 wheat hybrids were successively planted at two-week intervals (to expand the flowering period) in the field early in 2002, and the maize pollinator plants were subsequently grown in a glasshouse. When the wheat plants were at the proper stage (stage 50, Zadoks et al., 1974) they were emasculated and two to five days later the stigmas were pollinated with maize pollen. Two different modes of growth regulator application were used. In the first method, equal numbers of whole spikes were sprinkled with 100 mg/l 2,4-D solution or with a similar concentration of $AgNO_3$ solution 24 to 32 h after pollination (O'Donoughue and Bennet, 1994). In the second method the regulator was applied as a drop of 100 mg/l 2,4-D solution per floret 24 h after pollination. The immature embryos were rescued at 12–18 DAP and transferred to MS medium (Murashige and Skoog, 1962) without growth regulators at 22°C for three weeks in the dark. The responding embryos were then transferred to fresh MS medium in individual tubes (one plantlet per tube) at 20°C under a 16 h photoperiod until the stage of 2–3 leaves. At this stage the young plants were transferred to pots in a growth chamber (20/16°C day/night, 16 h photoperiod) to reach maturity.

Experiment 2 (first year)

The F_1 young plants were grown in pots in a growth chamber (20/16°C day/night, 16 h photoperiod) to reach maturity. The maize pollinator was grown in the field. The experiment lasted from August to December 2002. Only 2,4-D was used as growth regulator. One drop of 100 mg/l 2,4-D solution per floret was applied 24 h after pollination. At the same time, 3–5 ml of the same solution was injected into the upper internode of the stem. A second drop of the solution was applied at each floret. The stems of the treated florets were then cut at the first internode up from the ground and transferred to 100 mg/l 2,4-D solution supplemented with 40 g/l sucrose and 10 ml/l ethanol (combined treatment).

TABLE 1. Number of pollinated flowers, ovaries developed, haploid embryos and doubled-haploid plants produced from nine F_1 bread wheat hybrids

F_1 hybrids	Number of flowers pollinated	Ovaries developed		Embryos produced		Doubled haploids obtained	
		n	%	n	%	n	%
Acheloos × KVZ/Cgn	1389	1058	76.1^{c}	114	8.2^{cde}	16	1.2^{a}
KVZ/Cgn × Acheloos	1176	737	62.6^{e}	99	8.4^{bcd}	7	0.6^{ab}
Penios × Acheloos	1246	1012	81.2^{ab}	141	11.3^{a}	11	0.9^{a}
Acheloos × Penios	1251	883	70.6^{d}	86	6.8^{def}	7	0.6^{ab}
KVZ/Cgn × Vergina	1005	877	84.0^{a}	71	6.8^{def}	8	0.8^{ab}
KVZ/Cgn × Penios	1347	1058	78.5^{bc}	131	9.7^{abc}	18	1.3^{a}
Penios × KVZ/Cgn	1317	1037	78.7^{bc}	140	10.6^{ab}	13	1.0^{a}
Vergina × Acheloos	813	672	82.6^{a}	46	5.6^{f}	2	0.3^{b}
Acheloos × Vergina	1344	963	71.7^{d}	88	6.5^{ef}	4	0.3^{b}
Total/mean	10888	8297	76.2	916	8.4	86	0.8

Percentages bearing different letters differ significantly at P=0.05 (z-test).

The immature embryos were rescued 12–18 DAP and two different media were used: (a) MS/2 classical MS medium but with half macroelements, supplemented with 1 mg IAA and 1 mg kinetin per l medium; and (b) B5 medium (Gamborg et al., 1968) supplemented with 1 mg IAA and 1 mg kinetin per l medium. The haploid embryos were treated as in experiment 1.

Experiment 3 (second year)

The wheat maternal plants (9 F_1 hybrids) were planted in the field in January 2003 and the maize plants were grown in a chamber at 28–34/14–18°C day/night. Two modes of growth regulator application were used: (a) one drop per floret as in experiment 1 or (b) combined application as in experiment 2. MS/2 and B5 media were used as in experiment 2 and the haploid embryos were treated as in the previous experiments.

COLCHICINE APPLICATION

We applied 0.06% aqueous solution of colchicine supplemented with 2% dimethyl sulphoxide (DMSO) to roots of young plantlets according to the method described by Xynias and Roupakias (2000).

TRAITS MEASURED AND STATISTICAL ANALYSIS

The following traits were evaluated: number of developed ovaries, haploid embryos, green plants produced, and doubled haploid plants obtained. The evaluated traits were statistically analyzed using the chi-square two-way table format. Proportion comparisons were made by the z means test (Fasoulas, 2004)

RESULTS

The responses of the F_1 bread wheat plants to maize technique differed (Tab. 1). The percentage of developed ovaries ranged from moderate (62.6% in Kavkaz/Cgn × Acheloos) to high (84% in KVZ/Cgn × Vergina). The corresponding percentage of embryos produced ranged from 5.6% in Vergina × Acheloos to 11.3% in Penios × Acheloos. The percentage of obtained doubled haploids was low, ranging from 0.3% in Vergina × Acheloos and Acheloos × Vergina to 1.3% in KVZ/Cgn × Penios.

Five factors influenced the results: mode of growth regulator application, growth conditions of the wheat and maize plants, time of embryo rescue, embryo culture medium, and effect of genotype.

MODE OF GROWTH REGULATOR APPLICATION

The mode of growth regulator application affected the percentage of embryos produced and the corresponding percentages of doubled haploid plants produced (Tab. 2). No embryos were produced after sprinkling anthers with growth regulator. Drop application was more effective for embryo production (12.2% vs. 10.1% from combined application) but for doubled haploid plant production its effectiveness was the same as for combined application (Tab. 2).

GROWTH CONDITIONS OF THE WHEAT AND MAIZE PLANTS

The growth conditions of the parental crops significantly affected the number of developed ovaries (Tab. 2). However, the results of the field experiment in 2003 could be attributed to better manipulation

TABLE 2. Effect of mode of growth regulator application, growth conditions and time of embryo rescue on percentage of embryos and doubled-haploid plants produced

Factor	Number of pollinated flowers	Embryos produced		Doubled haploid plants	
		n	%	n	%
Mode of growth regulator application					
Sprinkle	2782	0	0	0	0
Drop	4409	541	12.3[a]	42	0.95[a]
Combined	3697	375	10.1[b]	44	1.19[a]
$P(\chi^2 > 345.12) = 0.0000$					
Growth conditions					
Field 2002	4435	119	2.7[c]	3	0.07[b]
Chamber	793	39	4.9[b]	2	0.25[b]
Field 2003	5660	758	13.4[a]	81	1.43[a]
$P(\chi^2 > 381.92) = 0.0000$					
Time of embryo rescue					
12 DAP*	2545	277	10.9[a]	16	0.06[a]
14 DAP	2631	258	9.8[a]	19	0.07[a]
16 DAP	2717	206	7.6[ab]	25	0.12[a]
18 DAP	2995	175	5.8[c]	26	0.15[a]
$P(\chi^2 > 48.687) = 0.0000$					

*DAP – days after pollination with maize pollen; percentages bearing different letters differ significantly at P=0.05 (z-test).

TABLE 3. Number of embryos cultured per cross and number (n) and percentage of haploid and doubled-haploid plants (plants after colchicine application) produced from each tested medium

Cross	Embryos cultured			Haploid plants produced						Doubled-haploid plants developed					
	MS	MS/2	B5							MS		MS/2		B5	
				n	%	n	%	n	%	n	%	n	%	n	%
Acheloos × KVZ	2	56	56	0	0	13	23	11	20	0	0	9	16.1	7	12.5
KVZ × Acheloos	16	46	37	0	0	6	13	8	22	0	0	3	6.5	4	10.8
Penios × Acheloos	22	68	51	0	0	12	18	7	14	0	0	7	10.3	4	7.8
Acheloos × Penios	11	35	40	2	18	5	14	6	15	1	9.1	3	8.6	3	7.5
KVZ × Vergina	10	30	31	1	10	9	30	7	23	1	10	4	13.3	3	9.7
KVZ × Penios	16	62	53	1	7.1	16	27	10	20	1	7.1	10	16.7	7	13.7
Penios × KVZ	9	59	72	0	0	8	14	13	18	0	0	6	10.2	7	9.7
Vergina × Acheloos	21	15	10	0	0	3	20	1	10	0	0	1	6.7	1	10.0
Acheloos × Vergina	14	40	34	0	0	3	7.5	4	12	0	0	2	5.0	2	5.9
Subtotal/mean	121	411	384	4	3.4[b]	75	18.3[a]	67	17.5[a]	3	2.5[b]	45	11[a]	38	9.9[a]
Total		916				146						86			

Percentages bearing different letters differ significantly at P=0.05 (z-test).

of the whole procedure. The number of embryos produced was also affected by the growth conditions of the maternal wheat plants: 758 embryos were produced from the field during 2003, 39 from the growth chamber and 119 from the field during 2002. The trend was similar for the percentages of doubled haploid plants obtained: 81 doubled haploid plants were produced from the field in 2003, 2 from the growth chamber and 3 from the field in 2002.

TIME OF EMBRYO RESCUE AND EMBRYO RESCUE MEDIUM

Time of embryo rescue had a slight effect on embryo production and no significant on the production of

the doubled haploid plants (Tab. 2). Immature embryos rescued at 18 DAP produced fewer embryos.

B5 and MS/2 media (both supplemented with 1 mg/l IAA and kinetin) did not differ in haploid and doubled haploid plant production (Tab. 3). MS medium without growth regulator supplementation performed worse for haploid and doubled haploid plant production. Applying colchicine solution to the roots of haploid plants resulted in 25%, 40% and 33% loss of the haploid plants originating from MS, MS/2 and B5 media respectively (Tab. 3).

EFFECT OF GENOTYPE

We obtained 146 haploid and 86 doubled haploid plants from all F_1 cross combinations (Tab. 3). Doubled haploid plant production was low. It ranged from 0.3% (Vergina × Acheloos, Acheloos × Vergina) to 1.3% (KVZ/Cgn × Penios) (Tab. 1).

DISCUSSION

The main criterion of the effectiveness of DH production techniques is the number of green fertile plants (doubled haploid plants) produced. This necessitates study of how this production is affected by the various factors referred to in the literature. The mode of growth regulator application affected the number of produced embryos, and two of the approaches applied (drop and combined application) proved equally effective (Tab. 2). This differs from the results reported by Cherkaoui et al. (2000), who found that combined application was better than drop application. In two other studies, sprinkling of the spike and drop application were equally effective (Almouslem et al., 1998; O'Donoughue and Bennett, 1994). According to Knox et al. (2000), the effectiveness of the method of growth regulator application depends on the genotype.

The growth conditions of the wheat plants affected all examined traits (field 2003 vs. field 2002, Tab. 2). The better results observed in 2003 can be attributed to application of fertilizer on warm days in May 2003 followed by adequate rain. O'Donoughue and Bennett (1994) also pointed to the role of growth conditions. The number of ovaries developed and the number of doubled haploid plants produced did not differ between 2002 field conditions and growth chamber conditions. In contrast, O'Donoughue and Bennett (1994) noted differences in haploid embryo production between plants grown in a chamber and plants grown in greenhouse conditions. Further research is needed to verify the effect of the stress conditions to which wheat plants are exposed during growth on haploid plant production (Knox et al., 2000). Matzk and Mahn (1994) suggested that the wheat and maize plants must be grown under optimal conditions because all differences in haploid plant production could be attributed to the variability in growth conditions of the parents and not to genotype-related reasons. Maize plants must be grown under controlled illumination (Almouslem et al., 1998), and special care must be taken to avoid insect infestation in maize plants, which reduces pollen viability (Knox et al., 2000).

The time of embryo rescue apparently affects the number of embryos produced more than the percentage of doubled haploid plants (Tab. 2). Embryo production was highest from embryos rescued at 12–14 DAP. Cherkaoui et al. (2000) made a similar finding. Reduction of haploid embryo production with the increase of days after pollination might be due to some degree of endosperm degradation (Inagaki and Snape, 1982; Suenaga, 1991). A null effect on doubled haploid production might be explained by the fact that embryos rescued on a later day after pollination are bigger and better differentiated and thus become more vigorous green plants (Cherkaoui et al., 2000).

The type of embryo rescue medium has a crucial effect on haploid production via maize technique in bread wheat. The percentage of doubled haploid plants originating from MS medium was 2.5% (Tab 3). This is much lower than the values reported in studies applying maize technique to durum wheat (Savaksan et al., 1997; Almouslem et al., 1998). MS medium without the addition of growth regulators gave a large number of embryos exhibiting very low germination response. This might be attributed partly to poor differentiation of the embryos, which inevitably leads to the development of inadequately formed plants. This phenomenon was studied by Ryczkowski et al. (1974), who suggested that increasing the osmotic pressure of the ovular sap could solve the problem. Guira (1993) attributed incomplete organogenesis to delayed embryo rescue; after culturing haploid embryos on B5 medium he reported production of green plants ranging from 40.5 to 91.9%, and attributed the above difference to the genotype effect. Cherkaoui et al. (2000) reported lower green plant production (20%) after culturing embryos on B5; in our present study it was reduced to ~10%. The superiority of MS/2 and B5 media over classical MS medium which we observed was also noted by Cherkaoui et al. (2000), who stated that halving the macroelements induces germination of large, well-developed embryos. Colchicine application resulted in loss of some of the haploid plants originating from the three media; the reduction was 40%, 33% and 25% for MS/2, B5 and MS media respectively. In a similar work, Savaksan et al. (1997) reported a smaller but also significant loss (25%) of haploid plants.

Our results on the effect of genotype are in line with previous reports (Sadasivaiah, 1999;

Cherkaoui et al., 2000; Wedzony et al. 2009) that the maize technique is less dependent on genotype: doubled haploid lines were produced from all the wheat F_1 hybrids, although some responded better than others. The observed differences in the androgenetic response of the germplasm we used could be attributed to differences in the genotypes' response to climatic conditions, as suggested by Matzk and Mann (1994) and O'Donoughue and Bennett (1994), or to the different genetic backgrounds of the evaluated F_1 wheat hybrids. It should be mentioned, however, that these F_1 wheat hybrids produced more DH plants after anther culture, which is a much less laborious approach (Zamani, 2001). We can suggest, therefore, that maize technique should be applied when production of DH plants is attempted in wheat genotypes that exhibit a poor response or no response to anther culture.

CONCLUSIONS

The two modes of growth regulator application (drop and combined) affected embryo production more than doubled haploid plant production. The same was found for the effect of time of embryo rescue. Field conditions had some effect on both embryo and doubled haploid plant production but the observed differences are more attributable to improved experience in handling maize technique. Two of the examined culture media (MS/2, B5) were equally effective. Adding colchicine to the media had a detrimental effect. Genotype had less of an effect on the outcome of maize technique than the other factors; the observed differences can be attributed to differences in the cultivars' response to climatic conditions or the different genetic backgrounds of the evaluated F_1 wheat hybrids.

AUTHORS' CONTRIBUTIONS

The authors of the present paper contributed equally and they all declare that there are no conflicts of interest.

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