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Production of Double Haploid Plants Using *In Vivo* Haploid Techniques in Corn

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ABSTRACT

This research was conducted at the breeding station of the Turkish breeding company Agromar A.Ş in the city of Bursa in Turkey during the 2013 and 2014 growing seasons. Used from within the same heterotic group crossings, 7 donor materials were obtained during the 2012 winter season in the greenhouse. The inducer line RWK-76xRWS, provided by University of Hohenheim, Germany, was used for generating haploid seeds. The donor and inducer crossing was performed during the 2013 summer season. The haploid selection and chromosome doubling were performed during the 2014 summer season. Seven donors were used for haploid induction which name are DNR1, DNR2, DNR3, DNR4, DNR5, DNR6, DNR7 respectively, from each donor different amount of ear crosses were performed (DNR1:16 ears, DNR2:10 ears, DNR3:10 ears, DNR4:12 ears, DNR5:11 ears, DNR6:13 ears, DNR7:11 ears). According to the present study, the average induction rate found ranged from 7.1 to 12.8%, and the average seedling survival rate in the greenhouse after colchicine application ranged from 57.9 to 77.6%. After transplanting to the field, 78.3-92.6% of these plants survived. As a result of this research, the chromosome doubling rate ranged from 22.5 to 48.3% depending on the donor material. These result indicates that maternal haploid selection visually is easy. Haploid induction rate (HIR) changes from donor to donor, its mean genotype and environment is effective for HIR. Average chromosome doubling rate is lower than other researchers' results, it is also effected by genotype and chromosome doubling methods.

Keywords: *Zea mays*; Haploid line; Double haploid line; *In vivo* induction

1. Introduction

The new phenomenon in maize breeding is called the double haploid technique, and the offspring of these plants are called double haploid lines (DHL). This technique is prevalent throughout the world and is being applied by maize breeders. Today, many private Turkish breeding firms and institutes use this method. The progress of science and technology and the economy have accelerated and advanced to allow creation of new plant varieties in the 20th century. The most critical stage of maize breeding is the improvement of inbred lines, which have the highest general and specific combining ability.

This kind of work takes a long time and requires a high budget and labor force. Inbred lines used for hybrid development require at least 7-10 years.

The key to increased genetic gains and accelerated development of improved varieties is reducing the time needed for inbred development. This can be most effectively achieved by application of double haploid (DH) technology (Prigge 2012). Currently, double haploid lines (DHL) produced by *in vivo* induction of maternal haploids are prevalent in maize (*Zea mays* L.) breeding (Gordillo & Geiger 2008; Rotarenco et al 2012; Battiselli et al 2013; Couto et al 2013). Haploids in maize can be obtained either through *in vitro* or *in vivo* techniques (Geiger 2009; Dang 2010; Dang et al 2011; Prasanna et al 2012). For maize, the widely used induction technique is *in vivo* induction of maternal haploids (Mureşeanu et al 2013). The limitations of in vitro haploid induction techniques in maize coupled with their requirement to have a good laboratory and skilled staff makes them less efficient methods (Chidzanga at al 2017). Two modes of *in vivo* haploid induction can be distinguished in maize, leading to maternal and paternal haploids (Röber et al 2005). In induction crosses aimed at paternal haploids, the inducer is used as the female and the donor plant as the male parent. Thus, the cytoplasm of paternal haploids originates from the inducer, but the chromosomes exclusively come from the donor plant. For production of maternal haploids, on the other hand, the inducer is used as the pollinator, leading to haploids carrying both the cytoplasm and chromosomes from the donor (Geiger 2009).

The *in vivo* production of maternal DHL involves the following four steps: (i) inducing haploidy by pollinating the source germplasm with pollen of the haploid inducer; (ii) identifying those seeds with haploid embryos based on a visual scorable morphological marker; (iii) duplicating chromosomes of putative haploids by treating the seedlings with a mitotic inhibitor; and (iv) self-pollinating DH plants to multiply their seed (Prigge 2012; Prasanna et al 2012; Tseng 2012). To induce maternal haploids, the donor plant is pollinated by a specific maize stock (line, single cross or population), which is called the inducer (Geiger & Gordillo 2009). For *in vivo* induction of maternal haploids, the pollen of maize inducer genotypes is used to pollinate the source germplasm from which DHL are to be developed (Prigge et al 2012). The haploid inducers are specialized genetic stocks that, when crossed to a diploid (normal) maize plant, result in progeny kernels in the ear segregating for diploid (2n) kernels and a certain fraction of haploid (n) kernels due to anomalous fertilization (Prasanna et al 2012). The goal of this research is to determine how the double haploid technique is applied and what sort of results are obtained.

2. Material and Methods

This research was conducted at the Agromar A.Ş breeding station department of the Plant Breeding and Genetics, Karacabey, district of Bursa, a province in Turkey (40°13'N, 28°22'E), during the growing seasons of 2013 and 2014. Plant materials were developed using from within the same heterotic group crossing in the 2012 winter season in greenhouse conditions. DHL were induced from F1 donor plants. Bernardo et al (2010) observed that DHL should be induced from F2 plants rather than from F1 plants. Seven donor genotypes were used for this study. The donor names are as follows: DNR1, DNR2, DNR3, DNR4, DNR5, DNR6, and DNR7. The DHL were obtained in two steps, as follows.

2.1. Generation of haploid plants (donor and ınducer crossing)

The donor and inducer crossings were performed during the 2013 summer season. In this study, the inducer line RWK-76xRWS provided by University of Hohenheim, Germany was used for generating haploid seeds. Averaged across a wide range of donors and environments, it has an induction rate of approximately 8%. A sister line, RWK-76, developed from the reciprocal cross (WS14xKEMS), even reached an average induction rate of 9-10%. This same rate was observed for the cross RWSxRWK-76. Although having related parents, this cross is much more vigorous and is better at shedding pollen than both of its parents; this cross is therefore easier to handle, particularly in adverse environments (Geiger 2009). The use of

F1 plants as inducers may be more advantageous because of their higher vigor, which generally is associated with more abundant pollen shedding (Röber et al 2005). Each plant was planted in 2 rows at a row spacing of 70x20 cm and a row length of 3 m. Each plot contained 32 plants. Because of the early maturity of the inducer line, the inducers were sowed two times: 4 and 7 days after planting of the donors. The haploid induction rates (HIR) of some inducer lines are given in Table 1 below (Roeber 2014).

Table 1- Haploid induction rates of some inducer line according to usage

Using type	*Line name*	*Haploid induction rate (%)*
Maternal	Stock 6	2.3-3.2
Maternal	WS14	2.0-5.0
Maternal	KEMS	6.3
Maternal	RWS	10.0
Maternal	UH400	8.0-9.0
Maternal	BRZO6	12.3
Maternal	B-432	13.9
Maternal	ZMS	0.6-3.4
Maternal	AC'R	5.5
Maternal	ACR	8.3
Maternal	KMS	0.8-2.9
Maternal	PK6	6.0
Maternal	AT-1	2.0-3.0
Maternal	AX6012	10.0
Paternal	W23ig	2.6-8.0

2.2. Identification of putative haploids and artificial chromosome doubling

The most efficient haploid identification marker is the 'red crown' or '*navoja*' kernel trait encoded by the dominant mutant allele R1-*nj* of the 'red color' gene R1. In the presence of dominant pigmentation genes A1 or A2 and C2, R1-*nj* causes deep pigmentation of the *aleurone* (endosperm tissue) in the crown (top) region of the kernel (Geiger 2009). The R1-*Navajo* (R1-*nj*) color marker facilitates easy and quick identification of haploid kernels at the seed stage during the *in vivo* haploid induction process in maize (Chaikam et al 2015). The R1-*nj* marker gene is widely used for screening haploids of dry seed. However, the expression of this gene has strong female influence: sometimes the screening of haploids can be very confusing or even impossible, especially in those cases where there are inhibitor genes (C1-I) in females (common for flint maize) (Rotarenco et al 2010). There are some newly methods provided by some researchers for haploid identification. Chaikam et al (2016) developed haploid inducer lines with triple anthocyanin color markers, including the expression of anthocyanin coloration in the seedling roots and leaf sheaths, in addition to the R1-*nj* gene. The other method is suggested by Melchinger et al (2014) that UH600 high oleic inducer line using for haploid identification. Jones at al (2012) recommend that near infrared spectroscopy for the haploid identification.

In the present study, haploid or diploid kernels were distinguished by means of the expression of the dominant anthocyanin marker gene R1-*nj* (Figure 1). Which mechanism is controlling maternal haploid induction? The answer is that there are two major ways that the maternal haploid inducer might produce kernels with haploid embryos. First, an abnormal fertilization might occur in which one sperm fertilizes the two polar nuclei and other sperm fails to fertilize the egg of an embryo sac, producing a kernel that has a triploid endosperm and haploid embryo. Second, the normal double fertilization event would occur, and then the chromosomes from the female parent are eliminated from the embryo after fertilization (Weber 2014). There are three types of seed obtained. The first one is diploid seed; kernels with purple coloration in the endosperm (*aleurone*) and the embryo (*scutellum*) are accepted as diploid. The second one is putative haploid kernels; purple endosperm but no coloration of the embryo is accepted as putative haploid. The last one is unpigmented kernels; without purple coloration of the embryo or endosperm is accepted as an outcross because of pollen contamination. Putative haploid seed is identified visually according to their *aleurone* and embryo color. Pigmented ears shelled individually and by HIR (haploid induction rate) are found according to each donor genotype. The haploid induction rate of the maize inducer denotes

the proportion of seeds with a haploid embryo detected in the total number of seeds harvested from source germplasm pollinated with inducer pollen (Prigge et al 2012).

Figure 1- Haploid and diploid kernels

In this research, the haploid induction rate (HIR), haploid germination rate (HGR), surviving seedling rate (SSR), surviving plant rate (SPR), and chromosome doubling rate (CDR) were calculated using the following formulas:

The genome of the haploid plant material should be doubled to obtain 100% homozygous plant materials after the putative haploids are identified. To achieve this, colchicine treatment is the most widely used procedure for chromosome doubling (Prasanna et al 2012; Mureşeanu et al 2013). Chromosome doubling of the selected haploid seedlings was performed according to methods of Deimling et al (1997). Haploid seeds were germinated in a growth chamber for 4 or 5 days. When coleoptiles reached 2-3 cm in length, the tops were cut and removed from the roots and immersed in a solution of 0.06% colchicine and 0.05% dimethyl sulfoxide (DMSO) for 12 hours at 18 °C. One day after the solution application, coleoptiles were washed for 20 minutes and transplanted into pots in the greenhouse. When the first cycle of DHL reached the 4- or 5-leaf-stage in greenhouse conditions, all of the seedlings were transferred to the field and transplanted for seed increase.

HIR= (Haploid kernels / Total kernels)*100 (1)

HGR= ((Haploid kernels - ungerminated kernels) / Total haploid kernels)*100 (2)

SSR= (Transplanted seedlings / Colchicine-treated seedlings)*100 (3)

SPR= (Surviving plant number / Transplanted seedling number)*100 (4)

CDR= (Fertile plant number / Selected plant number)*100 (5)

3. Results and Discussion

3.1. Haploid ınduction rate (HIR)

Seven donor genotypes were induced by the inducer line RWK-76xRWS in the summer season of 2013. The ears were shelled individually, and haploid kernels were selected according to visual selection. The mean haploid induction rate of each ear is given in Table 2.

There is a significant difference between donors at 5% level. The putative HIR changed from donor to donor such that DNR1 changed from 3.1 to 14.7%, DNR2 changed from 1.2 to 12.8%, DNR3 changed from 1.1 to 13.6%, DNR4 changed from 5.3 to 11.7%, DNR5 changed from 3.5 to 32.7%, DNR6 changed from 2.1 to 20.1%, and DNR7 changed from 2 to 19.8%. The highest average HIR belonged to DNR7 (12.8%) and lowest to DNR2 (7.1%), on average. Chalky (1994) reported that, on average, 27.4 kernels per ear of haploid plants were obtained in the first year of study and 26.3 in the second year from the inducer line ZMS. Cengiz (2016) reported that the highest HIR was established as 20.42% in the RWK-76 inducer line and that the lowest HIR was calculated as 17.75% in WS14. Cerit at al (2016) reported that the RWK-76 has highest HIR and the lowest one is Stock-6. Dang et al (2011) reported that the rates of putative haploids among progenies induced with the inducer lines RWK76 and RWK-76xRWS (15.7 and 15.0%, on average, respectively) were significantly lower ($P<0.05$) than those observed with the inducer line

Table 2- Putative haploid induction rate (%)

Ear no	*DNR1*	*DNR2*	*DNR3*	*DNR4*	*DNR5*	*DNR6*	*DNR7*
1	7.4	6.7	8.1	6.4	3.6	4.8	12.7
2	11.4	5.7	9.5	6.4	7.1	4.2	2.0
3	9.5	1.2	5.7	8.5	3.8	5.3	7.6
4	7.2	4.7	1.1	5.3	3.5	2.1	5.8
5	14.7	7.5	9.5	10.2	12.7	12.6	19.8
6	8.9	11.4	8.5	11.7	20.2	11.2	16.6
7	3.1	9.2	9.7	6.3	12.8	5.7	16.4
8	9.4	12.8	6.9	7.7	10.7	20.1	17.6
9	8.3	5.1	5.5	9.9	32.7	9.6	11.7
10	8.2	6.6	13.6	6.8	9.9	15.6	13.8
11	6.4			5.6	18.8	14.2	17.3
12	8.8			9.3		10.0	
13	8.3					7.1	
14	8.3						
15	14.1						
16	9.9						
Average	9.0 bc	7.1 c	7.8 c	7.8 c	12.3 ab	9.4 abc	12.8 a

significant at 5% probability levels

RWS (23.8%) for the same maternal plant material. Those induction rates are higher than those of the present study. Prasanna et al (2012) reported that the tropical inducer UHo induction rate is 8-10% and that of TAILs is 8-12%, which are similar to the results of the present research. The HIR of other inducer are given in the materials and methods section in Table 1.

3.2. Haploid germination rate (HGR)

Haploid kernels were treated with colchicine in the summer season of 2014. For this process, putative haploid kernels should be germinated to have seedlings ready before treating with colchicine. Haploid seeds were germinated in a growth chamber at 25 °C. The germination rates of each donor changed from 63.6 to 95.2%, with an average of 81.8% (Table 3.). The lowest HGR belonged to DNR6 (63.6%) and the highest to DNR7 (95.2%). At CIMMYT, a germination percentage of 85-90% is commonly achieved among the putative haploid kernels (Prasanna et al 2012). Our results are similar to those of this study.

Table 3- Haploid germination rate (%)

Donors	*Haploid kernels*	*Ungerminated kernels*	*HGR*
DNR1	406	31	92.4
DNR2	128	21	83.6
DNR3	209	57	72.7
DNR4	227	13	94.3
DNR5	211	62	70.6
DNR6	239	87	63.6
DNR7	294	14	95.2
Total	1714	Average	81.8

3.3. Colchicine treatment and surviving seedling rate (SSR)

Colchicine treatments were applied according to the methods of Deimling et al (1997). Other methods have been presented by Gayen et al (1994), who used three rates of colchicine in a DMSO solution. At CIMMYT, a solution of 0.04% colchicine and 0.5% DMSO is used for chromosomal doubling (Prasanna et al 2012). The application procedure is given in the materials and methods. The average SSR was 66.3% (Table 4). Cengiz (2016) reported

that 89% of plants lived of the 2178 seedlings that were planted the field. The highest donor SSR 77.6% belonged to DNR4; the lowest, 57.9%, belonged to DNR3.

Table 4- Surviving seedling rate (%)

Donors	*Colchicine-treated seedlings*	*Transplanted seedlings*	*SSR*
DNR1	375	243	64.8
DNR2	107	73	68.2
DNR3	152	88	57.9
DNR4	214	166	77.6
DNR5	149	87	58.4
DNR6	152	98	64.5
DNR7	280	204	72.9
		Average	66.3

3.4. Transplanted seedlings and surviving plant rate (SPR)

Surviving seedlings were transplanted to the field. Each seedling was transplanted to the field at a spacing of 70x20 cm. The surviving plant rates are given in Table 5. The average SPR was 86.8%. The highest SPR was 92.6%, which belonged to DNR1, and the lowest one was 78.3%, which belonged to DNR4.

Table 5- Surviving plant rate (%)

Donors	*Transplanted seedlings number*	*Surviving plants number*	*SPR*
DNR1	243	225	92.6
DNR2	73	66	90.4
DNR3	88	77	87.5
DNR4	166	130	78.3
DNR5	87	70	80.5
DNR6	98	89	90.8
DNR7	204	179	87.7
		Average	86.8

3.5. Chromosome doubling rate (CDR)

There were some misclassified plants that were identified in the field. They were determined by their agronomic attributes, such as purple stem, advanced vigor, etc. All of the misclassified plants were discarded before selfing. The first stage of DH lines is called D0, and second generation of DH lines is called D1 indicates the CDR percentages (Table 6). The average CDR found was 34.3%. The CDR changed for each donor, ranging from 22.5 to 48.3%. Doubling rates have reached 50%, 0-40%, and 55.31%, as reported by Vanous (2011), Tseng (2012) and Prasanna et al (2012), respectively. Cengiz (2016) reported that fertile plants made up 57% of live plants. These rates are higher than those of the present study. The chromosome doubling may vary depending on the genotype and application. The reason why CDR is different for many authors that the genotype and application methods for chromosome doubling is different. Beside haploid selection effects this rate.

Table 6- Chromosome doubling rate (%)

Donors	*Selected plants number*	*Fertile plant number*	*CDR*
DNR1	204	46	22.5
DNR2	52	15	28.8
DNR3	60	29	48.3
DNR4	115	49	42.6
DNR5	58	17	29.3
DNR6	80	25	31.3
DNR7	148	55	37.2
		Average	34.3

4. Conclusions

In the present study, the HIR, HGR, SSR, SPR, and CDR were determined. The values of these characteristics changed according to each donor genotype, and these terms can be used for this kind of research as common terms. The highest haploid induction rate obtained was 7.1-12.8% with the maternal inducer RWK-76xRWS. The average HGR found was 81.8%, and this rate is sufficient for utilizing haploid kernels. The SSR obtained in pots was lower than the SPR in the field because of the adverse effects of colchicine and acclimatization. As a result, the observed CDR was, on average, 34.3%. In conclusion, 1714 putative haploids were

identified based on the phenotypic marker system of the R1-*nj* allele. Among the 1714 putative haploids, only 87 D0 lines advanced to the D1 stage, which means only 5.1% of haploids can be advanced to the D1 stage. The most critical stage for DHL development is haploid identification. There are newly and efficient methods developed by the researcher for the identification of haploid induction like using high oleic inducer, near infrared spectroscopy and triple anthocyanin color markers inducer. These newly methods will helps to corn breeder for the selection of haploids. Most of the publication trace back maternal haploid system but the other important methods is paternal haploid system. This system specially work well in cms corn line development and backcross breeding. We suggest to corn breeders to utilize paternal haploid system.

To sum up DHL is invaluable and newly methods for corn breeders to have 100% homozygous line only in two generation. Accelerating the generation is less expensive than other corn breeding methods and less laboratory needed. It is recommended to corn breeders that they should use at least ten F1 donor ears crossing with inducer because of advanced D1 generation is only about 5 percent.

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