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DEVELOPMENT OF MAIZE (*Zea mays* L.) GENOTYPES BY USING *IN VIVO* DOUBLED HAPLOID TECHNIQUE

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ABSTRACT

Doubled haploid technology has become an increasingly popular and important tool for developing new maize (*Zea mays* L.) lines breeding. Although conventional plant breeding contributes significantly to maize development, it is time-consuming and has disadvantages such as using more labor and financial resources. Conventional maize breeding requires repeated self-pollination for about six to ten generations to obtain homozygous inbred lines, also 100% homozygous lines cannot be obtained at the end of this period. Completely homozygous lines can be produced in only two-three generations by using doubled haploid technology. *In vivo* induction of the maternal haploid method was used for the production of doubled haploid lines. Thirty-two different donors pollinated with haploid inducer line Stock6. Haploid seeds were identified visually by using dominant anthocyanin color marker genes *R1-nj*. Haploid induction rates (HIR) and chromosome doubling rate (CDR) were determined. Four hundred and eighty eight putative haploid seeds were obtained. The average haploid induction rate was calculated as 2.0%, the average chromosome doubling rate was 52.3%. Results of this study 20 doubled haploid lines were developed.

Keywords: Doubled haploid, haploid induction rate, *in vivo*, maize breeding.

INTRODUCTION

Maize is one of the most produced crops and plays a critical role in ensuring food, feedstock, and energy supply in the world. Also, it has been widely used for silage production over the past thirty years. The private sector and public sectors frequently introduce new maize varieties to the market because of the growing demand for maize (Mansfield and Mumm, 2014; Kusaksiz and Kusaksiz, 2018).

The development of high-quality, high-yielding, and highly adaptable varieties in maize is possible by consistently obtaining homozygous inbred lines with high combinability. Pure line development, which is the main subject of hybrid maize breeding, takes 6–10 years in classical methods, and it is sometimes impossible to obtain 100% homozygous lines at the end. In one generation, doubled haploid lines provide genetic homozygosity. The fact that haploids carry only a single copy of every gene allows for revealing recessive mutations. Haploid plants that have harmful genes either die or are weak and sterile and do not form seeds. In this way, the frequency of unfavorable harmful genes will be rapidly eliminated during the haploid stage. This process is similar to natural selection, but it provides an effective tool for eliminating undesirable genes and enriching good genes to rapidly enhance the genetic pool. Chromosome doubling of haploids will produce a DH line with 100% genetic homozygosity (Chang and Coe, 2009). Thus, doubled haploid (DH) allows for obtaining completely homozygous inbred lines in a short period of 2–3 generations. (Ren et al., 2017; Uliana Trentin et al., 2022).

Although maize (*Zea mays* L.) is a diploid plant (2n = 20), haploid individuals (n = 10) occur naturally at a ratio of one per 1000 seeds (Chase, 1949). Coe (1959) discovered Stock6, a haploid inducer that increased the frequency of haploid production compared with normal maize. If a genotype has a haploid induction rate (HIR) of at least 2%, it is considered an inducer.

Haploid can be produced using *in vitro* or *in vivo* techniques in maize. *In vitro* haploid production in maize is a time-consuming and costly process. However, DH line development based on *in vivo* is easier than *in vitro* with its high HIR value due to the presence of anthocyanin color marker in the genetic base of the inducer to enable easy identification of haploid at both the seed and seedling stages. The dominant mutant allele *R1-nj* of the " anthocyanin color" gene is the most efficient haploid identification marker. *R1-nj* gene causes pigmentation in the aleurone (endosperm) and the scutellum (embryo tissue) *R1-nj* (Navajo) is frequently used for haploid identification, and all haploid inducers now in use around the world contain *R1-nj* (Geiger and Gordillo, 2010).

The effectiveness of doubled haploid technic depends on the donor having colorless seeds, the inducer being homozygous for *R1-nj,* and preferably the dominant pigmentation genes (*A1* or *A2* and *C2*). If the donor genome is homozygous for *R1* or dominant anthocyanin inhibitors genes such as *C1-I, C2-Idf,* and *In1-D*, inhibit *R1-nj* expression. Flint, subtropical, tropical, and sweet corn groups have high frequencies of these alleles, resulting in high misclassification rates (Geiger, 2009; Ulina Trentin et al., 2022). Ulina Trentin et al. (2022) reported that dent hybrid showed higher inducibility as a donor than sweet and flint corn. Also, researchers stated that no statistical difference between the inducer lines. Additionally, novel haploid identification markers systems, such as the red root marker and high oil marker, are now being integrated into new haploid inducers. Thus, doubled haploid technique is accessible in germplasm such as flint or tropical material where the standard *R1-nj* marker is inhibited (Chaikam et al., 2019). Due to some limitations of the *R1-nj* color marker system, researchers have discovered other color markers that produce color in roots and stems, especially during germination, to reliably distinguish maternal haploids. *Pl1* (*Purple 1*) and *B1* (*Booster 1*) are two alleles that result in sunlight-independent purple pigmentation in the plant tissue (coleoptile and root). These anthocyanin genes were found suitable for cases where haploid sorting is not possible at the dry seed stage (Rotarenco et al., 2010). If *R1-nj* is poorly expressed in the scutellum, the coleoptile and root color gene *Pl* can be used to validate the putative haploids at the early seedling stage (Geiger, 2009). Thus, germinated haploid seeds can be distinguished from root coloration or field stem coloration. When hybridizing the source material with such an inducer line, the diploid seeds obtained will have colored (purple) roots and stems. Those considered to be haploid will not have this coloration (Coe and Sarkar, 1964).

In maize, there are two mechanisms of *in vivo* haploid induction, which result in maternal and paternal haploid. The inducer is used as a pollinator during maternal haploid induction. In commercial maize breeding, *in vivo* maternal haploid is routinely produced world-wide with a haploid inducer line such as Stock6 or other Stock6-derived inducers (Wang et al., 2019; Kalinowska et al., 2019).

Doubled haploid (DH) technology provides the most effective success in obtaining homozygous maize lines in a short time and at less cost. In this respect, the doubled haploid method has become an important technique for maize breeders. Therefore, our objectives were to (i) determine the haploid induction rate of Stock6; (ii) obtain 100% homozygous lines in a short period of 2 generations using the doubled haploid technique.

MATERIALS AND METHODS

This study was conducted at Bursa Uludag University Agriculture Faculty Research and Training Centre in Bursa, Turkey in 2019–2021. In the research, 32 genotypes were used as female parents (FAO 650–700 maturity group). All donor genotypes have a dent kernel type as well as colorless aleurone and embryo. Inducer line (Stock6) was used as the male parent, which was provided by the Maize Genetics Cooperation-Stock Center (USDA/ARS). Stock6 contains the *R1-nj*, *B1* and *Pl1* alleles together.

In this study, *in vivo* haploid induction methods were used. The inducer line is used as a pollinator to produce maternal haploid in this method (Rober et al., 2005). The steps of this method are as follows: (1) induction cross, (2) haploid (embryo) selection at the seed or seedling stage (3) haploid chromosome doubling, and selfing of double haploid plants to produce doubled haploid lines (Chaikam et al., 2019).

First-year in research, genotypes were planted in plots with 0.70 m row spacing, 0.20 m plant spacing, and 5 m row length. Inducer lines tassels (50% anthesis) and ears of 32 genotypes were covered with isolation papers. Three plants selected from genotypes in each row were pollinated with the pollen of Stock6. After the induction crosses, isolation papers were stored until harvest. When the ears had reached physiological maturity, the pollinated ears were harvested separately and dried in the sun.

Haploid seeds were identified via dominant anthocyanin color marker genes *R1-nj* (Meng et al., 2022). In addition, *in vivo* induced haploids were eliminated from diploids at the seedling or adult plant stage via the *Pl1* (*Purple 1*) and *B1* (*Booster 1*) alleles. These alleles showed coleoptile and root pigmentation. In practice, induction cross may result in the categories listed below (expression of *R1-nj*);

(1) Haploid seed has a red crown (regular triploid endosperm) and an unpigmented scutellum (haploid maternal embryo),

(2) F1 seed has the pigmentation of both aleurone and scutellum,

(3) If just the egg cell is fertilized and not the central cell, the seed has a pigmented (diploid) embryo and a non-pigmented,

(4) Seeds that have been (unintended) selfing or outcrossing with other colorless donors have no coloration (Figure 1) (Geiger, 2009).

Haploid seed numbers were determined. Haploid induction rates and chromosome doubling rate (CDR) were calculated according to the formula given below:

HIR = (Number of haploids / Total number of seeds) x 100 (Dong et al., 2013).

CDR = (Fertile plant number / Number of seeds applied colchicine) x 100 (Zararsız et al., 2019).

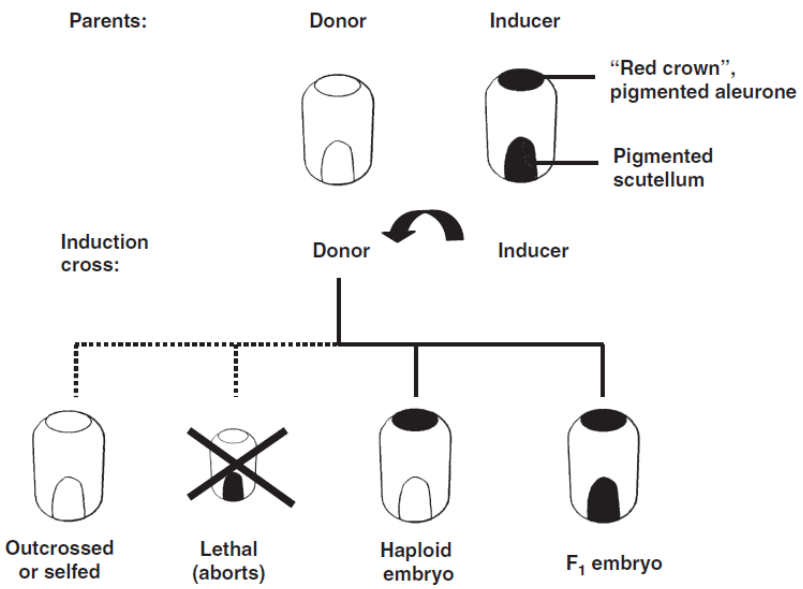

Figure 1. Use of the *R1-nj* haploid identification marker to distinguish haploid embryonated seeds after haploid induction hybridization (Geiger, 2009).

In the second year of the study, seeds considered haploid were germinated at 23°C in a dark climate chamber for chromosome doubling treatment. Since seeds with a haploid embryo include a typical triploid (3n) endosperm, they germinate similarly to seeds like a diploid embryo (Coe and Sarkar, 1964). Colchicine was applied 2–3 days after germination (at the coleoptile stage). The tip of the coleoptiles was cut off and immersed the whole seedling in a 0.06% colchicine solution plus 0.5% DSMO (dimethyl sulfoxide) for 12 h at 18°C (Gayen et al., 1994; Deimling et al., 1997). The seedlings were carefully washed in water after receiving the colchicine treatment. After they were grown in a greenhouse until they had five to six leaves. Then, the plants were transferred to the field a few weeks later (Figure 2). Self-pollination was performed on the first generation of doubled haploid plants (D_0).

Figure 2. Planting D_0 plants in the field (original).

RESULTS AND DISCUSSION

In this study, a total of 26412 seeds were obtained after induction crosses. Seeds were divided into 4 categories via the *R1-nj* marker system. Haploid seeds were distinguished by the anthocyanin color marker *R1-nj* such that they had a colored endosperm and an uncolored embryo. F1 seeds displayed color pigmentation of both embryo and endosperm, whereas outcrossed or self-pollinated seeds showed uncolored embryo and endosperm. Diploid endosperm seeds had a colored embryo and a colorless endosperm (Figure 3). Four hundred and eighty eight putative haploid seeds were obtained in our study. The number of other categories of seeds were given in Table 1.

The term inducibility is used to describe the influence of the donor parent on HIR. In our study, the value of the haploid induction rate of Stock6 varied between 0.16% and 3.58%, and the average haploid induction rate was calculated as 2.03%. Researchers have long reported that the rates of induction of source germplasm are different and their effects on HIR can be very high (Lashermes, 1988; Eder, 2002; De La Fuente et al., 2018). Eder (2002) observed HIR ranging from 2.7% to 8.0% in the pollination of 20 different donors (flint, dent, and flint x dent groups) with the same inducer. Coe (1959) reported that the haploid inducer line Stock6 has a 2.3%-3.2% maternal HIR. Cerit et al. (2016) found that Stock6 had a 1.28% haploid induction rate in their research to determine the haploid induction rates of different inducer lines. The HIR is higher under optimum growth conditions with the least stress factor (Geiger, 2009). Also, the HIR differs depending on the pollination time and method (Röber et al., 2005). In our study, hand-pollinating was performed on D_0 plants. Rotarenco et al. (2002) found that hand-pollination outperformed open-pollination in an isolated plot three days following the silk appearance. Similar to Rotarenco et al. (2002), hand-pollination was done three days after the

silk appearance in our research. Additionally, putative haploid seed selection accuracy depends on researchers who comprehend haploid identification by *R1-nj* expression on the aleurone and scutellum (Cengiz and Korkut, 2020).

Table 1. The number of seeds and the average number of haploid, F1, diploid, and outcross seed.

Genotypes	Putative haploid seed	F1	Lethal	Outcrossed	Total seed	HIR	F1	Lethal	Outcrossed	CDR
	Number of seeds					(%)				
G3	10	389	30	265	694	1.44	56.1	4.3	38.2	57
G5	2	656	20	278	956	0.20	68.6	2.1	29.1	100
G6	16	411	12	101	540	2.96	76.1	2.2	18.7	70
G8	17	407	7	112	543	3.13	75.0	1.3	20.6	36
G9	4	105	5	31	145	2.75	72.4	3.4	21.4	18
G10	10	213	3	53	279	3.58	76.3	1.1	19.0	40
G12	15	1051	114	208	1388	1.08	75.7	8.2	15.0	57
G13	46	977	7	405	1435	3.20	68.1	0.5	28.2	60
G14	12	936	52	321	1321	0.90	24.3	3.9	70.9	0
G16	9	150	2	108	269	3.34	55.8	0.7	40.1	50
G17	19	571	27	395	1012	1.87	56.4	2.7	39.0	55
G18	3	504	4	173	684	0.43	73,7	0.6	25.3	33
G19	19	1200	32	497	1748	1.08	68,6	1,8	28.4	33
G21	51	871	76	564	1562	3.26	55.8	4.9	36.1	87
G22	25	409	42	280	756	3.30	54.1	5.6	37.0	70
G24	10	573	9	232	824	1.21	69.5	1.1	28.2	40
G26	1	440	2	157	600	0.16	73.3	0.3	26.2	100
G30	6	683	35	244	968	0.61	70.6	3.6	25.2	13
G34	11	383	6	860	1260	0.87	30.4	0.5	68.3	80
G35	34	539	53	523	1149	2.95	46.9	4.6	45.5	25
G37	10	629	20	518	1177	0.84	53.4	1.7	44.0	50
G38	38	712	35	389	1174	3.23	60.6	3.0	33.1	44
G40	8	238	12	183	441	1.81	54.0	2.7	41.5	40
G41	17	233	8	310	568	2.99	41.0	1.4	54.6	0
G42	3	878	12	308	1201	0.24	73.1	1.0	25.6	67
G43	6	90	0	114	210	2.85	42.9	0.0	54.3	88
G44	24	325	43	353	745	3.22	43.6	5.8	47.4	77
G45	1	19	0	28	48	2.08	39.6	0.0	58.3	0
G46	34	643	59	315	1051	3.23	61.2	5.6	30.0	86
G47	5	772	52	146	975	0.51	79.2	5.3	15.0	50
G49	2	25	0	64	91	2.19	27.5	0.0	70.3	100
G51	20	398	31	149	598	3.34	66.6	5.2	24.9	50
Total	488	16430	810	8684	26412					
Average						2.03	59.1	2.7	36.2	52.3

Haploids are often sterile because meiotic divisions cannot occur, resulting in the non-formation of gametes. The haploid seedlings were treated with antimitotic chemicals to create artificial chromosomal doubling. Colchicine is commonly used in DH line development for chromosomal doubling (Melchinger et al., 2016; Chaikam et al., 2019). The average chromosome doubling rate (CDR) was calculated to be 52.3%. Seedlings of 3 genotypes (G14, G41, G45) did not survive after chromosome doubling treatment. According to the researchers, around 70%-80% of the haploid seedlings survive the colchicine treatment and 20%-30% of those produce selfed seeds (Rober et al., 2005).

Researchers have indicated that haploid plants are smaller and weaker than diploid homozygous lines (Auger et al., 2004; Cengiz and Korkut, 2020). Their short stature makes selfing processes more difficult. The fact that some plants are sterile or in general very little dusting complicates the selfing process. They are also more sensitive to stress conditions. Seeds that are considered haploid but misclassified were determined in the field according to some characteristics and no self-pollination was done in these plants. Seeds could not be obtained from some D_0 plants after self-pollination treatment. It has also been detected that some D_0 plants have completely sterile tassels. Chaikam et al. (2019) stated that the fertility of haploid tassels varies greatly and might range from just one

or a few anthers generating pollen to the entire tassel becoming fertile. According to researchers, self-pollination can be performed twice or three times on consecutive days on each D_0 plant with fertile tassels to guarantee good seed development.

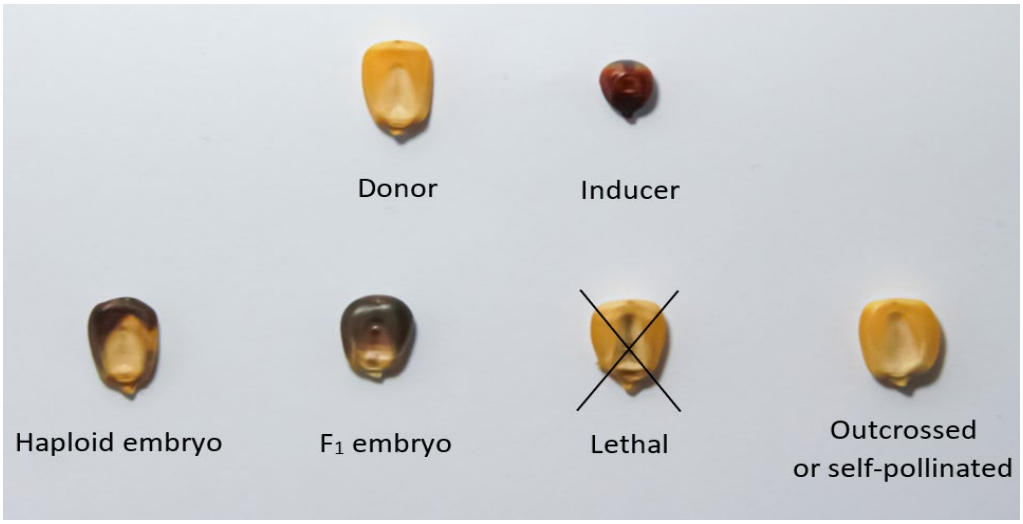

Figure 3. Expression of the seed color marker *R1-nj* (original).

Anthocyanin-colored seeds bearing ears were discarded at harvest time because they originated from non-haploid seeds. The seeds of each ear harvested from D_0 plants when they reach physiological maturity represent a fully homozygous doubled haploid line, often referred to as the D_1 generation (Chaikam et al., 2019).

CONCLUSION

The doubled haploid technology provides a great advantage in that homozygous lines can be obtained quickly. According to this study, the haploid induction rate and chromosome doubling rate of each genotype differed. Four hundred and eighty eight putative haploid seeds were obtained, and the average haploid induction rate was determined as 2.0%. As a result, 20 doubled haploid lines were produced, and we will cross-examine these lines in future projects to determine the best hybrid corn line/lines.

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